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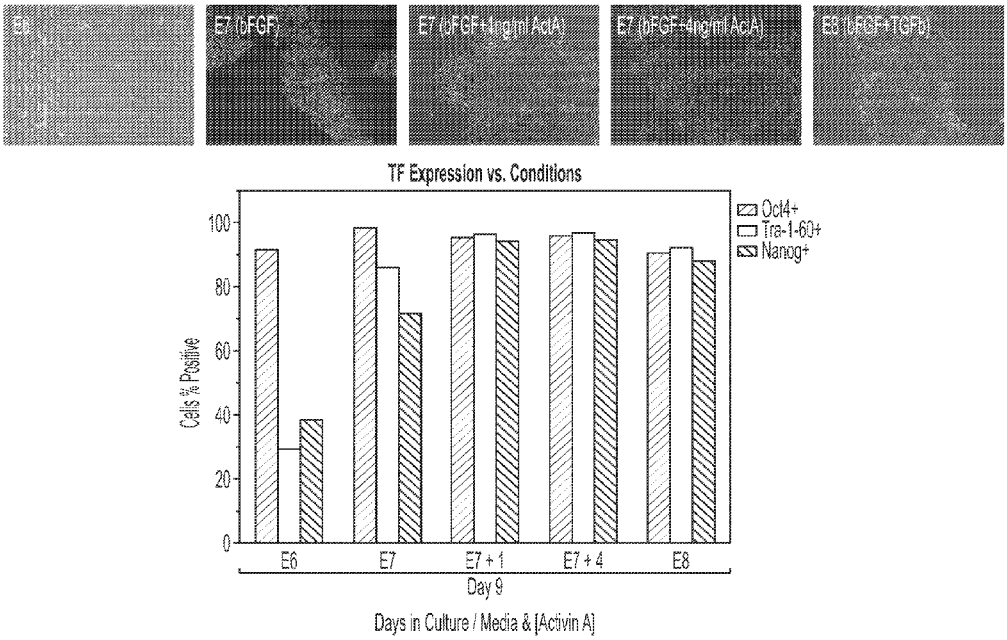

FIG. 1

(57) Abstract: Methods of culturing embryonic stem cells, induced pluripotent stem cells and/or differentiated cells in culture medium comprising activin are described. In one aspect, the disclosure features a pluripotent human stem cell, wherein the stem cell comprises: (i) a genomic edit that results in loss of function of Cytokine Inducible SH2 Containing Protein (CISH) and (ii) a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway, or a genomic edit that results in a loss of function of adenosine A2a receptor.

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ENGINEERED CELLS FOR THERAPY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/950,063, filed December 18, 2019, U.S. Provisional Application No. 63/025,735, filed May 15, 2020, and U.S. Provisional Application No. 63/115,592, filed November 18, 2020, the contents of all of which are hereby incorporated herein in their entirety.

BACKGROUND

[0002] There remains a need for engineered cells for therapeutic interventions, as well as for methods of culturing stem cells, such as embryonic stem cells and induced pluripotent cells, such that pluripotency is maintained.

SUMMARY

[0003] In one aspect, the disclosure features a pluripotent human stem cell, wherein the stem cell comprises: (i) a genomic edit that results in loss of function of Cytokine Inducible SH2 Containing Protein (CISH) and (ii) a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway, or a genomic edit that results in a loss of function of adenosine A2a receptor (ADORA2A). In some embodiments, the stem cell comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway and a genomic edit that results in a loss of function of ADORA2A.

[0004] In some embodiments, the stem cell comprises a genomic edit that results in a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor. In some embodiments, the TGF beta receptor is a TGF beta receptor II (TGFβRII).

[0005] In some embodiments, the stem cell expresses one or more pluripotency markers selected from the group consisting of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin, UTF-1, Oct4, Rex1, and Nanog.

[0006] In some embodiments, the disclosure features a differentiated cell, wherein the differentiated cell is a daughter cell of a pluripotent human stem cell described herein. In some embodiments, the differentiated cell is an immune cell. In some embodiments, the

differentiated cell is a lymphocyte. In some embodiments, the differentiated cell is a natural killer cell. In some embodiments, the stem cell is a human induced pluripotent stem cell (iPSC), and wherein the differentiated daughter cell is an iNK cell. In some embodiments, the cell: (a) does not express endogenous CD3, CD4, and/or CD8; and (b) expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, CD43, and/or CD45, or any combination thereof; (ii) NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 (CD16)); (iii) natural killer group-2 member D (NKG2D); (iv) CD69; (v) a natural cytotoxicity receptor; or any combination of two or more thereof.

[0007] In some embodiments, any of the cells described herein comprises one or more additional genomic edits. In some embodiments, the cell (1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16) (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) comprises at least one genomic edit that results in a loss of function of at least one of: (i) ADORA2A; (ii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iii) β-2 microglobulin (B2M); (iv) programmed cell death protein 1 (PD-1); (v) class II, major histocompatibility complex, transactivator (CIITA); (vi) natural killer cell receptor NKG2A (natural killer group 2A); (vii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (viii) cluster of differentiation 32B (CD32B, FCGR2B); (ix) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0008] In another aspect, the disclosure features a human induced pluripotent stem cell (iPSC), wherein the iPSC comprises a genomic edit that results in a loss of function of adenosine A2a receptor (ADORA2A). In some embodiments, the iPSC comprises a genomic

edit that results in a loss of function of an agonist of the TGF beta signaling pathway or a genomic edit that results in loss of function of Cytokine Inducible SH2 Containing Protein (CISH). In some embodiments, the iPSC comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway and a genomic edit that results in loss of function of CISH.

[0009] In some embodiments, the iPSC comprises a genomic edit that results in a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor. In some embodiments, TGF beta receptor is a TGF beta receptor II (TGFβRII).

[0010] In some embodiments, the iPSC expresses one or more pluripotency markers selected from the group consisting of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and Nanog.

[0011] In some embodiments, the disclosure features a differentiated cell, wherein the differentiated cell is a daughter cell of a human iPSC described herein. In some embodiments, the differentiated cell is an immune cell. In some embodiments, the differentiated cell is a lymphocyte. In some embodiments, the differentiated cell is a natural killer cell. In some embodiments, the differentiated daughter cell is an iNK cell. In some embodiments, the cell: (a) does not express endogenous CD3, CD4, and/or CD8; and (b) expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, CD43, and/or CD45, or any combination thereof; (ii) NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 (CD16)); (iii) natural killer group-2 member D (NKG2D); (iv) CD69; (v) a natural cytotoxicity receptor; or any combination of two or more thereof.

[0012] In some embodiments, any of the cells described herein comprises one or more additional genomic edits. In some embodiments, the cell: (1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of

an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) comprises at least one genomic edit that results in a loss of function of at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iii) β-2 microglobulin (B2M); (iv) programmed cell death protein 1 (PD-1); (v) class II, major histocompatibility complex, transactivator (CIITA); (vi) natural killer cell receptor NKG2A (natural killer group 2A); (vii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (viii) cluster of differentiation 32B (CD32B, FCGR2B); (ix) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0013] In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence according to any one of SEQ ID NO: 258-364, 1155, and 1162. In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 258-364, 1155, and 1162. In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, and (ii) a 5' extension sequence depicted in Table 3. In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0014] In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence according to any one

of SEQ ID NO: 29-257, 1157, and 1161. In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 29-257, 1157, and 1161. In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, and (ii) a 5' extension sequence depicted in Table 3. In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0015] In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence according to any one of SEQ ID NO: 827-1143, 1159, and 1163. In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 827-1143, 1159, and 1163. In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, and (ii) a 5' extension sequence depicted in Table 3. In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0016] In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence according to any one of SEQ ID NO: 258-364, 1155, and 1162. In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 258-364, 1155, and 1162. In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, and (ii) a 5' extension sequence depicted in Table 3. In some embodiments, a genomic edit resulting in loss of function of CISH in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0017] In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148), and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence according to any one of SEQ ID NO: 29-257, 1157, and 1161. In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 29-257, 1157, and 1161. In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148), and (ii) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, and (ii) a 5' extension sequence depicted in Table 3. In some embodiments, a genomic edit resulting in loss of function of TGFβRII in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148), and (ii) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0018] In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence according to any one of SEQ ID NO: 827-1143, 1159, and 1163. In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 827-1143, 1159, and 1163. In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, and (ii) a 5' extension sequence depicted in Table 3. In some embodiments, a genomic edit resulting in loss of function of ADORA2A in any of the cells described herein was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0019] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or human induced pluripotent stem cell) with one or more of: an RNA-guided nuclease and a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 258-364, 1155, and 1162; an RNA-guided nuclease and a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 29-257, 1157, and 1161; and/or an RNA-guided nuclease and a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 827-1143, 1159, and 1163.

[0020] In some embodiments, the method comprises contacting the cell with one or more of: (1) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, and (ii) a 5' extension sequence depicted in Table 3; (2) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, and (ii) a 5' extension sequence depicted in Table 3; and (3) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, and (ii) a 5' extension sequence depicted in Table 3.

[0021] In some embodiments, the method comprises contacting the cell with one or more of: (1) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; (2) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; and (3) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159

or 1163, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0022] In some embodiments, the RNA-guided nuclease is a Cas12a variant. In some embodiments, the Cas12a variant comprises one or more amino acid substitutions selected from M537R, F870L, and H800A. In some embodiments, the Cas12a variant comprises amino acid substitutions M537R, F870L, and H800A. In some embodiments, the Cas12a variant comprises an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148.

[0023] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or a human induced pluripotent stem cell) with one or more of: a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 258-364, 1155, and 1162; a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 29-257, 1157, and 1161; and/or a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease (e.g., a Cas12a variant, e.g., a Cas12a variant comprising 1, 2, or 3 of the amino acid substitutions selected from M537R, F870L, and H800A, e.g., a Cas12a variant comprising an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148) and (ii) a guide RNA comprising a targeting domain sequence comprising or consisting of a nucleotide sequence that is identical to, or differs by no more than 1, 2, or 3 nucleotides from, any one of SEQ ID NO: 827-1143, 1159, and 1163.

[0024] In some embodiments, the method comprises contacting the cell with one or more of: (1) an RNP comprising a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, and (ii) a 5' extension sequence depicted in Table 3; (2) an RNP comprising a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, and (ii) a 5' extension sequence depicted in Table 3; and (3) an RNP comprising a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, and (ii) a 5' extension sequence depicted in Table 3.

[0025] In some embodiments, the method comprises contacting the cell with one or more of: (1) an RNP comprising a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; (2) an RNP comprising a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; and (3) an RNP comprising a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence.

[0026] In some embodiments, the RNA-guided nuclease is a Cas12a variant. In some embodiments, the Cas12a variant comprises one or more amino acid substitutions selected from M537R, F870L, and H800A. In some embodiments, the Cas12a variant comprises amino acid substitutions M537R, F870L, and H800A. In some embodiments, the Cas12a variant comprises an amino acid sequence having 90%, 95%, or 100% identity to SEQ ID NO:1148.

[0027] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or a human induced pluripotent stem cell) with (i) a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1155 or 1162; a guide RNA comprises a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161; and a guide RNA comprises a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163; and (ii) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof).

[0028] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or a human induced pluripotent stem cell) with (1) an RNP comprising (i) a guide RNA comprising a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1155 or 1162; and (ii) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof); (2) an RNP comprising (i) a guide RNA comprises a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, and (ii) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof); and (3) an RNP comprising (i) a guide RNA comprises a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, and (ii) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof).

[0029] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or a human induced pluripotent stem cell) with (1) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, and (ii) a 5' extension sequence depicted in Table 3; (2) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, and (ii) a 5' extension sequence depicted in Table 3; (3) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide

sequence of SEQ ID NO: 1159 or 1163, and (ii) a 5' extension sequence depicted in Table 3; and (4) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof).

[0030] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or a human induced pluripotent stem cell) with (1) an RNP comprising (a) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, and (ii) a 5' extension sequence depicted in Table 3; and (b) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof); (2) an RNP comprising (a) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, and (ii) a 5' extension sequence depicted in Table 3; and (b) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof); and (3) an RNP comprising (a) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, and (ii) a 5' extension sequence depicted in Table 3; and (b) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof).

[0031] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or a human induced pluripotent stem cell) with (1) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; (2) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence (3) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide

sequence of SEQ ID NO: 1159 or 1163, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; and (4) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof).

[0032] In another aspect, the disclosure features a method of making a cell, e.g., a cell described herein, the method comprising contacting a cell (e.g., a pluripotent human stem cell or a human induced pluripotent stem cell) with (1) an RNP comprising (a) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO:1155 or 1162, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; and (b) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof); (2) an RNP comprising (a) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1157 or 1161, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; and (b) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof); and (3) an RNP comprising (a) a guide RNA comprising (i) a targeting domain sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1159 or 1163, (ii) a scaffold sequence comprising or consisting of the nucleotide sequence of SEQ ID NO: 1153 located 5' of the targeting domain sequence, and (iii) the nucleotide sequence of SEQ ID NO:1154 at the 5' of the scaffold sequence; and (b) an RNA-guided nuclease comprising an amino acid sequence having 90%, 95%, or 100% identity to one of SEQ ID NO:1144-1151 (or a portion thereof).

[0033] In another aspect, the disclosure features a pluripotent human stem cell, wherein the stem cell comprises a disruption in the transforming growth factor beta (TGF beta) signaling pathway. In some embodiments, the stem cell comprises a genetic modification that results in a loss of function of an agonist of the TGF beta signaling pathway. In some embodiments, the genetic modification is a genomic edit. In some

embodiments, the stem cell comprises a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor. In some embodiments, the TGF beta receptor is a TGF beta receptor II (TGFβRII).

[0034] In some embodiments, the stem cell further comprises a loss of function of an antagonist of interleukin signaling. In some embodiments, the stem cell further comprises a genomic modification that results in the loss of function of an antagonist of interleukin signaling. In some embodiments, the antagonist of interleukin signaling is an antagonist of the IL-15 signaling pathway and/or of the IL-2 signaling pathway.

[0035] In some embodiments, the stem cell comprises a loss of function of Cytokine Inducible SH2 Containing Protein (CISH). In some embodiments, the stem cell comprises a genomic modification that results in the loss of function of CISH.

[0036] In some embodiments, the stem cell expresses one or more pluripotency markers selected from the group consisting of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and Nanog.

[0037] In some embodiments, the stem cell comprises one or more additional genetic modifications. In some embodiments, the stem cell: (1) comprises at least one genetic modification characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) comprises at least one genetic modification that results in a loss of function of at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microglobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) class II, major histocompatibility complex, transactivator (CIITA); (vii) natural killer cell receptor NKG2A (natural killer group 2A); (viii) two or more HLA class II

histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (ix) cluster of differentiation 32B (CD32B, FCGR2B); (x) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0038] In some embodiments, the stem cell comprises a genetic modification in a TGFβRII gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:29-257, 1157, and 1161. In some embodiments, the stem cell comprises a genetic modification in a CISH gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:258-364, 1155, and 1162. In some embodiments, the stem cell comprises a genetic modification in a ADORA2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:827-1143, 1159, and 1163. In some embodiments, the stem cell comprises a genetic modification in a TIGIT gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:631-826. In some embodiments, the stem cell comprises a genetic modification in a B2M gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:365-576. In some embodiments, the stem cell comprises a genetic modification in a NKG2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:577-630.

[0039] In another aspect, the disclosure features a differentiated cell, wherein the differentiated cell is a daughter cell of a pluripotent human stem cell described herein. In some embodiments, the differentiated cell is an immune cell. In some embodiments, the differentiated cell is a lymphocyte. In some embodiments, the differentiated cell is a natural killer cell. In some embodiments, the stem cell is a human induced pluripotent stem cell (iPSC), and wherein the differentiated daughter cell is an induced Natural Killer (iNK) cell.

[0040] In some embodiments, the differentiated cell: (a) does not express endogenous CD3, CD4, and/or CD8; and (b) expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, CD43, and/or CD45, or any combination thereof; (ii) NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 (CD16)); (iii) natural killer group-2 member D (NKG2D); (iv) CD69; (v) a natural cytotoxicity receptor; or any combination of two or more thereof.

[0041] In some embodiments, the differentiated stem cell comprises one or more additional genetic modifications. In some embodiments, the differentiated stem cell: (1) comprises at least one genetic modification characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) comprises at least one genetic modification that results in a loss of function of at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microglobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) class II, major histocompatibility complex, transactivator (CIITA); (vii) natural killer cell receptor NKG2A (natural killer group 2A); (viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (ix) cluster of differentiation 32B (CD32B, FCGR2B); (x) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0042] In some embodiments, the differentiated stem cell comprises a genetic modification in a TGFβRII gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:29-257, 1157, and 1161. In some embodiments, the differentiated stem cell comprises a genetic modification in a CISH gene made using an

RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:258-364, 1155, and 1162. In some embodiments, the differentiated stem cell comprises a genetic modification in a ADORA2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:827-1143, 1159, and 1163. In some embodiments, the differentiated stem cell comprises a genetic modification in a TIGIT gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:631-826. In some embodiments, the differentiated stem cell comprises a genetic modification in a B2M gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:365-576. In some embodiments, the differentiated stem cell comprises a genetic modification in a NKG2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:577-630.

[0043] In another aspect, the disclosure features a method of culturing a pluripotent human stem cell, comprising culturing the stem cell in a medium comprising activin. In some embodiments, the pluripotent human stem cell is an embryonic stem cell or an induced pluripotent stem cell. In some embodiments, the pluripotent human stem cell does not express TGFβRII. In some embodiments, the pluripotent human stem cell is genetically engineered not to express TGFβRII. In some embodiments, the pluripotent human stem cell is genetically engineered to knock out a gene encoding TGFβRII.

[0044] In some embodiments, the activin is activin A. In some embodiments, the medium does not comprise TGFβ.

[0045] In some embodiments, the culturing is performed for a defined period of time (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 days, or more). In some embodiments, at one or more times during or following the culturing step, the pluripotent human stem cell maintains pluripotency (e.g., exhibits one or more pluripotency markers). In some embodiments, at one or more times during or following the culturing step, the pluripotent human stem cell expresses a detectable level of one or more of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81,

TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and Nanog. In some embodiments, at a time during or following the culturing step, the pluripotent human stem cell is differentiated into cells of endoderm, mesoderm, and/or ectoderm lineage. In some embodiments, the pluripotent human stem cell, or its progeny, is further differentiated into a natural killer (NK) cell.

[0046] In some embodiments, the pluripotent human stem cell is differentiated into an NK cell in a medium comprising human serum. In some embodiments, the medium comprises NKMACS + human serum (e.g., 5%, 10%, 15%, 20% or more human serum). In some embodiments, the NK cells exhibit improved cellular expansion, increased NK maturity (as exhibited by increased marker expression (e.g., CD45, CD56, CD16, and/or KIR)), and/or increased cytotoxicity, relative to an NK cell differentiated in a media without serum.

[0047] In some embodiments, the pluripotent human stem cell (1) comprises at least one genetic modification characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) comprises at least one genetic modification that results in a loss of function of at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microglobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) class II, major histocompatibility complex, transactivator (CIITA); (vii) natural killer cell receptor NKG2A (natural killer group 2A); (viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (ix) cluster of differentiation 32B (CD32B, FCGR2B); (x) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0048] In some embodiments, the pluripotent human stem cell comprises a genetic modification in a TGFβRII gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:29-257, 1157, and 1161. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a CISH gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:258-364, 1155, and 1162. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a ADORA2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:827-1143, 1159, and 1163. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a TIGIT gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:631-826. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a B2M gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:365-576. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a NKG2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:577-630.

[0049] In some embodiments, the method further comprises (1) genetically modifying the pluripotent human stem cell such that the pluripotent human stem cell expresses a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a non-naturally occurring variant of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) genetically modifying the pluripotent human stem cell to lose function of

at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microglobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) class II, major histocompatibility complex, transactivator (CIITA); (vii) natural killer cell receptor NKG2A (natural killer group 2A); (viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (ix) cluster of differentiation 32B (CD32B, FCGR2B); (x) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0050] In some embodiments, the method further comprises genetically modifying a TGFβRII gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:29-257, 1157, and 1161. In some embodiments, the method further comprises genetically modifying a CISH gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:258-364, 1155, and 11162. In some embodiments, the method further comprises genetically modifying a ADORA2A gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:827-1143, 1159, and 1163. In some embodiments, the method further comprises genetically modifying a TIGIT gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:631-826. In some embodiments, the method further comprises genetically modifying a B2M gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:365-576. In some embodiments, the method further comprises genetically modifying a NKG2A gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:577-630.

[0051] In another aspect, the disclosure features a cell culture comprising (i) a pluripotent human stem cell and (ii) a cell culture medium comprising activin, wherein the pluripotent human stem cell comprises a disruption in the transforming growth factor beta

(TGF beta) signaling pathway. In some embodiments, the stem cell comprises a genetic modification that results in a loss of function of an agonist of the TGF beta signaling pathway. In some embodiments, the genetic modification is a genomic edit. In some embodiments, the stem cell comprises a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor. In some embodiments, the TGF beta receptor is a TGF beta receptor II (TGFβRII).

[0052] In some embodiments, the pluripotent human stem cell: (1) comprises at least one genetic modification characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) comprises at least one genetic modification that results in a loss of function of at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microglobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) class II, major histocompatibility complex, transactivator (CIITA); (vii) natural killer cell receptor NKG2A (natural killer group 2A); (viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (ix) cluster of differentiation 32B (CD32B, FCGR2B); (x) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0053] In some embodiments, the pluripotent human stem cell comprises a genetic modification in a TGFβRII gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:29-257, 1157, and 1161. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a CISH gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that

is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:258-364, 1155, and 1162. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a ADORA2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:827-1143, 1159, and 1163. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a TIGIT gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:631-826. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a B2M gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:365-576. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a NKG2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:577-630.

[0054] In another aspect, the method comprises a method of increasing a level of iNK cell activity comprising: (i) providing a pluripotent human stem cell comprising a disruption in the transforming growth factor beta (TGF beta) signaling pathway; and (ii) differentiating the pluripotent human stem cell into an iNK cell, wherein the iNK cell has a higher level of cell activity as compared to an iNK cell not comprising a disruption of the TGF beta signaling pathway.

[0055] In some embodiments, the iNK is differentiated from a pluripotent human stem cell cultured in a medium comprising activin. In some embodiments, the method further comprises culturing the pluripotent human stem cell in a medium comprising activin before and/or during the differentiating step.

[0056] In some embodiments, the pluripotent human stem cell is differentiated into an NK cell in a medium comprising human serum. In some embodiments, the medium comprises NKMACS + human serum (e.g., 5%, 10%, 15%, 20% or more human serum). In some embodiments, the NK cells exhibit improved cellular expansion, increased NK maturity

(as exhibited by increased marker expression (e.g., CD45, CD56, CD16, and/or KIR)), and/or increased cytotoxicity, relative to an NK cell differentiated in a media without serum.

[0057] In some embodiments, the method further comprises disrupting the transforming growth factor beta (TGF beta) signaling pathway in the pluripotent human stem cell. In some embodiments, the stem cell comprises a genetic modification that results in a loss of function of an agonist of the TGF beta signaling pathway. In some embodiments, the genetic modification is a genomic edit. In some embodiments, the stem cell comprises a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor. In some embodiments, the TGF beta receptor is a TGF beta receptor II (TGFβRII).

[0058] In some embodiments, the pluripotent human stem cell: (1) comprises at least one genetic modification characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) comprises at least one genetic modification that results in a loss of function of at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microglobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) class II, major histocompatibility complex, transactivator (CIITA); (vii) natural killer cell receptor NKG2A (natural killer group 2A); (viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (ix) cluster of differentiation 32B (CD32B, FCGR2B); (x) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0059] In some embodiments, the pluripotent human stem cell comprises a genetic modification in a TGFβRII gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3

nucleotides from, any one of SEQ ID NOs:29-257, 1157, and 1161. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a CISH gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:258-364, 1155, and 1162. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a ADORA2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:827-1143, 1159, and 1163. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a TIGIT gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:631-826. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a B2M gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:365-576. In some embodiments, the pluripotent human stem cell comprises a genetic modification in a NKG2A gene made using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:577-630.

[0060] In some embodiments, the method further comprises (1) genetically modifying the pluripotent human stem cell such that the pluripotent human stem cell expresses a nucleic acid sequence encoding: (i) a chimeric antigen receptor (CAR); (ii) a FcγRIII (CD16) or a variant (e.g., non-naturally occurring variant) of FcγRIII (CD16); (iii) interleukin 15 (IL-15); (iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; (v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) genetically modifying the pluripotent human stem cell to lose function of at least one of: (i) cytokine inducible SH2 containing protein (CISH); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microglobulin (B2M); (v) programmed cell death protein 1

(PD-1); (vi) class II, major histocompatibility complex, transactivator (CIITA); (vii) natural killer cell receptor NKG2A (natural killer group 2A); (viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (ix) cluster of differentiation 32B (CD32B, FCGR2B); (x) T cell receptor alpha constant (TRAC); or any combination of two or more thereof.

[0061] In some embodiments, the method further comprises genetically modifying a TGFβRII gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:29-257, 1157, and 1161. In some embodiments, the method further comprises genetically modifying a CISH gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:258-364, 1155, and 1162. In some embodiments, the method further comprises genetically modifying a ADORA2A gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:827-1143, 1159, and 1163. In some embodiments, the method further comprises genetically modifying a TIGIT gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:631-826. In some embodiments, the method further comprises genetically modifying a B2M gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:365-576. In some embodiments, the method further comprises genetically modifying a NKG2A gene using an RNA-guided nuclease and a gRNA molecule comprising a targeting domain sequence that is the same as, or differs by no more than 3 nucleotides from, any one of SEQ ID NOs:577-630.

[0062] In another aspect, the disclosure features a method of culturing a stem cell, for example, a human stem cell, such as, e.g., a human embryonic stem cell, a human induced pluripotent stem cell, or a human pluripotent stem cell, comprising culturing the stem cell in a medium that comprises activin, e.g., activin A. In some embodiments, the stem cell is an embryonic stem cell or an induced pluripotent stem cell. In some embodiments, the stem cell

comprises a modification, e.g., a genetic modification, that disrupts a TGF (transforming growth factor) signaling pathway in the stem cell. In some embodiments, the genetic modification is a modification that disrupts (e.g., reduces or abolishes) TGF beta signaling in the stem cell. For example, in some embodiments, the modification is a modification of a gene encoding a protein of the TGF beta signaling pathway, such as a TGF beta receptor. In some embodiments, the modification results in a loss of function and/or a loss of expression of the protein of the TGF beta signaling pathway. In some embodiments, the modification results in a knockout of the protein of the TGF beta signaling pathway. In some embodiments, the stem cell does not express a functional TGFβ receptor protein, e.g., the stem cell does not express a TGFβRII protein or does not express a functional TGFβRII protein. In some embodiments, the stem cell expresses a dominant negative variant of an agonist of a protein of the TGF beta signaling pathway, e.g., a dominant negative variant of TGFβRII. In some embodiments, the stem cell over-expresses an antagonist of the TGF beta signaling pathway. In some embodiments, the stem cell does not express TGFβRII. In some embodiments, the stem cell is genetically engineered not to express TGFβRII. In some embodiments, the stem cell is genetically engineered to knock out a gene encoding TGFβRII. In some embodiments, the genetic modification is a modification that enhances (e.g., maintains or increases) IL-15 signaling in the stem cell. For example, in some embodiments, the modification is a modification of a gene encoding a protein that acts on the IL-15 signaling pathway, such as Cytokine Inducible SH2 Containing Protein (CISH), a negative regulator of IL-15 signaling. In some embodiments, the modification results in a loss of function and/or a loss of expression of the protein that acts on the IL-15 signaling pathway. In some embodiments, the modification results in a knockout of the protein that acts on the IL-15 signaling. In some embodiments, the stem cell does not express a functional CISH gene, e.g., the stem cell does not express a CISH protein or does not express a functional CISH protein. In some embodiments, the stem cell does not express CISH. In some embodiments, the stem cell is genetically engineered not to express CISH. In some embodiments, the stem cell is genetically engineered to knock out a gene encoding CISH (i.e., CISH, cytokine-inducible SH2-containing protein). In some embodiments, the stem cell does not express TGFβRII or CISH. In some embodiments, the stem cell is genetically engineered not to express each of TGFβRII or CISH. In some embodiments, the stem cell is genetically engineered to knock out a gene encoding TGFβRII and a gene encoding CISH in

the same cell (double KO). In some embodiments, the stem cell has been edited, e.g., via CRISPR/Cas editing or other suitable technology, to disrupt a gene encoding a gene product involved in TGF signaling, e.g., in TGF beta signaling, such as, for example, a gene encoding a TGF beta RII protein, or e.g., IL-15 signaling, such as, for example, a gene encoding a CIS protein, within the genome of the cell. In some embodiments, e.g., in embodiments, where two copies or alleles of the gene encoding a gene product involved in TGF signaling and/or IL-15 signaling is present in the cell, the cell is modified (e.g., edited), so that both copies or alleles are modified, e.g., in that expression of the gene, or of a functional gene product encoded by the gene, is disrupted, decreased, or abolished from both alleles.

[0063] In some embodiments, the activin is activin A. In some embodiments, the medium does not comprise TGFβ.

[0064] In some embodiments, the culturing is performed for a defined period of time (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 days, or more). In some embodiments, at one or more times during or following the culturing step, the human stem cell maintains pluripotency (e.g., exhibits one or more measure of pluripotency). In some embodiments, at one or more times during or following the culturing step, the human stem cell expresses a detectable level of one or more of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and Nanog. In some embodiments, at a time during or following the culturing step, the human stem cell retains the capacity to differentiate into cells of endoderm, mesoderm, and ectoderm germ layers.

[0065] In another aspect, the disclosure features a cell culture comprising (i) an embryonic stem cell or an induced pluripotent stem cell and (ii) a cell culture medium comprising activin, wherein the embryonic stem cell or an induced pluripotent stem cell is genetically engineered not to express TGFβRII and/or CISH.

[0066] In some embodiments, an RNA-guided nuclease is a Cas12a variant. In some embodiments, the Cas12a variant comprises amino acid substitutions selected from M537R, F870L, and H800A. In some embodiments, the Cas12a variant comprises amino acid substitutions M537R, F870L, and H800A. In some embodiments, the Cas12a variant comprises an amino acid sequence according to SEQ ID NO: 1148.

BRIEF DESCRIPTION OF THE DRAWING

[0067] The present teachings described herein will be more fully understood from the following description of various illustrative embodiments, when read together with the accompanying drawings. It should be understood that the drawings described below are for illustration purposes only and are not intended to limit the scope of the present teachings in any way.

[0068] FIG. 1 shows microscopy of cell morphology and flow cytometry of pluripotency markers of human induced pluripotent stem cells (hiPSCs) grown in various media in the absence or presence of Activin A (1 ng/ml or 4 ng/ml ActA).

[0069] FIG. 2 shows morphology of TGFβRII knockout hiPSCs (clone 7) or CISH/TGFβRII DKO hiPSCs (clone 7) cultured in media with or without Activin A (1 ng/mL, 2 ng/mL, 4 ng/mL, or 10 ng/mL).

[0070] FIG. 3 shows morphology of TGFβRII knockout hiPSCs (clone 9) cultured in media with our without Activin A (1 ng/mL, 2 ng/mL, 4 ng/mL, or 10 ng/mL).

[0071] FIG. 4A shows the bulk editing rates at the CISH and TGFβRII loci for single knockout and double knockout hiPSCs.

[0072] FIG. 4B shows expression of Oct4 and SSEA4 in TGFβRII knockout hiPSCs, CISH knockout hiPSCs, and double knockout hiPSCs cultured in Activin A.

[0073] FIG. 5 shows expression of Nanog and Tra-1-60 in TGFβRII knockout hiPSCs, CISH knockout hiPSCs, and double knockout hiPSCs cultured in Activin A.

[0074] FIG. 6 is a schematic of the procedure related to the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies Inc.).

[0075] FIG. 7A shows expression of differentiation markers of TGFβRII knockout hiPSCs, CISH knockout hiPSCs, and double knockout hiPSCs cultured in Activin A.

[0076] FIG. 7B shows karyotypes of TGFβRII / CISH double knockout hiPSCs cultured in Activin A.

[0077] FIG. 7C shows an expanded Activin A concentration curve performed on an unedited parental PSC line, an edited TGFβRII KO clone (C7), and an additional representative (unedited) cell line designated RUCDR. The minimum concentration of Activin A required to maintain each line varied slightly with the TGFβRII KO clone requiring a higher baseline amount of Activin A as compared to the parental control (0.5 ng/ml vs 0.1 ng/ml).

[0078] Figure 7D shows the stemness marker expression in an unedited parental PSC line, an edited TGFβRII KO clone (C7), and an unedited RUCDR cell line, when cultured with the base medias alone (no supplemental Activin A). The TGFβRII KO iPSCs did not maintain stemness marker expression while the two unedited lines were able to maintain stemness marker expression in E8.

[0079] FIG. 8A is a schematic representation of an exemplary method for creating edited iPSC clones, followed by the differentiation to and characterization of enhanced CD56+ iNK cells.

[0080] FIG. 8B is a schematic of an iNK cell differentiation process utilizing STEMDiff APEL2 during the second stage of the differentiation process.

[0081] FIG. 8C is a schematic of an iNK cell differentiation process utilizing NK-MACS with 15% serum during the second stage of the differentiation process.

[0082] FIG. 8D shows the fold-expansion of unedited PCS-derived iNK cells and the percentage of iNK cells expressing CD45 and CD56 at day 39 of differentiation when differentiated using NK-MACS or Apel2 methods as depicted in FIG 8C and FIG. 8B respectively.

[0083] FIG. 8E shows in the upper panel a heat map of the surface expression phenotypes (measured as a percentage of the population) of differentiated iNK cells derived from unedited PCS iPSCs when differentiated using NK-MACS or APEL2 methods as depicted in FIG 8C and FIG. 8B respectively. The bottom panel displays representative histogram plots to illustrate the differences in the iNKs generated by the two methods.

[0084] FIG. 8F shows a heat map of the surface expression phenotypes (measured as a percentage of the population) of differentiated edited iNKs (TGFβRII knockout, CISH knockout, and double knockout (DKO)) and unedited parental iPSCs (WT) when differentiated using NK-MACS or APEL2 methods as depicted in FIG 8C and FIG. 8B respectively.

[0085] FIG. 8G shows unedited iNK cell effector function when differentiated using NK-MACS or APEL2 methods as depicted in FIG 8C and FIG. 8B respectively.

[0086] FIG. 9 shows differentiation phenotypes of edited clones (TGFβRII knockout, CISH knockout, and double knockout) as compared to parental wild type clones.

[0087] FIG. 10 shows surface expression phenotype of edited iNKs (TGFβRII knockout, CISH knockout, and double knockout) as compared to parental clone iNKs and wild type cells.

[0088] FIG. 11A shows surface expression phenotype of edited iNKs (TGFβRII knockout, CISH knockout, and double knockout) as compared to parental clone iNKs ("WT") and peripheral blood-derived natural killer cells.

[0089] FIG. 11B is a flow cytometry histogram plot that shows the surface expression phenotype of edited iNK cells (TGFβRII/CISH double knockout) as compared to parental clone iNK cells ("unedited iNK cells").

[0090] FIG. 11C shows surface expression phenotypes (measured as a percentage of the population) of edited iNK cells (TGFβRII/CISH double knockout) as compared to parental clone iNK cells ("unedited iNK cells") at day 25, day 32, and day 39 post-hiPSC differentiation (average values from at least 5 separate differentiations).

[0091] FIG. 11D shows pSTAT3 expression phenotypes (measured as a percentage of the population) of edited CD56+ iNK cells ("CISH KO iNKs") as compared to parental clone CD56+ iNK cells ("unedited iNKs") at 10 minutes and 120 minutes following IL-15 induced activation. Briefly, the day 39 or day 40 iNKs are plated the day before in a cytokine starve condition. The next day the cells are stimulated with 10 ng/ml of IL15 for the length of time indicated. The cells are fixed immediately at the end of the time point, stained for CD56

followed by an intracellular stain. The cells were processed on a NovoCyte Quanteon and the data was analyzed in FlowJo. Data shown is a representative experiment of >3 experiments performed.

[0092] FIG. 11E shows pSMAD2/3 expression phenotypes (measured as a percentage of the population) of edited CD56+ iNK cells (TGFβRII/CISH double knockout, "DKO iNKs") as compared to parental clone CD56+ iNK cells ("unedited iNK cells") at 10 minutes and 120 minutes following IL-15 and TGF-β induced activation Briefly, the day 39 or day 40 iNKs were plated the day before in a cytokine starve condition. The next day the cells were stimulated with 10 ng/ml of IL-15 and 50 ng/ml of TGF-β for the length of time indicated. The cells were fixed immediately at the end of the time point, stained for CD56 followed by an intracellular stain. The cells were processed on a NovoCyte Quanteon and the data was analyzed in FlowJo. Data shown is a representative experiment of >3 experiments performed.

[0093] FIG. 11F shows IFN-γ expression phenotypes (measured as a percentage of the population) of edited CD56+ iNK cells (TGFβRII/CISH double knockout, "DKO IFNg") as compared to parental clone CD56+ iNK cells (unedited iNKs, "WT IFNg") with or without phorbol myristate acetate (PMA) and ionomycin (IMN) stimulation. The data is representative. It is generated from a single differentiation and each condition in the assay is run with 2 technical replicates. **$p<0.05$ vs unedited iNK cells (paired t test).

[0094] FIG. 11G shows TNF-α expression phenotypes (measured as a percentage of the population) of edited CD56+ iNK cells (TGFβRII/CISH double knockout, "DKO TNF a") as compared to parental clone CD56+ iNK cells (unedited iNK cells, "WT TNFa") with or without Phorbol myristate acetate (PMA) and Ionomycin (IMN) stimulation. The data is representative. It is generated from a single differentiation and each condition in the assay is run with 2 technical replicates. **$p<0.05$ vs unedited iNK cells (paired t test).

[0095] FIG. 12A is a schematic representation of an exemplary solid tumor cell killing assay, depicting the use of edited iNK cells (TGFβRII/CISH double knockout) to kill SK-OV-3 ovarian cells in the presence or absence of IL-15 and TGF-β.

[0096] FIG. 12B shows the results of a solid tumor killing assay as described in FIG 12A. iNK cells function to reduce tumor cell spheroid size. Certain edited iNK cells (CISH

single knockout, "CISH_2, 4, 5, and 8") were not significantly different from the parental clone iNK cells ("WT_2"), while certain edited iNK cells (TGFβRII single knockout, "TGFβRII_7", and TGFβRII/CISH double knockout "DKO") functioned significantly better at effector-target (E:T) ratios of 1 or greater when measured in the presence of TGF- β as compared to parental clone iNK cells ("WT_2"). ****$p<0.0001$ vs unedited iNK cells (two-way ANOVA, Sidak's multiple comparisons test).

[0097] FIG. 12C shows edited iNK cell effector function as compared to unedited iNK cells.

[0098] FIG. 13 shows the results of an *in-vitro* serial killing assay, where iNK cells are serially challenged with hematological cancer cells (e.g., Nalm6 cells) in the presence of 10 ng/ml of IL-15 and 10 ng/ml of TGF-β; the X axis represents time, with tumor cells being added every 48hours, while the Y axis represents killing efficacy as measured by normalized total red object area (e.g., presence of tumor cells). The data shows that edited iNK cells (TGFβRII/CISH double knockout) continue to kill hematological cancer cells while unedited iNK cells lose this function at equivalent time points.

[0099] FIG. 14 shows surface expression phenotypes (measured as a percentage of the population) of certain edited iNK clonal cells (CISH single knockout "CISH_C2, C4, C5, and C8", TGFβRII single knockout "TGFβRII-C7", and TGFβRII/CISH double knockout "DKO-C1") as compared to parental clone iNK cells ("WT") at day 25, day 32, and day 39 post-hiPSC differentiation when cultured in the presence of 1 ng/mL or 10 ng/mL IL-15.

[0100] FIG. 15A is a schematic of an *in-vivo* tumor killing assay. Mice were intraperitoneally inoculated with 1 x 10^6 SKOV3-luc cells, mice are randomized, and 4 days later, 20 x 10^6 iNK cells were introduced intraperitoneally. Mice were followed for up to 60 days post-tumor implantation. The X axis represents time since implantation, while the Y axis represents killing efficacy as measured by total bioluminescence (p/s).

[0101] FIG. 15B shows the results of an *in-vivo* tumor killing assay as described in FIG. 15A. An individual mouse is represented by each horizontal line. The data show that both unedited iNK cells ("unedited iNK") and DKO edited iNK cells (TGFβRII/CISH double knockout) prevent tumor growth better than vehicle, while edited iNK cells kill tumor cells

significantly better than vehicle *in-vivo*. Each experimental group had 9 animals each. ***p< 0.001, ****p<0.0001 by a 2-way ANOVA analysis.

[0102] FIG. 15C shows the averaged results with standard error of the mean of the *in-vivo* tumor killing assay described in FIG 15B. Populations of mice are represented by each horizontal line. The data show that DKO edited iNK cells (TGFβRII/CISH double knockout) prevent tumor growth and kill tumor cells significantly better than vehicle or unedited iNK cells *in-vivo*. ***p<0.001, ****p<0.0001 by a 2-way ANOVA analysis.

[0103] FIG. 16A shows surface expression phenotypes (measured as a percentage of the population) of bulk edited iNK cells (left panel - ADORA2A single knockout) or certain edited iNK clonal cells (right panel - ADORA2A single knockout) as compared to parental clone iNK cells ("PCS_WT") at day 25, day 32, and day 39 or at day 28, day 36, and day 39 post-hiPSC differentiation. Representative data from multiple differentiations.

[0104] FIG. 16B shows cyclic AMP (cAMP) concentration phenotypes following 5′-(N-Ethylcarboxamido)adenosine ("NECA", adenosine agonist) activation for edited iNK clonal cells (ADORA2A single knockout) as compared to parental clone iNK cells ("unedited iNKs"). The Y axis represents average cAMP concentration in nM (a proxy for ADORA2A activation), while the X axis represents NECA concentration in nM.

[0105] FIG. 16C shows the results of an *in-vitro* serial killing assay, where iNK cells are serially challenged with hematological cancer cells (e.g., Nalm6 cells) in the presence of 100μM NECA, and 10 ng/ml of IL-15; the X axis represents time, with tumor cells being added every 48hours, while the Y axis represents killing efficacy as measured by total red object area (e.g., presence of tumor cells). The data shows that edited iNK cells ("ADORA2A KO iNK") kill hematological cancer cells more effectively than unedited iNK cells ("Ctrl iNK") under conditions that mimic adenosine suppression.

[0106] FIG. 17A shows surface expression phenotypes (measured as a percentage of the population) of certain edited iNK clonal cells (TGFβRII/CISH/ADORA2A triple knockout, "CRA_6" and "CR+A_8") as compared to parental clone iNK cells ("WT_2") at day 25, day 32, and day 39 post-hiPSC differentiation. Data is representative of multiple differentiations.

[0107] FIG. 17B shows cyclic AMP (cAMP) concentration phenotypes following NECA (adenosine agonist) activation for edited iNK clonal cells (TGFβRII/CISH/ADORA2A triple knockout, "TKO iNKs") as compared to parental clone iNK cells ("unedited iNKs"). The Y axis represents average cAMP concentration in nM (a proxy for ADORA2A activation), while the X axis represents NECA concentration in nM.

[0108] FIG. 17C shows the results of a solid tumor killing assay as described in FIG 12A without IL-15. iNK cells function to reduce tumor cell spheroid size. The Y axis measures total integrated red object (e.g., presence of tumor cells), while the X axis represents the effector to target (E:T) cell ratio. The edited iNK cells (ADORA2A single knockout "ADORA2A", TGFβRII/CISH double knockout "DKO", or TGFβRII/CISH/ADORA2A triple knockout "TKO") had lower EC50 rates when measured in the presence of TGF- β as compared to parental clone iNK cells ("Control") (average values from at least 3 separate differentiations).

[0109] FIG. 18 shows the results of guide RNA selection assays for the loci TGFβRII, CISH, ADORA2A, TIGIT, and NKG2A utilizing in-vitro editing in iPSCs.

DETAILED DESCRIPTION

[0110] Some aspects of the disclosure are based, at least in part, on the recognition that, surprisingly, stem cells, e.g., embryonic stem cells or induced pluripotent stem cells, can be cultured in a culture medium that includes activin A, and that the presence of activin in the culture media abrogates a requirement for the presence of a TGF signaling agonist, e.g., of TGF beta, in the culture medium. Some aspects of the present disclosure relate to the recognition that, surprisingly, stem cells, including human stem cells, such as, for example, human embryonic stem cells or human induced pluripotent stem cells, retain their pluripotency when cultured in media comprising activin, e.g., activin A, even in the absence of a TGF beta signaling agonist, such as, for example, TGF beta, in the culture medium. Additionally, the disclosure is based, in part, on the recognition that, surprisingly, iPSCs lacking TGFβIIR (e.g., genetically knocked out, for example, via gene editing) can be cultured in a culture medium that includes activin, and that such cells not only grow but maintain their pluripotency. The present disclosure additionally encompasses cell cultures

comprising embryonic stem cells and a culture medium comprising activin, as well as methods of culturing such stem cells and/or progeny thereof.

Definitions and Abbreviations

[0111] Unless otherwise specified, each of the following terms have the meaning set forth in this section.

[0112] The indefinite articles "a" and "an" refer to at least one of the associated noun, and are used interchangeably with the terms "at least one" and "one or more." The conjunctions "or" and "and/or" are used interchangeably as non-exclusive disjunctions.

[0113] The term "cancer" (also used interchangeably with the terms, "hyperproliferative" and "neoplastic"), as used herein, refers to cells having the capacity for autonomous growth, *i.e.*, an abnormal state or condition characterized by rapidly proliferating cell growth. Cancerous disease states may be categorized as pathologic, *i.e.*, characterizing or constituting a disease state, *e.g.*, malignant tumor growth, or may be categorized as non-pathologic, *i.e.*, a deviation from normal but not associated with a disease state, *e.g.*, cell proliferation associated with wound repair. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. In some embodiments, "cancer" includes malignancies of or affecting various organ systems, such as lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract. In some embodiments, "cancer" includes adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and/or cancer of the esophagus.

[0114] As used herein, the term "carcinoma" is refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. The term carcinoma, as used herein, is well-recognized in the art. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. In some embodiments, carcinoma also includes carcinosarcomas, *e.g.*, which include malignant tumors composed of carcinomatous and sarcomatous tissues. In some embodiments, an

“adenocarcinoma” is a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures. In some embodiments, a “sarcoma” is art recognized and refers to malignant tumors of mesenchymal derivation.

[0115] The term “differentiation” as used herein is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a blood cell or a muscle cell. In some embodiments, a differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. For example, an iPSC can be differentiated into various more differentiated cell types, for example, a neural or a hematopoietic stem cell, a lymphocyte, a cardiomyocyte, and other cell types, upon treatment with suitable differentiation factors in the cell culture medium. In some embodiments, suitable methods, differentiation factors, and cell culture media for the differentiation of pluri- and multipotent cell types into more differentiated cell types are well known to those of skill in the art. In some embodiments, the term "committed", is applied to the process of differentiation to refer to a cell that has proceeded through a differentiation pathway to a point where, under normal circumstances, it would or will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type (other than a specific cell type or subset of cell types) nor revert to a less differentiated cell type.

[0116] The terms “differentiation marker,” "differentiation marker gene," or "differentiation gene," as used herein refers to genes or proteins whose expression are indicative of cell differentiation occurring within a cell, such as a pluripotent cell. In some embodiments, differentiation marker genes include, but are not limited to, the following genes: CD34, CD4, CD8, CD3, CD56 (NCAM), CD49, CD45; NK cell receptor (cluster of differentiation 16 (CD16)), natural killer group-2 member D (NKG2D), CD69, NKp30, NKp44, NKp46, CD158b, FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GAT A3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1, BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene

(Brachyury), ZIC1, GATA1, GATA2, HDAC4, HDAC5, HDAC7, HDAC9, NOTCH1, NOTCH2, NOTCH4, PAX5, RBPJ, RUNX1, STAT1 and STAT3.

[0117] The terms "differentiation marker gene profile," or "differentiation gene profile," "differentiation gene expression profile," "differentiation gene expression signature," "differentiation gene expression panel," "differentiation gene panel," or "differentiation gene signature" as used herein refer to expression or levels of expression of a plurality of differentiation marker genes.

[0118] The term "edited iNK cell" as used herein refers to a natural killer cell which has been modified to change at least one expression product of at least one gene at some point in the development of the cell. In some embodiments, a modification can be introduced using, e.g., gene editing techniques such as CRISPR-Cas or, e.g., dominant-negative constructs. In some embodiments, an iNK cell is edited at a time point before it has differentiated into an iNK cell, e.g., at a precursor stage, at a stem cell stage, etc. In some embodiments, an edited iNK cell is compared to a non-edited iNK cell (an NK cell produced by differentiating an iPSC cell, which iPSC cell and/or iNK cell do not have modifications, e.g., genetic modifications).

[0119] The term "embryonic stem cell" as used herein refers to pluripotent stem cells derived from the inner cell mass of the embryonic blastocyst. In some embodiments, embryonic stem cells are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In some such embodiments, embryonic stem cells do not contribute to the extra-embryonic membranes or the placenta, *i.e.*, are not totipotent.

[0120] The term "endogenous," as used herein in the context of nucleic acids (*e.g.*, genes, protein-encoding genomic regions, promoters), refers to a native nucleic acid or protein in its natural location, *e.g.*, within the genome of a cell.

[0121] The term "exogenous," as used herein in the context of nucleic acids, *e.g.*, expression constructs, cDNAs, indels, and nucleic acid vectors, refers to nucleic acids that have artificially been introduced into the genome of a cell using, for example, gene-editing or genetic engineering techniques, *e.g.,* CRISPR-based editing techniques.

[0122] The term “genome editing system” refers to any system having RNA-guided DNA editing activity.

[0123] The terms “guide RNA” and “gRNA” refer to any nucleic acid that promotes the specific association (or “targeting”) of an RNA-guided nuclease such as a Cas9 or a Cpf1 (Cas12a) to a target sequence such as a genomic or episomal sequence in a cell.

[0124] The terms "hematopoietic stem cell," or "definitive hematopoietic stem cell" as used herein, refer to CD34-positive stem cells. In some embodiments, CD34-positive stem cells are capable of giving rise to mature myeloid and/or lymphoid cell types. In some embodiments, the myeloid and/or lymphoid cell types include, for example, T cells, natural killer cells and/or B cells.

[0125] The terms "induced pluripotent stem cell" or “iPSC” as used herein to refer to a stem cell obtained from a differentiated somatic (*e.g.*, adult, neonatal, or fetal) cell by a process referred to as reprogramming (e.g., dedifferentiation). In some embodiments, reprogrammed cells are capable of differentiating into tissues of all three germ or dermal layers: mesoderm, endoderm, and ectoderm. iPSCs are not found in nature.

[0126] The term "multipotent stem cell" as used herein refers to a cell that has the developmental potential to differentiate into cells of one or more germ layers (ectoderm, mesoderm and endoderm), but not all three germ layers. Thus, in some embodiments, a multipotent cell may also be termed a "partially differentiated cell." Multipotent cells are well-known in the art, and examples of multipotent cells include adult stem cells, such as for example, hematopoietic stem cells and neural stem cells. In some embodiments, "multipotent" indicates that a cell may form many types of cells in a given lineage, but not cells of other lineages. For example, a multipotent hematopoietic cell can form the many different types of blood cells (red, white, platelets, *etc.*), but it cannot form neurons. Accordingly, in some embodiments, "multipotency" refers to a state of a cell with a degree of developmental potential that is less than totipotent and pluripotent.

[0127] The term "pluripotent" as used herein refers to ability of a cell to form all lineages of the body or soma (*i.e.,* the embryo proper) or a given organism (e.g., human). For example, embryonic stem cells are a type of pluripotent stem cells that are able to form cells

from each of the three germs layers, the ectoderm, the mesoderm, and the endoderm. Generally, pluripotency may be described as a continuum of developmental potencies ranging from an incompletely or partially pluripotent cell (*e.g.*, an epiblast stem cell or EpiSC), which is unable to give rise to a complete organism to the more primitive, more pluripotent cell, which is able to give rise to a complete organism (*e.g.*, an embryonic stem cell or an induced pluripotent stem cell).

[0128] The term "pluripotency" as used herein refers to a cell that has the developmental potential to differentiate into cells of all three germ layers (Ectoderm, mesoderm, and endoderm). In some embodiments, pluripotency can be determined, in part, by assessing pluripotency characteristics of the cells. In some embodiments, pluripotency characteristics include, but are not limited to: (i) pluripotent stem cell morphology; (ii) the potential for unlimited self-renewal; (iii) expression of pluripotent stem cell markers including, but not limited to SSEA1 (mouse only), SSEA3/4, SSEA5, TRA1- 60/81, TRAl-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD90, CD105, OCT4, NANOG, SOX2, CD30 and/or CD50; (iv) ability to differentiate to all three somatic lineages (ectoderm, mesoderm and endoderm); (v) teratoma formation consisting of the three somatic lineages; and (vi) formation of embryoid bodies consisting of cells from the three somatic lineages.

[0129] The term "pluripotent stem cell morphology" as used herein refers to the classical morphological features of an embryonic stem cell. In some embodiments, normal embryonic stem cell morphology is characterized as small and round in shape, with a high nucleus-to-cytoplasm ratio, the notable presence of nucleoli, and typical intercell spacing.

[0130] The term "polynucleotide" (including, but not limited to "nucleotide sequence", "nucleic acid", "nucleic acid molecule", "nucleic acid sequence", and "oligonucleotide") as used herein refer to a series of nucleotide bases (also called "nucleotides") in DNA and RNA, and mean any chain of two or more nucleotides. In some embodiments, polynucleotides, nucleotide sequences, nucleic acids *etc.* can be chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. In some such embodiments, modifications can occur at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, its hybridization parameters, *etc.* In general, a nucleotide sequence typically carries genetic information,

including, but not limited to, the information used by cellular machinery to make proteins and enzymes. In some embodiments, a nucleotide sequence and/or genetic information comprises double- or single-stranded genomic DNA, RNA, any synthetic and genetically manipulated polynucleotide, and/or sense and/or antisense polynucleotides. In some embodiments, nucleic acids containing modified bases.

[0131] Conventional IUPAC notation is used in nucleotide sequences presented herein, as shown in Table 1, below (*see also* Cornish-Bowden A, Nucleic Acids Res. 1985 May 10; 13(9):3021-30, incorporated by reference herein). It should be noted, however, that "T" denotes "Thymine or Uracil" in those instances where a sequence may be encoded by either DNA or RNA, for example in gRNA targeting domains.

Table 1: IUPAC nucleic acid notation

Character	**Base**
A	Adenine
T	Thymine or Uracil
G	Guanine
C	Cytosine
U	Uracil
K	G or T/U
M	A or C
R	A or G
Y	C or T/U
S	C or G
W	A or T/U
B	C, G or T/U
V	A, C or G
H	A, C or T/U

D	A, G or T/U
N	A, C, G or T/U

[0132] The terms "potency" or "developmental potency" as used herein refers to the sum of all developmental options accessible to the cell (*i.e.*, the developmental potency), particularly, for example in the context of cellular developmental potential, In some embodiments, the continuum of cell potency includes, but is not limited to, totipotent cells, pluripotent cells, multipotent cells, oligopotent cells, unipotent cells, and terminally differentiated cells.

[0133] The terms "prevent," "preventing," and "prevention" as used herein refer to the prevention of a disease in a mammal, *e.g.,* in a human, including (a) avoiding or precluding the disease; (b) affecting the predisposition toward the disease; or (c) preventing or delaying the onset of at least one symptom of the disease.

[0134] The terms "protein," "peptide" and "polypeptide" as used herein are used interchangeably to refer to a sequential chain of amino acids linked together via peptide bonds. The terms include individual proteins, groups or complexes of proteins that associate together, as well as fragments or portions, variants, derivatives and analogs of such proteins. Unless otherwise specified, peptide sequences are presented herein using conventional notation, beginning with the amino or N-terminus on the left, and proceeding to the carboxyl or C-terminus on the right. Standard one-letter or three-letter abbreviations can be used.

[0135] The terms "reprogramming" or "dedifferentiation" or "increasing cell potency" or "increasing developmental potency" as used herein refer to a method of increasing potency of a cell or dedifferentiating a cell to a less differentiated state. For example, in some embodiments, a cell that has an increased cell potency has more developmental plasticity (*i.e.,* can differentiate into more cell types) compared to the same cell in the non-reprogrammed state. That is, in some embodiments, , a reprogrammed cell is one that is in a less differentiated state than the same cell in a non- reprogrammed state. In some embodiments, "reprogramming" refers to de-differentiating a somatic cell, or a multipotent stem cell, into a pluripotent stem cell, also referred to as an induced pluripotent stem cell, or

iPSC. Suitable methods for the generation of iPSCs from somatic or multipotent stem cells are well known to those of skill in the art.

[0136] The terms "RNA-guided nuclease" and "RNA-guided nuclease molecule" are used interchangeably herein. In some embodiments, the RNA-guided nuclease is a RNA-guided DNA endonuclease enzyme. In some embodiments, the RNA-guided nuclease is a CRISPR nuclease. Non-limiting examples of RNA-guided nucleases are listed in Table 2 below, and the methods and compositions disclosed herein can use any combination of RNA-guided nucleases disclosed herein, or known to those of ordinary skill in the art. Those of ordinary skill in the art will be aware of additional nucleases and nuclease variants suitable for use in the context of the present disclosure, and it will be understood that the present disclosure is not limited in this respect.

Table 2. RNA-Guided Nucleases

Nuclease	**Length (a.a.)**	**PAM**	**Reference**
SpCas9	1368	NGG	Cong *et al.*, Science. 2013;339(6121):819-23
SaCas9	1053	NNGRRT	Ran *et al.*, Nature. 2015;520(7546):186-91.
(KKH) SaCas9	1067	NNNRRT	Kleinstiver *et al.*, Nat Biotechnol. 2015;33(12):1293-1298
AsCpf1 (AsCas12a)	1353	TTTV	Zetsche *et al.*, Nat Biotechnol. 2017;35(1):31-34.
LbCpf1 (LbCas12a)	1274	TTTV	Zetsche *et al.*, Cell. 2015;163(3):759-71.
CasX	980	TTC	Burstein *et al.*, Nature. 2017;542(7640):237-241.
CasY	1200	TA	Burstein *et al.*, Nature. 2017;542(7640):237-241.
Cas12h1	870	RTR	Yan *et al.*, Science. 2019;363(6422):88-91.
Cas12i1	1093	TTN	Yan *et al.*, Science. 2019;363(6422):88-91.

Cas12c1	unknown	TG	Yan *et al.*, Science. 2019;363(6422):88-91.
Cas12c2	unknown	TN	Yan *et al.*, Science. 2019;363(6422):88-91.
eSpCas9	1423	NGG	Chen *et al.*, Nature. 2017;550(7676):407-410.
Cas9-HF1	1367	NGG	Chen *et al.*, Nature. 2017;550(7676):407-410.
HypaCas9	1404	NGG	Chen *et al.*, Nature. 2017;550(7676):407-410.
dCas9-Fokl	1623	NGG	U.S. Patent No. 9,322,037
Sniper-Cas9	1389	NGG	Lee *et al.*, Nat Commun. 2018;9(1):3048.
xCas9	1786	NGG, NG, GAA, GAT	Wang *et al.*, Plant Biotechnol J. 2018; pbi.13053.
AaCas12b	1129	TTN	Teng *et al.* Cell Discov. 2018;4:63.
evoCas9	1423	NGG	Casini *et al.*, Nat Biotechnol. 2018;36(3):265-271.
SpCas9-NG	1423	NG	Nishimasu *et al.*, Science. 2018;361(6408):1259-1262.
VRQR	1368	NGA	Li *et al.*, The CRISPR Journal, 2018; 01:01
VRER	1372	NGCG	Kleinstiver *et al.*, Nature. 2016;529(7587):490-5.
NmeCas9	1082	NNNNGATT	Amrani *et al.*, Genome Biol. 2018;19(1):214.
CjCas9	984	NNNNRYAC	Kim *et al.*, Nat Commun. 2017;8:14500.
BhCas12b	1108	ATTN	Strecker *et al.*, Nat Commun. 2019 Jan 22;10(1):212.
BhCas12b V4	1108	ATTN	Strecker *et al.*, Nat Commun. 2019 Jan 22;10(1):212.
CasΦ	700-800	TBN (where B is G, T, or C)	Pausch *et al.*, Science 2020;369(6501):333-337.

[0137] Additional suitable RNA-guided nucleases, e.g., Cas9 and Cas12 nucleases, will be apparent to the skilled artisan in view of the present disclosure, and the disclosure is

not limited by the exemplary suitable nucleases provided herein. In some embodiments, a suitable nuclease is a Cas9 or Cpf1 (Cas12a) nuclease. In some embodiments, the disclosure also embraces nuclease variants, e.g., Cas9 or Cpf1 nuclease variants. In some embodiments, a nuclease is a nuclease variant, which refers to a nuclease comprising an amino acid sequence characterized by one or more amino acid substitutions, deletions, or additions as compared to the wild type amino acid sequence of the nuclease. In some embodiments, a suitable nuclease and/or nuclease variant may also include purification tags (e.g., polyhistidine tags) and/or signaling peptides, e.g., comprising or consisting of a nuclear localization signal sequence. Some non-limiting examples of suitable nucleases and nuclease variants are described in more detail elsewhere herein and also include those described in PCT application PCT/US2019/22374, filed March 14, 2019, and entitled "*Systems and Methods for the Treatment of Hemoglobinopathies*," the entire contents of which are incorporated herein by reference. In some embodiments, the RNA-guided nuclease is an *Acidaminococcus sp.* Cpf1 variant (AsCpf1 variant). In some embodiments, suitable Cpf1 nuclease variants, including suitable AsCpf1 variants will be known or apparent to those of ordinary skill in the art based on the present disclosure, and include, but are not limited to , the Cpf1 variants disclosed herein or otherwise known in the art. For example, in some embodiments, the RNA-guided nuclease is a *Acidaminococcus sp.* Cpf1 RR variant (AsCpf1-RR). In another embodiment, the RNA-guided nuclease is a Cpf1 RVR variant. For example, suitable Cpf1 variants include those having an M537R substitution, an H800A substitution, and/or an F870L substitution, or any combination thereof (numbering scheme according to AsCpf1 wild-type sequence).

[0138] The term "subject" as used herein means a human or non-human animal. In some embodiments a human subject can be any age (*e.g.,* a fetus, infant, child, young adult, or adult). In some embodiments a human subject may be at risk of or suffer from a disease, or may be in need of alteration of a gene or a combination of specific genes. Alternatively, in some embodiments, a subject may be a non-human animal, which may include, but is not limited to, a mammal. In some embodiments, a non-human animal is a non-human primate, a rodent (*e.g.*, a mouse, rat, hamster, guinea pig, *etc.*), a rabbit, , a dog, a cat, and so on. In certain embodiments of this disclosure, the non-human animal subject is livestock, *e.g.,* avow, a horse, a sheep, a goat, etc.. In certain embodiments, the non-human animal subject is poultry, e.g., a chicken, a turkey, a duck, etc. .

[0139] The terms "treatment," "treat," and "treating," as used herein refer to a clinical intervention aimed to reverse, alleviate, delay the onset of, or inhibit the progress, ameliorate, reduce severity of, prevent or delay the recurrence of a disease, disorder, or condition or one or more symptoms thereof, and/or improve one or more symptoms of a disease, disorder, or condition as described herein. In some embodiments, a condition includes an injury. In some embodiments, an injury may be acute or chronic (e.g., tissue damage from an underlying disease or disorder that causes, e.g., secondary damage such as tissue injury). In some embodiments, treatment, *e.g.*, in the form of a modified NK cell or a population of modified NK cells as described herein, may be administered to a subject after one or more symptoms have developed and/or after a disease has been diagnosed. Treatment may be administered in the absence of symptoms, *e.g.*, to prevent or delay onset of a symptom or inhibit onset or progression of a disease. For example, in some embodiments, treatment may be administered to a susceptible individual prior to the onset of symptoms (*e.g.*, in light of genetic or other susceptibility factors). In some embodiments, treatment may also be continued after symptoms have resolved, for example to prevent or delay their recurrence. In some embodiments, treatment results in improvement and/or resolution of one or more symptoms of a disease, disorder or condition.

[0140] The term "variant" as used herein refers to an entity such as a polypeptide, polynucleotide or small molecule that shows significant structural identity with a reference entity but differs structurally from the reference entity in the presence or level of one or more chemical moieties as compared with the reference entity. In many embodiments, a variant also differs functionally from its reference entity. In general, whether a particular entity is properly considered to be a "variant" of a reference entity is based on its degree of structural identity with the reference entity.

Stem Cells

[0141] Methods of the disclosure can be used to culture stem cells. Stem cells are typically cells that have the capacity to produce unaltered daughter cells (self-renewal; cell division produces at least one daughter cell that is identical to the parent cell) and to give rise to specialized cell types (potency). Stem cells include, but are not limited to, embryonic stem (ES) cells, embryonic germ (EG) cells, germline stem (GS) cells, human mesenchymal stem cells (hMSCs), adipose tissue-derived stem cells (ADSCs), multipotent adult progenitor cells

(MAPCs), multipotent adult germline stem cells (maGSCs) and unrestricted somatic stem cell (USSCs). Generally, stem cells can divide without limit. After division, the stem cell may remain as a stem cell, become a precursor cell, or proceed to terminal differentiation. A precursor cell is a cell that can generate a fully differentiated functional cell of at least one given cell type. Generally, precursor cells can divide. After division, a precursor cell can remain a precursor cell, or may proceed to terminal differentiation.

[0142] Pluripotent stem cells are generally known in the art. The present disclosure provides technologies (e.g., systems, compositions, methods, etc.) related to pluripotent stem cells. In some embodiments, pluripotent stem cells are stem cells that: (a) are capable of inducing teratomas when transplanted in immunodeficient (SCID) mice; (b) are capable of differentiating to cell types of all three germ layers (e.g., can differentiate to ectodermal, mesodermal, and endodermal cell types); and/or (c) express one or more markers of embryonic stem cells (e.g., human embryonic stem cells express Oct 4, alkaline phosphatase, SSEA-3 surface antigen,SSEA-4 surface antigen, nanog, TRA-1-60, TRA-1-81, SOX2, REX1, etc.). In some aspects, human pluripotent stem cells do not show expression of differentiation markers. In some embodiments, ES cells and/or iPSCs cultured using methods of the disclosure maintain their pluripotency (e.g., (a) are capable of inducing teratomas when transplanted in immunodeficient (SCID) mice; (b) are capable of differentiating to cell types of all three germ layers (e.g., can differentiate to ectodermal, mesodermal, and endodermal cell types); and/or (c) express one or more markers of embryonic stem cells).

[0143] In some embodiments, ES cells (e.g., human ES cells) can be derived from the inner cell mass of blastocysts or morulae. In some embodiments, ES cells can be isolated from one or more blastomeres of an embryo, e.g., without destroying the remainder of the embryo. In some embodiments, ES cells can be produced by somatic cell nuclear transfer. In some embodiments, ES cells can be derived from fertilization of an egg cell with sperm or DNA, nuclear transfer, parthenogenesis, or by means to generate ES cells, e.g., with homozygosity in the HLA region. In some embodiments, human ES cells can be produced or derived from a zygote, blastomeres, or blastocyst-staged mammalian embryo produced by the fusion of a sperm and egg cell, nuclear transfer, parthenogenesis, or the reprogramming of chromatin and subsequent incorporation of the reprogrammed chromatin into a plasma membrane to produce an embryonic cell. Exemplary human ES cells are known in the art

and include, but are not limited to, MAO1, MAO9, ACT-4, No. 3, H1, H7, H9, H14 and ACT30 ES cells. In some embodiments, human ES cells, regardless of their source or the particular method used to produce them, can be identified based on, e.g., (i) the ability to differentiate into cells of all three germ layers, (ii) expression of at least Oct-4 and alkaline phosphatase, and/or (iii) ability to produce teratomas when transplanted into immunocompromised animals. In some embodiments, ES cells have been serially passaged as cell lines.

iPSCs

[0144] Induced pluripotent stem cells (iPSC) are a type of pluripotent stem cell artificially derived from a non-pluripotent cell, such as an adult somatic cell (e.g., a fibroblast cell or other suitable somatic cell), by inducing expression of certain genes. iPSCs can be derived from any organism, such as a mammal. In some embodiments, iPSCs are produced from mice, rats, rabbits, guinea pigs, goats, pigs, cows, non-human primates or humans. iPSCs are similar to ES cells in many respects, such as the expression of certain stem cell genes and proteins, chromatin methylation patterns, doubling time, embryoid body formation, teratoma formation, viable chimera formation, potency and/or differentiability. Various suitable methods for producing iPSCs are known in the art. In some embodiments, iPSCs can be derived by transfection of certain stem cell-associated genes (such asOct-3/4 (Pouf51) and Sox2) into non-pluripotent cells, such as adult fibroblasts. Transfection can be achieved through viral vectors, such as retroviruses, lentiviruses, or adenoviruses. Additional suitable reprogramming methods include the use of vectors that do not integrate into the genome of the host cell, e.g., episomal vectors, or the delivery of reprogramming factors directly via encoding RNA or as proteins has also been described. For example, cells can be transfected with Oct3/4, Sox2, Klf4, and/or c-Myc using a retroviral system or with OCT4, SOX2, NANOG, and/or LIN28 using a lentiviral system. After 3-4 weeks, small numbers of transfected cells begin to become morphologically and biochemically similar to pluripotent stem cells, and can be isolated through morphological selection, doubling time, or through a reporter gene and antibiotic selection. In one example, iPSCs from adult human cells are generated by the method described by Yu et al. (Science 318(5854):1224 (2007)) or Takahashi et al. (Cell 131:861-72 (2007)). In some embodiments, iPSCs are generated by a commercial source. In some embodiments, iPSCs are generated by a vendor. In some

embodiments, iPSCs are generated by a contract research organization. Numerous suitable methods for reprogramming are known to those of skill in the art, and the present disclosure is not limited in this respect.

Genetically Engineered Stem Cells

[0145] In some embodiments, a stem cell (e.g., iPSC) described herein is genetically engineered to introduce a disruption in one or more targets described herein. For example, in some embodiments, a stem cell (e.g., iPSC) can be genetically engineered to knockout all or a portion of one or more target gene, introduce a frameshift in one or more target genes, and/or cause a truncation of an encoded gene product (e.g., by introducing a premature stop codon). In some embodiments, a stem cell (e.g., iPSC) can be genetically engineered to knockout all or a portion of a target gene using a gene-editing system, e.g., as described herein. In some such embodiments, a gene-editing system may be or comprise a CRISPR system, a zinc finger nuclease system, a TALEN, and/or a meganuclease.

TGF signaling

[0146] In certain embodiments, the disclosure provides a genetically engineered stem cell, and/or progeny cell, comprising a disruption in TGF signaling, e.g., TGF beta signaling. This is useful, for example, in circumstances where it is desirable to generate a differentiated cell from pluripotent stem cell, wherein TGF signaling, e.g., TGF beta signaling is disrupted in the differentiated cell.

[0147] For example, TGF beta signaling inhibits or decreases the survival and/or activity of some differentiated cell types that are useful for therapeutic applications, e.g., TGF beta signaling is a negative regulator of natural killer cells, which can be used in immunotherapeutic applications. In some embodiments, it is desirable to generate a clinically effective number of natural killer cells comprising a genetic modification that disrupts TGF beta signaling, thus avoiding the negative effect of TGF beta on the clinical effectiveness of such cells. It is advantageous, in some embodiments, to source such NK cells from a pluripotent stem cell, instead, for example, from mature NK cells obtained from a donor. Modifying the stem cell instead of the differentiated cell has, among others, the advantage of allowing for clonal derivation, characterization, and/or expansion of a specific genotype, e.g., a specific stem cell clone harboring a specific genetic modification (e.g., a targeted disruption

of TGFβRII in the absence of any undesired (e.g., off-target) modifications). In some embodiments, the stem cell, e.g., the human iPSC, is genetically engineered not to express one or more TGFβ receptor, e.g., TGFβRII, or to express a dominant negative variant of a TGFβ receptor, e.g., a dominant negative TGFβRII variant. Exemplary sequences of TGFβRII are set forth in KR710923.1, NM_001024847.2, and NM_003242.5. An exemplary dominant negative TGFβRII is disclosed in Immunity. 2000 Feb;12(2):171-81.

Additional Loss-of-Function Modifications

[0148] In certain embodiments, the disclosure provides a genetically engineered stem cell, and/or progeny cell, that additionally or alternatively comprises a disruption in interleukin signaling, e.g., IL-15 signaling. IL-15 is a cytokine with structural similarity to Interleukin-2 (IL-2), which binds to and signals through a complex composed of IL-2/IL-15 receptor beta chain (CD122) and the common gamma chain (gamma-C, CD132). Exemplary sequences of IL-15 are provided in NG_029605.2. Disruption of IL-15 signaling may be useful, for example, in circumstances where it is desirable to generate a differentiated cell from a pluripotent stem cell, but with certain signaling pathways (e.g., IL-15) disrupted in the differentiated cell. IL-15 signaling can inhibit or decrease survival and/or activity of some types of differentiated cells, such as cells that may be useful for therapeutic applications. For example, IL-15 signaling is a negative regulator of natural killer (NK) cells. CISH (encoded by the CISH gene) is downstream of the IL-15 receptor and can act as a negative regulator of IL-15 signaling in NK cells. As used herein, the term "CISH" refers to the Cytokine Inducible SH2 Containing Protein (see, e.g., Delconte et al., Nat Immunol. 2016 Jul;17(7):816-24; exemplary sequences for CISH are set forth as NG_023194.1). In some embodiments, disruption of CISH regulation may increase activation of Jak/STAT pathways, leading to increased survival, proliferation and/or effector functions of NK cells. Thus, in some embodiments, genetically engineered NK cells (e.g., iNK cells, e.g., generated from genetically engineered hiPSCs comprising a disruption of CISH regulation) exhibit greater responsiveness to IL-15-mediated signaling than non-genetically engineered NK cells. In some such embodiments, genetically engineered NK cells exhibit greater effector function relative to non-genetically engineered NK cells.

[0149] In some embodiments, a genetically engineered stem cell and/or progeny cell, additionally or alternatively, comprises a disruption and/or loss of function in one or more of

B2M, NKG2A, PD1, TIGIT, ADORA2a, CIITA, HLA class II histocompatibility antigen alpha chain genes, HLA class II histocompatibility antigen beta chain genes, CD32B, or TRAC.

[0150] As used herein, the term "B2M" (β2 microglobulin) refers to a serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells. Exemplary sequences for B2M are set forth as NG_012920.2.

[0151] As used herein, the term "NKG2A" (natural killer group 2A) refers to a protein belonging to the killer cell lectin-like receptor family, also called NKG2 family, which is a group of transmembrane proteins preferentially expressed in NK cells. This family of proteins is characterized by the type II membrane orientation and the presence of a C-type lectin domain. See, e.g., Kamiya-T et al., J Clin Invest 2019 https://doi.org/10.1172/JCI123955. Exemplary sequences for NKG2A are set forth as AF461812.1.

[0152] As used herein, the term "PD1" (Programmed cell death protein 1), also known CD279 (cluster of differentiation 279), refers to a protein found on the surface of cells that has a role in regulating the immune system's response to the cells of the human body by down-regulating the immune system and promoting self-tolerance by suppressing T cell inflammatory activity. PD1 is an immune checkpoint and guards against autoimmunity. Exemplary sequences for PD1 are set forth as NM_005018.3.

[0153] As used herein, the term "TIGIT" (T cell immunoreceptor with Ig and ITIM domains) refers to a member of the PVR (poliovirus receptor) family of immunoglobulin proteins. The product of this gene is expressed on several classes of T cells including follicular B helper T cells (TFH). Exemplary sequences for TIGIT are set forth in NM_173799.4.

[0154] As used herein, the term "ADORA2A" refers to the adenosine A2a receptor, a member of the guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) superfamily, which is subdivided into classes and subtypes. This protein, an adenosine receptor of A2A subtype, uses adenosine as the preferred endogenous agonist and

preferentially interacts with the G(s) and G(olf) family of G proteins to increase intracellular cAMP levels. Exemplary sequences of ADORA2a are provided in NG_052804.1.

[0155] As used herein, the term "CIITA" refers to the protein located in the nucleus that acts as a positive regulator of class II major histocompatibility complex gene transcription, and is referred to as the "master control factor" for the expression of these genes. The protein also binds GTP and uses GTP binding to facilitate its own transport into the nucleus. Mutations in this gene have been associated with bare lymphocyte syndrome type II (also known as hereditary MHC class II deficiency or HLA class II-deficient combined immunodeficiency), increased susceptibility to rheumatoid arthritis, multiple sclerosis, and possibly myocardial infarction. See, e.g., Chang et al., J Exp Med 180:1367-1374; and Chang et al., Immunity. 1996 Feb;4(2):167-78, the entire contents of each of which are incorporated by reference herein. An exemplary sequence of CIITA is set forth as NG_009628.1.

[0156] In some embodiments, two or more HLA class II histocompatibility antigen alpha chain genes and/or two or more HLA class II histocompatibility antigen beta chain genes are disrupted, e.g., knocked out, e.g., by genomic editing. For example, in some embodiments, two or more HLA class II histocompatibility antigen alpha chain genes selected from HLA-DQA1, HLA-DRA, HLA-DPA1, HLA-DMA, HLA-DQA2, and HLA-DOA are disrupted, e.g., knocked out. For another example, in some embodiments, two or more HLA class II histocompatibility antigen beta chain genes selected from HLA-DMB, HLA-DOB, HLA-DPB1, HLA-DQB1, HLA-DQB3, HLA-DQB2, HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5 are disrupted, e.g., knocked out. See, e.g., Crivello et al., J Immunol January 2019, ji1800257; DOI: https://doi.org/10.4049/jimmunol.1800257, the entire contents of which are incorporated herein by reference.

[0157] As used herein, the term "CD32B" (cluster of differentiation 32B) refers to a low affinity immunoglobulin gamma Fc region receptor II-b protein that, in humans, is encoded by the FCGR2B gene. See, e.g., Rankin-CT et al., Blood 2006 108(7):2384-91, the entire contents of which are incorporated herein by reference.

[0158] As used herein, the term "TRAC" refers to the T-cell receptor alpha subunit (constant), encoded by the TRAC locus.

Gain-of-Function Modifications

[0159] In some embodiments, a genetically engineered stem cell and/or progeny cell, additionally or alternatively, comprises a genetic modification that leads to expression of one or more of a CAR; a non-naturally occurring variant of FcγRIII (CD16); interleukin 15 (IL-15); an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor; interleukin 12 (IL-12); an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor; human leukocyte antigen G (HLA-G); human leukocyte antigen E (HLA-E); or leukocyte surface antigen cluster of differentiation CD47 (CD47).

[0160] As used herein, the term "chimeric antigen receptor" or "CAR" refers to a receptor protein that has been modified to give cells expressing the CAR the new ability to target a specific protein. Within the context of the disclosure, an cell modified to comprise a CAR may be used for immunotherapy to target and destroy cells associated with a disease or disorder, *e.g.*, cancer cells.

[0161] CARs of interest include, but are not limited to, a CAR targeting mesothelin, EGFR, HER2 and/or MICA/B. To date, mesothelin-targeted CAR T-cell therapy has shown early evidence of efficacy in a phase I clinical trial of subjects having mesothelioma, non-small cell lung cancer, and breast cancer (NCT02414269). Similarly, CARs targeting EGFR, HER2 and MICA/B have shown promise in early studies (see, *e.g.,* Li et al. (2018), *Cell Death & Disease*, 9(177); Han et al. (2018) Am. J. Cancer Res., 8(1):106-119; and Demoulin 2017) Future Oncology, 13(8); the entire contents of each of which are expressly incorporated herein by reference in their entireties).

[0162] CARs are well-known to those of ordinary skill in the art and include those described in, for example: WO13/063419 (mesothelin), WO15/164594 (EGFR), WO13/063419 (HER2), WO16/154585 (MICA and MICB), the entire contents of each of which are expressly incorporated herein by reference in their entireties. Any suitable CAR, NK-CAR, or other binder that targets a cell, *e.g.*, an NK cell, to a target cell, *e.g.*, a cell associated with a disease or disorder, may be expressed in the modified NK cells provided herein. Exemplary CARs, and binders, include, but are not limited to, CARs and binders that bind BCMA, CD19, CD22, CD20, CD33, CD123, androgen receptor, PSMA, PSCA, Muc1, HPV viral peptides (i.e., E7), EBV viral peptides, CD70, WT1, CEA, EGFRvIII, IL13Rα2,

and GD2, CA125, CD7, EpCAM, Muc16, CD30. Additional suitable CARs and binders for use in the modified NK cells provided herein will be apparent to those of skill in the art based on the present disclosure and the general knowledge in the art. Such additional suitable CARs include those described in Figure 3 of Davies and Maher, *Adoptive T-cell Immunotherapy of Cancer Using Chimeric Antigen Receptor-Grafted T Cells*, Archivum Immunologiae et Therapiae Experimentalis 58(3):165-78 (2010), the entire contents of which are incorporated herein by reference.

[0163] As used herein, the term "CD16" refers to a receptor (FcγRIII) for the Fc portion of immunoglobulin G, and it is involved in the removal of antigen-antibody complexes from the circulation, as well as other antibody-dependent responses.

[0164] As used herein, the term "IL-15/IL15RA" or "Interleukin-15" (IL-15) refers to a cytokine with structural similarity to Interleukin-2 (IL-2). Like IL-2, IL-15 binds to and signals through a complex composed of IL-2/IL-15 receptor beta chain (CD122) and the common gamma chain (gamma-C, CD132). IL-15 is secreted by mononuclear phagocytes (and some other cells) following infection by virus(es). This cytokine induces cell proliferation of natural killer cells; cells of the innate immune system whose principal role is to kill virally infected cells. IL-15 Receptor alpha (IL15RA) specifically binds IL-15 with very high affinity, and is capable of binding IL-15 independently of other subunits. It is suggested that this property allows IL-15 to be produced by one cell, endocytosed by another cell, and then presented to a third party cell. IL15RA is reported to enhance cell proliferation and expression of apoptosis inhibitor BCL2L1/BCL2-XL and BCL2. Exemplary sequences of IL-15 are provided in NG_029605.2, and exemplary sequences of IL-15RA are provided in NM_002189.4. In some embodiments, the IL-15R variant is a constitutively active IL-15R variant. In some embodiments, the constitutively active IL-15R variant is a fusion between IL-15R and an IL-15R agonist, e.g., an IL-15 protein or IL-15R-binding fragment thereof. In some embodiments, the IL-15R agonist is IL-15, or an IL-15R-binding variant thereof. Exemplary suitable IL-15R variants include, without limitation, those described, e.g., in Mortier E et al, 2006; The Journal of Biological Chemistry 2006 281: 1612-1619; or in Bessard-A et al., Mol Cancer Ther. 2009 Sep;8(9):2736-45, the entire contents of each of which are incorporated by reference herein.

[0165] As used herein, the term "IL-12" refers to interleukin-12, a cytokine that acts on T and natural killer cells. In some embodiments, a genetically engineered stem cell and/or progeny cell comprises a genetic modification that leads to expression of one or more of an interleukin 12 (IL12) pathway agonist, e.g., IL-12, interleukin 12 receptor (IL-12R) or a variant thereof (e.g., a constitutively active variant of IL-12R, e.g., an IL-12R fused to an IL-12R agonist (IL-12RA).

[0166] As used herein, the term "HLA-G" refers to the HLA non-classical class I heavy chain paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). The heavy chain is anchored in the membrane. HLA-G is expressed on fetal derived placental cells. HLA-G is a ligand for NK cell inhibitory receptor KIR2DL4, and therefore expression of this HLA by the trophoblast defends it against NK cell-mediated death. See e.g., Favier et al., Tolerogenic Function of Dimeric Forms of HLA-G Recombinant Proteins: A Comparative Study In Vivo PLOS One 2011, the entire contents of which are incorporated herein by reference. An exemplary sequence of HLA-G is set forth as NG_029039.1.

[0167] As used herein, the term "HLA-E" refers to the HLA class I histocompatibility antigen, alpha chain E, also sometimes referred to as MHC class I antigen E. The HLA-E protein in humans is encoded by the HLA-E gene. The human HLA-E is a non-classical MHC class I molecule that is characterized by a limited polymorphism and a lower cell surface expression than its classical paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). The heavy chain is anchored in the membrane. HLA-E binds a restricted subset of peptides derived from the leader peptides of other class I molecules. HLA-E expressing cells escape allogeneic responses and lysis by NK cells. See e.g., Geornalusse-G et al., Nature Biotechnology 2017 35(8), the entire contents of which are incorporated herein by reference. Exemplary sequences of the HLA-E protein are provided in NM_005516.6.

[0168] As used herein, the term "CD47," also sometimes referred to as "integrin associated protein" (IAP), refers to a transmembrane protein that in humans is encoded by the CD47 gene. CD47 belongs to the immunoglobulin superfamily, partners with membrane integrins, and also binds the ligands thrombospondin-1 (TSP-1) and signal-regulatory protein alpha (SIRPα). CD47 acts as a signal to macrophages that allows CD47-expressing cells to

escape macrophage attack. See, e.g., Deuse-T, et al., Nature Biotechnology 2019 37: 252–258, the entire contents of which are incorporated herein by reference.

Generation of iNK cells

[0169] In some embodiments, the present disclosure provides methods of generating iNK cells (e.g., genetically modified iNK cells) that are derived from stem cells described herein.

[0170] In some embodiments, genetic modifications (e.g., genomic edits) present in an iNK cell of the present disclosure can be made at any stage during the reprogramming process from donor cell to iPSC, during the iPSC stage, and/or at any stage of the process of differentiating the iPSC to an iNK state, *e.g.*, at an intermediary state, such as, for example, an iPSC-derived HSC state, or even up to or at the final iNK cell state.

[0171] For example, one or more genomic edits present in an edited iNK cell of the present disclosure may be made at one or more different cell stages (e.g., reprogramming from donor to iPSC, differentiation of iPSC to iNK). In some embodiments, one or more genomic edits present in modified genetically modified iNK cell provided herein is made before reprogramming a donor cell to an iPSC state. In some embodiments, all edits present in a genetically modified iNK cell provided herein are made at the same time, in close temporal proximity, and/or at the same cell stage of the reprogramming/differentiation process, *e.g.*, at the donor cell stage, during the reprogramming process, at the iPSC stage, or during the differentiation process, e.g., from iPSC to iNK. In some embodiments, two or more edits present in a genetically modified iNK cell provided herein are made at different times and/or at different cell stages of the reprogramming/differentiation process from donor cell to iPSC to iNK. For example, in some embodiments, a first edit is made at the donor cell stage and a second (different) edit is made at the iPSC stage. In some embodiments, a first edit is made at the reprogramming stage (e.g., donor to iPSC) and a second (different) edit is made at the iPSC stage.

[0172] A variety of cell types can be used as a donor cell that can be subjected to reprogramming, differentiation, and/or genomic editing strategies described herein. For example, the donor cell can be a pluripotent stem cell or a differentiated cell, *e.g.*, a somatic cell, such as, for example, a fibroblast or a T lymphocyte. In some embodiments, donor cells

are manipulated (e.g., subjected to reprogramming, differentiation, and/or genomic editing) to generate iNK cells described herein.

[0173] A donor cell can be from any suitable organism. For example, in some embodiments, the donor cell is a mammalian cell, e.g., a human cell or a non-human primate cell. In some embodiments, the donor cell is a somatic cell. In some embodiments, the donor cell is a stem cell or progenitor cell. In certain embodiments, the donor cell is not or was not part of a human embryo and its derivation does not involve destruction of a human embryo.

[0174] In some embodiments, an edited iNK cell is derived from an iPSC, which in turn is derived from a somatic donor cell. Any suitable somatic cell can be used in the generation of iPSCs, and in turn, the generation of iNK cells. Suitable strategies for deriving iPSCs from various somatic donor cell types have been described and are known in the art. In some embodiments, a somatic donor cell is a fibroblast cell. In some embodiments, a somatic donor cell is a mature T cell.

[0175] For example, in some embodiments, a somatic donor cell, from which an iPSC, and subsequently an iNK cell is derived, is a developmentally mature T cell (a T cell that has undergone thymic selection). One hallmark of developmentally mature T cells is a rearranged T cell receptor locus. During T cell maturation, the TCR locus undergoes V(D)J rearrangements to generate complete V-domain exons. These rearrangements are retained throughout reprogramming of a T cells to an iPSC, and throughout differentiation of the resulting iPSC to a somatic cell.

[0176] In certain embodiments, a somatic donor cell is a $CD8^+$ T cell, a $CD8^+$ naïve T cell, a $CD4^+$ central memory T cell, a $CD8^+$ central memory T cell, a $CD4^+$ effector memory T cell, a $CD4^+$ effector memory T cell, a $CD4^+$ T cell, a $CD4^+$ stem cell memory T cell, a $CD8^+$ stem cell memory T cell, a $CD4^+$ helper T cell, a regulatory T cell, a cytotoxic T cell, a natural killer T cell, a CD4+ naïve T cell, a TH17 $CD4^+$ T cell, a TH1 $CD4^+$ T cell, a TH2 $CD4^+$ T cell, a TH9 $CD4^+$ T cell, a $CD4^+$ $Foxp3^+$ T cell, a $CD4^+$ $CD25^+$ $CD127^-$ T cell, or a $CD4^+$ $CD25^+$ $CD127^-$ $Foxp3^+$ T cell.

[0177] T cells can be advantageous for the generation of iPSCs. For example, T cells can be edited with relative ease, *e.g.*, by CRISPR-based methods or other gene-editing

methods. Additionally, the rearranged TCR locus allows for genetic tracking of individual cells and their daughter cells. For example, if the reprogramming, expansion, culture, and/or differentiation strategies involved in the generation of NK cells a clonal expansion of a single cell, the rearranged TCR locus can be used as a genetic marker unambiguously identifying a cell and its daughter cells. This, in turn, allows for the characterization of a cell population as truly clonal, or for the identification of mixed populations, or contaminating cells in a clonal population. Another potential advantage of using T cells in generating iNK cells carrying multiple edits is that certain karyotypic aberrations associated with chromosomal translocations are selected against in T cell culture. Such aberrations can pose a concern when editing cells by CRISPR technology, and in particular when generating cells carrying multiple edits. Using T cell derived iPSCs as a starting point for the derivation of therapeutic lymphocytes can allow for the expression of a pre-screened TCR in the lymphocytes, *e.g.*, via selecting the T cells for binding activity against a specific antigen, *e.g.*, a tumor antigen, reprogramming the selected T cells to iPSCs, and then deriving lymphocytes from these iPSCs that express the TCR (*e.g.*, T cells). This strategy can allow for activating the TCR in other cell types, *e.g.*, by genetic or epigenetic strategies. Additionally, T cells retain at least part of their "epigenetic memory" throughout the reprogramming process, and thus subsequent differentiation of the same or a closely related cell type, such as iNK cells can be more efficient and/or result in higher quality cell populations as compared to approaches using non-related cells, such as fibroblasts, as a starting point for iNK derivation.

[0178] In some embodiments, a donor cell being manipulated, *e.g.*, a cell being reprogrammed and/or undergoing genomic editing, is one or more of a long-term hematopoietic stem cell, a short term hematopoietic stem cell, a multipotent progenitor cell, a lineage restricted progenitor cell, a lymphoid progenitor cell, a myeloid progenitor cell, a common myeloid progenitor cell, an erythroid progenitor cell, a megakaryocyte erythroid progenitor cell, a retinal cell, a photoreceptor cell, a rod cell, a cone cell, a retinal pigmented epithelium cell, a trabecular meshwork cell, a cochlear hair cell, an outer hair cell, an inner hair cell, a pulmonary epithelial cell, a bronchial epithelial cell, an alveolar epithelial cell, a pulmonary epithelial progenitor cell, a striated muscle cell, a cardiac muscle cell, a muscle satellite cell, a neuron, a neuronal stem cell, a mesenchymal stem cell, an induced pluripotent stem (iPS) cell, an embryonic stem cell, a fibroblast, a monocyte-derived macrophage or dendritic cell, a megakaryocyte, a neutrophil, an eosinophil, a basophil, a mast cell, a

reticulocyte, a B cell, *e.g.,* a progenitor B cell, a Pre B cell, a Pro B cell, a memory B cell, a plasma B cell, a gastrointestinal epithelial cell, a biliary epithelial cell, a pancreatic ductal epithelial cell, an intestinal stem cell, a hepatocyte, a liver stellate cell, a Kupffer cell, an osteoblast, an osteoclast, an adipocyte, a preadipocyte, a pancreatic islet cell (*e.g.,* a beta cell, an alpha cell, a delta cell), a pancreatic exocrine cell, a Schwann cell, or an oligodendrocyte.

[0179] In some embodiments, a donor cell is one or more of a circulating blood cell, *e.g.,* a reticulocyte, megakaryocyte erythroid progenitor (MEP) cell, myeloid progenitor cell (CMP/GMP), lymphoid progenitor (LP) cell, hematopoietic stem/progenitor cell (HSC), or endothelial cell (EC). In some embodiments, a donor cell is one or more of a bone marrow cell (*e.g.,* a reticulocyte, an erythroid cell (*e.g.,* erythroblast), an MEP cell, myeloid progenitor cell (CMP/GMP), LP cell, erythroid progenitor (EP) cell, HSC, multipotent progenitor (MPP) cell, endothelial cell (EC), hemogenic endothelial (HE) cell, or mesenchymal stem cell). In some embodiments, a donor cell is one or more of a myeloid progenitor cell (*e.g.,* a common myeloid progenitor (CMP) cell or granulocyte macrophage progenitor (GMP) cell). In some embodiments, a donor cell is one or more of a lymphoid progenitor cell, *e.g.,* a common lymphoid progenitor (CLP) cell. In some embodiments, a donor cell is one or more of an erythroid progenitor cell (*e.g.,* an MEP cell). In some embodiments, a donor cell is one or more of a hematopoietic stem/progenitor cell (*e.g.,* a long term HSC (LT-HSC), short term HSC (ST-HSC), MPP cell, or lineage restricted progenitor (LRP) cell). In certain embodiments, the donor cell is a $CD34^+$ cell, $CD34^+CD90^+$ cell, $CD34^+CD38^-$ cell, $CD34^+CD90^+CD49f^+CD38^-CD45RA^-$ cell, $CD105^+$ cell, $CD31^+$, or $CD133^+$ cell, or a $CD34^+CD90^+$ $CD133^+$ cell. In some embodiments, a donor cell is one or more of an umbilical cord blood $CD34^+$ HSPC, umbilical cord venous endothelial cell, umbilical cord arterial endothelial cell, amniotic fluid $CD34^+$ cell, amniotic fluid endothelial cell, placental endothelial cell, or placental hematopoietic $CD34^+$ cell. In some embodiments, a donor cell is one or more of a mobilized peripheral blood hematopoietic $CD34^+$ cell (after the patient is treated with a mobilization agent, *e.g.,* G-CSF or Plerixafor). In some embodiments, a donor cell is a peripheral blood endothelial cell. In some embodiments, a donor cell is a peripheral blood natural killer cell.

[0180] In some embodiments, a donor cell is a dividing cell. In some embodiments, a donor cell is a non-dividing cell.

[0181] In some embodiments, a genetically modified (e.g., edited) iNK cell resulting from one or more methods and/or strategies described herein, are administered to a subject in need thereof, *e.g.*, in the context of an immuno-oncology therapeutic approach. In some embodiments, donor cells, or any cells of any stage of the reprogramming, differentiating, and/or editing strategies provided herein, can be maintained in culture or stored (*e.g.*, frozen in liquid nitrogen) using any suitable method known in the art, *e.g.*, for subsequent characterization or administration to a subject in need thereof.

Genome editing systems

[0182] Genome editing systems of the present disclosure may be used, for example, to edit stem cells. In some embodiments, genome editing systems of the present disclosure include at least two components adapted from naturally occurring CRISPR systems: a guide RNA (gRNA) and an RNA-guided nuclease. These two components form a complex that is capable of associating with a specific nucleic acid sequence and editing the DNA in or around that nucleic acid sequence, for instance by making one or more of a single-strand break (an SSB or nick), a double-strand break (a DSB) and/or a point mutation.

[0183] Naturally occurring CRISPR systems are organized evolutionarily into two classes and five types (Makarova et al. Nat Rev Microbiol. 2011 Jun; 9(6): 467–477 ("Makarova")), and while genome editing systems of the present disclosure may adapt components of any type or class of naturally occurring CRISPR system, the embodiments presented herein are generally adapted from Class 2, and type II or V CRISPR systems. Class 2 systems, which encompass types II and V, are characterized by relatively large, multidomain RNA-guided nuclease proteins (e.g., Cas9 or Cpf1) and one or more guide RNAs (e.g., a crRNA and, optionally, a tracrRNA) that form ribonucleoprotein (RNP) complexes that associate with (i.e., target) and cleave specific loci complementary to a targeting (or spacer) sequence of the crRNA. Genome editing systems according to the present disclosure similarly target and edit cellular DNA sequences, but differ significantly from CRISPR systems occurring in nature. For example, the unimolecular guide RNAs described herein do not occur in nature, and both guide RNAs and RNA-guided nucleases according to this disclosure may incorporate any number of non-naturally occurring modifications.

[0184] Genome editing systems can be implemented (e.g. administered or delivered to a cell or a subject) in a variety of ways, and different implementations may be suitable for distinct applications. For instance, a genome editing system is implemented, in certain embodiments, as a protein/RNA complex (a ribonucleoprotein, or RNP), which can be included in a pharmaceutical composition that optionally includes a pharmaceutically acceptable carrier and/or an encapsulating agent, such as a lipid or polymer micro- or nano-particle, micelle, liposome, etc. In certain embodiments, a genome editing system is implemented as one or more nucleic acids encoding the RNA-guided nuclease and guide RNA components described above (optionally with one or more additional components); in certain embodiments, the genome editing system is implemented as one or more vectors comprising such nucleic acids, for instance a viral vector such as an adeno-associated virus; and in certain embodiments, the genome editing system is implemented as a combination of any of the foregoing. Additional or modified implementations that operate according to the principles set forth herein will be apparent to the skilled artisan and are within the scope of this disclosure.

[0185] It should be noted that the genome editing systems of the present disclosure can be targeted to a single specific nucleotide sequence, or may be targeted to — and capable of editing in parallel — two or more specific nucleotide sequences through the use of two or more guide RNAs. The use of multiple gRNAs is referred to as "multiplexing" throughout this disclosure, and can be employed to target multiple, unrelated target sequences of interest, or to form multiple SSBs or DSBs within a single target domain and, in some cases, to generate specific edits within such target domain. For example, International Patent Publication No. WO 2015/138510 by Maeder et al. ("Maeder") describes a genome editing system for correcting a point mutation (C.2991+1655A to G) in the human CEP290 gene that results in the creation of a cryptic splice site, which in turn reduces or eliminates the function of the gene. The genome editing system of Maeder utilizes two guide RNAs targeted to sequences on either side of (i.e., flanking) the point mutation, and forms DSBs that flank the mutation. This, in turn, promotes deletion of the intervening sequence, including the mutation, thereby eliminating the cryptic splice site and restoring normal gene function.

[0186] As another example, WO 2016/073990 by Cotta-Ramusino, et al. ("Cotta-Ramusino") describes a genome editing system that utilizes two gRNAs in combination with

a Cas9 nickase (a Cas9 that makes a single strand nick such as *S. pyogenes* D10A), an arrangement termed a "dual-nickase system." The dual-nickase system of Cotta-Ramusino is configured to make two nicks on opposite strands of a sequence of interest that are offset by one or more nucleotides, which nicks combine to create a double strand break having an overhang (5' in the case of Cotta-Ramusino, though 3' overhangs are also possible). The overhang, in turn, can facilitate homology directed repair events in some circumstances. And, as another example, WO 2015/070083 by Palestrant et al. ("Palestrant") describes a gRNA targeted to a nucleotide sequence encoding Cas9 (referred to as a "governing RNA"), which can be included in a genome editing system comprising one or more additional gRNAs to permit transient expression of a Cas9 that might otherwise be constitutively expressed, for example in some virally transduced cells. These multiplexing applications are intended to be exemplary, rather than limiting, and the skilled artisan will appreciate that other applications of multiplexing are generally compatible with the genome editing systems described here.

[0187] Genome editing systems can, in some instances, form double strand breaks that are repaired by cellular DNA double-strand break mechanisms such as NHEJ or HDR. These mechanisms are described throughout the literature, for example by Davis & Maizels, PNAS, 111(10):E924-932, March 11, 2014 ("Davis") (describing Alt-HDR); Frit et al. DNA Repair 17(2014) 81-97 ("Frit") (describing Alt-NHEJ); and Iyama and Wilson III, DNA Repair (Amst.) 2013-Aug; 12(8): 620-636 ("Iyama") (describing canonical HDR and NHEJ pathways generally).

[0188] Where genome editing systems operate by forming DSBs, such systems optionally include one or more components that promote or facilitate a particular mode of double-strand break repair or a particular repair outcome. For instance, Cotta-Ramusino also describes genome editing systems in which a single stranded oligonucleotide "donor template" is added; the donor template is incorporated into a target region of cellular DNA that is cleaved by the genome editing system, and can result in a change in the target sequence.

[0189] In certain embodiments, genome editing systems modify a target sequence, or modify expression of a target gene in or near the target sequence, without causing single- or double-strand breaks. For example, a genome editing system may include an RNA-guided nuclease fused to a functional domain that acts on DNA, thereby modifying the target

sequence or its expression. As one example, an RNA-guided nuclease can be connected to (e.g., fused to) a cytidine deaminase functional domain, and may operate by generating targeted C-to-A substitutions. Exemplary nuclease/deaminase fusions are described in Komor et al. Nature 533, 420–424 (19 May 2016) ("Komor"). Alternatively, a genome editing system may utilize a cleavage-inactivated (i.e., a "dead") nuclease, such as a dead Cas9 (dCas9), and may operate by forming stable complexes on one or more targeted regions of cellular DNA, thereby interfering with functions involving the targeted region(s) including, without limitation, mRNA transcription, chromatin remodeling, etc.

Guide RNA (gRNA) molecules

[0190] Guide RNAs (gRNAs) of the present disclosure may be unimolecular (comprising a single RNA molecule, and referred to alternatively as chimeric), or modular (comprising more than one, and typically two, separate RNA molecules, such as a crRNA and a tracrRNA, which are usually associated with one another, for instance by duplexing). gRNAs and their component parts are described throughout the literature, for instance in Briner et al. (Molecular Cell 56(2), 333-339, October 23, 2014 ("Briner")), and in Cotta-Ramusino.

[0191] In bacteria and archaea, type II CRISPR systems generally comprise an RNA-guided nuclease protein such as Cas9, a CRISPR RNA (crRNA) that includes a 5' region that is complementary to a foreign sequence, and a trans-activating crRNA (tracrRNA) that includes a 5' region that is complementary to, and forms a duplex with, a 3' region of the crRNA. While not intending to be bound by any theory, it is thought that this duplex facilitates the formation of — and is necessary for the activity of — the Cas9/gRNA complex. As type II CRISPR systems were adapted for use in gene editing, it was discovered that the crRNA and tracrRNA could be joined into a single unimolecular or chimeric guide RNA, in one non-limiting example, by means of a four nucleotide (e.g., GAAA) "tetraloop" or "linker" sequence bridging complementary regions of the crRNA (at its 3' end) and the tracrRNA (at its 5' end). (Mali et al. Science. 2013 Feb 15; 339(6121): 823–826 ("Mali"); Jiang et al. Nat Biotechnol. 2013 Mar; 31(3): 233–239 ("Jiang"); and Jinek et al., 2012 Science Aug. 17; 337(6096): 816-821 ("Jinek 2012")).

[0192] Guide RNAs, whether unimolecular or modular, include a "targeting domain" that is fully or partially complementary to a target domain within a target sequence, such as a DNA sequence in the genome of a cell where editing is desired. Targeting domains are referred to by various names in the literature, including without limitation "guide sequences" (Hsu et al., Nat Biotechnol. 2013 Sep; 31(9): 827–832, ("Hsu")), "complementarity regions" (Cotta-Ramusino), "spacers" (Briner) and generically as "crRNAs" (Jiang). Irrespective of the names they are given, targeting domains are typically 10-30 nucleotides in length, and in certain embodiments are 16-24 nucleotides in length (for instance, 16, 17, 18, 19, 20, 21, 22, 23 or 24 nucleotides in length), and are at or near the 5' terminus of in the case of a Cas9 gRNA, and at or near the 3' terminus in the case of a Cpf1 gRNA.

[0193] In addition to the targeting domains, gRNAs typically (but not necessarily, as discussed below) include a plurality of domains that may influence the formation or activity of gRNA/Cas9 complexes. For instance, as mentioned above, the duplexed structure formed by first and secondary complementarity domains of a gRNA (also referred to as a repeat:anti-repeat duplex) interacts with the recognition (REC) lobe of Cas9 and can mediate the formation of Cas9/gRNA complexes. (Nishimasu et al., Cell 156, 935-949, February 27, 2014 ("Nishimasu 2014") and Nishimasu et al., Cell 162, 1113-1126, August 27, 2015 ("Nishimasu 2015")). It should be noted that the first and/or second complementarity domains may contain one or more poly-A tracts, which can be recognized by RNA polymerases as a termination signal. The sequence of the first and second complementarity domains are, therefore, optionally modified to eliminate these tracts and promote the complete in vitro transcription of gRNAs, for instance through the use of A-G swaps as described in Briner, or A-U swaps. These and other similar modifications to the first and second complementarity domains are within the scope of the present disclosure.

[0194] Along with the first and second complementarity domains, Cas9 gRNAs typically include two or more additional duplexed regions that are involved in nuclease activity in vivo but not necessarily in vitro. (Nishimasu 2015). A first stem-loop one near the 3' portion of the second complementarity domain is referred to variously as the "proximal domain," (Cotta-Ramusino) "stem loop 1" (Nishimasu 2014 and 2015) and the "nexus" (Briner). One or more additional stem loop structures are generally present near the 3' end of the gRNA, with the number varying by species: s. pyogenes gRNAs typically include two 3'

stem loops (for a total of four stem loop structures including the repeat:anti-repeat duplex), while *S. aureus* and other species have only one (for a total of three stem loop structures). A description of conserved stem loop structures (and gRNA structures more generally) organized by species is provided in Briner.

[0195] While the foregoing description has focused on gRNAs for use with Cas9, it should be appreciated that other RNA-guided nucleases have been (or may in the future be) discovered or invented which utilize gRNAs that differ in some ways from those described to this point. For instance, Cpf1 ("CRISPR from Prevotella and Franciscella 1") is a recently discovered RNA-guided nuclease that does not require a tracrRNA to function. (Zetsche et al., 2015, Cell 163, 759–771 October 22, 2015 ("Zetsche I")). A gRNA for use in a Cpf1 genome editing system generally includes a targeting domain and a complementarity domain (alternately referred to as a "handle"). It should also be noted that, in gRNAs for use with Cpf1, the targeting domain is usually present at or near the 3' end, rather than the 5' end as described above in connection with Cas9 gRNAs (the handle is at or near the 5' end of a Cpf1 gRNA).

[0196] Those of skill in the art will appreciate, however, that although structural differences may exist between gRNAs from different prokaryotic species, or between Cpf1 and Cas9 gRNAs, the principles by which gRNAs operate are generally consistent. Because of this consistency of operation, gRNAs can be defined, in broad terms, by their targeting domain sequences, and skilled artisans will appreciate that a given targeting domain sequence can be incorporated in any suitable gRNA, including a unimolecular or chimeric gRNA, or a gRNA that includes one or more chemical modifications and/or sequential modifications (substitutions, additional nucleotides, truncations, etc.). Thus, for economy of presentation in this disclosure, gRNAs may be described solely in terms of their targeting domain sequences.

[0197] More generally, skilled artisans will appreciate that some aspects of the present disclosure relate to systems, methods and compositions that can be implemented using multiple RNA-guided nucleases. For this reason, unless otherwise specified, the term gRNA should be understood to encompass any suitable gRNA that can be used with any RNA-guided nuclease, and not only those gRNAs that are compatible with a particular species of Cas9 or Cpf1. By way of illustration, the term gRNA can, in certain embodiments, include a gRNA for use with any RNA-guided nuclease occurring in a Class 2 CRISPR

system, such as a type II or type V or CRISPR system, or an RNA-guided nuclease derived or adapted therefrom.

gRNA design

[0198] Methods for selection and validation of target sequences as well as off-target analyses have been described previously, e.g., in Mali; Hsu; Fu et al., 2014 Nat Biotechnol 32(3): 279-84, Heigwer et al., 2014 Nat methods 11(2):122-3; Bae et al. (2014) Bioinformatics 30(10): 1473-5; and Xiao A et al. (2014) Bioinformatics 30(8): 1180-1182. As a non-limiting example, gRNA design may involve the use of a software tool to optimize the choice of potential target sequences corresponding to a user's target sequence, e.g., to minimize total off-target activity across the genome. While off-target activity is not limited to cleavage, the cleavage efficiency at each off-target sequence can be predicted, e.g., using an experimentally-derived weighting scheme. These and other guide selection methods are described in detail in Maeder and Cotta-Ramusino.

[0199] For example, methods for selection and validation of target sequences as well as off-target analyses can be performed using cas-offinder (Bae S, Park J, Kim J-S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics. 2014;30:1473–5). Cas-offinder is a tool that can quickly identify all sequences in a genome that have up to a specified number of mismatches to a guide sequence.

[0200] As another example, methods for scoring how likely a given sequence is to be an off-target (e.g., once candidate target sequences are identified) can be performed. An exemplary score includes a Cutting Frequency Determination (CFD) score, as described by Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol. 2016;34:184–91.

gRNA modifications

[0201] In certain embodiments, gRNAs as used herein may be modified or unmodified gRNAs. In certain embodiments, a gRNA may include one or more modifications. In certain embodiments, the one or more modifications may include a

phosphorothioate linkage modification, a phosphorodithioate (PS2) linkage modification, a 2'-O-methyl modification, or combinations thereof. In certain embodiments, the one or more modifications may be at the 5' end of the gRNA, at the 3' end of the gRNA, or combinations thereof.

[0202] In certain embodiments, a gRNA modification may comprise one or more phosphorodithioate (PS2) linkage modifications.

[0203] In some embodiments, a gRNA used herein includes one or more or a stretch of deoxyribonucleic acid (DNA) bases, also referred to herein as a "DNA extension." In some embodiments, a gRNA used herein includes a DNA extension at the 5' end of the gRNA, the 3' end of the gRNA, or a combination thereof. In certain embodiments, the DNA extension may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 DNA bases long. For example, in certain embodiments, the DNA extension may be 1, 2, 3, 4, 5, 10, 15, 20, or 25 DNA bases long. In certain embodiments, the DNA extension may include one or more DNA bases selected from adenine (A), guanine (G), cytosine (C), or thymine (T). In certain embodiments, the DNA extension includes the same DNA bases. For example, the DNA extension may include a stretch of adenine (A) bases. In certain embodiments, the DNA extension may include a stretch of thymine (T) bases. In certain embodiments, the DNA extension includes a combination of different DNA bases. In certain embodiments, a DNA extension may comprise a sequence set forth in Table 3.

[0204] Exemplary suitable 5' extensions for Cpf1 guide RNAs are provided in Table 3 below:

Table 3: Exemplary Cpf1 gRNA 5' Extensions

SEQ ID NO:	5' extension sequence	5' modification
1	rCrUrUrUrU	+5 RNA
2	rArArGrArCrCrUrUrUrU	+10 RNA

3	rArUrGrUrGrUrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrUrU	+25 RNA
4	rArGrGrCrCrArGrCrUrUrGrCrCrGrGrUrUrUrUrUrUrArGrUrCrGrUrGrCrUrGrCrUrUrCrArUrGrUrGrUrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrUrU	+60 RNA
5	CTTTT	+5 DNA
6	AAGACCTTTT	+10 DNA
7	ATGTGTTTTTGTCAAAAGACCTTTT	+25 DNA
8	AGGCCAGCTTGCCGGTTTTTTAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAGACCTTTT	+60 DNA
9	TTTTTGTCAAAAGACCTTTT	+20 DNA
10	GCTTCATGTGTTTTTGTCAAAAGACCTTTT	+30 DNA
11	GCCGGTTTTTTAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAGACCTTTT	+50 DNA
12	TAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAGACCTTTT	+40 DNA
13	C*C*GAAGTTTTCTTCGGTTTT	+20 DNA + 2xPS
14	T*T*TTTCCGAAGTTTTCTTCGGTTTT	+25 DNA + 2xPS
15	A*A*CGCTTTTTCCGAAGTTTTCTTCGGTTTT	+30 DNA + 2xPS
16	G*C*GTTGTTTTCAACGCTTTTTCCGAAGTTTTCTTCGGTTTT	+41 DNA + 2xPS
17	G*G*CTTCTTTTGAAGCCTTTTTGCGTTGTTTTCAACGCTTTTTCCGAAGTTTTCTTCGGTTTT	+62 DNA + 2xPS
18	A*T*GTGTTTTTGTCAAAAGACCTTTT	+25 DNA + 2xPS
19	AAAAAAAAAAAAAAAAAAAAAAAAA	+25 A
20	TTTTTTTTTTTTTTTTTTTTTTTTT	+25 T

21	mA*mU*rGrUrGrUrUrUrUrGrUrCrArArArArGrArCrCr UrUrUrU	+25 RNA + 2xPS
22	mA*mA*rArArArArArArArArArArArArArArArArArArAr ArArArA	PolyA RNA + 2xPS
23	mU*mU*rUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUr UrUrUrU	PolyU RNA + 2xPS
All bases are in upper case Lowercase "r" represents RNA, 2'-hydroxy; bases not modified by an "r" are DNA All bases are linked via standard phosphodiester bonds except as noted: "*" represents phosphorothioate modification "PS" represents phosphorothioate modification		

[0205] In certain embodiments, a gRNA used herein includes a DNA extension as well as a chemical modification, e.g., one or more phosphorothioate linkage modifications, one or more phosphorodithioate (PS2) linkage modifications, one or more 2'-O-methyl modifications, or one or more additional suitable chemical gRNA modification disclosed herein, or combinations thereof. In certain embodiments, the one or more modifications may be at the 5' end of the gRNA, at the 3' end of the gRNA, or combinations thereof.

[0206] Without wishing to be bound by theory, it is contemplated that any DNA extension may be used with any gRNA disclosed herein, so long as it does not hybridize to the target nucleic acid being targeted by the gRNA and it also exhibits an increase in editing at the target nucleic acid site relative to a gRNA which does not include such a DNA extension.

[0207] In some embodiments, a gRNA used herein includes one or more or a stretch of ribonucleic acid (RNA) bases, also referred to herein as an "RNA extension." In some embodiments, a gRNA used herein includes an RNA extension at the 5' end of the gRNA, the 3' end of the gRNA, or a combination thereof. In certain embodiments, the RNA extension may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51,

52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 RNA bases long. For example, in certain embodiments, the RNA extension may be 1, 2, 3, 4, 5, 10, 15, 20, or 25 RNA bases long. In certain embodiments, the RNA extension may include one or more RNA bases selected from adenine (rA), guanine (rG), cytosine (rC), or uracil (rU), in which the "r" represents RNA, 2'-hydroxy. In certain embodiments, the RNA extension includes the same RNA bases. For example, the RNA extension may include a stretch of adenine (rA) bases. In certain embodiments, the RNA extension includes a combination of different RNA bases. In certain embodiments, a gRNA used herein includes an RNA extension as well as one or more phosphorothioate linkage modifications, one or more phosphorodithioate (PS2) linkage modifications, one or more 2'-O-methyl modifications, one or more additional suitable gRNA modification, e.g., chemical modification, disclosed herein, or combinations thereof. In certain embodiments, the one or more modifications may be at the 5' end of the gRNA, at the 3' end of the gRNA, or combinations thereof. In certain embodiments, a gRNA including a RNA extension may comprise a sequence set forth herein.

[0208] It is contemplated that gRNAs used herein may also include an RNA extension and a DNA extension. In certain embodiments, the RNA extension and DNA extension may both be at the 5' end of the gRNA, the 3' end of the gRNA, or a combination thereof. In certain embodiments, the RNA extension is at the 5' end of the gRNA and the DNA extension is at the 3' end of the gRNA. In certain embodiments, the RNA extension is at the 3' end of the gRNA and the DNA extension is at the 5' end of the gRNA.

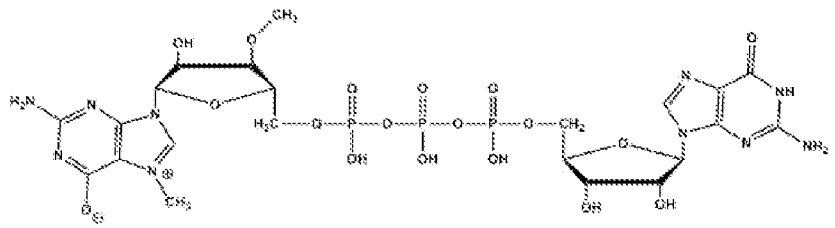
[0209] In some embodiments, a gRNA which includes a modification, e.g., a DNA extension at the 5' end and/or a chemical modification as disclosed herein, is complexed with a RNA-guided nuclease, e.g., an AsCpf1 nuclease, to form an RNP, which is then employed to edit a target cell, e.g., a pluripotent stem cell or a daughter cell thereof.

[0210] Additional suitable gRNA modifications will be apparent to those of ordinary skill in the art based on the present disclosure. Suitable gRNA modifications include, for example, those described in PCT application PCT/US2018/054027, filed on October 2, 2018, and entitled "*MODIFIED CPF1 GUIDE RNA*;" in PCT application PCT/US2015/000143, filed on December 3, 2015, and entitled "*GUIDE RNA WITH CHEMICAL*

MODIFICATIONS;" in PCT application PCT/US2016/026028, filed April 5, 2016, and entitled "*CHEMICALLY MODIFIED GUIDE RNAS FOR CRISPR/CAS-MEDIATED GENE REGULATION*;" and in PCT application PCT/US2016/053344, filed on September 23, 2016, and entitled "*NUCLEASE-MEDIATED GENOME EDITING OF PRIMARY CELLS AND ENRICHMENT THEREOF*;" the entire contents of each of which are incorporated herein by reference.

[0211] Certain exemplary modifications discussed in this section can be included at any position within a gRNA sequence including, without limitation at or near the 5' end (e.g., within 1-10, 1-5, or 1-2 nucleotides of the 5' end) and/or at or near the 3' end (e.g., within 1-10, 1-5, or 1-2 nucleotides of the 3' end). In some cases, modifications are positioned within functional motifs, such as the repeat-anti-repeat duplex of a Cas9 gRNA, a stem loop structure of a Cas9 or Cpf1 gRNA, and/or a targeting domain of a gRNA.

[0212] As one example, the 5' end of a gRNA can include a eukaryotic mRNA cap structure or cap analog (e.g., a G(5')ppp(5')G cap analog, a m7G(5')ppp(5')G cap analog, or a 3'-O-Me-m7G(5')ppp(5')G anti reverse cap analog (ARCA)), as shown below:

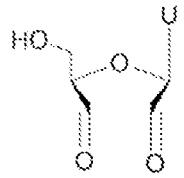


The cap or cap analog can be included during either chemical or enzymatic synthesis of the gRNA.

[0213] Along similar lines, the 5' end of the gRNA can lack a 5' triphosphate group. For instance, *in vitro* transcribed gRNAs can be phosphatase-treated (e.g., using calf intestinal alkaline phosphatase) to remove a 5' triphosphate group.

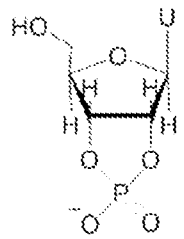
[0214] Another common modification involves the addition, at the 3' end of a gRNA, of a plurality (e.g., 1-10, 10-20, or 25-200) of adenine (A) residues referred to as a polyA tract. The polyA tract can be added to a gRNA during chemical or enzymatic synthesis, using a polyadenosine polymerase (e.g., E. coli Poly(A)Polymerase).

[0215] Guide RNAs can be modified at a 3' terminal U ribose. For example, the two terminal hydroxyl groups of the U ribose can be oxidized to aldehyde groups and a concomitant opening of the ribose ring to afford a modified nucleoside as shown below:



wherein "U" can be an unmodified or modified uridine.

[0216] The 3' terminal U ribose can be modified with a 2'3' cyclic phosphate as shown below:



wherein "U" can be an unmodified or modified uridine.

[0217] Guide RNAs can contain 3' nucleotides that can be stabilized against degradation, e.g., by incorporating one or more of the modified nucleotides described herein. In certain embodiments, uridines can be replaced with modified uridines, e.g., 5-(2-amino)propyl uridine, and 5-bromo uridine, or with any of the modified uridines described herein; adenosines and guanosines can be replaced with modified adenosines and guanosines, e.g., with modifications at the 8-position, e.g., 8-bromo guanosine, or with any of the modified adenosines or guanosines described herein.

[0218] In certain embodiments, sugar-modified ribonucleotides can be incorporated into a gRNA, e.g., wherein the 2' OH-group is replaced by a group selected from H, -OR, -R (wherein R can be, e.g., alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), halo, -SH, -SR (wherein R can be, e.g., alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), amino (wherein amino can be, e.g., NH_2, alkylamino, dialkylamino, heterocyclyl, arylamino, diarylamino, heteroarylamino, diheteroarylamino, or amino acid); or cyano (-CN). In certain

embodiments, the phosphate backbone can be modified as described herein, e.g., with a phosphothioate (PhTx) group. In certain embodiments, one or more of the nucleotides of the gRNA can each independently be a modified or unmodified nucleotide including, but not limited to 2'-sugar modified, such as, 2'-O-methyl, 2'-O-methoxyethyl, or 2'-Fluoro modified including, e.g., 2'-F or 2'-O-methyl, adenosine (A), 2'-F or 2'-O-methyl, cytidine (C), 2'-F or 2'-O-methyl, uridine (U), 2'-F or 2'-O-methyl, thymidine (T), 2'-F or 2'-O-methyl, guanosine (G), 2'-O-methoxyethyl-5-methyluridine (Teo), 2'-O-methoxyethyladenosine (Aeo), 2'-O-methoxyethyl-5-methylcytidine (m5Ceo), and any combinations thereof.

[0219] Guide RNAs can also include "locked" nucleic acids (LNA) in which the 2' OH-group can be connected, e.g., by a C1-6 alkylene or C1-6 heteroalkylene bridge, to the 4' carbon of the same ribose sugar. Any suitable moiety can be used to provide such bridges, including without limitation methylene, propylene, ether, or amino bridges; O-amino (wherein amino can be, e.g., NH_2, alkylamino, dialkylamino, heterocyclyl, arylamino, diarylamino, heteroarylamino, or diheteroarylamino, ethylenediamine, or polyamino) and aminoalkoxy or $O(CH_2)_n$-amino (wherein amino can be, e.g., NH_2, alkylamino, dialkylamino, heterocyclyl, arylamino, diarylamino, heteroarylamino, or diheteroarylamino, ethylenediamine, or polyamino).

[0220] In certain embodiments, a gRNA can include a modified nucleotide which is multicyclic (e.g., tricyclo; and "unlocked" forms, such as glycol nucleic acid (GNA) (e.g., R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), or threose nucleic acid (TNA, where ribose is replaced with α-L-threofuranosyl-(3'→2')).

[0221] Generally, gRNAs include the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary modified gRNAs can include, without limitation, replacement of the oxygen in ribose (e.g., with sulfur (S), selenium (Se), or alkylene, such as, e.g., methylene or ethylene); addition of a double bond (e.g., to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (e.g., to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (e.g., to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for example, anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone). Although the majority of sugar analog alterations are localized to the 2' position, other sites

are amenable to modification, including the 4' position. In certain embodiments, a gRNA comprises a 4'-S, 4'-Se or a 4'-C-aminomethyl-2'-O-Me modification.

[0222] In certain embodiments, deaza nucleotides, e.g., 7-deaza-adenosine, can be incorporated into a gRNA. In certain embodiments, O- and N-alkylated nucleotides, e.g., N6-methyl adenosine, can be incorporated into a gRNA. In certain embodiments, one or more or all of the nucleotides in a gRNA are deoxynucleotides.

[0223] Guide RNAs can also include one or more cross-links between complementary regions of the crRNA (at its 3' end) and the tracrRNA (at its 5' end) (e.g., within a "tetraloop" structure and/or positioned in any stem loop structure occurring within a gRNA). A variety of linkers are suitable for use. For example, guide RNAs can include common linking moieties including, without limitation, polyvinylether, polyethylene, polypropylene, polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyglycolide (PGA), polylactide (PLA), polycaprolactone (PCL), and copolymers thereof.

[0224] In some embodiments, a bifunctional cross-linker is used to link a 5' end of a first gRNA fragment and a 3' end of a second gRNA fragment, and the 3' or 5' ends of the gRNA fragments to be linked are modified with functional groups that react with the reactive groups of the cross-linker. In general, these modifications comprise one or more of amine, sulfhydryl, carboxyl, hydroxyl, alkene (e.g., a terminal alkene), azide and/or another suitable functional group. Multifunctional (e.g. bifunctional) cross-linkers are also generally known in the art, and may be either heterofunctional or homofunctional, and may include any suitable functional group, including without limitation isothiocyanate, isocyanate, acyl azide, an NHS ester, sulfonyl chloride, tosyl ester, tresyl ester, aldehyde, amine, epoxide, carbonate (e.g., Bis(p-nitrophenyl) carbonate), aryl halide, alkyl halide, imido ester, carboxylate, alkyl phosphate, anhydride, fluorophenyl ester, HOBt ester, hydroxymethyl phosphine, O-methylisourea, DSC, NHS carbamate, glutaraldehyde, activated double bond, cyclic hemiacetal, NHS carbonate, imidazole carbamate, acyl imidazole, methylpyridinium ether, azlactone, cyanate ester, cyclic imidocarbonate, chlorotriazine, dehydroazepine, 6-sulfo-cytosine derivatives, maleimide, aziridine, TNB thiol, Ellman's reagent, peroxide, vinylsulfone, phenylthioester, diazoalkanes, diazoacetyl, epoxide, diazonium, benzophenone, anthraquinone, diazo derivatives, diazirine derivatives, psoralen derivatives, alkene, phenyl boronic acid, etc. In some embodiments, a first gRNA fragment comprises a first reactive

group and the second gRNA fragment comprises a second reactive group. For example, the first and second reactive groups can each comprise an amine moiety, which are crosslinked with a carbonate-containing bifunctional crosslinking reagent to form a urea linkage. In other instances, (a) the first reactive group comprises a bromoacetyl moiety and the second reactive group comprises a sulfhydryl moiety, or (b) the first reactive group comprises a sulfhydryl moiety and the second reactive group comprises a bromoacetyl moiety, which are crosslinked by reacting the bromoacetyl moiety with the sulfhydryl moiety to form a bromoacetyl-thiol linkage. These and other cross-linking chemistries are known in the art, and are summarized in the literature, including by Greg T. Hermanson, Bioconjugate Techniques, 3rd Ed. 2013, published by Academic Press.

[0225] Additional suitable gRNA modifications will be apparent to those of ordinary skill in the art based on the present disclosure. Suitable gRNA modifications include, for example, those described in PCT application PCT/US2018/054027, filed on October 2, 2018, and entitled "*MODIFIED CPF1 GUIDE RNA*;" in PCT application PCT/US2015/000143, filed on December 3, 2015, and entitled "*GUIDE RNA WITH CHEMICAL MODIFICATIONS*;" in PCT application PCT/US2016/026028, filed April 5, 2016, and entitled "*CHEMICALLY MODIFIED GUIDE RNAS FOR CRISPR/CAS-MEDIATED GENE REGULATION*;" and in PCT application PCT/US2016/053344, filed on September 23, 2016, and entitled "*NUCLEASE-MEDIATED GENOME EDITING OF PRIMARY CELLS AND ENRICHMENT THEREOF*;" the entire contents of each of which are incorporated herein by reference.

Exemplary gRNAs

[0226] Non-limiting examples of guide RNAs suitable for certain embodiments embraced by the present disclosure are provided herein, for example, in the Tables below. Those of ordinary skill in the art will be able to envision suitable guide RNA sequences for a specific nuclease, e.g., a Cas9 or Cpf-1 nuclease, from the disclosure of the targeting domain sequence, either as a DNA or RNA sequence. For example, a guide RNA comprising a targeting sequence consisting of RNA nucleotides would include the RNA sequence corresponding to the targeting domain sequence provided as a DNA sequence, and this contain uracil instead of thymidine nucleotides. For example, a guide RNA comprising a targeting domain sequence consisting of RNA nucleotides, and described by the DNA

sequence TCTGCAGAAATGTTCCCCGT (SEQ ID NO: 24) would have a targeting domain of the corresponding RNA sequence UCUGCAGAAAUGUUCCCCGU (SEQ ID NO: 25). As will be apparent to the skilled artisan, such a targeting sequence would be linked to a suitable guide RNA scaffold, e.g., a crRNA scaffold sequence or a chimeric crRNA/tracrRNA scaffold sequence. Suitable gRNA scaffold sequences are known to those of ordinary skill in the art. For AsCpf1, for example, a suitable scaffold sequence comprises the sequence UAAUUUCUACUCUUGUAGAU (SEQ ID NO: 26) added to the 5'- terminus of the targeting domain. In the example above, this would result in a Cpf1 guide RNA of the sequence UAAUUUCUACUCUUGUAGAUUCUGCAGAAAUGUUCCCCGU (SEQ ID NO: 27). Those of skill in the art would further understand how to modify such a guide RNA, e.g., by adding a DNA extension (e.g., in the example above, adding a 25-mer DNA extension as described herein would result, for example, in a guide RNA of the sequence ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArU rUrCrUrGrCrArGrArArArUrGrUrUrCrCrCrCrGrU) (SEQ ID NO: 28). It will be understood that the exemplary targeting sequences provided herein are not limiting, and additional suitable sequences, e.g., variants of the specific sequences disclosed herein, will be apparent to the skilled artisan based on the present disclosure in view of the general knowledge in the art.

[0227] In some embodiments the gRNA for use in the disclosure is a gRNA targeting TGFβRII (TGFβRII gRNA). In some embodiments, the gRNA targeting TGFβRII is one or more of the gRNAs described in Table 4.

Table 4: Exemplary TGFβRII gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme	SEQ ID NO:
TGFBR24326	CAGGACGATGTGCAGCGGCC	20	AsCpf1 RR	29
TGFBR24327	ACCGCACGTTCAGAAGTCGG	20	AsCpf1 RR	30
TGFBR24328	ACAACTGTGTAAATTTTGTG	20	AsCpf1 RR	31
TGFBR24329	CAACTGTGTAAATTTTGTGA	20	AsCpf1 RR	32
TGFBR24330	ACCTGTGACAACCAGAAATC	20	AsCpf1 RR	33
TGFBR24331	CCTGTGACAACCAGAAATCC	20	AsCpf1 RR	34

TGFBR24332	TGTGGCTTCTCACAGATGGA	20	AsCpf1 RR	35
TGFBR24333	TCTGTGAGAAGCCACAGGAA	20	AsCpf1 RR	36
TGFBR24334	AAGCTCCCCTACCATGACTT	20	AsCpf1 RR	37
TGFBR24335	GAATAAAGTCATGGTAGGGG	20	AsCpf1 RR	38
TGFBR24336	AGAATAAAGTCATGGTAGGG	20	AsCpf1 RR	39
TGFBR24337	CTACCATGACTTTATTCTGG	20	AsCpf1 RR	40
TGFBR24338	TACCATGACTTTATTCTGGA	20	AsCpf1 RR	41
TGFBR24339	TAATGCACTTTGGAGAAGCA	20	AsCpf1 RR	42
TGFBR24340	TTCATAATGCACTTTGGAGA	20	AsCpf1 RR	43
TGFBR24341	AAGTGCATTATGAAGGAAAA	20	AsCpf1 RR	44
TGFBR24342	TGTGTTCCTGTAGCTCTGAT	20	AsCpf1 RR	45
TGFBR24343	TGTAGCTCTGATGAGTGCAA	20	AsCpf1 RR	46
TGFBR24344	AGTGACAGGCATCAGCCTCC	20	AsCpf1 RR	47
TGFBR24345	AGTGGTGGCAGGAGGCTGAT	20	AsCpf1 RR	48
TGFBR24346	AGGTTGAACTCAGCTTCTGC	20	AsCpf1 RR	49
TGFBR24347	CAGGTTGAACTCAGCTTCTG	20	AsCpf1 RR	50
TGFBR24348	ACCTGGGAAACCGGCAAGAC	20	AsCpf1 RR	51
TGFBR24349	CGTCTTGCCGGTTTCCCAGG	20	AsCpf1 RR	52
TGFBR24350	GCGTCTTGCCGGTTTCCCAG	20	AsCpf1 RR	53
TGFBR24351	TGAGCTTCCGCGTCTTGCCG	20	AsCpf1 RR	54
TGFBR24352	GCGAGCACTGTGCCATCATC	20	AsCpf1 RR	55
TGFBR24353	GGATGATGGCACAGTGCTCG	20	AsCpf1 RR	56
TGFBR24354	AGGATGATGGCACAGTGCTC	20	AsCpf1 RR	57
TGFBR24355	CGTGTGCCAACAACATCAAC	20	AsCpf1 RR	58
TGFBR24356	GCTCAATGGGCAGCAGCTCT	20	AsCpf1 RR	59

TGFBR24357	ACCAGGGTGTCCAGCTCAAT	20	AsCpf1 RR	60
TGFBR24358	CACCAGGGTGTCCAGCTCAA	20	AsCpf1 RR	61
TGFBR24359	CCACCAGGGTGTCCAGCTCA	20	AsCpf1 RR	62
TGFBR24360	GCTTGGCCTTATAGACCTCA	20	AsCpf1 RR	63
TGFBR24361	GAGCAGTTTGAGACAGTGGC	20	AsCpf1 RR	64
TGFBR24362	AGAGGCATACTCCTCATAGG	20	AsCpf1 RR	65
TGFBR24363	CTATGAGGAGTATGCCTCTT	20	AsCpf1 RR	66
TGFBR24364	AAGAGGCATACTCCTCATAG	20	AsCpf1 RR	67
TGFBR24365	TATGAGGAGTATGCCTCTTG	20	AsCpf1 RR	68
TGFBR24366	GATTGATGTCTGAGAAGATG	20	AsCpf1 RR	69
TGFBR24367	CTCCTCAGCCGTCAGGAACT	20	AsCpf1 RR	70
TGFBR24368	GTTCCTGACGGCTGAGGAGC	20	AsCpf1 RR	71
TGFBR24369	GCTCCTCAGCCGTCAGGAAC	20	AsCpf1 RR	72
TGFBR24370	TGACGGCTGAGGAGCGGAAG	20	AsCpf1 RR	73
TGFBR24371	TCTTCCGCTCCTCAGCCGTC	20	AsCpf1 RR	74
TGFBR24372	AACTCCGTCTTCCGCTCCTC	20	AsCpf1 RR	75
TGFBR24373	CAACTCCGTCTTCCGCTCCT	20	AsCpf1 RR	76
TGFBR24374	CCAACTCCGTCTTCCGCTCC	20	AsCpf1 RR	77
TGFBR24375	ACGCCAAGGGCAACCTACAG	20	AsCpf1 RR	78
TGFBR24376	CGCCAAGGGCAACCTACAGG	20	AsCpf1 RR	79
TGFBR24377	AGCTGATGACATGCCGCGTC	20	AsCpf1 RR	80
TGFBR24378	GGGCGAGGGAGCTGCCCAGC	20	AsCpf1 RR	81
TGFBR24379	CGGGCGAGGGAGCTGCCCAG	20	AsCpf1 RR	82
TGFBR24380	CCGGGCGAGGGAGCTGCCCA	20	AsCpf1 RR	83
TGFBR24381	TCGCCCGGGGGATTGCTCAC	20	AsCpf1 RR	84

TGFBR24382	ACATGGAGTGTGATCACTGT	20	AsCpf1 RR	85
TGFBR24383	CAGTGATCACACTCCATGTG	20	AsCpf1 RR	86
TGFBR24384	TGTGGGAGGCCCAAGATGCC	20	AsCpf1 RR	87
TGFBR24385	TGTGCACGATGGGCATCTTG	20	AsCpf1 RR	88
TGFBR24386	CGAGGATATTGGAGCTCTTG	20	AsCpf1 RR	89
TGFBR24387	ATATCCTCGTGAAGAACGAC	20	AsCpf1 RR	90
TGFBR24388	GACGCAGGGAAAGCCCAAAG	20	AsCpf1 RR	91
TGFBR24389	CTGCGTCTGGACCCTACTCT	20	AsCpf1 RR	92
TGFBR24390	TGCGTCTGGACCCTACTCTG	20	AsCpf1 RR	93
TGFBR24391	CAGACAGAGTAGGGTCCAGA	20	AsCpf1 RR	94
TGFBR24392	GCCAGCACGATCCCACCGCA	20	AsCpf1 RVR	95
TGFBR24393	AAGGAAAAAAAAAAGCCTGG	20	AsCpf1 RVR	96
TGFBR24394	ACACCAGCAATCCTGACTTG	20	AsCpf1 RVR	97
TGFBR24395	ACTAGCAACAAGTCAGGATT	20	AsCpf1 RVR	98
TGFBR24396	GCAACTCCCAGTGGTGGCAG	20	AsCpf1 RVR	99
TGFBR24397	TGTCATCATCATCTTCTACT	20	AsCpf1 RVR	100
TGFBR24398	GACCTCAGCAAAGCGACCTT	20	AsCpf1 RVR	101
TGFBR24399	AGGCCAAGCTGAAGCAGAAC	20	AsCpf1 RVR	102
TGFBR24400	AGGAGTATGCCTCTTGGAAG	20	AsCpf1 RVR	103
TGFBR24401	CCTCTTGGAAGACAGAGAAG	20	AsCpf1 RVR	104
TGFBR24402	TTCTCATGCTTCAGATTGAT	20	AsCpf1 RVR	105
TGFBR24403	CTCGTGAAGAACGACCTAAC	20	AsCpf1 RVR	106
TGFbR2036	GGCCGCTGCACATCGTCCTG	20	SpyCas9	107
TGFbR2037	GCGGGGTCTGCCATGGGTCG	20	SpyCas9	108
TGFbR2038	AGTTGCTCATGCAGGATTTC	20	SpyCas9	109

TGFbR2039	CCAGAATAAAGTCATGGTAG	20	SpyCas9	110
TGFbR2040	CCCCTACCATGACTTTATTC	20	SpyCas9	111
TGFbR2041	AAGTCATGGTAGGGGAGCTT	20	SpyCas9	112
TGFbR2042	AGTCATGGTAGGGGAGCTTG	20	SpyCas9	113
TGFbR2043	ATTGCACTCATCAGAGCTAC	20	SpyCas9	114
TGFbR2044	CCTAGAGTGAAGAGATTCAT	20	SpyCas9	115
TGFbR2045	CCAATGAATCTCTTCACTCT	20	SpyCas9	116
TGFbR2046	AAAGTCATGGTAGGGGAGCT	20	SpyCas9	117
TGFbR2047	GTGAGCAATCCCCCGGGCGA	20	SpyCas9	118
TGFbR2048	GTCGTTCTTCACGAGGATAT	20	SpyCas9	119
TGFbR2049	GCCGCGTCAGGTACTCCTGT	20	SpyCas9	120
TGFbR2050	GACGCGGCATGTCATCAGCT	20	SpyCas9	121
TGFbR2051	GCTTCTGCTGCCGGTTAACG	20	SpyCas9	122
TGFbR2052	GTGGATGACCTGGCTAACAG	20	SpyCas9	123
TGFbR2053	GTGATCACACTCCATGTGGG	20	SpyCas9	124
TGFbR2054	GCCCATTGAGCTGGACACCC	20	SpyCas9	125
TGFbR2055	GCGGTCATCTTCCAGGATGA	20	SpyCas9	126
TGFbR2056	GGGAGCTGCCCAGCTTGCGC	20	SpyCas9	127
TGFbR2057	GTTGATGTTGTTGGCACACG	20	SpyCas9	128
TGFbR2058	GGCATCTTGGGCCTCCCACA	20	SpyCas9	129
TGFbR2059	GCGGCATGTCATCAGCTGGG	20	SpyCas9	130
TGFbR2060	GCTCCTCAGCCGTCAGGAAC	20	SpyCas9	131
TGFbR2061	GCTGGTGTTATATTCTGATG	20	SpyCas9	132
TGFbR2062	CCGACTTCTGAACGTGCGGT	20	SpyCas9	133
TGFbR2063	TGCTGGCGATACGCGTCCAC	20	SpyCas9	134

TGFbR2064	CCCGACTTCTGAACGTGCGG	20	SpyCas9	135
TGFbR2065	CCACCGCACGTTCAGAAGTC	20	SpyCas9	136
TGFbR2066	TCACCCGACTTCTGAACGTG	20	SpyCas9	137
TGFbR2067	CCCACCGCACGTTCAGAAGT	20	SpyCas9	138
TGFbR2068	CGAGCAGCGGGGTCTGCCAT	20	SpyCas9	139
TGFbR2069	ACGAGCAGCGGGGTCTGCCA	20	SpyCas9	140
TGFbR2070	AGCGGGGTCTGCCATGGGTC	20	SpyCas9	141
TGFbR2071	CCTGAGCAGCCCCCGACCCA	20	SpyCas9	142
TGFbR2072	CCATGGGTCGGGGGCTGCTC	20	SpyCas9	143
TGFbR2073	AACGTGCGGTGGGATCGTGC	20	SpyCas9	144
TGFbR2074	GGACGATGTGCAGCGGCCAC	20	SpyCas9	145
TGFbR2075	GTCCACAGGACGATGTGCAG	20	SpyCas9	146
TGFbR2076	CATGGGTCGGGGGCTGCTCA	20	SpyCas9	147
TGFbR2077	CAGCGGGGTCTGCCATGGGT	20	SpyCas9	148
TGFbR2078	ATGGGTCGGGGGCTGCTCAG	20	SpyCas9	149
TGFbR2079	CGGGGTCTGCCATGGGTCGG	20	SpyCas9	150
TGFbR2080	AGGAAGTCTGTGTGGCTGTA	20	SpyCas9	151
TGFbR2081	CTCCATCTGTGAGAAGCCAC	20	SpyCas9	152
TGFbR2082	ATGATAGTCACTGACAACAA	20	SpyCas9	153
TGFbR2083	GATGCTGCAGTTGCTCATGC	20	SpyCas9	154
TGFbR2084	ACAGCCACACAGACTTCCTG	20	SpyCas9	155
TGFbR2085	GAAGCCACAGGAAGTCTGTG	20	SpyCas9	156
TGFbR2086	TTCCTGTGGCTTCTCACAGA	20	SpyCas9	157
TGFbR2087	CTGTGGCTTCTCACAGATGG	20	SpyCas9	158
TGFbR2088	TCACAAAATTTACACAGTTG	20	SpyCas9	159

TGFbR2089	GACAACATCATCTTCTCAGA	20	SpyCas9	160
TGFbR2090	TCCAGAATAAAGTCATGGTA	20	SpyCas9	161
TGFbR2091	GGTAGGGGAGCTTGGGGTCA	20	SpyCas9	162
TGFbR2092	TTCTCCAAAGTGCATTATGA	20	SpyCas9	163
TGFbR2093	CATCTTCCAGAATAAAGTCA	20	SpyCas9	164
TGFbR2094	CACATGAAGAAAGTCTCACC	20	SpyCas9	165
TGFbR2095	TTCCAGAATAAAGTCATGGT	20	SpyCas9	166
TGFbR2096	TTTTCCTTCATAATGCACTT	20	SpyCas9	167
TGFBR24024	CACAGTTGTGGAAACTTGAC	20	AsCpf1	168
TGFBR24039	CCCAACTCCGTCTTCCGCTC	20	AsCpf1	169
TGFBR24040	GGCTTTCCCTGCGTCTGGAC	20	AsCpf1	170
TGFBR24036	CTGAGGTCTATAAGGCCAAG	20	AsCpf1	171
TGFBR24026	TGATGTGAGATTTTCCACCT	20	AsCpf1	172
TGFBR24038	CCTATGAGGAGTATGCCTCT	20	AsCpf1	173
TGFBR24033	AAGTGACAGGCATCAGCCTC	20	AsCpf1	174
TGFBR24028	CCATGACCCCAAGCTCCCCT	20	AsCpf1	175
TGFBR24031	CTTCATAATGCACTTTGGAG	20	AsCpf1	176
TGFBR24032	TTCATGTGTTCCTGTAGCTC	20	AsCpf1	177
TGFBR24029	TTCTGGAAGATGCTGCTTCT	20	AsCpf1	178
TGFBR24035	CCCACCAGGGTGTCCAGCTC	20	AsCpf1	179
TGFBR24037	AGACAGTGGCAGTCAAGATC	20	AsCpf1	180
TGFBR24041	CCTGCGTCTGGACCCTACTC	20	AsCpf1	181
TGFBR24025	CACAACTGTGTAAATTTTGT	20	AsCpf1	182
TGFBR24030	GAGAAGCAGCATCTTCCAGA	20	AsCpf1	183
TGFBR24027	TGGTTGTCACAGGTGGAAAA	20	AsCpf1	184

TGFBR24034	CCAGGTTGAACTCAGCTTCT	20	AsCpf1	185
TGFBR24043	ATCACAAAATTTACACAGTTG	21	SauCas9	186
TGFBR24065	GGCATCAGCCTCCTGCCACCA	21	SauCas9	187
TGFBR24110	GTTAGCCAGGTCATCCACAGA	21	SauCas9	188
TGFBR24099	GCTGGGCAGCTCCCTCGCCCG	21	SauCas9	189
TGFBR24064	CAGGAGGCTGATGCCTGTCAC	21	SauCas9	190
TGFBR24094	GAGGAGCGGAAGACGGAGTTG	21	SauCas9	191
TGFBR24108	CGTCTGGACCCTACTCTGTCT	21	SauCas9	192
TGFBR24058	TTTTTCCTTCATAATGCACTT	21	SauCas9	193
TGFBR24075	CCATTGAGCTGGACACCCTGG	21	SauCas9	194
TGFBR24057	CTTCTCCAAAGTGCATTATGA	21	SauCas9	195
TGFBR24103	GCCCAAGATGCCCATCGTGCA	21	SauCas9	196
TGFBR24060	TCATGTGTTCCTGTAGCTCTG	21	SauCas9	197
TGFBR24048	GTGATGCTGCAGTTGCTCATG	21	SauCas9	198
TGFBR24087	TCTCATGCTTCAGATTGATGT	21	SauCas9	199
TGFBR24081	TCCCTATGAGGAGTATGCCTC	21	SauCas9	200
TGFBR24044	CATCACAAAATTTACACAGTT	21	SauCas9	201
TGFBR24077	ATTGAGCTGGACACCCTGGTG	21	SauCas9	202
TGFBR24080	CAGTCAAGATCTTTCCCTATG	21	SauCas9	203
TGFBR24046	AGGATTTCTGGTTGTCACAGG	21	SauCas9	204
TGFBR24101	TCCACAGTGATCACACTCCAT	21	SauCas9	205
TGFBR24079	AGCAGAACACTTCAGAGCAGT	21	SauCas9	206
TGFBR24072	CCGGCAAGACGCGGAAGCTCA	21	SauCas9	207
TGFBR24074	GATGTCAGAGCGGTCATCTTC	21	SauCas9	208
TGFBR24062	TCATTGCACTCATCAGAGCTA	21	SauCas9	209

TGFBR24054	CTTCCAGAATAAAGTCATGGT	21	SauCas9	210
TGFBR24045	AGATTTTCCACCTGTGACAAC	21	SauCas9	211
TGFBR24049	ACTGCAGCATCACCTCCATCT	21	SauCas9	212
TGFBR24098	AGCTGGGCAGCTCCCTCGCCC	21	SauCas9	213
TGFBR24090	TGACGGCTGAGGAGCGGAAGA	21	SauCas9	214
TGFBR24076	CATTGAGCTGGACACCCTGGT	21	SauCas9	215
TGFBR24078	AGCAAAGCGACCTTTCCCCAC	21	SauCas9	216
TGFBR24067	CGCGTTAACCGGCAGCAGAAG	21	SauCas9	217
TGFBR24063	GAAATATGACTAGCAACAAGT	21	SauCas9	218
TGFBR24107	AGACAGAGTAGGGTCCAGACG	21	SauCas9	219
TGFBR24047	CAGGATTTCTGGTTGTCACAG	21	SauCas9	220
TGFBR24096	CTCCTGTAGGTTGCCCTTGGC	21	SauCas9	221
TGFBR24105	ACAGAGTAGGGTCCAGACGCA	21	SauCas9	222
TGFBR24056	GCTTCTCCAAAGTGCATTATG	21	SauCas9	223
TGFBR24068	GCAGCAGAAGCTGAGTTCAAC	21	SauCas9	224
TGFBR24093	TGAGGAGCGGAAGACGGAGTT	21	SauCas9	225
TGFBR24055	CTTTGGAGAAGCAGCATCTTC	21	SauCas9	226
TGFBR24053	CTCCCCTACCATGACTTTATT	21	SauCas9	227
TGFBR24106	GACAGAGTAGGGTCCAGACGC	21	SauCas9	228
TGFBR24092	CTGAGGAGCGGAAGACGGAGT	21	SauCas9	229
TGFBR24102	GGGCATCTTGGGCCTCCCACA	21	SauCas9	230
TGFBR24082	CCAAGAGGCATACTCCTCATA	21	SauCas9	231
TGFBR24051	AGAATGACGAGAACATAACAC	21	SauCas9	232
TGFBR24097	CCTGACGCGGCATGTCATCAG	21	SauCas9	233
TGFBR24073	AGCGAGCACTGTGCCATCATC	21	SauCas9	234

TGFBR24104	GCAGGTTAGGTCGTTCTTCAC	21	SauCas9	235
TGFBR24050	ACCTCCATCTGTGAGAAGCCA	21	SauCas9	236
TGFBR24052	TAAAGTCATGGTAGGGGAGCT	21	SauCas9	237
TGFBR24061	TCAGAGCTACAGGAACACATG	21	SauCas9	238
TGFBR24086	TCTCAGACATCAATCTGAAGC	21	SauCas9	239
TGFBR24066	CATCAGCCTCCTGCCACCACT	21	SauCas9	240
TGFBR24089	CGCTCCTCAGCCGTCAGGAAC	21	SauCas9	241
TGFBR24071	AACCTGGGAAACCGGCAAGAC	21	SauCas9	242
TGFBR24095	TCCACGCCAAGGGCAACCTAC	21	SauCas9	243
TGFBR24100	GAGGTGAGCAATCCCCCGGGC	21	SauCas9	244
TGFBR24069	CAGCAGAAGCTGAGTTCAACC	21	SauCas9	245
TGFBR24083	TCCAAGAGGCATACTCCTCAT	21	SauCas9	246
TGFBR24070	AGCAGAAGCTGAGTTCAACCT	21	SauCas9	247
TGFBR24088	CCAGTTCCTGACGGCTGAGGA	21	SauCas9	248
TGFBR24085	AGGAGTATGCCTCTTGGAAGA	21	SauCas9	249
TGFBR24084	TTCCAAGAGGCATACTCCTCA	21	SauCas9	250
TGFBR24042	CAACTGTGTAAATTTTGTGAT	21	SauCas9	251
TGFBR24059	TGAAGGAAAAAAAAAAGCCTG	21	SauCas9	252
TGFBR24091	CGTCTTCCGCTCCTCAGCCGT	21	SauCas9	253
TGFBR24109	CCAGGTCATCCACAGACAGAG	21	SauCas9	254
TGFBR2736	GCCTAGAGTGAAGAGATTCAT	21	SpyCas9	255
TGFBR2737	GTTCTCCAAAGTGCATTATGA	21	SpyCas9	256
TGFBR2738	GCATCTTCCAGAATAAAGTCA	21	SpyCas9	257
TGFBR2739	TGATGTGAGATTTTCCACCTG	21	Cas12a	1172

[0228] In some embodiments the gRNA for use in the disclosure is a gRNA targeting CISH (CISH gRNA). In some embodiments, the gRNA targeting CISH is one or more of the gRNAs described in Table 5.

Table 5: Exemplary CISH gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme	SEQ ID NO:
CISH0873	CAACCGTCTGGTGGCCGACG	20	SpyCas9	258
CISH0874	CAGGATCGGGGCTGTCGCTT	20	SpyCas9	259
CISH0875	TCGGGCCTCGCTGGCCGTAA	20	SpyCas9	260
CISH0876	GAGGTAGTCGGCCATGCGCC	20	SpyCas9	261
CISH0877	CAGGTGTTGTCGGGCCTCGC	20	SpyCas9	262
CISH0878	GGAGGTAGTCGGCCATGCGC	20	SpyCas9	263
CISH0879	GGCATACTCAATGCGTACAT	20	SpyCas9	264
CISH0880	CCGCCTTGTCATCAACCGTC	20	SpyCas9	265
CISH0881	AGGATCGGGGCTGTCGCTTC	20	SpyCas9	266
CISH0882	CCTTGTCATCAACCGTCTGG	20	SpyCas9	267
CISH0883	TACTCAATGCGTACATTGGT	20	SpyCas9	268
CISH0884	GGGTTCCATTACGGCCAGCG	20	SpyCas9	269
CISH0885	GGCACTGCTTCTGCGTACAA	20	SpyCas9	270
CISH0886	GGTTGATGACAAGGCGGCAC	20	SpyCas9	271
CISH0887	TGCTGGGGCCTTCCTCGAGG	20	SpyCas9	272
CISH0888	TTGCTGGCTGTGGAGCGGAC	20	SpyCas9	273
CISH0889	TTCTCCTACCTTCGGGAATC	20	SpyCas9	274
CISH0890	GACTGGCTTGGGCAGTTCCA	20	SpyCas9	275
CISH0891	CATGCAGCCCTTGCCTGCTG	20	SpyCas9	276
CISH0892	AGCAAAGGACGAGGTCTAGA	20	SpyCas9	277

CISH0893	GCCTGCTGGGGCCTTCCTCG	20	SpyCas9	278
CISH0894	CAGACTCACCAGATTCCCGA	20	SpyCas9	279
CISH0895	ACCTCGTCCTTTGCTGGCTG	20	SpyCas9	280
CISH0896	CTCACCAGATTCCCGAAGGT	20	SpyCas9	281
CISH7048	TACGCAGAAGCAGTGCCCGC	20	AsCpf1	282
CISH7049	AGGTGTACAGCAGTGGCTGG	20	AsCpf1	283
CISH7050	GGTGTACAGCAGTGGCTGGT	20	AsCpf1	284
CISH7051	CGGATGTGGTCAGCCTTGTG	20	AsCpf1	285
CISH7052	CACTGACAGCGTGAACAGGT	20	AsCpf1	286
CISH7053	ACTGACAGCGTGAACAGGTA	20	AsCpf1	287
CISH7054	GCTCACTCTCTGTCTGGGCT	20	AsCpf1	288
CISH7055	CTGGCTGTGGAGCGGACTGG	20	AsCpf1	289
CISH7056	GCTCTGACTGTACGGGGCAA	20	AsCpf1 RR	290
CISH7057	AGCTCTGACTGTACGGGGCA	20	AsCpf1 RR	291
CISH7058	ACAGTACCCCTTCCAGCTCT	20	AsCpf1 RR	292
CISH7059	CGTCGGCCACCAGACGGTTG	20	AsCpf1 RR	293
CISH7060	CCAGCCACTGCTGTACACCT	20	AsCpf1 RR	294
CISH7061	ACCCCGGCCCTGCCTATGCC	20	AsCpf1 RR	295
CISH7062	GGTATCAGCAGTGCAGGAGG	20	AsCpf1 RR	296
CISH7063	GATGTGGTCAGCCTTGTGCA	20	AsCpf1 RR	297
CISH7064	GGATGTGGTCAGCCTTGTGC	20	AsCpf1 RR	298
CISH7065	GGCCACGCATCCTGGCCTTT	20	AsCpf1 RR	299
CISH7066	GAAAGGCCAGGATGCGTGGC	20	AsCpf1 RR	300
CISH7067	ACTGCTTGTCCAGGCCACGC	20	AsCpf1 RR	301
CISH7068	TCTGGACTCCAACTGCTTGT	20	AsCpf1 RR	302

CISH7069	GTCTGGACTCCAACTGCTTG	20	AsCpf1 RR	303
CISH7070	GCTTCCGTCTGGACTCCAAC	20	AsCpf1 RR	304
CISH7071	GACGGAAGCTGGAGTCGGCA	20	AsCpf1 RR	305
CISH7072	CGCTGTCAGTGAAAACCACT	20	AsCpf1 RR	306
CISH7073	CTGACAGCGTGAACAGGTAG	20	AsCpf1 RR	307
CISH7074	TTACGGCCAGCGAGGCCCGA	20	AsCpf1 RR	308
CISH7075	ATTACGGCCAGCGAGGCCCG	20	AsCpf1 RR	309
CISH7076	GGAATCTGGTGAGTCTGAGG	20	AsCpf1 RR	310
CISH7077	CCCTCAGACTCACCAGATTC	20	AsCpf1 RR	311
CISH7078	CGAAGGTAGGAGAAGGTCTT	20	AsCpf1 RR	312
CISH7079	GAAGGTAGGAGAAGGTCTTG	20	AsCpf1 RR	313
CISH7080	GCACCTTTGGCTCACTCTCT	20	AsCpf1 RR	314
CISH7081	TCGAGGAGGTGGCAGAGGGT	20	AsCpf1 RR	315
CISH7082	TGGAACTGCCCAAGCCAGTC	20	AsCpf1 RR	316
CISH7083	AGGGACGGGGCCCACAGGGG	20	AsCpf1 RR	317
CISH7084	GGGACGGGGCCCACAGGGGC	20	AsCpf1 RR	318
CISH7085	CTCCACAGCCAGCAAAGGAC	20	AsCpf1 RR	319
CISH7086	CAGCCAGCAAAGGACGAGGT	20	AsCpf1 RR	320
CISH7087	CTGCCTTCTAGACCTCGTCC	20	AsCpf1 RR	321
CISH7088	CCTAAGGAGGATGCGCCTAG	20	AsCpf1 RVR	322
CISH7089	TGGCCTCCTGCACTGCTGAT	20	AsCpf1 RVR	323
CISH7090	AGCAGTGCAGGAGGCCACAT	20	AsCpf1 RVR	324
CISH7091	CCGACTCCAGCTTCCGTCTG	20	AsCpf1 RVR	325
CISH7092	GGGGTTCCATTACGGCCAGC	20	AsCpf1 RVR	326
CISH7093	CACAGCAGATCCTCCTCTGG	20	AsCpf1 RVR	327

CISH7094	ATTGCCCCGTACAGTCAGAG	20	SauCas9	328
CISH7095	CCCGTACAGTCAGAGCTGGA	20	SauCas9	329
CISH7096	TGGTGGAGGAGCAGGCAGTG	20	SauCas9	330
CISH7097	TCCTTAGGCATAGGCAGGGC	20	SauCas9	331
CISH7098	CGGCCCTGCCTATGCCTAAG	20	SauCas9	332
CISH7099	TAGGCATAGGCAGGGCCGGG	20	SauCas9	333
CISH7100	AGGCAGGGCCGGGGTGGGAG	20	SauCas9	334
CISH7101	GCAGGATCGGGGCTGTCGCT	20	SauCas9	335
CISH7102	CTGCACAAGGCTGACCACAT	20	SauCas9	336
CISH7103	TGCACAAGGCTGACCACATC	20	SauCas9	337
CISH7104	CTGACCACATCCGGAAAGGC	20	SauCas9	338
CISH7105	GGCCACGCATCCTGGCCTTT	20	SauCas9	339
CISH7106	GCGTGGCCTGGACAAGCAGT	20	SauCas9	340
CISH7107	GACAAGCAGTTGGAGTCCAG	20	SauCas9	341
CISH7108	GTTGGAGTCCAGACGGAAGC	20	SauCas9	342
CISH7109	ATGCGTACATTGGTGGGGCC	20	SauCas9	343
CISH7110	TGGCCCCACCAATGTACGCA	20	SauCas9	344
CISH7111	GCTACCTGTTCACGCTGTCA	20	SauCas9	345
CISH7112	TGACAGCGTGAACAGGTAGC	20	SauCas9	346
CISH7113	GTCGGGCCTCGCTGGCCGTA	20	SauCas9	347
CISH7114	GCACTTGCCTAGGCTGGTAT	20	SauCas9	348
CISH7115	GGGAATCTGGTGAGTCTGAG	20	SauCas9	349
CISH7116	CTCACCAGATTCCCGAAGGT	20	SauCas9	350
CISH7117	CTCCTACCTTCGGGAATCTG	20	SauCas9	351
CISH7118	CAAGACCTTCTCCTACCTTC	20	SauCas9	352

CISH7119	CCAAGACCTTCTCCTACCTT	20	SauCas9	353
CISH7120	GCCAAGACCTTCTCCTACCT	20	SauCas9	354
CISH7121	TATGCACAGCAGATCCTCCT	20	SauCas9	355
CISH7122	CAAAGGTGCTGGACCCAGAG	20	SauCas9	356
CISH7123	GGCTCACTCTCTGTCTGGGC	20	SauCas9	357
CISH7124	AGGGTACCCCAGCCCAGACA	20	SauCas9	358
CISH7125	AGAGGGTACCCCAGCCCAGA	20	SauCas9	359
CISH7126	GTACCCTCTGCCACCTCCTC	20	SauCas9	360
CISH7127	CCTTCCTCGAGGAGGTGGCA	20	SauCas9	361
CISH7128	ATGACTGGCTTGGGCAGTTC	20	SauCas9	362
CISH7129	GGCCCCTGTGGGCCCCGTCC	20	SauCas9	363
CISH7130	AGGACGAGGTCTAGAAGGCA	20	SauCas9	364
CISH7131	ACTGACAGCGTGAACAGGTAG	21	Cas12a	1173

[0229] In some embodiments, the gRNA for use in the disclosure is a gRNA targeting B2M (B2M gRNA). In some embodiments, the gRNA targeting B2M is one or more of the gRNAs described in Table 6.

Table 6: Exemplary B2M gRNAs

gRNA name	gRNA Targeting Domain Target sequence (DNA)	Length	Enzyme	SEQ ID NO:
B2M1	TATAAGTGGAGGCGTCGCGC	20	SpyCas9	365
B2M2	GGGCACGCGTTTAATATAAG	20	SpyCas9	366
B2M3	ACTCACGCTGGATAGCCTCC	20	SpyCas9	367
B2M4	GGCCGAGATGTCTCGCTCCG	20	SpyCas9	368
B2M5	CACGCGTTTAATATAAGTGG	20	SpyCas9	369
B2M6	AAGTGGAGGCGTCGCGCTGG	20	SpyCas9	370

B2M7	GAGTAGCGCGAGCACAGCTA	20	SpyCas9	371
B2M8	AGTGGAGGCGTCGCGCTGGC	20	SpyCas9	372
B2M9	GCCCGAATGCTGTCAGCTTC	20	SpyCas9	373
B2M10	CGCGAGCACAGCTAAGGCCA	20	SpyCas9	374
B2M11	CTCGCGCTACTCTCTCTTTC	20	SpyCas9	375
B2M12	GGCCACGGAGCGAGACATCT	20	SpyCas9	376
B2M13	CGTGAGTAAACCTGAATCTT	20	SpyCas9	377
B2M14	AGTCACATGGTTCACACGGC	20	SpyCas9	378
B2M15	AAGTCAACTTCAATGTCGGA	20	SpyCas9	379
B2M16	CAGTAAGTCAACTTCAATGT	20	SpyCas9	380
B2M17	ACCCAGACACATAGCAATTC	20	SpyCas9	381
B2M18	GCATACTCATCTTTTTCAGT	20	SpyCas9	382
B2M19	ACAGCCCAAGATAGTTAAGT	20	SpyCas9	383
B2M20	GGCATACTCATCTTTTTCAG	20	SpyCas9	384
B2M21	TTCCTGAAGCTGACAGCATT	20	SpyCas9	385
B2M22	TCACGTCATCCAGCAGAGAA	20	SpyCas9	386
B2M23	CAGCCCAAGATAGTTAAGTG	20	SpyCas9	387
B2M-c1	AAUUCUCUCUCCAUUCUU	18	AsCpf1	388
B2M-c2	AAUUCUCUCUCCAUUCUUC	19	AsCpf1	389
B2M-c3	AAUUCUCUCUCCAUUCUUCA	20	AsCpf1	390
B2M-c4	AAUUCUCUCUCCAUUCUUCAG	21	AsCpf1	391
B2M-c5	AAUUCUCUCUCCAUUCUUCAGU	22	AsCpf1	392
B2M-c6	AAUUCUCUCUCCAUUCUUCAGUA	23	AsCpf1	393
B2M-c7	AAUUCUCUCUCCAUUCUUCAGUAA	24	AsCpf1	394
B2M-c8	ACUUUCCAUUCUCUGCUG	18	AsCpf1	395

B2M-c9	ACUUUCCAUUCUCUGCUGG	19	AsCpf1	396
B2M-c10	ACUUUCCAUUCUCUGCUGGA	20	AsCpf1	397
B2M-c11	ACUUUCCAUUCUCUGCUGGAU	21	AsCpf1	398
B2M-c12	ACUUUCCAUUCUCUGCUGGAUG	22	AsCpf1	399
B2M-c13	ACUUUCCAUUCUCUGCUGGAUGA	23	AsCpf1	400
B2M-c14	ACUUUCCAUUCUCUGCUGGAUGAC	24	AsCpf1	401
B2M-c15	AGCAAGGACUGGUCUUUC	18	AsCpf1	402
B2M-c16	AGCAAGGACUGGUCUUUCU	19	AsCpf1	403
B2M-c17	AGCAAGGACUGGUCUUUCUA	20	AsCpf1	404
B2M-c18	AGCAAGGACUGGUCUUUCUAU	21	AsCpf1	405
B2M-c19	AGCAAGGACUGGUCUUUCUAUC	22	AsCpf1	406
B2M-c20	AGCAAGGACUGGUCUUUCUAUCU	23	AsCpf1	407
B2M-c21	AGCAAGGACUGGUCUUUCUAUCUC	24	AsCpf1	408
B2M-c22	AGUGGGGGUGAAUUCAGU	18	AsCpf1	409
B2M-c23	AGUGGGGGUGAAUUCAGUG	19	AsCpf1	410
B2M-c24	AGUGGGGGUGAAUUCAGUGU	20	AsCpf1	411
B2M-c25	AGUGGGGGUGAAUUCAGUGUA	21	AsCpf1	412
B2M-c26	AGUGGGGGUGAAUUCAGUGUAG	22	AsCpf1	413
B2M-c27	AGUGGGGGUGAAUUCAGUGUAGU	23	AsCpf1	414
B2M-c28	AGUGGGGGUGAAUUCAGUGUAGUA	24	AsCpf1	415
B2M-c29	AUCCAUCCGACAUUGAAG	18	AsCpf1	416
B2M-c30	AUCCAUCCGACAUUGAAGU	19	AsCpf1	417
B2M-c31	AUCCAUCCGACAUUGAAGUU	20	AsCpf1	418
B2M-c32	AUCCAUCCGACAUUGAAGUUG	21	AsCpf1	419
B2M-c33	AUCCAUCCGACAUUGAAGUUGA	22	AsCpf1	420

B2M-c34	AUCCAUCCGACAUUGAAGUUGAC	23	AsCpf1	421
B2M-c35	AUCCAUCCGACAUUGAAGUUGACU	24	AsCpf1	422
B2M-c36	CAAUUCUCUCUCCAUUCU	18	AsCpf1	423
B2M-c37	CAAUUCUCUCUCCAUUCUU	19	AsCpf1	424
B2M-c38	CAAUUCUCUCUCCAUUCUUC	20	AsCpf1	425
B2M-c39	CAAUUCUCUCUCCAUUCUUCA	21	AsCpf1	426
B2M-c40	CAAUUCUCUCUCCAUUCUUCAG	22	AsCpf1	427
B2M-c41	CAAUUCUCUCUCCAUUCUUCAGU	23	AsCpf1	428
B2M-c42	CAAUUCUCUCUCCAUUCUUCAGUA	24	AsCpf1	429
B2M-c43	CAGUGGGGGUGAAUUCAG	18	AsCpf1	430
B2M-c44	CAGUGGGGGUGAAUUCAGU	19	AsCpf1	431
B2M-c45	CAGUGGGGGUGAAUUCAGUG	20	AsCpf1	432
B2M-c46	CAGUGGGGGUGAAUUCAGUGU	21	AsCpf1	433
B2M-c47	CAGUGGGGGUGAAUUCAGUGUA	22	AsCpf1	434
B2M-c48	CAGUGGGGGUGAAUUCAGUGUAG	23	AsCpf1	435
B2M-c49	CAGUGGGGGUGAAUUCAGUGUAGU	24	AsCpf1	436
B2M-c50	CAUUCUCUGCUGGAUGAC	18	AsCpf1	437
B2M-c51	CAUUCUCUGCUGGAUGACG	19	AsCpf1	438
B2M-c52	CAUUCUCUGCUGGAUGACGU	20	AsCpf1	439
B2M-c53	CAUUCUCUGCUGGAUGACGUG	21	AsCpf1	440
B2M-c54	CAUUCUCUGCUGGAUGACGUGA	22	AsCpf1	441
B2M-c55	CAUUCUCUGCUGGAUGACGUGAG	23	AsCpf1	442
B2M-c56	CAUUCUCUGCUGGAUGACGUGAGU	24	AsCpf1	443
B2M-c57	CCCGAUAUUCCUCAGGUA	18	AsCpf1	444
B2M-c58	CCCGAUAUUCCUCAGGUAC	19	AsCpf1	445

B2M-c59	CCCGAUAUUCCUCAGGUACU	20	AsCpf1	446
B2M-c60	CCCGAUAUUCCUCAGGUACUC	21	AsCpf1	447
B2M-c61	CCCGAUAUUCCUCAGGUACUCC	22	AsCpf1	448
B2M-c62	CCCGAUAUUCCUCAGGUACUCCA	23	AsCpf1	449
B2M-c63	CCCGAUAUUCCUCAGGUACUCCAA	24	AsCpf1	450
B2M-c64	CCGAUAUUCCUCAGGUAC	18	AsCpf1	451
B2M-c65	CCGAUAUUCCUCAGGUACU	19	AsCpf1	452
B2M-c66	CCGAUAUUCCUCAGGUACUC	20	AsCpf1	453
B2M-c67	CCGAUAUUCCUCAGGUACUCC	21	AsCpf1	454
B2M-c68	CCGAUAUUCCUCAGGUACUCCA	22	AsCpf1	455
B2M-c69	CCGAUAUUCCUCAGGUACUCCAA	23	AsCpf1	456
B2M-c70	CCGAUAUUCCUCAGGUACUCCAAA	24	AsCpf1	457
B2M-c71	CUCACGUCAUCCAGCAGA	18	AsCpf1	458
B2M-c72	CUCACGUCAUCCAGCAGAG	19	AsCpf1	459
B2M-c73	CUCACGUCAUCCAGCAGAGA	20	AsCpf1	460
B2M-c74	CUCACGUCAUCCAGCAGAGAA	21	AsCpf1	461
B2M-c75	CUCACGUCAUCCAGCAGAGAAU	22	AsCpf1	462
B2M-c76	CUCACGUCAUCCAGCAGAGAAUG	23	AsCpf1	463
B2M-c77	CUCACGUCAUCCAGCAGAGAAUGG	24	AsCpf1	464
B2M-c78	CUGAAUUGCUAUGUGUCU	18	AsCpf1	465
B2M-c79	CUGAAUUGCUAUGUGUCUG	19	AsCpf1	466
B2M-c80	CUGAAUUGCUAUGUGUCUGG	20	AsCpf1	467
B2M-c81	CUGAAUUGCUAUGUGUCUGGG	21	AsCpf1	468
B2M-c82	CUGAAUUGCUAUGUGUCUGGGU	22	AsCpf1	469
B2M-c83	CUGAAUUGCUAUGUGUCUGGGUU	23	AsCpf1	470

B2M-c84	CUGAAUUGCUAUGUGUCUGGGUUU	24	AsCpf1	471
B2M-c85	GAGUACCUGAGGAAUAUC	18	AsCpf1	472
B2M-c86	GAGUACCUGAGGAAUAUCG	19	AsCpf1	473
B2M-c87	GAGUACCUGAGGAAUAUCGG	20	AsCpf1	474
B2M-c88	GAGUACCUGAGGAAUAUCGGG	21	AsCpf1	475
B2M-c89	GAGUACCUGAGGAAUAUCGGGA	22	AsCpf1	476
B2M-c90	GAGUACCUGAGGAAUAUCGGGAA	23	AsCpf1	477
B2M-c91	GAGUACCUGAGGAAUAUCGGGAAA	24	AsCpf1	478
B2M-c92	UAUCUCUUGUACUACACU	18	AsCpf1	479
B2M-c93	UAUCUCUUGUACUACACUG	19	AsCpf1	480
B2M-c94	UAUCUCUUGUACUACACUGA	20	AsCpf1	481
B2M-c95	UAUCUCUUGUACUACACUGAA	21	AsCpf1	482
B2M-c96	UAUCUCUUGUACUACACUGAAU	22	AsCpf1	483
B2M-c97	UAUCUCUUGUACUACACUGAAUU	23	AsCpf1	484
B2M-c98	UAUCUCUUGUACUACACUGAAUUC	24	AsCpf1	485
B2M-c99	UCAAUUCUCUCUCCAUUC	18	AsCpf1	486
B2M-c100	UCAAUUCUCUCUCCAUUCU	19	AsCpf1	487
B2M-c101	UCAAUUCUCUCUCCAUUCUU	20	AsCpf1	488
B2M-c102	UCAAUUCUCUCUCCAUUCUUC	21	AsCpf1	489
B2M-c103	UCAAUUCUCUCUCCAUUCUUCA	22	AsCpf1	490
B2M-c104	UCAAUUCUCUCUCCAUUCUUCAG	23	AsCpf1	491
B2M-c105	UCAAUUCUCUCUCCAUUCUUCAGU	24	AsCpf1	492
B2M-c106	UCACAGCCCAAGAUAGUU	18	AsCpf1	493
B2M-c107	UCACAGCCCAAGAUAGUUA	19	AsCpf1	494
B2M-c108	UCACAGCCCAAGAUAGUUAA	20	AsCpf1	495

B2M-c109	UCACAGCCCAAGAUAGUUAAG	21	AsCpf1	496
B2M-c110	UCACAGCCCAAGAUAGUUAAGU	22	AsCpf1	497
B2M-c111	UCACAGCCCAAGAUAGUUAAGUG	23	AsCpf1	498
B2M-c112	UCACAGCCCAAGAUAGUUAAGUGG	24	AsCpf1	499
B2M-c113	UCAGUGGGGGUGAAUUCA	18	AsCpf1	500
B2M-c114	UCAGUGGGGGUGAAUUCAG	19	AsCpf1	501
B2M-c115	UCAGUGGGGGUGAAUUCAGU	20	AsCpf1	502
B2M-c116	UCAGUGGGGGUGAAUUCAGUG	21	AsCpf1	503
B2M-c117	UCAGUGGGGGUGAAUUCAGUGU	22	AsCpf1	504
B2M-c118	UCAGUGGGGGUGAAUUCAGUGUA	23	AsCpf1	505
B2M-c119	UCAGUGGGGGUGAAUUCAGUGUAG	24	AsCpf1	506
B2M-c120	UGGCCUGGAGGCUAUCCA	18	AsCpf1	507
B2M-c121	UGGCCUGGAGGCUAUCCAG	19	AsCpf1	508
B2M-c122	UGGCCUGGAGGCUAUCCAGC	20	AsCpf1	509
B2M-c123	UGGCCUGGAGGCUAUCCAGCG	21	AsCpf1	510
B2M-c124	UGGCCUGGAGGCUAUCCAGCGU	22	AsCpf1	511
B2M-c125	UGGCCUGGAGGCUAUCCAGCGUG	23	AsCpf1	512
B2M-c126	UGGCCUGGAGGCUAUCCAGCGUGA	24	AsCpf1	513
B2M-c127	AUAGAUCGAGACAUGUAA	18	AsCpf1	514
B2M-c128	AUAGAUCGAGACAUGUAAG	19	AsCpf1	515
B2M-c129	AUAGAUCGAGACAUGUAAGC	20	AsCpf1	516
B2M-c130	AUAGAUCGAGACAUGUAAGCA	21	AsCpf1	517
B2M-c131	AUAGAUCGAGACAUGUAAGCAG	22	AsCpf1	518
B2M-c132	AUAGAUCGAGACAUGUAAGCAGC	23	AsCpf1	519
B2M-c133	AUAGAUCGAGACAUGUAAGCAGCA	24	AsCpf1	520

B2M-c134	CAUAGAUCGAGACAUGUA	18	AsCpf1	521
B2M-c135	CAUAGAUCGAGACAUGUAA	19	AsCpf1	522
B2M-c136	CAUAGAUCGAGACAUGUAAG	20	AsCpf1	523
B2M-c137	CAUAGAUCGAGACAUGUAAGC	21	AsCpf1	524
B2M-c138	CAUAGAUCGAGACAUGUAAGCA	22	AsCpf1	525
B2M-c139	CAUAGAUCGAGACAUGUAAGCAG	23	AsCpf1	526
B2M-c140	CAUAGAUCGAGACAUGUAAGCAGC	24	AsCpf1	527
B2M-c141	CUCCACUGUCUUUUUCAU	18	AsCpf1	528
B2M-c142	CUCCACUGUCUUUUUCAUA	19	AsCpf1	529
B2M-c143	CUCCACUGUCUUUUUCAUAG	20	AsCpf1	530
B2M-c144	CUCCACUGUCUUUUUCAUAGA	21	AsCpf1	531
B2M-c145	CUCCACUGUCUUUUUCAUAGAU	22	AsCpf1	532
B2M-c146	CUCCACUGUCUUUUUCAUAGAUC	23	AsCpf1	533
B2M-c147	CUCCACUGUCUUUUUCAUAGAUCG	24	AsCpf1	534
B2M-c148	UCAUAGAUCGAGACAUGU	18	AsCpf1	535
B2M-c149	UCAUAGAUCGAGACAUGUA	19	AsCpf1	536
B2M-c150	UCAUAGAUCGAGACAUGUAA	20	AsCpf1	537
B2M-c151	UCAUAGAUCGAGACAUGUAAG	21	AsCpf1	538
B2M-c152	UCAUAGAUCGAGACAUGUAAGC	22	AsCpf1	539
B2M-c153	UCAUAGAUCGAGACAUGUAAGCA	23	AsCpf1	540
B2M-c154	UCAUAGAUCGAGACAUGUAAGCAG	24	AsCpf1	541
B2M-c155	UCCACUGUCUUUUUCAUA	18	AsCpf1	542
B2M-c156	UCCACUGUCUUUUUCAUAG	19	AsCpf1	543
B2M-c157	UCCACUGUCUUUUUCAUAGA	20	AsCpf1	544
B2M-c158	UCCACUGUCUUUUUCAUAGAU	21	AsCpf1	545

B2M-c159	UCCACUGUCUUUUUCAUAGAUC	22	AsCpf1	546
B2M-c160	UCCACUGUCUUUUUCAUAGAUCG	23	AsCpf1	547
B2M-c161	UCCACUGUCUUUUUCAUAGAUCGA	24	AsCpf1	548
B2M-c162	UCUCCACUGUCUUUUUCA	18	AsCpf1	549
B2M-c163	UCUCCACUGUCUUUUUCAU	19	AsCpf1	550
B2M-c164	UCUCCACUGUCUUUUUCAUA	20	AsCpf1	551
B2M-c165	UCUCCACUGUCUUUUUCAUAG	21	AsCpf1	552
B2M-c166	UCUCCACUGUCUUUUUCAUAGA	22	AsCpf1	553
B2M-c167	UCUCCACUGUCUUUUUCAUAGAU	23	AsCpf1	554
B2M-c168	UCUCCACUGUCUUUUUCAUAGAUC	24	AsCpf1	555
B2M-c169	UUCUCCACUGUCUUUUUC	18	AsCpf1	556
B2M-c170	UUCUCCACUGUCUUUUUCA	19	AsCpf1	557
B2M-c171	UUCUCCACUGUCUUUUUCAU	20	AsCpf1	558
B2M-c172	UUCUCCACUGUCUUUUUCAUA	21	AsCpf1	559
B2M-c173	UUCUCCACUGUCUUUUUCAUAG	22	AsCpf1	560
B2M-c174	UUCUCCACUGUCUUUUUCAUAGA	23	AsCpf1	561
B2M-c175	UUCUCCACUGUCUUUUUCAUAGAU	24	AsCpf1	562
B2M-c176	UUUCUCCACUGUCUUUUU	18	AsCpf1	563
B2M-c177	UUUCUCCACUGUCUUUUUC	19	AsCpf1	564
B2M-c178	UUUCUCCACUGUCUUUUUCA	20	AsCpf1	565
B2M-c179	UUUCUCCACUGUCUUUUUCAU	21	AsCpf1	566
B2M-c180	UUUCUCCACUGUCUUUUUCAUA	22	AsCpf1	567
B2M-c181	UUUCUCCACUGUCUUUUUCAUAG	23	AsCpf1	568
B2M-c182	UUUCUCCACUGUCUUUUUCAUAGA	24	AsCpf1	569
B2M-c183	UUUUCUCCACUGUCUUUU	18	AsCpf1	570

B2M-c184	UUUUCUCCACUGUCUUUUU	19	AsCpf1	571
B2M-c185	UUUUCUCCACUGUCUUUUUC	20	AsCpf1	572
B2M-c186	UUUUCUCCACUGUCUUUUUCA	21	AsCpf1	573
B2M-c187	UUUUCUCCACUGUCUUUUUCAU	22	AsCpf1	574
B2M-c188	UUUUCUCCACUGUCUUUUUCAUA	23	AsCpf1	575
B2M-c189	UUUUCUCCACUGUCUUUUUCAUAG	24	AsCpf1	576

[0230] In some embodiments, the gRNA for use in the disclosure is a gRNA targeting PD1. gRNAs targeting B2M and PD1 for use in the disclosure are further described in WO2015161276 and WO2017152015 by Welstead et al.; both incorporated in their entirety herein by reference.

[0231] In some embodiments, the gRNA for use in the disclosure is a gRNA targeting NKG2A (NKG2A gRNA). In some embodiments, the gRNA targeting NKG2A is one or more of the gRNAs described in Table 7.

Table 7: Exemplary NKG2A gRNAs

Name	**gRNA Targeting Domain Sequence (DNA)**	**Length**	**Enzyme**	**SEQ ID NO:**
NKG2A55	GAGGTAAAGCGTTTGCATTTG	21	AsCpf1	577
NKG2A56	CCTCTAAAGCTTATGCTTACA	21	AsCpf1	578
NKG2A57	AGTCGATTTACTTGTAGCACT	21	AsCpf1	579
NKG2A58	CTTGTAGCACTGCACAGTTAA	21	AsCpf1	580
NKG2A59	TCCATTACAGGATAAAAGACT	21	AsCpf1	581
NKG2A60	CTCCATTACAGGATAAAAGAC	21	AsCpf1	582
NKG2A61	TCTCCATTACAGGATAAAAGA	21	AsCpf1	583
NKG2A62	ATCCTGTAATGGAGAAAAATC	21	AsCpf1	584
NKG2A63	TCCTGTAATGGAGAAAAATCC	21	AsCpf1	585

NKG2A136	AAACATGAGTAAGTTGTTTTG	21	AsCpf1	586
NKG2A137	GCTTTCAAACATGAGTAAGTT	21	AsCpf1	587
NKG2A138	AAAGCCAAACCATTCATTGTC	21	AsCpf1	588
NKG2A139	GTAACAGCAGTCATCATCCAT	21	AsCpf1	589
NKG2A140	ACCATCCTCATGGATTGGTGT	21	AsCpf1	590
NKG2A141	TGTCCATCATTTCACCATCCT	21	AsCpf1	591
NKG2A142	GAAATTTCTGTCCATCATTTC	21	AsCpf1	592
NKG2A143	AGAAATTTCTGTCCATCATTT	21	AsCpf1	593
NKG2A144	TTTTAGAAATTTCTGTCCATC	21	AsCpf1	594
NKG2A145	CTTTTAGAAATTTCTGTCCAT	21	AsCpf1	595
NKG2A146	TTTTCTTTTAGAAATTTCTGT	21	AsCpf1	596
NKG2A147	TAAAAGAAAAGAAAGAATTTT	21	AsCpf1	597
NKG2A270	AAACATTTACATCTTACCATT	21	AsCpf1	598
NKG2A271	CATCTTACCATTTCTTCTTCA	21	AsCpf1	599
NKG2A272	TATAGATAATGAAGAAGAAAT	21	AsCpf1	600
NKG2A273	TTCTTCATTATCTATAGAAAG	21	AsCpf1	601
NKG2A274	CTGGCCTGTACTTCGAAGAAC	21	AsCpf1	602
NKG2A275	CTTACCAATGTAGTAACAACT	21	AsCpf1	603
NKG2A276	GCACGTCATTGTGGCCATTGT	21	AsCpf1	604
NKG2A277	TTTAGCACGTCATTGTGGCCA	21	AsCpf1	605
NKG2A414	CCATCAGCTCCAGAGAAGCTC	21	AsCpf1	606
NKG2A415	TCTCCCTGCAGATTTACCATC	21	AsCpf1	607
NKG2A437	AAATGCTTTACCTTTGCAGTG	21	AsCpf1	608
NKG2A438	AATGCTTTACCTTTGCAGTGA	21	AsCpf1	609
NKG2A439	CCTTTGCAGTGATAGGTTTTG	21	AsCpf1	610

NKG2A440	CAGTGATAGGTTTTGTCATTC	21	AsCpf1	611
NKG2A441	AAGGGAATGACAAAACCTATC	21	AsCpf1	612
NKG2A442	CAAGGGAATGACAAAACCTAT	21	AsCpf1	613
NKG2A443	GTCATTCCCTTGAAAATCCTG	21	AsCpf1	614
NKG2A444	TCATTCCCTTGAAAATCCTGA	21	AsCpf1	615
NKG2A445	TGAAGGTTTAATTCCGCATAG	21	AsCpf1	616
NKG2A446	GAAGGTTTAATTCCGCATAGG	21	AsCpf1	617
NKG2A447	AAGGTTTAATTCCGCATAGGT	21	AsCpf1	618
NKG2A448	ATTCCGCATAGGTTATTTCCT	21	AsCpf1	619
NKG2A449	GCAACTGAACAGGAAATAACC	21	AsCpf1	620
NKG2A450	AGCAACTGAACAGGAAATAAC	21	AsCpf1	621
NKG2A451	CTGTTCAGTTGCTAAAATGGA	21	AsCpf1	622
NKG2A452	TATTGCCTTTAGGTTTTCGTT	21	AsCpf1	623
NKG2A453	ATTGCCTTTAGGTTTTCGTTG	21	AsCpf1	624
NKG2A454	TTGCCTTTAGGTTTTCGTTGC	21	AsCpf1	625
NKG2A455	GGTTTTCGTTGCTGCCTCTTT	21	AsCpf1	626
NKG2A456	CGTTGCTGCCTCTTTGGGTTT	21	AsCpf1	627
NKG2A457	GTTGCTGCCTCTTTGGGTTTG	21	AsCpf1	628
NKG2A458	GGTTTGGGGGCAGATTCAGGT	21	AsCpf1	629
NKG2A459	GGGGCAGATTCAGGTCTGAGT	21	AsCpf1	630
NKG2A460	GCAACTGAACAGGAAATAACC	21	Cas12a	1176

[0232] In some embodiments, the gRNA for use in the disclosure is a gRNA targeting TIGIT (TIGIT gRNA). In some embodiments, the gRNA targeting TIGIT is one or more of the gRNAs described in Table 8.

Table 8. TIGIT gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme	SEQ ID NO:
TIGIT4170	TCTGCAGAAATGTTCCCCGT	20	AsCpf1	631
TIGIT4171	TGCAGAGAAAGGTGGCTCTA	20	AsCpf1	632
TIGIT4172	TAATGCTGACTTGGGGTGGC	20	AsCpf1	633
TIGIT4173	TAGGACCTCCAGGAAGATTC	20	AsCpf1	634
TIGIT4174	TAGTCAACGCGACCACCACG	20	AsCpf1	635
TIGIT4175	TCCTGAGGTCACCTTCCACA	20	AsCpf1	636
TIGIT4176	TATTGTGCCTGTCATCATTC	20	AsCpf1	637
TIGIT4177	TGACAGGCACAATAGAAACAA	21	SauCas9	638
TIGIT4178	GACAGGCACAATAGAAACAAC	21	SauCas9	639
TIGIT4179	AAACAACGGGGAACATTTCTG	21	SauCas9	640
TIGIT4180	ACAACGGGGAACATTTCTGCA	21	SauCas9	641
TIGIT4181	TGATAGAGCCACCTTTCTCTG	21	SauCas9	642
TIGIT4182	GGGTCACTTGTGCCGTGGTGG	21	SauCas9	643
TIGIT4183	GGCACAAGTGACCCAGGTCAA	21	SauCas9	644
TIGIT4184	GTCCTGCTGCTCCCAGTTGAC	21	SauCas9	645
TIGIT4185	TGGCCATTTGTAATGCTGACT	21	SauCas9	646
TIGIT4186	TGGCACATCTCCCCATCCTTC	21	SauCas9	647
TIGIT4187	CATCTCCCCATCCTTCAAGGA	21	SauCas9	648
TIGIT4188	CCACTCGATCCTTGAAGGATG	21	SauCas9	649
TIGIT4189	GGCCACTCGATCCTTGAAGGA	21	SauCas9	650
TIGIT4190	CCTGGGGCCACTCGATCCTTG	21	SauCas9	651
TIGIT4191	GACTGGAGGGTGAGGCCCAGG	21	SauCas9	652
TIGIT4192	ATCGTTCACGGTCAGCGACTG	21	SauCas9	653

TIGIT4193	GTCGCTGACCGTGAACGATAC	21	SauCas9	654
TIGIT4194	CGCTGACCGTGAACGATACAG	21	SauCas9	655
TIGIT4195	GCATCTATCACACCTACCCTG	21	SauCas9	656
TIGIT4196	CCTACCCTGATGGGACGTACA	21	SauCas9	657
TIGIT4197	TACCCTGATGGGACGTACACT	21	SauCas9	658
TIGIT4198	CCCTGATGGGACGTACACTGG	21	SauCas9	659
TIGIT4199	TTCTCCCAGTGTACGTCCCAT	21	SauCas9	660
TIGIT4200	GGAGAATCTTCCTGGAGGTCC	21	SauCas9	661
TIGIT4201	CATGGCTCCAAGCAATGGAAT	21	SauCas9	662
TIGIT4202	CGCGGCCATGGCTCCAAGCAA	21	SauCas9	663
TIGIT4203	TCGCGGCCATGGCTCCAAGCA	21	SauCas9	664
TIGIT4204	CATCGTGGTGGTCGCGTTGAC	21	SauCas9	665
TIGIT4205	AAAGCCCTCAGAATCCATTCT	21	SauCas9	666
TIGIT4206	CATTCTGTGGAAGGTGACCTC	21	SauCas9	667
TIGIT4207	TTCTGTGGAAGGTGACCTCAG	21	SauCas9	668
TIGIT4208	CCTGAGGTCACCTTCCACAGA	21	SauCas9	669
TIGIT4209	TTCTCCTGAGGTCACCTTCCA	21	SauCas9	670
TIGIT4210	AGGAGAAAATCAGCTGGACAG	21	SauCas9	671
TIGIT4211	GGAGAAAATCAGCTGGACAGG	21	SauCas9	672
TIGIT4212	GCCCCAGTGCTCCCTCACCCC	21	SauCas9	673
TIGIT4213	TGGACACAGCTTCCTGGGGGT	21	SauCas9	674
TIGIT4214	TCTGCCTGGACACAGCTTCCT	21	SauCas9	675
TIGIT4215	AGCTGCACCTGCTGGGCTCTG	21	SauCas9	676
TIGIT4216	GCTGGGCTCTGTGGAGAGCAG	21	SauCas9	677
TIGIT4217	TGGGCTCTGTGGAGAGCAGCG	21	SauCas9	678

TIGIT4218	CTGCATGACTACTTCAATGTC	21	SauCas9	679
TIGIT4219	AATGTCCTGAGTTACAGAAGC	21	SauCas9	680
TIGIT4220	TGGGTAACTGCAGCTTCTTCA	21	SauCas9	681
TIGIT4221	GACAGGCACAATAGAAACAA	20	SpyCas9	682
TIGIT4222	ACAGGCACAATAGAAACAAC	20	SpyCas9	683
TIGIT4223	CAGGCACAATAGAAACAACG	20	SpyCas9	684
TIGIT4224	GGGAACATTTCTGCAGAGAA	20	SpyCas9	685
TIGIT4225	AACATTTCTGCAGAGAAAGG	20	SpyCas9	686
TIGIT4226	ATGTCACCTCTCCTCCACCA	20	SpyCas9	687
TIGIT4227	CTTGTGCCGTGGTGGAGGAG	20	SpyCas9	688
TIGIT4228	GGTCACTTGTGCCGTGGTGG	20	SpyCas9	689
TIGIT4229	CACCACGGCACAAGTGACCC	20	SpyCas9	690
TIGIT4230	CTGGGTCACTTGTGCCGTGG	20	SpyCas9	691
TIGIT4231	GACCTGGGTCACTTGTGCCG	20	SpyCas9	692
TIGIT4232	CACAAGTGACCCAGGTCAAC	20	SpyCas9	693
TIGIT4233	ACAAGTGACCCAGGTCAACT	20	SpyCas9	694
TIGIT4234	CCAGGTCAACTGGGAGCAGC	20	SpyCas9	695
TIGIT4235	CTGCTGCTCCCAGTTGACCT	20	SpyCas9	696
TIGIT4236	CCTGCTGCTCCCAGTTGACC	20	SpyCas9	697
TIGIT4237	GGAGCAGCAGGACCAGCTTC	20	SpyCas9	698
TIGIT4238	CATTACAAATGGCCAGAAGC	20	SpyCas9	699
TIGIT4239	GGCCATTTGTAATGCTGACT	20	SpyCas9	700
TIGIT4240	GCCATTTGTAATGCTGACTT	20	SpyCas9	701
TIGIT4241	CCATTTGTAATGCTGACTTG	20	SpyCas9	702
TIGIT4242	TTTGTAATGCTGACTTGGGG	20	SpyCas9	703

TIGIT4243	CCCCAAGTCAGCATTACAAA	20	SpyCas9	704
TIGIT4244	GCACATCTCCCCATCCTTCA	20	SpyCas9	705
TIGIT4245	CCCATCCTTCAAGGATCGAG	20	SpyCas9	706
TIGIT4246	CACTCGATCCTTGAAGGATG	20	SpyCas9	707
TIGIT4247	CCACTCGATCCTTGAAGGAT	20	SpyCas9	708
TIGIT4248	GCCACTCGATCCTTGAAGGA	20	SpyCas9	709
TIGIT4249	TTCAAGGATCGAGTGGCCCC	20	SpyCas9	710
TIGIT4250	TGGGGCCACTCGATCCTTGA	20	SpyCas9	711
TIGIT4251	GATCGAGTGGCCCCAGGTCC	20	SpyCas9	712
TIGIT4252	AGTGGCCCCAGGTCCCGGCC	20	SpyCas9	713
TIGIT4253	GTGGCCCCAGGTCCCGGCCT	20	SpyCas9	714
TIGIT4254	GAGGCCCAGGCCGGGACCTG	20	SpyCas9	715
TIGIT4255	TGAGGCCCAGGCCGGGACCT	20	SpyCas9	716
TIGIT4256	GTGAGGCCCAGGCCGGGACC	20	SpyCas9	717
TIGIT4257	TGGAGGGTGAGGCCCAGGCC	20	SpyCas9	718
TIGIT4258	CTGGAGGGTGAGGCCCAGGC	20	SpyCas9	719
TIGIT4259	GCGACTGGAGGGTGAGGCCC	20	SpyCas9	720
TIGIT4260	CGGTCAGCGACTGGAGGGTG	20	SpyCas9	721
TIGIT4261	GTTCACGGTCAGCGACTGGA	20	SpyCas9	722
TIGIT4262	CGTTCACGGTCAGCGACTGG	20	SpyCas9	723
TIGIT4263	TATCGTTCACGGTCAGCGAC	20	SpyCas9	724
TIGIT4264	TCGCTGACCGTGAACGATAC	20	SpyCas9	725
TIGIT4265	CGCTGACCGTGAACGATACA	20	SpyCas9	726
TIGIT4266	GCTGACCGTGAACGATACAG	20	SpyCas9	727
TIGIT4267	GTACTCCCCTGTATCGTTCA	20	SpyCas9	728

TIGIT4268	ATCTATCACACCTACCCTGA	20	SpyCas9	729
TIGIT4269	TCTATCACACCTACCCTGAT	20	SpyCas9	730
TIGIT4270	TACCCTGATGGGACGTACAC	20	SpyCas9	731
TIGIT4271	ACCCTGATGGGACGTACACT	20	SpyCas9	732
TIGIT4272	AGTGTACGTCCCATCAGGGT	20	SpyCas9	733
TIGIT4273	TCCCAGTGTACGTCCCATCA	20	SpyCas9	734
TIGIT4274	CTCCCAGTGTACGTCCCATC	20	SpyCas9	735
TIGIT4275	GTACACTGGGAGAATCTTCC	20	SpyCas9	736
TIGIT4276	CACTGGGAGAATCTTCCTGG	20	SpyCas9	737
TIGIT4277	CTGAGCTTTCTAGGACCTCC	20	SpyCas9	738
TIGIT4278	AGGTTCCAGATTCCATTGCT	20	SpyCas9	739
TIGIT4279	AAGCAATGGAATCTGGAACC	20	SpyCas9	740
TIGIT4280	GATTCCATTGCTTGGAGCCA	20	SpyCas9	741
TIGIT4281	TGGCTCCAAGCAATGGAATC	20	SpyCas9	742
TIGIT4282	GCGGCCATGGCTCCAAGCAA	20	SpyCas9	743
TIGIT4283	TGGAGCCATGGCCGCGACGC	20	SpyCas9	744
TIGIT4284	AGCCATGGCCGCGACGCTGG	20	SpyCas9	745
TIGIT4285	GACCACCAGCGTCGCGGCCA	20	SpyCas9	746
TIGIT4286	GCAGATGACCACCAGCGTCG	20	SpyCas9	747
TIGIT4287	CATCTGCACAGCAGTCATCG	20	SpyCas9	748
TIGIT4288	CTGCACAGCAGTCATCGTGG	20	SpyCas9	749
TIGIT4289	AGCCCTCAGAATCCATTCTG	20	SpyCas9	750
TIGIT4290	CTCAGAATCCATTCTGTGGA	20	SpyCas9	751
TIGIT4291	TTCCACAGAATGGATTCTGA	20	SpyCas9	752
TIGIT4292	CTTCCACAGAATGGATTCTG	20	SpyCas9	753

TIGIT4293	ATTCTGTGGAAGGTGACCTC	20	SpyCas9	754
TIGIT4294	TGAGGTCACCTTCCACAGAA	20	SpyCas9	755
TIGIT4295	GACCTCAGGAGAAAATCAGC	20	SpyCas9	756
TIGIT4296	CAGGAGAAAATCAGCTGGAC	20	SpyCas9	757
TIGIT4297	GTCCAGCTGATTTTCTCCTG	20	SpyCas9	758
TIGIT4298	GAGAAAATCAGCTGGACAGG	20	SpyCas9	759
TIGIT4299	AATCAGCTGGACAGGAGGAA	20	SpyCas9	760
TIGIT4300	CCCAGTGCTCCCTCACCCCC	20	SpyCas9	761
TIGIT4301	CTGGGGGTGAGGGAGCACTG	20	SpyCas9	762
TIGIT4302	CCTGGGGGTGAGGGAGCACT	20	SpyCas9	763
TIGIT4303	TCCTGGGGGTGAGGGAGCAC	20	SpyCas9	764
TIGIT4304	ACACAGCTTCCTGGGGGTGA	20	SpyCas9	765
TIGIT4305	GACACAGCTTCCTGGGGGTG	20	SpyCas9	766
TIGIT4306	ACCCCCAGGAAGCTGTGTCC	20	SpyCas9	767
TIGIT4307	GCCTGGACACAGCTTCCTGG	20	SpyCas9	768
TIGIT4308	TGCCTGGACACAGCTTCCTG	20	SpyCas9	769
TIGIT4309	CTGCCTGGACACAGCTTCCT	20	SpyCas9	770
TIGIT4310	TCTGCCTGGACACAGCTTCC	20	SpyCas9	771
TIGIT4311	CAGGCAGAAGCTGCACCTGC	20	SpyCas9	772
TIGIT4312	AGGCAGAAGCTGCACCTGCT	20	SpyCas9	773
TIGIT4313	CAGCAGGTGCAGCTTCTGCC	20	SpyCas9	774
TIGIT4314	GCTGCACCTGCTGGGCTCTG	20	SpyCas9	775
TIGIT4315	TGCTCTCCACAGAGCCCAGC	20	SpyCas9	776
TIGIT4316	CTGGGCTCTGTGGAGAGCAG	20	SpyCas9	777
TIGIT4317	TGGGCTCTGTGGAGAGCAGC	20	SpyCas9	778

TIGIT4318	GGGCTCTGTGGAGAGCAGCG	20	SpyCas9	779
TIGIT4319	CTGTGGAGAGCAGCGGGGAG	20	SpyCas9	780
TIGIT4320	ATTGAAGTAGTCATGCAGCT	20	SpyCas9	781
TIGIT4321	TGTCCTGAGTTACAGAAGCC	20	SpyCas9	782
TIGIT4322	GTCCTGAGTTACAGAAGCCT	20	SpyCas9	783
TIGIT4323	TACCCAGGCTTCTGTAACTC	20	SpyCas9	784
TIGIT4324	TGAAGAAGCTGCAGTTACCC	20	SpyCas9	785
TIGIT4325	TGCAGCTTCTTCACAGAGAC	20	SpyCas9	786
TIGIT5053	GTTGTTTCTATTGTGCCTGT	20	AsCpf1 RR	787
TIGIT5054	CGTTGTTTCTATTGTGCCTG	20	AsCpf1 RR	788
TIGIT5055	CCGTTGTTTCTATTGTGCCT	20	AsCpf1 RR	789
TIGIT5056	CCACGGCACAAGTGACCCAG	20	AsCpf1 RR	790
TIGIT5057	AGTTGACCTGGGTCACTTGT	20	AsCpf1 RR	791
TIGIT5058	AAGTCAGCATTACAAATGGC	20	AsCpf1 RR	792
TIGIT5059	CATCCTTCAAGGATCGAGTG	20	AsCpf1 RR	793
TIGIT5060	ATCCTTCAAGGATCGAGTGG	20	AsCpf1 RR	794
TIGIT5061	AGGATCGAGTGGCCCCAGGT	20	AsCpf1 RR	795
TIGIT5062	AGGTCCCGGCCTGGGCCTCA	20	AsCpf1 RR	796
TIGIT5063	GGCCTGGGCCTCACCCTCCA	20	AsCpf1 RR	797
TIGIT5064	CGGTCAGCGACTGGAGGGTG	20	AsCpf1 RR	798
TIGIT5065	GTCGCTGACCGTGAACGATA	20	AsCpf1 RR	799
TIGIT5066	TGTATCGTTCACGGTCAGCG	20	AsCpf1 RR	800
TIGIT5067	CTGTATCGTTCACGGTCAGC	20	AsCpf1 RR	801
TIGIT5068	ATCAGGGTAGGTGTGATAGA	20	AsCpf1 RR	802
TIGIT5069	AGTGTACGTCCCATCAGGGT	20	AsCpf1 RR	803

TIGIT5070	GGAAGATTCTCCCAGTGTAC	20	AsCpf1 RR	804
TIGIT5071	TGGAGGTCCTAGAAAGCTCA	20	AsCpf1 RR	805
TIGIT5072	AGCAATGGAATCTGGAACCT	20	AsCpf1 RR	806
TIGIT5073	AGATTCCATTGCTTGGAGCC	20	AsCpf1 RR	807
TIGIT5074	GATTCCATTGCTTGGAGCCA	20	AsCpf1 RR	808
TIGIT5075	ATTGCTTGGAGCCATGGCCG	20	AsCpf1 RR	809
TIGIT5076	TTGCTTGGAGCCATGGCCGC	20	AsCpf1 RR	810
TIGIT5077	CAGAATGGATTCTGAGGGCT	20	AsCpf1 RR	811
TIGIT5078	ACAGAATGGATTCTGAGGGC	20	AsCpf1 RR	812
TIGIT5079	TTCTGTGGAAGGTGACCTCA	20	AsCpf1 RR	813
TIGIT5080	GCTGATTTTCTCCTGAGGTC	20	AsCpf1 RR	814
TIGIT5081	TCCTGTCCAGCTGATTTTCT	20	AsCpf1 RR	815
TIGIT5082	TTCCTCCTGTCCAGCTGATT	20	AsCpf1 RR	816
TIGIT5083	TGGGGGTGAGGGAGCACTGG	20	AsCpf1 RR	817
TIGIT5084	AGTGCTCCCTCACCCCCAGG	20	AsCpf1 RR	818
TIGIT5085	TCACCCCCAGGAAGCTGTGT	20	AsCpf1 RR	819
TIGIT5086	CAGGAAGCTGTGTCCAGGCA	20	AsCpf1 RR	820
TIGIT5087	AGGAAGCTGTGTCCAGGCAG	20	AsCpf1 RR	821
TIGIT5088	GGCAGAAGCTGCACCTGCTG	20	AsCpf1 RR	822
TIGIT5089	CAGAGCCCAGCAGGTGCAGC	20	AsCpf1 RR	823
TIGIT5090	GCTGCTCTCCACAGAGCCCA	20	AsCpf1 RR	824
TIGIT5091	CGCTGCTCTCCACAGAGCCC	20	AsCpf1 RR	825
TIGIT5092	ATGTCCTGAGTTACAGAAGC	20	AsCpf1 RR	826
TIGIT5093	TGCAGAGAAAGGTGGCTCTAT	21	Cas12a	1175

[0233] In some embodiments the gRNA for use in the disclosure is a gRNA targeting ADORA2a (ADORA2a gRNA). In some embodiments, the gRNA targeting ADORA2a is one or more of the gRNAs described in Table 9.

Table 9. ADORA2a gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme	SEQ ID NO:
ADORA2A337	GAGCACACCCACTGCGATGT	20	SpyCas9	827
ADORA2A338	GATGGCCAGGAGACTGAAGA	20	SpyCas9	828
ADORA2A339	CTGCTCACCGGAGCGGGATG	20	SpyCas9	829
ADORA2A340	GTCTGTGGCCATGCCCATCA	20	SpyCas9	830
ADORA2A341	TCACCGGAGCGGGATGCGGA	20	SpyCas9	831
ADORA2A342	GTGGCAGGCAGCGCAGAACC	20	SpyCas9	832
ADORA2A343	AGCACACCAGCACATTGCCC	20	SpyCas9	833
ADORA2A344	CAGGTTGCTGTTGAGCCACA	20	SpyCas9	834
ADORA2A345	CTTCATTGCCTGCTTCGTCC	20	SpyCas9	835
ADORA2A346	GTACACCGAGGAGCCCATGA	20	SpyCas9	836
ADORA2A347	GATGGCAATGTAGCGGTCAA	20	SpyCas9	837
ADORA2A348	CTCCTCGGTGTACATCACGG	20	SpyCas9	838
ADORA2A349	CGAGGAGCCCATGATGGGCA	20	SpyCas9	839
ADORA2A350	GGGCTCCTCGGTGTACATCA	20	SpyCas9	840
ADORA2A351	CTTTGTGGTGTCACTGGCGG	20	SpyCas9	841
ADORA2A352	CCGCTCCGGTGAGCAGGGCC	20	SpyCas9	842
ADORA2A353	GGGTTCTGCGCTGCCTGCCA	20	SpyCas9	843
ADORA2A354	GGACGAAGCAGGCAATGAAG	20	SpyCas9	844
ADORA2A355	GTGCTGATGGTGATGGCAAA	20	SpyCas9	845
ADORA2A356	AGCGCAGAACCCGGTGCTGA	20	SpyCas9	846

ADORA2A357	GAGCTCCATCTTCAGTCTCC	20	SpyCas9	847
ADORA2A358	TGCTGATGGTGATGGCAAAG	20	SpyCas9	848
ADORA2A359	GGCGGCGGCCGACATCGCAG	20	SpyCas9	849
ADORA2A360	AATGAAGAGGCAGCCGTGGC	20	SpyCas9	850
ADORA2A361	GGGCAATGTGCTGGTGTGCT	20	SpyCas9	851
ADORA2A362	CATGCCCATCATGGGCTCCT	20	SpyCas9	852
ADORA2A363	AATGTAGCGGTCAATGGCGA	20	SpyCas9	853
ADORA2A364	AGTAGTTGGTGACGTTCTGC	20	SpyCas9	854
ADORA2A365	AGCGGTCAATGGCGATGGCC	20	SpyCas9	855
ADORA2A366	CGCATCCCGCTCCGGTGAGC	20	SpyCas9	856
ADORA2A367	GCATCCCGCTCCGGTGAGCA	20	SpyCas9	857
ADORA2A368	TGGGCAATGTGCTGGTGTGC	20	SpyCas9	858
ADORA2A369	CAACTACTTTGTGGTGTCAC	20	SpyCas9	859
ADORA2A370	CGCTCCGGTGAGCAGGGCCG	20	SpyCas9	860
ADORA2A371	GATGGTGATGGCAAAGGGGA	20	SpyCas9	861
ADORA2A372	GGTGTACATCACGGTGGAGC	20	SpyCas9	862
ADORA2A373	GAACGTCACCAACTACTTTG	20	SpyCas9	863
ADORA2A374	CAGTGACACCACAAAGTAGT	20	SpyCas9	864
ADORA2A375	GGCCATCCTGGGCAATGTGC	20	SpyCas9	865
ADORA2A376	CCCGGCCCTGCTCACCGGAG	20	SpyCas9	866
ADORA2A377	CACCAGCACATTGCCCAGGA	20	SpyCas9	867
ADORA2A378	TTTGCCATCACCATCAGCAC	20	SpyCas9	868
ADORA2A379	CTCCACCGTGATGTACACCG	20	SpyCas9	869
ADORA2A380	GGAGCTGGCCATTGCTGTGC	20	SpyCas9	870
ADORA2A381	CAGGATGGCCAGCACAGCAA	20	SpyCas9	871

ADORA2A382	GAACCCGGTGCTGATGGTGA	20	SpyCas9	872
ADORA2A383	TGGAGCTCTGCGTGAGGACC	20	SpyCas9	873
ADORA2A384	CCCGCTCCGGTGAGCAGGGC	20	SpyCas9	874
ADORA2A385	AGGCAATGAAGAGGCAGCCG	20	SpyCas9	875
ADORA2A386	CCGGCCCTGCTCACCGGAGC	20	SpyCas9	876
ADORA2A387	GCGGCGGCCGACATCGCAGT	20	SpyCas9	877
ADORA2A388	GGTGCTGATGGTGATGGCAA	20	SpyCas9	878
ADORA2A389	CTACTTTGTGGTGTCACTGG	20	SpyCas9	879
ADORA2A390	TACACCGAGGAGCCCATGAT	20	SpyCas9	880
ADORA2A391	TCTGTGGCCATGCCCATCAT	20	SpyCas9	881
ADORA2A392	ATTGCTGTGCTGGCCATCCT	20	SpyCas9	882
ADORA2A393	CGTGAGGACCAGGACGAAGC	20	SpyCas9	883
ADORA2A394	TTGCCATCACCATCAGCACC	20	SpyCas9	884
ADORA2A395	GGATGCGGATGGCAATGTAG	20	SpyCas9	885
ADORA2A396	TTGCCATCCGCATCCCGCTC	20	SpyCas9	886
ADORA2A397	TGAAGATGGAGCTCTGCGTG	20	SpyCas9	887
ADORA2A398	CATTGCTGTGCTGGCCATCC	20	SpyCas9	888
ADORA2A399	TGCTGGTGTGCTGGGCCGTG	20	SpyCas9	889
ADORA2A820	GGCTCCTCGGTGTACATCACG	21	SauCas9	890
ADORA2A821	GAGCTCTGCGTGAGGACCAGG	21	SauCas9	891
ADORA2A822	GATGGAGCTCTGCGTGAGGAC	21	SauCas9	892
ADORA2A823	CCAGCACACCAGCACATTGCC	21	SauCas9	893
ADORA2A824	AGGACCAGGACGAAGCAGGCA	21	SauCas9	894
ADORA2A825	TGCCATCCGCATCCCGCTCCG	21	SauCas9	895
ADORA2A826	GTGTGGCTCAACAGCAACCTG	21	SauCas9	896

ADORA2A827	AGCTCCACCGTGATGTACACC	21	SauCas9	897
ADORA2A828	GTAGCGGTCAATGGCGATGGC	21	SauCas9	898
ADORA2A829	CGGTGCTGATGGTGATGGCAA	21	SauCas9	899
ADORA2A830	CCCTGCTCACCGGAGCGGGAT	21	SauCas9	900
ADORA2A831	GTGACGTTCTGCAGGTTGCTG	21	SauCas9	901
ADORA2A832	GCTCCACCGTGATGTACACCG	21	SauCas9	902
ADORA2A833	ACTGAAGATGGAGCTCTGCGT	21	SauCas9	903
ADORA2A834	CCAGCTCCACCGTGATGTACA	21	SauCas9	904
ADORA2A835	CCTTTGCCATCACCATCAGCA	21	SauCas9	905
ADORA2A836	CCGGTGCTGATGGTGATGGCA	21	SauCas9	906
ADORA2A837	CCTGGGCAATGTGCTGGTGTG	21	SauCas9	907
ADORA2A838	AGGCAGCCGTGGCAGGCAGCG	21	SauCas9	908
ADORA2A839	GCGATGGCCAGGAGACTGAAG	21	SauCas9	909
ADORA2A840	CGATGGCCAGGAGACTGAAGA	21	SauCas9	910
ADORA2A841	TCCCGCTCCGGTGAGCAGGGC	21	SauCas9	911
ADORA2A842	TGCTTCGTCCTGGTCCTCACG	21	SauCas9	912
ADORA2A843	ACCAGGACGAAGCAGGCAATG	21	SauCas9	913
ADORA2A844	ATGTACACCGAGGAGCCCATG	21	SauCas9	914
ADORA2A845	TCGTCTGTGGCCATGCCCATC	21	SauCas9	915
ADORA2A846	TCAATGGCGATGGCCAGGAGA	21	SauCas9	916
ADORA2A847	GGTGCTGATGGTGATGGCAAA	21	SauCas9	917
ADORA2A848	TAGCGGTCAATGGCGATGGCC	21	SauCas9	918
ADORA2A849	TCCGCATCCCGCTCCGGTGAG	21	SauCas9	919
ADORA2A850	CTGGCGGCGGCCGACATCGCA	21	SauCas9	920
ADORA2A851	GCCATTGCTGTGCTGGCCATC	21	SauCas9	921

ADORA2A852	ATCCCGCTCCGGTGAGCAGGG	21	SauCas9	922
ADORA2A853	AGACTGAAGATGGAGCTCTGC	21	SauCas9	923
ADORA2A854	CCCCGGCCCTGCTCACCGGAG	21	SauCas9	924
ADORA2A855	ATGGTGATGGCAAAGGGGATG	21	SauCas9	925
ADORA2A856	GCTCCTCGGTGTACATCACGG	21	SauCas9	926
ADORA2A248	TGTCGATGGCAATAGCCAAG	20	SpyCas9	927
ADORA2A249	AGAAGTTGGTGACGTTCTGC	20	SpyCas9	928
ADORA2A250	TTCGCCATCACCATCAGCAC	20	SpyCas9	929
ADORA2A251	GAAGAAGAGGCAGCCATGGC	20	SpyCas9	930
ADORA2A252	CACAAGCACGTTACCCAGGA	20	SpyCas9	931
ADORA2A253	CAACTTCTTCGTGGTATCTC	20	SpyCas9	932
ADORA2A254	CAGGATGGCCAGCACAGCAA	20	SpyCas9	933
ADORA2A255	AATTCCACTCCGGTGAGCCA	20	SpyCas9	934
ADORA2A256	AGCGCAGAAGCCAGTGCTGA	20	SpyCas9	935
ADORA2A257	GTGCTGATGGTGATGGCGAA	20	SpyCas9	936
ADORA2A258	GGAGCTGGCCATTGCTGTGC	20	SpyCas9	937
ADORA2A259	AATAGCCAAGAGGCTGAAGA	20	SpyCas9	938
ADORA2A260	CTCCTCGGTGTACATCATGG	20	SpyCas9	939
ADORA2A261	GGACAAAGCAGGCGAAGAAG	20	SpyCas9	940
ADORA2A262	TCTGGCGGCGGCTGACATCG	20	SpyCas9	941
ADORA2A263	TGGGTAACGTGCTTGTGTGC	20	SpyCas9	942
ADORA2A264	GATGTACACCGAGGAGCCCA	20	SpyCas9	943
ADORA2A265	TAACCCCTGGCTCACCGGAG	20	SpyCas9	944
ADORA2A266	TCACCGGAGTGGAATTCGGA	20	SpyCas9	945
ADORA2A267	GCGGCGGCTGACATCGCGGT	20	SpyCas9	946

ADORA2A268	GATGGTGATGGCGAATGGGA	20	SpyCas9	947
ADORA2A269	GGCTTCTGCGCTGCCTGCCA	20	SpyCas9	948
ADORA2A270	ATTCCACTCCGGTGAGCCAG	20	SpyCas9	949
ADORA2A271	GGTGTACATCATGGTGGAGC	20	SpyCas9	950
ADORA2A272	ATTGCTGTGCTGGCCATCCT	20	SpyCas9	951
ADORA2A273	CTCCACCATGATGTACACCG	20	SpyCas9	952
ADORA2A274	GGCGGCGGCTGACATCGCGG	20	SpyCas9	953
ADORA2A275	TACACCGAGGAGCCCATGGC	20	SpyCas9	954
ADORA2A276	GGGTAACGTGCTTGTGTGCT	20	SpyCas9	955
ADORA2A277	CAGGTTGCTGTTGATCCACA	20	SpyCas9	956
ADORA2A278	TGAAGATGGAACTCTGCGTG	20	SpyCas9	957
ADORA2A279	GATGGCGATGTATCTGTCGA	20	SpyCas9	958
ADORA2A280	CTTCTTCGCCTGCTTTGTCC	20	SpyCas9	959
ADORA2A281	AGGCGAAGAAGAGGCAGCCA	20	SpyCas9	960
ADORA2A282	TGCTTGTGTGCTGGGCCGTG	20	SpyCas9	961
ADORA2A283	GAAGCCAGTGCTGATGGTGA	20	SpyCas9	962
ADORA2A284	CGTGAGGACCAGGACAAAGC	20	SpyCas9	963
ADORA2A285	TGGAACTCTGCGTGAGGACC	20	SpyCas9	964
ADORA2A286	CATTGCTGTGCTGGCCATCC	20	SpyCas9	965
ADORA2A287	TTCTCCCGCCATGGGCTCCT	20	SpyCas9	966
ADORA2A288	TGGCTCACCGGAGTGGAATT	20	SpyCas9	967
ADORA2A289	TGCTGATGGTGATGGCGAAT	20	SpyCas9	968
ADORA2A290	CTTCGTGGTATCTCTGGCGG	20	SpyCas9	969
ADORA2A291	AGCACACAAGCACGTTACCC	20	SpyCas9	970
ADORA2A292	GGGCTCCTCGGTGTACATCA	20	SpyCas9	971

ADORA2A293	GTACACCGAGGAGCCCATGG	20	SpyCas9	972
ADORA2A294	GAACGTCACCAACTTCTTCG	20	SpyCas9	973
ADORA2A295	TCGCCATCCGAATTCCACTC	20	SpyCas9	974
ADORA2A296	GAGTTCCATCTTCAGCCTCT	20	SpyCas9	975
ADORA2A297	GAATTCCACTCCGGTGAGCC	20	SpyCas9	976
ADORA2A298	CAGAGATACCACGAAGAAGT	20	SpyCas9	977
ADORA2A299	CTTCTTCGTGGTATCTCTGG	20	SpyCas9	978
ADORA2A695	CAGTGCTGATGGTGATGGCGA	21	SauCas9	979
ADORA2A696	CGAATTCCACTCCGGTGAGCC	21	SauCas9	980
ADORA2A697	CCGAATTCCACTCCGGTGAGC	21	SauCas9	981
ADORA2A698	GCTGAAGATGGAACTCTGCGT	21	SauCas9	982
ADORA2A699	CGTGCTTGTGTGCTGGGCCGT	21	SauCas9	983
ADORA2A700	GTGAGGACCAGGACAAAGCAG	21	SauCas9	984
ADORA2A701	TCGATGGCAATAGCCAAGAGG	21	SauCas9	985
ADORA2A702	CATCGACAGATACATCGCCAT	21	SauCas9	986
ADORA2A703	GTACACCGAGGAGCCCATGGC	21	SauCas9	987
ADORA2A704	GCTCCACCATGATGTACACCG	21	SauCas9	988
ADORA2A705	AAGCCAGTGCTGATGGTGATG	21	SauCas9	989
ADORA2A706	CACCGCGATGTCAGCCGCCGC	21	SauCas9	990
ADORA2A707	AGGCTGAAGATGGAACTCTGC	21	SauCas9	991
ADORA2A708	GCCGCCGCCAGAGATACCACG	21	SauCas9	992
ADORA2A709	AGCTCCACCATGATGTACACC	21	SauCas9	993
ADORA2A710	AGGCAGCCATGGCAGGCAGCG	21	SauCas9	994
ADORA2A711	CCTGGCTCACCGGAGTGGAAT	21	SauCas9	995
ADORA2A712	CCAGCTCCACCATGATGTACA	21	SauCas9	996

ADORA2A713	ACCAGGACAAAGCAGGCGAAG	21	SauCas9	997
ADORA2A714	CCTGGGTAACGTGCTTGTGTG	21	SauCas9	998
ADORA2A715	AGGACCAGGACAAAGCAGGCG	21	SauCas9	999
ADORA2A716	TCAGCCGCCGCCAGAGATACC	21	SauCas9	1000
ADORA2A717	GGCTCCTCGGTGTACATCATG	21	SauCas9	1001
ADORA2A718	CTGGCGGCGGCTGACATCGCG	21	SauCas9	1002
ADORA2A719	GATGGAACTCTGCGTGAGGAC	21	SauCas9	1003
ADORA2A720	GCTCCTCGGTGTACATCATGG	21	SauCas9	1004
ADORA2A721	TGTACACCGAGGAGCCCATGG	21	SauCas9	1005
ADORA2A722	GCCATTGCTGTGCTGGCCATC	21	SauCas9	1006
ADORA2A723	CAATAGCCAAGAGGCTGAAGA	21	SauCas9	1007
ADORA2A724	ATGGTGATGGCGAATGGGATG	21	SauCas9	1008
ADORA2A725	ATGTACACCGAGGAGCCCATG	21	SauCas9	1009
ADORA2A726	GTGTGGATCAACAGCAACCTG	21	SauCas9	1010
ADORA2A727	TGCTTTGTCCTGGTCCTCACG	21	SauCas9	1011
ADORA2A728	GTAACCCCTGGCTCACCGGAG	21	SauCas9	1012
ADORA2A729	CCAGCACACAAGCACGTTACC	21	SauCas9	1013
ADORA2A730	TATCTGTCGATGGCAATAGCC	21	SauCas9	1014
ADORA2A731	GCAATAGCCAAGAGGCTGAAG	21	SauCas9	1015
ADORA2A732	AGTGCTGATGGTGATGGCGAA	21	SauCas9	1016
ADORA2A733	ACACCGAGGAGCCCATGGCGG	21	SauCas9	1017
ADORA2A734	CGCCATCCGAATTCCACTCCG	21	SauCas9	1018
ADORA2A4111	TGGTGTCACTGGCGGCGGCC	20	AsCpf1	1019
ADORA2A4112	CCATCACCATCAGCACCGGG	20	AsCpf1	1020
ADORA2A4113	CCATCGGCCTGACTCCCATG	20	AsCpf1	1021

ADORA2A4114	GCTGACCGCAGTTGTTCCAA	20	AsCpf1	1022
ADORA2A4115	AGGATGTGGTCCCCATGAAC	20	AsCpf1	1023
ADORA2A4116	CCTGTGTGCTGGTGCCCCTG	20	AsCpf1	1024
ADORA2A4117	CGGATCTTCCTGGCGGCGCG	20	AsCpf1	1025
ADORA2A4118	CCCTCTGCTGGCTGCCCCTA	20	AsCpf1	1026
ADORA2A4119	TTCTGCCCCGACTGCAGCCA	20	AsCpf1	1027
ADORA2A4120	AAGGCAGCTGGCACCAGTGC	20	AsCpf1	1028
ADORA2A4121	TAAGGGCATCATTGCCATCTG	21	SauCas9	1029
ADORA2A4122	CGGCCTGACTCCCATGCTAGG	21	SauCas9	1030
ADORA2A4123	GCAGTTGTTCCAACCTAGCAT	21	SauCas9	1031
ADORA2A4124	CCGCAGTTGTTCCAACCTAGC	21	SauCas9	1032
ADORA2A4125	CAAGAACCACTCCCAGGGCTG	21	SauCas9	1033
ADORA2A4126	CTTGGCCCTCCCCGCAGCCCT	21	SauCas9	1034
ADORA2A4127	CACTTGGCCCTCCCCGCAGCC	21	SauCas9	1035
ADORA2A4128	GGCCAAGTGGCCTGTCTCTTT	21	SauCas9	1036
ADORA2A4129	TTCATGGGGACCACATCCTCA	21	SauCas9	1037
ADORA2A4130	TGAAGTACACCATGTAGTTCA	21	SauCas9	1038
ADORA2A4131	CTGGTGCCCCTGCTGCTCATG	21	SauCas9	1039
ADORA2A4132	GCTCATGCTGGGTGTCTATTT	21	SauCas9	1040
ADORA2A4133	CTTCAGCTGTCGTCGCGCCGC	21	SauCas9	1041
ADORA2A4134	CGCGACGACAGCTGAAGCAGA	21	SauCas9	1042
ADORA2A4135	GATGGAGAGCCAGCCTCTGCC	21	SauCas9	1043
ADORA2A4136	GCGTGGCTGCAGTCGGGGCAG	21	SauCas9	1044
ADORA2A4137	ACGATGGCCAGGTACATGAGC	21	SauCas9	1045
ADORA2A4138	CTCTCCCACACCAATTCGGTT	21	SauCas9	1046

ADORA2A4139	GATTCACAACCGAATTGGTGT	21	SauCas9	1047
ADORA2A4140	GGGATTCACAACCGAATTGGT	21	SauCas9	1048
ADORA2A4141	CGTAGATGAAGGGATTCACAA	21	SauCas9	1049
ADORA2A4142	GGATACGGTAGGCGTAGATGA	21	SauCas9	1050
ADORA2A4143	TCATCTACGCCTACCGTATCC	21	SauCas9	1051
ADORA2A4144	CGGATACGGTAGGCGTAGATG	21	SauCas9	1052
ADORA2A4145	GCGGAAGGTCTGGCGGAACTC	21	SauCas9	1053
ADORA2A4146	AATGATCTTGCGGAAGGTCTG	21	SauCas9	1054
ADORA2A4147	GACGTGGCTGCGAATGATCTT	21	SauCas9	1055
ADORA2A4148	TTGCTGCCTCAGGACGTGGCT	21	SauCas9	1056
ADORA2A4149	CAAGGCAGCTGGCACCAGTGC	21	SauCas9	1057
ADORA2A4150	CGGGCACTGGTGCCAGCTGCC	21	SauCas9	1058
ADORA2A4151	CTTGGCAGCTCATGGCAGTGA	21	SauCas9	1059
ADORA2A4152	CCGTCTCAACGGCCACCCGCC	21	SauCas9	1060
ADORA2A4153	CACACTCCTGGCGGGTGGCCG	21	SauCas9	1061
ADORA2A4154	TGCCGTTGGCCCACACTCCTG	21	SauCas9	1062
ADORA2A4155	CCATTGGGCCTCCGCTCAGGG	21	SauCas9	1063
ADORA2A4156	CATAGCCATTGGGCCTCCGCT	21	SauCas9	1064
ADORA2A4157	AATGGCTATGCCCTGGGGCTG	21	SauCas9	1065
ADORA2A4158	ATGCCCTGGGGCTGGTGAGTG	21	SauCas9	1066
ADORA2A4159	GCCCTGGGGCTGGTGAGTGGA	21	SauCas9	1067
ADORA2A4160	TGGTGAGTGGAGGGAGTGCCC	21	SauCas9	1068
ADORA2A4161	GAGGGAGTGCCCAAGAGTCCC	21	SauCas9	1069
ADORA2A4162	AGGGAGTGCCCAAGAGTCCCA	21	SauCas9	1070
ADORA2A4163	GTCTGGGAGGCCCGTGTTCCC	21	SauCas9	1071

ADORA2A4164	CATGGCTAAGGAGCTCCACGT	21	SauCas9	1072
ADORA2A4165	GAGCTCCTTAGCCATGAGCTC	21	SauCas9	1073
ADORA2A4166	GCTCCTTAGCCATGAGCTCAA	21	SauCas9	1074
ADORA2A4167	GGCCTAGATGACCCCCTGGCC	21	SauCas9	1075
ADORA2A4168	CCCCCTGGCCCAGGATGGAGC	21	SauCas9	1076
ADORA2A4169	CTCCTGCTCCATCCTGGGCCA	21	SauCas9	1077
ADORA2A4416	CCGTGATGTACACCGAGGAG	20	AsCpf1 RR	1078
ADORA2A4417	CTTTGCCATCACCATCAGCA	20	AsCpf1 RR	1079
ADORA2A4418	TTTGCCATCACCATCAGCAC	20	AsCpf1 RR	1080
ADORA2A4419	TTGCCTGCTTCGTCCTGGTC	20	AsCpf1 RR	1081
ADORA2A4420	TCCTGGTCCTCACGCAGAGC	20	AsCpf1 RR	1082
ADORA2A4421	TCTTCAGTCTCCTGGCCATC	20	AsCpf1 RR	1083
ADORA2A4422	GTCTCCTGGCCATCGCCATT	20	AsCpf1 RR	1084
ADORA2A4423	ACCTAGCATGGGAGTCAGGC	20	AsCpf1 RR	1085
ADORA2A4424	AACCTAGCATGGGAGTCAGG	20	AsCpf1 RR	1086
ADORA2A4425	ATGCTAGGTTGGAACAACTG	20	AsCpf1 RR	1087
ADORA2A4426	GCAGCCCTGGGAGTGGTTCT	20	AsCpf1 RR	1088
ADORA2A4427	CGCAGCCCTGGGAGTGGTTC	20	AsCpf1 RR	1089
ADORA2A4428	AGGGCTGCGGGGAGGGCCAA	20	AsCpf1 RR	1090
ADORA2A4429	TGGGGACCACATCCTCAAAG	20	AsCpf1 RR	1091
ADORA2A4430	CATGAACTACATGGTGTACT	20	AsCpf1 RR	1092
ADORA2A4431	ATGAACTACATGGTGTACTT	20	AsCpf1 RR	1093
ADORA2A4432	ACTTCTTTGCCTGTGTGCTG	20	AsCpf1 RR	1094
ADORA2A4433	TGCTGCTCATGCTGGGTGTC	20	AsCpf1 RR	1095
ADORA2A4434	CAAATAGACACCCAGCATGA	20	AsCpf1 RR	1096

ADORA2A4435	GCTGTCGTCGCGCCGCCAGG	20	AsCpf1 RR	1097
ADORA2A4436	TGGCGGCGCGACGACAGCTG	20	AsCpf1 RR	1098
ADORA2A4437	TCTGCTTCAGCTGTCGTCGC	20	AsCpf1 RR	1099
ADORA2A4438	GGCAGAGGCTGGCTCTCCAT	20	AsCpf1 RR	1100
ADORA2A4439	CGGCAGAGGCTGGCTCTCCA	20	AsCpf1 RR	1101
ADORA2A4440	CCGGCAGAGGCTGGCTCTCC	20	AsCpf1 RR	1102
ADORA2A4441	CACTGCAGAAGGAGGTCCAT	20	AsCpf1 RR	1103
ADORA2A4442	TGCTGCCAAGTCACTGGCCA	20	AsCpf1 RR	1104
ADORA2A4443	ACAATGATGGCCAGTGACTT	20	AsCpf1 RR	1105
ADORA2A4444	TACACATCATCAACTGCTTC	20	AsCpf1 RR	1106
ADORA2A4445	CTTTCTTCTGCCCCGACTGC	20	AsCpf1 RR	1107
ADORA2A4446	GACTGCAGCCACGCCCCTCT	20	AsCpf1 RR	1108
ADORA2A4447	TCTCTGGCTCATGTACCTGG	20	AsCpf1 RR	1109
ADORA2A4448	CAACCGAATTGGTGTGGGAG	20	AsCpf1 RR	1110
ADORA2A4449	ACACCAATTCGGTTGTGAAT	20	AsCpf1 RR	1111
ADORA2A4450	GTTGTGAATCCCTTCATCTA	20	AsCpf1 RR	1112
ADORA2A4451	TTCATCTACGCCTACCGTAT	20	AsCpf1 RR	1113
ADORA2A4452	TCTACGCCTACCGTATCCGC	20	AsCpf1 RR	1114
ADORA2A4453	CGAGTTCCGCCAGACCTTCC	20	AsCpf1 RR	1115
ADORA2A4454	GCCAGACCTTCCGCAAGATC	20	AsCpf1 RR	1116
ADORA2A4455	CCAGACCTTCCGCAAGATCA	20	AsCpf1 RR	1117
ADORA2A4456	GCAAGATCATTCGCAGCCAC	20	AsCpf1 RR	1118
ADORA2A4457	CAAGATCATTCGCAGCCACG	20	AsCpf1 RR	1119
ADORA2A4458	CAGCCACGTCCTGAGGCAGC	20	AsCpf1 RR	1120
ADORA2A4459	AGGCAGCTGGCACCAGTGCC	20	AsCpf1 RR	1121

ADORA2A4460	TCACTGCCATGAGCTGCCAA	20	AsCpf1 RR	1122
ADORA2A4461	TCTCAACGGCCACCCGCCAG	20	AsCpf1 RR	1123
ADORA2A4462	CTCAGGGTGGGGAGCACTGC	20	AsCpf1 RR	1124
ADORA2A4463	CACCCTGAGCGGAGGCCCAA	20	AsCpf1 RR	1125
ADORA2A4464	ACCCTGAGCGGAGGCCCAAT	20	AsCpf1 RR	1126
ADORA2A4465	AGGGCATAGCCATTGGGCCT	20	AsCpf1 RR	1127
ADORA2A4466	CTCACCAGCCCCAGGGCATA	20	AsCpf1 RR	1128
ADORA2A4467	TCCACTCACCAGCCCCAGGG	20	AsCpf1 RR	1129
ADORA2A4468	TGGGACTCTTGGGCACTCCC	20	AsCpf1 RR	1130
ADORA2A4469	CTGGGACTCTTGGGCACTCC	20	AsCpf1 RR	1131
ADORA2A4470	CCTGGGACTCTTGGGCACTC	20	AsCpf1 RR	1132
ADORA2A4471	AGGGGAACACGGGCCTCCCA	20	AsCpf1 RR	1133
ADORA2A4472	CGTCTGGGAGGCCCGTGTTC	20	AsCpf1 RR	1134
ADORA2A4473	AGACGTGGAGCTCCTTAGCC	20	AsCpf1 RR	1135
ADORA2A4474	TTGAGCTCATGGCTAAGGAG	20	AsCpf1 RR	1136
ADORA2A4475	CTGGCCTAGATGACCCCCTG	20	AsCpf1 RR	1137
ADORA2A4476	TGGCCTAGATGACCCCCTGG	20	AsCpf1 RR	1138
ADORA2A4477	TCCTGGGCCAGGGGGTCATC	20	AsCpf1 RR	1139
ADORA2A4478	CTGGCCCAGGATGGAGCAGG	20	AsCpf1 RR	1140
ADORA2A4479	TGGCCCAGGATGGAGCAGGA	20	AsCpf1 RR	1141
ADORA2A4480	CGCGAGTTCCGCCAGACCTT	20	AsCpf1 RVR	1142
ADORA2A4481	CCCTGGGGCTGGTGAGTGGA	20	AsCpf1RVR	1143
ADORA2A4482	CCATCGGCCTGACTCCCATGC	21	Cas12a	1174

[0234] It will be understood that the exemplary gRNAs disclosed herein are provided to illustrate non-limiting embodiments embraced by the present disclosure. Additional

suitable gRNA sequences will be apparent to the skilled artisan based on the present disclosure, and the disclosure is not limited in this respect.

RNA-guided nucleases

[0235] RNA-guided nucleases according to the present disclosure include, but are not limited to, naturally-occurring Class 2 CRISPR nucleases such as Cas9, and Cpf1, as well as other nucleases derived or obtained therefrom. In functional terms, RNA-guided nucleases are defined as those nucleases that: (a) interact with (e.g., complex with) a gRNA; and (b) together with the gRNA, associate with, and optionally cleave or modify, a target region of a DNA that includes (i) a sequence complementary to the targeting domain of the gRNA and, optionally, (ii) an additional sequence referred to as a "protospacer adjacent motif," or "PAM," which is described in greater detail below. As the following examples will illustrate, RNA-guided nucleases can be defined, in broad terms, by their PAM specificity and cleavage activity, even though variations may exist between individual RNA-guided nucleases that share the same PAM specificity or cleavage activity. Skilled artisans will appreciate that some aspects of the present disclosure relate to systems, methods and compositions that can be implemented using any suitable RNA-guided nuclease having a certain PAM specificity and/or cleavage activity. For this reason, unless otherwise specified, the term RNA-guided nuclease should be understood as a generic term, and not limited to any particular type (e.g., Cas9 vs. Cpf1), species (e.g., *S. pyogenes* vs. *S. aureus*) or variation (e.g., full-length vs. truncated or split; naturally-occurring PAM specificity vs. engineered PAM specificity, etc.) of RNA-guided nuclease.

[0236] The PAM sequence takes its name from its sequential relationship to the "protospacer" sequence that is complementary to gRNA targeting domains (or "spacers"). Together with protospacer sequences, PAM sequences define target regions or sequences for specific RNA-guided nuclease / gRNA combinations.

[0237] Various RNA-guided nucleases may require different sequential relationships between PAMs and protospacers. In general, Cas9s recognize PAM sequences that are 3' of the protospacer. Cpf1, on the other hand, generally recognizes PAM sequences that are 5' of the protospacer.

[0238] In addition to recognizing specific sequential orientations of PAMs and protospacers, RNA-guided nucleases can also recognize specific PAM sequences. *S. aureus* Cas9, for instance, recognizes a PAM sequence of NNGRRT or NNGRRV, wherein the N residues are immediately 3' of the region recognized by the gRNA targeting domain. *S. pyogenes* Cas9 recognizes NGG PAM sequences. *F. novicida* Cpf1 recognizes a TTN PAM sequence. PAM sequences have been identified for a variety of RNA-guided nucleases, and a strategy for identifying novel PAM sequences has been described by Shmakov et al., 2015, Molecular Cell 60, 385–397, November 5, 2015. It should also be noted that engineered RNA-guided nucleases can have PAM specificities that differ from the PAM specificities of reference molecules (for instance, in the case of an engineered RNA-guided nuclease, the reference molecule may be the naturally occurring variant from which the RNA-guided nuclease is derived, or the naturally occurring variant having the greatest amino acid sequence homology to the engineered RNA-guided nuclease).

[0239] In addition to their PAM specificity, RNA-guided nucleases can be characterized by their DNA cleavage activity: naturally-occurring RNA-guided nucleases typically form DSBs in target nucleic acids, but engineered variants have been produced that generate only SSBs (discussed above) Ran & Hsu, et al., Cell 154(6), 1380–1389, September 12, 2013 ("Ran")), or that that do not cut at all.

Cas9

[0240] Crystal structures have been determined for *S. pyogenes* Cas9 (Jinek et al., Science 343(6176), 1247997, 2014 ("Jinek 2014"), and for *S. aureus* Cas9 in complex with a unimolecular guide RNA and a target DNA (Nishimasu 2014; Anders et al., Nature. 2014 Sep 25;513(7519):569-73 ("Anders 2014"); and Nishimasu 2015).

[0241] A naturally occurring Cas9 protein comprises two lobes: a recognition (REC) lobe and a nuclease (NUC) lobe; each of which comprise particular structural and/or functional domains. The REC lobe comprises an arginine-rich bridge helix (BH) domain, and at least one REC domain (e.g., a REC1 domain and, optionally, a REC2 domain). The REC lobe does not share structural similarity with other known proteins, indicating that it is a unique functional domain. While not wishing to be bound by any theory, mutational analyses suggest specific functional roles for the BH and REC domains: the BH domain appears to

play a role in gRNA:DNA recognition, while the REC domain is thought to interact with the repeat:anti-repeat duplex of the gRNA and to mediate the formation of the Cas9/gRNA complex.

[0242] The NUC lobe comprises a RuvC domain, an HNH domain, and a PAM-interacting (PI) domain. The RuvC domain shares structural similarity to retroviral integrase superfamily members and cleaves the non-complementary (i.e., bottom) strand of the target nucleic acid. It may be formed from two or more split RuvC motifs (such as RuvC I, RuvCII, and RuvCIII in *S. pyogenes* and *S. aureus*). The HNH domain, meanwhile, is structurally similar to HNN endonuclease motifs, and cleaves the complementary (i.e., top) strand of the target nucleic acid. The PI domain, as its name suggests, contributes to PAM specificity.

[0243] While certain functions of Cas9 are linked to (but not necessarily fully determined by) the specific domains set forth above, these and other functions may be mediated or influenced by other Cas9 domains, or by multiple domains on either lobe. For instance, in *S. pyogenes* Cas9, as described in Nishimasu 2014, the repeat:antirepeat duplex of the gRNA falls into a groove between the REC and NUC lobes, and nucleotides in the duplex interact with amino acids in the BH, PI, and REC domains. Some nucleotides in the first stem loop structure also interact with amino acids in multiple domains (PI, BH and REC1), as do some nucleotides in the second and third stem loops (RuvC and PI domains).

Cpf1

[0244] The crystal structure of *Acidaminococcus sp.* Cpf1 in complex with crRNA and a dsDNA target including a TTTN PAM sequence has been solved by Yamano et al. (Cell. 2016 May 5; 165(4): 949–962 ("Yamano"), incorporated by reference herein). Cpf1, like Cas9, has two lobes: a REC (recognition) lobe, and a NUC (nuclease) lobe. The REC lobe includes REC1 and REC2 domains, which lack similarity to any known protein structures. The NUC lobe, meanwhile, includes three RuvC domains (RuvC-I, -II and -III) and a BH domain. However, in contrast to Cas9, the Cpf1 REC lobe lacks an HNH domain, and includes other domains that also lack similarity to known protein structures: a structurally unique PI domain, three Wedge (WED) domains (WED-I, -II and -III), and a nuclease (Nuc) domain.

[0245] While Cas9 and Cpf1 share similarities in structure and function, it should be appreciated that certain Cpf1 activities are mediated by structural domains that are not analogous to any Cas9 domains. For instance, cleavage of the complementary strand of the target DNA appears to be mediated by the Nuc domain, which differs sequentially and spatially from the HNH domain of Cas9. Additionally, the non-targeting portion of Cpf1 gRNA (the handle) adopts a pseudoknot structure, rather than a stem loop structure formed by the repeat:antirepeat duplex in Cas9 gRNAs.

Nuclease variants

[0246] The RNA-guided nucleases described herein have activities and properties that can be useful in a variety of applications, but the skilled artisan will appreciate that RNA-guided nucleases can also be modified in certain instances, to alter cleavage activity, PAM specificity, or other structural or functional features.

[0247] Turning first to modifications that alter cleavage activity, mutations that reduce or eliminate the activity of domains within the NUC lobe have been described above. Exemplary mutations that may be made in the RuvC domains, in the Cas9 HNH domain, or in the Cpf1 Nuc domain are described in Ran & Hsu, *et al.*, (Cell 154(6), 1380–1389, September 12, 2013), and Yamano, et al. (Cell. 2016 May 5; 165(4): 949–962); as well as in WO 2016/073990 by Cotta-Ramusino, the entire contents of each of which are incorporated herein by reference. In general, mutations that reduce or eliminate activity in one of the two nuclease domains result in RNA-guided nucleases with nickase activity, but it should be noted that the type of nickase activity varies depending on which domain is inactivated. As one example, inactivation of a RuvC domain or of a Cas9 HNH domain results in a nickase .

[0248] Modifications of PAM specificity relative to naturally occurring Cas9 reference molecules has been described by Kleinstiver *et al.* for both *S. pyogenes* (Kleinstiver *et al.*, Nature. 2015 Jul 23;523(7561):481-5); and *S. aureus* (Kleinstiver *et al.*, Nat Biotechnol. 2015 Dec; 33(12): 1293–1298). Kleinstiver *et al.* have also described modifications that improve the targeting fidelity of Cas9 (Nature, 2016 January 28; 529, 490-495). Each of these references is incorporated by reference herein.

[0249] RNA-guided nucleases have been split into two or more parts, as described by Zetsche *et al.* (Nat Biotechnol. 2015 Feb;33(2):139-42, incorporated by reference), and by Fine *et al.* (Sci Rep. 2015 Jul 1;5:10777, incorporated by reference).

[0250] RNA-guided nucleases can be, in certain embodiments, size-optimized or truncated, for instance via one or more deletions that reduce the size of the nuclease while still retaining gRNA association, target and PAM recognition, and cleavage activities. In certain embodiments, RNA guided nucleases are bound, covalently or non-covalently, to another polypeptide, nucleotide, or other structure, optionally by means of a linker. Exemplary bound nucleases and linkers are described by Guilinger *et al.*, Nature Biotechnology 32, 577–582 (2014), which is incorporated by reference herein.

[0251] RNA-guided nucleases also optionally include a tag, such as, but not limited to, a nuclear localization signal, to facilitate movement of RNA-guided nuclease protein into the nucleus. In certain embodiments, the RNA-guided nuclease can incorporate C- and/or N-terminal nuclear localization signals. Nuclear localization sequences are known in the art and are described in Maeder and elsewhere.

[0252] The foregoing list of modifications is intended to be exemplary in nature, and the skilled artisan will appreciate, in view of the instant disclosure, that other modifications may be possible or desirable in certain applications. For brevity, therefore, exemplary systems, methods and compositions of the present disclosure are presented with reference to particular RNA-guided nucleases, but it should be understood that the RNA-guided nucleases used may be modified in ways that do not alter their operating principles. Such modifications are within the scope of the present disclosure.

[0253] Exemplary suitable nuclease variants include, but are not limited to , AsCpf1 variants comprising an M537R substitution, an H800A substitution, and/or an F870L substitution, or any combination thereof (numbering scheme according to AsCpf1 wild-type sequence). In some embodiments, an ASCpf1 variant comprises an M537R substitution, an H800A substitution, and an F870L substitution. Other suitable modifications of the AsCpf1 amino acid sequence are known to those of ordinary skill in the art. Some exemplary sequences of wild-type AsCpf1 and AsCpf1 variants are provided below:

[0254] His-AsCpf1-sNLS-sNLS H800A amino acid sequence (SEQ ID NO: 1144):

MGHHHHHHGSTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYK
ELKPIIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAI
HDYFIGRTDNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFD
KFTTYFSGFYENRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFE
NVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNL
AIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNEN
VLETAEALFNELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKS
AKEKVQRSLKHEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQE
EKEILKSQLDSLLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNY
ATKKPYSVEKFKLNFQMPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRY
KALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFI
EPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKT
TSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKD
FAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAARLGEK
MLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRR
FTSDKFFFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDST
GKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIV
DLMIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKV
GGVLNPYQLTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHES
RKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDA
KGTPFIAGKRIVPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDD
SHAIDTMVALIRSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADA
NGAYHIALKGQLLLNHLKESKDLKLQNGISNQDWLAYIQELRNGSPKKKRKVGSPK
KKRKV

[0255] Cpf1 variant 1 amino acid sequence (SEQ ID NO: 1145):

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK
TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDN
LTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYE
NRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVS
TSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAH
IIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFN
ELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLK
HEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDS
LLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEK
FKLNFQRPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKT
SEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDL
NNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSS
QYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPN
LHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQ
KTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFLFHV
PITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLN
TIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAV

VVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQL
TDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDF
LHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRI
VPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALI
RSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKG
QLLLNHLKESKDLKLQNGISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRK
VGGSGGSGGSGGSGGSGGSGGSGGSLEHHHHHH

[0256] Cpf1 variant 2 amino acid sequence (SEQ ID NO: 1146):

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK
TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDN
LTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYE
NRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVS
TSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAH
IIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFN
ELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLK
HEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDS
LLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEK
FKLNFQMPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKT
SEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDL
NNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSS
QYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPN
LHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQ
KTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFFFHV
PITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLN
TIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAV
VVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQL
TDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDF
LHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRI
VPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALI
RSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKG
QLLLNHLKESKDLKLQNGISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRK
VGGSGGSGGSGGSGGSGGSGGSGGSLEHHHHHH

[0257] Cpf1 variant 3 amino acid sequence (SEQ ID NO: 1147):

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK
TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDN
LTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYE
NRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVS
TSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAH
IIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFN

ELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLK
HEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDS
LLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEK
FKLNFQRPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKT
SEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDL
NNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSS
QYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPN
LHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAARLGEKMLNKKLKDQ
KTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFLFHV
PITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLN
TIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAV
VVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQL
TDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDF
LHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRI
VPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALI
RSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKG
QLLLNHLKESKDLKLQNGISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRK
VGGSGGSGGSGGSGGSGGSGGSGGSLEHHHHHH

[0258] Cpf1 variant 4 amino acid sequence (SEQ ID NO: 1148):

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK
TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDN
LTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYE
NRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVS
TSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAH
IIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFN
ELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLK
HEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDS
LLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEK
FKLNFQRPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKT
SEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDL
NNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSS
QYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPN
LHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAARLGEKMLNKKLKDQ
KTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFLFHV
PITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLN
TIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAV
VVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQL
TDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDF
LHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRI
VPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALI
RSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKG
QLLLNHLKESKDLKLQNGISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRK
V

[0259] Cpf1 variant 5 amino acid sequence (SEQ ID NO: 1149):

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK
TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDN
LTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYE
NRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVS
TSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAH
IIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFN
ELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLK
HEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDS
LLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEK
FKLNFQRPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKT
SEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDL
NNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSS
QYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPN
LHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQ
KTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFLFHV
PITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLN
TIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAV
VVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQL
TDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDF
LHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRI
VPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALI
RSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKG
QLLLNHLKESKDLKLQNGISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRK
V

[0260] Cpf1 variant 6 amino acid sequence (SEQ ID NO: 1150):

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK
TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDN
LTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYE
NRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVS
TSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAH
IIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFN
ELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLK
HEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDS
LLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEK
FKLNFQRPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKT
SEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDL
NNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSS
QYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPN
LHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQ
KTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFLFHV
PITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLN
TIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAV
VVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQL
TDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDF

LHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRI
VPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALI
RSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKG
QLLLNHLKESKDLKLQNGISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRK
VGGSGGSGGSGGSGGSGGSGGSGGSLEHHHHHH

[0261] Cpf1 variant 7 amino acid sequence (SEQ ID NO: 1151):

MGRDPGKPIPNPLLGLDSTAPKKKRKVGIHGVPAATQFEGFTNLYQVSKTLRFELIPQ
GKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYKTYADQCLQLVQLDWENLSAAIDS
YRKEKTEETRNALIEEQATYRNAIHDYFIGRTDNLTDAINKRHAEIYKGLFKAELFNG
KVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENRKNVFSAEDISTAIPHRIVQDNFP
KFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQIDLYN
QLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFIL
EEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLTHIFISHKKLETISSALCD
HWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEIISAAGKELSEAFKQK
TSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAVDESNEVDPEF
SARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQMPTLASGWDVNKEKNN
GAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCS
TQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQK
GYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRI
AEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNG
QAELFYRPKSRMKRMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDL
SDEARALLPNVITKEVSHEIIKDRRFTSDKFFFHVPITLNYQAANSPSKFNQRVNAYLK
EHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQA
WSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQ
FEKMLIDKLNCLVLKDYPAEKVGGVLNPYQLTDQFTSFAKMGTQSGFLFYVPAPYTS
KIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRG
LPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHRFTGRYRDLYPANELIALL
EEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNAATGEDYINSPVRDL
NGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNGISNQDWL
AYIQELRNPKKKRKVKLAAALEHHHHHH

[0262] Exemplary AsCpf1 wild-type amino acid sequence (SEQ ID NO: 1152):

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK
TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDN
LTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYE
NRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVS
TSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAH
IIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFN
ELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLK
HEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDS
LLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEK
FKLNFQMPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKT
SEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDL
NNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSS
QYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPN

LHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQ
KTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFFFHV
PITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLN
TIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAV
VVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQL
TDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDF
LHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRI
VPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALI
RSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKG
QLLLNHLKESKDLKLQNGISNQDWLAYIQELRN

[0263] Additional suitable nucleases and nuclease variants will be apparent to the skilled artisan based on the present disclosure in view of the knowledge in the art. Exemplary suitable nucleases may include, but are not limited to, those provided in Table 2 herein.

Nucleic acids encoding RNA-guided nucleases

[0264] Nucleic acids encoding RNA-guided nucleases, e.g., Cas9, Cpf1 or functional fragments thereof, are provided herein. Exemplary nucleic acids encoding RNA-guided nucleases have been described previously (see, e.g., Cong 2013; Wang 2013; Mali 2013; Jinek 2012).

[0265] In some cases, a nucleic acid encoding an RNA-guided nuclease can be a synthetic nucleic acid sequence. For example, the synthetic nucleic acid molecule can be chemically modified. In certain embodiments, an mRNA encoding an RNA-guided nuclease will have one or more (e.g., all) of the following properties: it can be capped; polyadenylated; and substituted with 5-methylcytidine and/or pseudouridine.

[0266] Synthetic nucleic acid sequences can also be codon optimized, e.g., at least one non-common codon or less-common codon has been replaced by a common codon. For example, the synthetic nucleic acid can direct the synthesis of an optimized messenger mRNA, e.g., optimized for expression in a mammalian expression system, e.g., described herein. Examples of codon optimized Cas9 coding sequences are presented in Cotta-Ramusino.

[0267] In addition, or alternatively, a nucleic acid encoding an RNA-guided nuclease may comprise a nuclear localization sequence (NLS). Nuclear localization sequences are known in the art.

[0268] As an example, the nucleic acid sequence for Cpf1 variant 4 is set forth below as SEQ ID NO: 1177

ATGACCCAGTTTGAAGGTTTCACCAATCTGTATCAGGTTAGCAAAACCCTGCGTTTTGAACT
GATTCCGCAGGGTAAAACCCTGAAACATATTCAAGAACAGGGCTTCATCGAAGAGGATAAAG
CACGTAACGATCACTACAAAGAACTGAAACCGATTATCGACCGCATCTATAAAACCTATGCA
GATCAGTGTCTGCAGCTGGTTCAGCTGGATTGGGAAAATCTGAGCGCAGCAATTGATAGTTA
TCGCAAAGAAAAAACCGAAGAAACCCGTAATGCACTGATTGAAGAACAGGCAACCTATCGTA
ATGCCATCCATGATTATTTCATTGGTCGTACCGATAATCTGACCGATGCAATTAACAAACGT
CACGCCGAAATCTATAAAGGCCTGTTTAAAGCCGAACTGTTTAATGGCAAAGTTCTGAAACA
GCTGGGCACCGTTACCACCACCGAACATGAAAATGCACTGCTGCGTAGCTTTGATAAATTCA
CCACCTATTTCAGCGGCTTTTATGAGAATCGCAAAAACGTGTTTAGCGCAGAAGATATTAGC
ACCGCAATTCCGCATCGTATTGTGCAGGATAATTTCCCGAAATTCAAAGAGAACTGCCACAT
TTTTACCCGTCTGATTACCGCAGTTCCGAGCCTGCGTGAACATTTTGAAAACGTTAAAAAAG
CCATCGGCATCTTTGTTAGCACCAGCATTGAAGAAGTTTTTAGCTTCCCGTTTTACAATCAG
CTGCTGACCCAGACCCAGATTGATCTGTATAACCAACTGCTGGGTGGTATTAGCCGTGAAGC
AGGCACCGAAAAAATCAAAGGTCTGAATGAAGTGCTGAATCTGGCCATTCAGAAAAATGATG
AAACCGCACATATTATTGCAAGCCTGCCGCATCGTTTTATTCCGCTGTTCAAACAAATTCTG
AGCGATCGTAATACCCTGAGCTTTATTCTGGAAGAATTCAAATCCGATGAAGAGGTGATTCA
GAGCTTTTGCAAATACAAAACGCTGCTGCGCAATGAAAATGTTCTGGAAACTGCCGAAGCAC
TGTTTAACGAACTGAATAGCATTGATCTGACCCACATCTTTATCAGCCACAAAAAACTGGAA
ACCATTTCAAGCGCACTGTGTGATCATTGGGATACCCTGCGTAATGCCCTGTATGAACGTCG
TATTAGCGAACTGACCGGTAAAATTACCAAAAGCGCGAAAGAAAAAGTTCAGCGCAGTCTGA
AACATGAGGATATTAATCTGCAAGAGATTATTAGCGCAGCCGGTAAAGAACTGTCAGAAGCA
TTTAAACAGAAAACCAGCGAAATTCTGTCACATGCACATGCAGCACTGGATCAGCCGCTGCC
GACCACCCTGAAAAAACAAGAAGAAAAAGAAATCCTGAAAAGCCAGCTGGATAGCCTGCTGG
GTCTGTATCATCTGCTGGACTGGTTTGCAGTTGATGAAAGCAATGAAGTTGATCCGGAATTT
AGCGCACGTCTGACCGGCATTAAACTGGAAATGGAACCGAGCCTGAGCTTTTATAACAAAGC
CCGTAATTATGCCACCAAAAAACCGTATAGCGTCGAAAAATTCAAACTGAACTTTCAGCGTC
CGACCCTGGCAAGCGGTTGGGATGTTAATAAAGAAAAAAACAACGGTGCCATCCTGTTCGTG

AAAAATGGCCTGTATTATCTGGGTATTATGCCGAAACAGAAAGGTCGTTATAAAGCGCTGAG
CTTTGAACCGACGGAAAAAACCAGTGAAGGTTTTGATAAAATGTACTACGACTATTTTCCGG
ATGCAGCCAAAATGATTCCGAAATGTAGCACCCAGCTGAAAGCAGTTACCGCACATTTTCAG
ACCCATACCACCCCGATTCTGCTGAGCAATAACTTTATTGAACCGCTGGAAATCACCAAAGA
GATCTACGATCTGAATAACCCGGAAAAAGAGCCGAAAAAATTCCAGACCGCATATGCAAAAA
AAACCGGTGATCAGAAAGGTTATCGTGAAGCGCTGTGTAAATGGATTGATTTCACCCGTGAT
TTTCTGAGCAAATACACCAAAACCACCAGTATCGATCTGAGCAGCCTGCGTCCGAGCAGCCA
GTATAAAGATCTGGGCGAATATTATGCAGAACTGAATCCGCTGCTGTATCATATTAGCTTTC
AGCGTATTGCCGAGAAAGAAATCATGGACGCAGTTGAAACCGGTAAACTGTACCTGTTCCAG
ATCTACAATAAAGATTTTGCCAAAGGCCATCATGGCAAACCGAATCTGCATACCCTGTATTG
GACCGGTCTGTTTAGCCCTGAAAATCTGGCAAAAACCTCGATTAAACTGAATGGTCAGGCGG
AACTGTTTTATCGTCCGAAAAGCCGTATGAAACGTATGGCAGCTCGTCTGGGTGAAAAAATG
CTGAACAAAAAACTGAAAGACCAGAAAACCCCGATCCCGGATACACTGTATCAAGAACTGTA
TGATTATGTGAACCATCGTCTGAGCCATGATCTGAGTGATGAAGCACGTGCCCTGCTGCCGA
ATGTTATTACCAAAGAAGTTAGCCACGAGATCATTAAAGATCGTCGTTTTACCAGCGACAAA
TTCCTGTTTCATGTGCCGATTACCCTGAATTATCAGGCAGCAAATAGCCCGAGCAAATTTAA
CCAGCGTGTTAATGCATATCTGAAAGAACATCCAGAAACGCCGATTATTGGTATTGATCGTG
GTGAACGTAACCTGATTTATATCACCGTTATTGATAGCACCGGCAAAATCCTGGAACAGCGT
AGCCTGAATACCATTCAGCAGTTTGATTACCAGAAAAAACTGGATAATCGCGAGAAAGAACG
TGTTGCAGCACGTCAGGCATGGTCAGTTGTTGGTACAATTAAAGACCTGAAACAGGGTTATC
TGAGCCAGGTTATTCATGAAATTGTGGATCTGATGATTCACTATCAGGCCGTTGTTGTGCTG
GAAAACCTGAATTTTGGCTTTAAAAGCAAACGTACCGGCATTGCAGAAAAAGCAGTTTATCA
GCAGTTCGAGAAAATGCTGATTGACAAACTGAATTGCCTGGTGCTGAAAGATTATCCGGCTG
AAAAAGTTGGTGGTGTTCTGAATCCGTATCAGCTGACCGATCAGTTTACCAGCTTTGCAAAA
ATGGGCACCCAGAGCGGATTTCTGTTTTATGTTCCGGCACCGTATACGAGCAAAATTGATCC
GCTGACCGGTTTTGTTGATCCGTTTGTTTGGAAAACCATCAAAAACCATGAAAGCCGCAAAC
ATTTTCTGGAAGGTTTCGATTTTCTGCATTACGACGTTAAAACGGGTGATTTCATCCTGCAC
TTTAAAATGAATCGCAATCTGAGTTTTCAGCGTGGCCTGCCTGGTTTTATGCCTGCATGGGA
TATTGTGTTTGAGAAAAACGAAACACAGTTCGATGCAAAAGGCACCCCGTTTATTGCAGGTA
AACGTATTGTTCCGGTGATTGAAAATCATCGTTTCACCGGTCGTTATCGCGATCTGTATCCG
GCAAATGAACTGATCGCACTGCTGGAAGAGAAAGGTATTGTTTTTCGTGATGGCTCAAACAT
TCTGCCGAAACTGCTGGAAAATGATGATAGCCATGCAATTGATACCATGGTTGCACTGATTC
GTAGCGTTCTGCAGATGCGTAATAGCAATGCAGCAACCGGTGAAGATTACATTAATAGTCCG

GTTCGTGATCTGAATGGTGTTTGTTTTGATAGCCGTTTTCAGAATCCGGAATGGCCGATGGA
TGCAGATGCAAATGGTGCATATCATATTGCACTGAAAGGACAGCTGCTGCTGAACCACCTGA
AAGAAAGCAAAGATCTGAAACTGCAAAACGGCATTAGCAATCAGGATTGGCTGGCATATATC
CAAGAACTGCGTAACGGTCGTAGCAGTGATGATGAAGCAACCGCAGATAGCCAGCATGCAGC
ACCGCCTAAAAAGAAACGTAAAGTT

Activin

[0269] The TGF-β superfamily consists of more than 45 members including activins, inhibins, myostatin, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and nodal (see, e.g., Morianos et al., Journal of Autoimmunity 104:102314 (2019)). Activins are found either as homodimers or heterodimers of βA or/and βB subunits linked with disulfide bonds. There are three functional isoforms of activins: activin-A (βAβA), activin B (βBβB) and activin AB (βAβB) (Xia et al., J. Endocrinol. 202:1-12 (2009)). The βC and βE subunits are found in mammals and the βB subunit in Xenopus laevis. Transcripts of the βA and βB subunits are detected in nearly every tissue in the human body and exhibit increased expression in the reproductive system, while the βC and βE subunits are predominantly expressed in the liver (Woodruff, Biochem. Pharmacol. 55:953-963 (1998)). Activin-A is a cytokine of approximately 25 kDa and represents the most extensively investigated protein among the family of activins. Activin-A was initially identified as a gonadal protein that induces the biosynthesis and secretion of the follicle-stimulating hormone from the pituitary (Hedger et al., Cytokine Growth Factor Rev. 24:285-295 (2013)). It is highly conserved among vertebrates, reaching up to 95% homology between species. Activin-A regulates fundamental biologic processes, such as, haematopoiesis, embryonic development, stem cell maintenance and pluripotency, tissue repair and fibrosis (Kariyawasam et al., Clin. Exp. Allergy 41:1505-1514 (2011)).

[0270] Activin, e.g., Activin A, is well known and commercially available (from, e.g., STEMCELL Technologies Inc., Cambridge, MA).

Culture Methods

[0271] In general, an ES cell (e.g., an ES cell genetically engineered not to express one or more TGFβ receptor, e.g., TGFβRII) can be cultured to maintain pluripotency by

culturing such ES cells in media that contains activin, e.g., a particular, effective level of activin (e.g., during one or more stages of culture).

[0272] In some embodiments, ES cells described herein are cultured (e.g., at one or more stages of culture) in a medium that includes activin, e.g., an elevated level of activin, to maintain pluripotency of the cells. In some embodiments, a level of one or more ES markers (e.g., SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and/or Nanog) in a sample of cells from the culture is increased relative to the corresponding level(s) in a sample of cells cultured using the same medium that does not include activin, e.g., an elevated level of activin. In some embodiments, the increased level of one or more ES marker is higher than the corresponding level(s) by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, or more, of the corresponding level.

[0273] As used herein, an "elevated level of activin" means a higher concentration of activin than is present in a standard medium, a starting medium, a medium used at one or more stages of culture, and/or in a medium in which ES cells are cultured. In some embodiments, activin is not present in a standard and/or starting medium, a medium used at one or more other stages of culture, and/or in a medium in which ES cells are cultured, and an "elevated level" is any amount of activin. A medium can include an elevated level of activin initially (i.e., at the start of a culture), and/or medium can be supplemented with activin to achieve an elevated level of activin at a particular time or times (e.g., at one or more stages) during culturing.

[0274] In some embodiments, an elevated level of activin is an increase of at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 550%, 600%, 650%, 700%, 750%, 800%, 850%, 900%, 950%, 1000% or more, relative to a level of activin in a standard medium, a starting medium, a medium during one or more stages of culture, and/or in a medium in which ES cells are cultured.

[0275] In some embodiments, an elevated level of activin is about 0.5 ng/mL, 1 ng/mL, 2 ng/mL, 3 ng/mL, 4 ng/mL, 5 ng/mL, 10 ng/mL, 15 ng/mL, 20 ng/mL, 25 ng/mL, 30 ng/mL, 35 ng/mL, 40 ng/mL, 45 ng/mL, 50 ng/mL, 60 ng/mL, 70 ng/mL, 80 ng/mL, 90

ng/mL, 100 ng/mL, or more, activin. In some embodiments, an elevated level of activin is about 0.5 ng/mL to about 20 ng/mL activin, about 0.5 ng/mL to about 10 ng/mL activin, about 4 ng/mL to about 10 ng/mL activin.

[0276] Cells can be cultured in a variety of cell culture media known in the art, which are modified according to the disclosure to include activin as described herein. Cell culture medium is understood by those of skill in the art to refer to a nutrient solution in which cells, such as animal or mammalian cells, are grown. A cell culture medium generally includes one or more of the following components: an energy source (e.g., a carbohydrate such as glucose); amino acids; vitamins; lipids or free fatty acids; and trace elements, e.g., inorganic compounds or naturally occurring elements in the micromolar range. Cell culture medium can also contain additional components, such as hormones and other growth factors (e.g., insulin, transferrin, epidermal growth factor, serum, and the like); signaling factors (e.g., interleukin 15 (IL-15), transforming growth factor beta (TGF-β), and the like); salts (e.g., calcium, magnesium and phosphate); buffers (e.g., HEPES); nucleosides and bases (e.g., adenosine, thymidine, hypoxanthine); antibiotics (e.g., gentamycin); and cell protective agents (e.g., a Pluronic polyol (Pluronic F68)).

[0277] Media that has been prepared or commercially available can be modified according to the present disclosure for utilization in the methods described herein. Nonlimiting examples of such media include Minimal Essential Medium (MEM, Sigma, St. Louis, Mo.); Ham's F10 Medium (Sigma); Dulbecco's Modified Eagles Medium (DMEM, Sigma); RPM I-1640 Medium (Sigma); HyClone cell culture medium (HyClone, Logan, Utah); Power CHO2 (Lonza Inc., Allendale, NJ); and chemically-defined (CD) media, which are formulated for particular cell types. In some embodiments, a culture medium is an E8 medium described in, e.g., Chen et al., Nat. Methods 8:424-429 (2011)). In some embodiments, a cell culture medium includes activin but lacks TGFβ.

[0278] Cell culture conditions (including pH, O_2, CO_2, agitation rate and temperature) suitable for ES cells are those that are known in the art, such as described in Schwartz et al., Methods Mol. Biol. 767:107-123 (2011) and Chen et al., Nat. Methods 8:424-429 (2011).

[0279] In some embodiments, cells are cultured in one or more stages, and cells can be cultured in medium having an elevated level of activin in one or more stages. For

example, a culture method can include a first stage (e.g., using a medium having a reduced level of or no activin) and a second stage (e.g., using a medium having an elevated level of activin). In some embodiments, a culture method can include a first stage (e.g., using a medium having an elevated level of activin) and a second stage (e.g., using a medium having a reduced level of activin). In some embodiments, a culture method includes more than two stages, e.g., 3, 4, 5, 6, or more stages, and any stage can include medium having an elevated level of activin or a reduced level of activin. The length of culture is not limiting. For example, a culture method can be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more days. In some embodiments, a culture method includes at least two stages. For example, a first stage can include culturing cells in medium having a reduced level of activin (e.g., for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more days), and a second stage can include culturing cells in medium having an elevated level of activin (e.g., for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more days). In some embodiments, a first stage can include culturing cells in medium having an elevated level of activin (e.g., for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more days), and a second stage can include culturing cells in medium having a reduced level of activin (e.g., for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more days).

[0280] In particular methods, levels of one or more ES marker (e.g., SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and/or Nanog) expressed in a sample of cells from a cell culture are monitored during one or more times (e.g., one or more stages) of cell culture, thereby allowing adjustment (e.g., increasing or decreasing the amount of activin in the culture) stopping the culture, and/or harvesting the cells from the culture.

Methods of Characterization

Methods of characterizing cells including characterizing cellular phenotype are known to those of skill in the art. In some embodiments, one or more such methods may include, but not be limited to, for example, morphological analyses and flow cytometry. Cellular lineage and identity markers are known to those of skill in the art. One or more such markers may be combined with one or more characterization methods to determine a composition of a cell population or phenotypic identity of one or more cells. For example, in some embodiments, cells of a particular population will be characterized using flow cytometry. In some such embodiments, a sample of a population of cells will be evaluated for presence and proportion

of one or more cell surface markers and/or one or more intracellular markers. As will be understood by those of skill in the art, such cell surface markers may be representative of different lineages. For example, pluripotent cells may be identified by one or more of any number of markers known to be associated with such cells, such as, for example, CD34. Further, in some embodiments, cells may be identified by markers that indicate some degree of differentiation. Such markers will be known to one of skill in the art. For example, in some embodiments, markers of differentiated cells may include those associated with differentiated hematopoietic cells such as, e.g., CD43, CD45 (differentiated hematopoietic cells). In some embodiments, markers of differentiated cells may be associated with NK cell phenotypes such as, e.g., CD56 (also known as neural cell adhesion molecule), NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 (CD16), natural killer group-2 member A (NKG2A), natural killer group-2 member D (NKG2D), CD69, a natural cytotoxicity receptor (e.g., NCR1, NCR2, NCR3, NKp30, NKp44, NKp46, and/or CD158b), killer immunoglobulin-like receptor (KIR), and CD94 (also known as killer cell lectin-like receptor subfamily D, member 1 (KLRD1)) etc. In some embodiments, markers may be T cell markers (e.g., CD3, CD4, CD8, etc.).

Methods of Use

[0281] A variety of diseases, disorders and/or conditions may be treated through use of technologies provided by the present disclosure. For example, in some embodiments, a disease, disorder and/or condition may be treated by introducing modified cells as described herein (e.g., edited iNK cells) to a subject. Examples of diseases that may be treated include, but not limited to, cancer, e.g., solid tumors, e.g., of the brain, prostate, breast, lung, colon, uterus, skin, liver, bone, pancreas, ovary, testes, bladder, kidney, head, neck, stomach, cervix, rectum, larynx, or esophagus; and hematological malignancies, e.g., acute and chronic leukemias, lymphomas, e.g., B-cell lymphomas including Hodgkin's and non-Hodgkin lymphomas , multiple myeloma and myelodysplastic syndromes.

[0282] In some embodiments, the present disclosure provides methods of treating a subject in need thereof by administering to the subject a composition comprising any of the cells described herein. In some embodiments, a therapeutic agent or composition may be administered before, during, or after the onset of a disease, disorder, or condition (including, e.g., an injury).

[0283] In particular embodiments, the subject has a disease, disorder, or condition, that can be treated by a cell therapy. In some embodiments, a subject in need of cell therapy is a subject with a disease, disorder and/or condition, whereby a cell therapy, *e.g.*, a therapy in which a composition comprising a cell described herein, is administered to the subject, whereby the cell therapy treats at least one symptom associated with the disease, disorder, and/or condition. In some embodiments, a subject in need of cell therapy includes, but is not limited to, a candidate for bone marrow or stem cell transplant, a subject who has received chemotherapy or irradiation therapy, a subject who has or is at risk of having a hyperproliferative disorder or a cancer, *e.g.*, a hyperproliferative disorder or a cancer of hematopoietic system, a subject having or at risk of developing a tumor, *e.g.*, a solid tumor, and/or a subject who has or is at risk of having a viral infection or a disease associated with a viral infection.

Pharmaceutical Compositions

[0284] In some embodiments, the present disclosure provides pharmaceutical compositions comprising one or more genetically modified cells described herein, e.g., an edited iNK cell described herein. In some embodiments, a pharmaceutical composition further comprises a pharmaceutically acceptable excipient. In some embodiments, a pharmaceutical composition comprises isolated pluripotent stem cell-derived hematopoietic lineage cells comprising at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs, e.g., genetically modified (e.g., edited) T cells, NK cells, NKT cells, CD34+ HE cells or HSCs. In some embodiments, a pharmaceutical composition comprises isolated pluripotent stem cell-derived hematopoietic lineage cells comprising about 95% to about 100% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs, e.g., genetically modified (e.g., edited) T cells, NK cells, NKT cells, CD34+ HE cells or HSCs.

[0285] In some embodiments, a pharmaceutical composition of the present disclosure comprises an isolated population of pluripotent stem cell-derived hematopoietic lineage cells, wherein the isolated population has less than about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 15%, 20%, 25%, or 30% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs, e.g., genetically modified (e.g., edited) T cells, NK cells, NKT cells, CD34+ HE cells or HSCs. In some embodiments, an isolated population of pluripotent stem cell-derived hematopoietic lineage

cells has more than about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 15%, 20%, 25%, or 30% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs, e.g., genetically modified (e.g., edited) T cells, NK cells, NKT cells, CD34+ HE cells or HSCs. In some embodiments, an isolated population of pluripotent stem cell-derived hematopoietic lineage cells has about 0.1% to about 1%, about 1% to about 3%, about 3% to about 5%, about 10%- about 15%, about 15%-20%, about 20%-25%, about 25%-30%, about 30%-35%, about 35%-40%, about 40%-45%, about 45%-50%, about 60%-70%, about 70%-80%, about 80%-90%, about 90%-95%, or about 95% to about 100% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs, e.g., genetically modified (e.g., edited) T cells, NK cells, NKT cells, CD34+ HE cells or HSCs.

[0286] In some embodiments, an isolated population of pluripotent stem cell-derived hematopoietic lineage cells comprises about 0.1%, about 1%, about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% , about 99%, or about 100% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs, e.g., genetically modified (e.g., edited) T cells, NK cells, NKT cells, CD34+ HE cells or HSCs.

[0287] As one of ordinary skill in the art would understand, both autologous and allogeneic cells can be used in adoptive cell therapies. Autologous cell therapies generally have reduced infection, low probability for GVHD, and rapid immune reconstitution relative to other cell therapies. Allogeneic cell therapies generally have an immune mediated graft-versus-malignancy (GVM) effect, and low rate of relapse relative to other cell therapies. Based on the specific condition(s) of the subject in need of the cell therapy, one of ordinary skill in the art would be able to determine which specific type of therapy(ies) to administer.

[0288] In some embodiments, a pharmaceutical composition comprises pluripotent stem cell-derived hematopoietic lineage cells that are allogeneic to a subject. In some embodiments, a pharmaceutical composition comprises pluripotent stem cell-derived hematopoietic lineage cells that are autologous to a subject. For autologous transplantation, the isolated population of pluripotent stem cell-derived hematopoietic lineage cells can be either a complete or partial HLA-match with patient subject. In some embodiments, the pluripotent stem cell-derived hematopoietic lineage cells are not HLA-matched to a subject.

[0289] In some embodiments, pluripotent stem cell-derived hematopoietic lineage cells can be administered to a subject without being expanded ex vivo or in vitro prior to administration. In particular embodiments, an isolated population of derived hematopoietic lineage cells is modulated and treated ex vivo using one or more agent to obtain immune cells with improved therapeutic potential. In some embodiments, the modulated population of derived hematopoietic lineage cells can be washed to remove the treatment agent(s), and the improved population can be administered to a subject without further expansion of the population in vitro. In some embodiments, an isolated population of derived hematopoietic lineage cells is expanded prior to modulating the isolated population with one or more agents.

[0290] In some embodiments, an isolated population of derived hematopoietic lineage cells can be genetically modified (e.g., by recombinant methods) to express TCR, CAR or other proteins. For genetically engineered derived hematopoietic lineage cells that express recombinant TCR or CAR, whether prior to or after genetic modification of the cells, the cells can be activated and expanded using methods as described, for example, in U.S. Pat. Nos. 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005.

Cancers

[0291] Any cancer can be treated using a composition described herein. Exemplary therapeutic targets of the present disclosure include cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, eye, gastrointestine, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, a cancer may specifically be of the following non-limiting histological type: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma;

chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; Paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; Leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; Kaposi sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; Ewing sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; B-cell lymphoma; Hodgkin's disease; Hodgkin's

lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

[0292] In some embodiments, the cancer is a breast cancer. In some embodiments, the cancer is colon cancer. In some embodiments, the cancer is gastric cancer. In some embodiments, the cancer is RCC. In another embodiment, the cancer is non-small cell lung cancer (NSCLC).

[0293] In some embodiments, solid cancer indications that can be treated with iNK cells (e.g., genetically modified iNK cells, e.g., edited iNK cells) provided herein, either alone or in combination with one or more additional cancer treatment modality, include: bladder cancer, hepatocellular carcinoma, prostate cancer, ovarian/uterine cancer, pancreatic cancer, mesothelioma, melanoma, glioblastoma, HPV-associated and/or HPV-positive cancers such as cervical and HPV+ head and neck cancer, oral cavity cancer, cancer of the pharynx, thyroid cancer, gallbladder cancer, and soft tissue sarcomas. In some embodiments, hematological cancer indications that can be treated with the iNK cells (e.g., genetically modified iNK cells, e.g., edited iNK cells) provided herein, either alone or in combination with one or more additional cancer treatment modalities, include: ALL, CLL, NHL, DLBCL, AML, CML, and multiple myeloma (MM).

[0294] Examples of cellular proliferative and/or differentiative disorders of the lung include, but are not limited to, tumors such as bronchogenic carcinoma, including paraneoplastic syndromes, bronchioloalveolar carcinoma, neuroendocrine tumors, such as bronchial carcinoid, miscellaneous tumors, metastatic tumors, and pleural tumors, including solitary fibrous tumors (pleural fibroma) and malignant mesothelioma.

[0295] Examples of cellular proliferative and/or differentiative disorders of the breast include, but are not limited to, proliferative breast disease including, *e.g.*, epithelial hyperplasia, sclerosing adenosis, and small duct papillomas; tumors, *e.g.*, stromal tumors

such as fibroadenoma, phyllodes tumor, and sarcomas, and epithelial tumors such as large duct papilloma; carcinoma of the breast including in situ (noninvasive) carcinoma that includes ductal carcinoma in situ (including Paget's disease) and lobular carcinoma in situ, and invasive (infiltrating) carcinoma including, but not limited to, invasive ductal carcinoma, invasive lobular carcinoma, medullary carcinoma, colloid (mucinous) carcinoma, tubular carcinoma, and invasive papillary carcinoma, and miscellaneous malignant neoplasms. Disorders in the male breast include, but are not limited to, gynecomastia and carcinoma.

[0296] Examples of cellular proliferative and/or differentiative disorders involving the colon include, but are not limited to, tumors of the colon, such as non-neoplastic polyps, adenomas, familial syndromes, colorectal carcinogenesis, colorectal carcinoma, and carcinoid tumors.

[0297] Examples of cancers or neoplastic conditions, in addition to the ones described above, include, but are not limited to, a fibrosarcoma, myosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, gastric cancer, esophageal cancer, rectal cancer, pancreatic cancer, ovarian cancer, prostate cancer, uterine cancer, cancer of the head and neck, skin cancer, brain cancer, squamous cell carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular cancer, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, leukemia, lymphoma, or Kaposi sarcoma.

[0298] Exemplary useful additional cancer treatment modalities include, but are not limited to: chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine,

trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfanide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gamma1I and calicheamicin omegal1 (see, *e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®) and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane,

trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2′,2″-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, *e.g.*, paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANET™), and doxetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; cyclosporine, sirolimus, rapamycin, rapalogs, ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU, leucovovin; anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene (EVISTA®), droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (FARESTON®); anti-progesterones; estrogen receptor down-regulators (ERDs); estrogen receptor antagonists such as fulvestrant (FASLODEX®); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as leuprolide acetate (LUPRON® and ELIGARD®), goserelin acetate, buserelin acetate and tripterelin; other anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, megestrol acetate (MEGASE®), exemestane (AROMASIN®), formestanie, fadrozole, vorozole (RIVISOR®), letrozole

(FEMARA®), and anastrozole (ARIMIDEX®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); aptamers, described for example in U.S. Pat. No. 6,344,321, which is herein incorporated by reference in its entirety; anti HGF monoclonal antibodies (*e.g.*, AV299 from Aveo, AMG102, from Amgen); truncated mTOR variants (*e.g.*, CGEN241 from Compugen); protein kinase inhibitors that block mTOR induced pathways (*e.g.*, ARQ197 from Arqule, XL880 from Exelexis, SGX523 from SGX Pharmaceuticals, MP470 from Supergen, PF2341066 from Pfizer); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); COX-2 inhibitors such as celecoxib (CELEBREX®; 4-(5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl) benzenesulfonamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[0299] Other compounds that are effective in treating cancer are known in the art and described herein that are suitable for use with the compositions and methods of the present disclosure as additional cancer treatment modalities are described, for example, in the "Physicians' Desk Reference, 62nd edition. Oradell, N.J.: Medical Economics Co., 2008 ", Goodman & Gilman's "The Pharmacological Basis of Therapeutics, Eleventh Edition. McGraw-Hill, 2005", "Remington: The Science and Practice of Pharmacy, 20th Edition. Baltimore, Md.: Lippincott Williams & Wilkins, 2000.", and "The Merck Index, Fourteenth Edition. Whitehouse Station, N.J.: Merck Research Laboratories, 2006", incorporated herein by reference in relevant parts.

[0300] Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By "consisting of is meant including, and limited to, whatever follows the phrase "consisting of:" Thus, the phrase "consisting of indicates that the

listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

[0301] These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

[0302] The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. The contents of database entries, e.g., NCBI nucleotide or protein database entries provided herein, are incorporated herein in their entirety. Where database entries are subject to change over time, the contents as of the filing date of the present application are incorporated herein by reference. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

[0303] The disclosure is further illustrated by the following examples. The examples are provided for illustrative purposes only. They are not to be construed as limiting the scope or content of the disclosure in any way.

EXAMPLES

Example 1: Generating edited iPSC cells using Cas12a and testing effect of Activin A on pluripotency

[0304] To generate natural killer cells from pluripotent stem cells, a representative induced pluripotent stem cell (iPSC) line was generated and designated "PCS-201". This line was generated by reprogramming adult male human primary dermal fibroblasts purchased from ATCC (ATCC® PCS-201-012) using a commercially available non-modified RNA reprogramming kit (Stemgent/Reprocell, USA). The reprogramming kit contains non-modified reprogramming mRNAs (OCT4, SOX2, KLF4, cMYC, NANOG, and LIN28) with immune evasion mRNAs (E3, K3, and B18R) and double-stranded microRNAs (miRNAs) from the 302/367 clusters. Fibroblasts were seeded in fibroblast expansion medium (DMEM/F12 with 10% FBS). The next day, media was switched to Nutristem medium and daily overnight transfections were performed for 4 days (day 1 to 4). Primary iPSC colonies appeared on day 7 and were picked on day 10-14. Picked colonies were expanded clonally to achieve a sufficient number of cells to establish a master cell bank. The parental line chosen from this process and used for the subsequent experiments passed standard quality controls, including confirmation of stemness marker expression, normal karyotype and pluripotency.

[0305] To generate edited iPSC cells, the PCS-201 (PCS) cells were electroporated with a Cas12a RNP designed to cut at the target gene of interest. Briefly, the cells were treated 24 hours prior to transfection with a ROCK inhibitor (Y27632). On the day of transfection, a single cell solution was generated using accutase and 500,000 PCS iPS cells were resuspended in the appropriate electroporation buffer and Cas12a RNP at a final concentration of 2μM. When two RNPs were added simultaneously, the total RNP concentration was 4 μM (2+2). This solution was electroporated using a Lonza 4D electroporator system. Following electroporation, the cells were plated in 6-well plates in mTESR media containing CloneR (Stemcell Technologies). The cells were allowed to grow for 3-5 days with daily media changes, and the CloneR was removed from the media by 48 hours post electroporation. To pick single colonies, the expanded cells were plated at a low density in 10 cm plates after resuspending them in a single cell suspension. Rock inhibitor was used to support the cells during single cell plating for 3-5 days post plating depending on the size of the colonies on the plate. After 7-10 days, sufficiently sized colonies with

acceptable morphology were picked and plated into 24-well plates. The picked colonies were expanded to sufficient numbers to allow harvesting of genomic DNA for subsequent analysis and for cell line cryopreservation. Editing was confirmed by NGS and selected clones were expanded further and banked. Ultimately, karyotyping, stemness flow, and differentiation assays were performed on a subset of selected clones.

[0306] Two target genes of interest were CISH and TGFβRII, both of which were hypothesized to enhance natural killer cell function. As the TGFβ:TGFβRII pathway is believed to be involved in the maintenance of pluripotency, it was hypothesized that a functional deletion of TGFβRII in iPSCs could lead to differentiation and prevent generation of TGFβRII edited iPSCs. Due to the convergence of Activin receptor signaling and TGFβRII signaling in regulating SMAD2/3 and other intracellular molecules, it was hypothesized that Activin A could replace TGFβ in commercially available pluripotent stem cell medias to generate edited lines. To test this hypothesis, the pluripotency of unedited and TGFβRII edited iPSCs grown with Activin A was assessed. Several different culture medias were utilized: "E6" (Essential 6™ Medium, #A1516401, ThermoFisher), which lacks TGFβ, "E7", which was E6 supplemented with 100 ng/ml of bFGF (Peprotech, #100-18B), "E8" (Essential 8™ Medium, #A1517001, ThermoFisher), and "E7 + ActA", which was E6 supplemented with 100 ng/ml of bFGF and varying concentrations of Activin A (Peprotech #120-14P). Typically, E6 and E7 medias are typically insufficient to maintain the stemness and pluripotency of PSCs over multiple passages in culture.

[0307] In order to determine whether Activin A could maintain PCS iPSCs in the absence of exogenous TGFβ, unedited PCS iPSCs were plated on a LaminStem™ 521 (Biological Industries) coated 6-well plate and cultured in E6, E7, E8 or E7+ActA (with Activin A at two different concentrations – 1 ng/ml and 4 ng/ml). After 2 passages, the cells were assessed for morphology and stemness marker expression. Morphology was assessed using a standard phase contrast setting on an inverted microscope. Colonies with defined edges and non-differentiated cells typical of iPSC colonies, were deemed to be stem like. To confirm the morphological observations, the expression of standard iPS cell stemness markers was measured using intracellular flow cytometry. Briefly, cells were dissociated, stained for extracellular markers, and then fixed overnight and permeabilized using the reagents and standard protocol from the Foxp3/Transcription Factor Staining Buffer Set

(eBioscience™). Cells were stained for flow cytometric analysis with anti-human TRA-1-60-R_AF®488 (Biolegend®; Clone TRA-1-60-R), anti-Human Nanog_AF®647 (BD Pharmingen™; Clone N31-355), and anti-Oct4 (Oct3)_PE (Biolegend®; Clone 3A2A20),. Cells were recorded on a NovoCyte Quanteon Flow Cytometer (Agilent) and analyzed using FlowJo (FlowJo, LLC). As shown in Figure 1, both 1 ng/mL and 4 ng/ml of Activin A was sufficient to maintain pluripotency with equivalent stemness marker expression to the cells grown in E8. As expected, cells grown in E6 and E7 (which lacked TGFβ) did not maintain stemness gene expression to the same degree as E8, indicating the loss of iPSC stemness in the absence of TGFβ or Activin A. These results suggest that Activin A can supplement iPSC stemness in the absence of TGFβ signaling.

[0308] Given the demonstration that Activin A could support iPSC stemness in the absence of TGFβ, TGFβRII knockout ("KO") iPSCs , CISH KO iPSCs, and TGFβRII/CISH double knockout ("DKO") iPSC lines were generated. Specifically, iPSCs were edited using an RNP having an engineered Cas12a with three amino acid substitutions (M537R, F870L, and H800A (SEQ ID NO: 1148)) and a gRNA specific for CISH or TGFβRII. To make CISH/TGFβRII DKO iPSCs, iPSCs were treated with an RNP targeting CISH and an RNP targeting TGFβRII simultaneously. The particular guide RNA sequences of Table 10 were used for editing of CISH and TGFβRII. Both guides were generated with a targeting domain consisting of RNA, an AsCpf1 scaffold of the sequence UAAUUUCUACUCUUGUAGAU (SEQ ID NO: 1153) located 5' of the targeting domain, and a 25-mer DNA extension of the sequence ATGTGTTTTTGTCAAAAGACCTTTT (SEQ ID NO: 1154) at the 5' terminus of the scaffold sequence.

Table 10. Guide RNA sequences

Target	gRNA Targeting Domain Sequence	Full Length gRNA Sequence
CISH 7050	GGUGUACAGCAGUGGCUGGU (SEQ ID NO: 1155)	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrGrGrUrGrUrArCrArGrCrArGrUrGrGrCrUrGrGrU (SEQ ID NO: 1156)
TGFβRII 24026	UGAUGUGAGAUUUUCCACCU (SEQ ID NO: 1157)	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrUrGrArUrGrUrGrArGrArU

		rUrUrUrCrCrArCrCrU (SEQ ID NO: 1158)

[0309] The edited clones were generated as described above with a minor modification for the cells treated with TGFβRII RNPs. Briefly, TGFβRII-edited PCS iPSCs and TGFβRII/CISH edited PCS iPSCs were plated after electroporation at the 6-well stage in the mTESR supplemented with 10 ng/ml of Activin A in order to support the generation of edited clones. The cells were cultured with 10 ng/ml of Activin A through the cell colony picking and early expansion stages. Colonies assessed as having the correct single KO (CISH KO or TGFβRII KO) or double KO (CISH/TGFβRII DKO) were picked and expanded (clonal selection).

[0310] To determine the optimal concentration of Activin A for culturing of TGFβRII KO and TGFβRII/CISH DKO iPSCs, a slightly expanded concentration curve was tested as shown Figure 2. Similar to the assessment performed previously, the iPSCs were cultured in a Matrigel-treated 6-well plate with concentrations of 1 ng/ml, 2 ng/ml, 4 ng/ml and 10 ng/ml Activin A. As shown in Figure 2, TGFβRII KO or CISH/TGFβRII DKO cells cultured in E7 medium supplemented with 4 ng/mL Activin A for 19 days (over 5 passages) maintained a wild type morphology. Figure 3 shows the morphology of TGFβRII KO PCS-201 hiPSC Clone 9.

[0311] As shown in Figure 4A, the initial editing efficiency of the iPSCs treated simultaneously with the CISH and TGFβRII RNPs (prior to clonal selection) was high, with 95% of the CISH alleles edited and 78% of the TGFβRII alleles edited. Unedited iPSC controls did not have indels at either loci. iPSCs that were treated with CISH or TGFβRII RNPs individually showed 93% and 82% editing rates prior to clone selection (depicted in Figure 4A). The KO cell lines (CISH KO iPSCs, TGFβRII KO iPSCs, and CISH/TGFβRII DKO iPSCs) were subsequently assessed for the presence of pluripotency markers Oct4, SSEA4, Nanog, and Tra-1-60 after culturing in the presence of supplemental Activin A. As shown in Figures 4B and 5, culturing the KO cell lines in Activin A maintained expression of these pluripotency markers.

[0312] The KO iPSC lines cultured in Activin A were next assessed for their capacity to differentiate using the STEMdiff™ Trilineage Differentiation Kit assay (from STEMCELL Technologies Inc., Vancouver, BC, CA) as depicted schematically in Figure 6. As shown in Figure 7A, culturing the single KO (TGFβRII KO iPSCs or CISH KO iPSCs) and DKO (TGFβRII/CISH DKO iPSCs) cell lines in media with supplemental Activin A maintained their ability to differentiate into early progenitors of all 3 germ layers, as shown by expression of ectoderm (OTX2), mesoderm (brachyury), and endoderm (GATA4) markers (Figure 7A). The unedited PCS control cells were also able to express each of these markers.

[0313] The edited iPSCs were next karyotyped to determine whether the Cas12a editing caused large genetic abnormalities, such as translocations. As shown in Figure 7B, the cells had normal karyotypes with no translocation between the cut sites.

[0314] To further support the results described above, an expanded Activin A concentration curve was performed on the unedited parental PSC line, an edited TGFβRII KO iPSC clone (C7), and an additional representative (unedited) cell line designated RUCDR (RUCDR Infinite Biologics group, Piscaway NJ). At the outset, the iPSCs were seeded at 1e5 cells per well in a 1x LaminStem™ 521 (Biological Industries) coated 12-well plate. Cells were then passaged 10 times over ~40-50 days using 0.5 mM EDTA in 1x PBS dissociation and Y-27632 (Biological Industries) until wells achieved >75% confluency. Cells were cultured in Essential 6™ Medium (Gibco), TeSR™-E7™, and TeSR™-E8™ (StemCell Technologies) for controls and titrated using TeSR™-E7™ supplemented with *E. coli*-derived recombinant human/murine/rat Activin A (PeproTech) spanning a 4-log concentration dosage (0.001 – 10 ng/mL). Following 5 and 10 passages, cells were dissociated and then fixed overnight and permeabilized using the reagents and standard protocol from the Foxp3/Transcription Factor Staining Buffer Set (eBioscience™). Cells were stained for flow cytometric analysis with anti-human TRA-1-60-R_AF®488 (Biolegend®; Clone TRA-1-60-R), anti Sox2_PerCP-Cy™5.5 (BD Pharmingen™; Clone O30-678), anti Human Nanog_AF®647 (BD Pharmingen™; Clone N31-355), anti-Oct4 (Oct3)_PE (Biolegend®; Clone 3A2A20), and anti-human SSEA-4_PE/Dazzle™ 594 (Biolegend®; Clone MC-813-70). Cells were recorded on a NovoCyte Quanteon Flow Cytometer (Agilent) and analyzed using FlowJo (FlowJo, LLC). Figure 7C shows the titration curves for the tested iPSC lines. The minimum concentration of Activin A required

to maintain each line varied slightly, with the TGFβRII KO iPSCs requiring a higher baseline amount of Activin A as compared to the parental control (0.5 ng/ml vs 0.1 ng/ml). In all 3 cell lines, 4 ng/ml was well above the minimum amount of Activin A necessary to maintain stemness marker expression over an extended culture period. Figure 7D shows the stemness marker expression in the cells culture with the base medias alone (no Activin A). As expected, the TGFβRII KO iPSCs did not maintain expression, while the two unedited lines were able to maintain stemness marker expression in E8.

Example 2: Differentiation of edited CISH KO, TGFβRII KO, and CISH/TGFβRII DKO iPSCs into iNK cells exhibiting enhanced function

[0315] Figure 8A depicts a schematic of an exemplary workflow for development of a CRISPR-Cas12a-edited iPSC platform for generation of enhanced CD56+ iNK cells. As shown in Figure 8A, the CISH and TGFβRII genes are targeted in iPSCs via delivery of RNPs to the cells using electroporation to generate CISH/TGFβRII DKO iPSCs. iPSCs with the desired edits at both the CISH and TGFβRII genes can then be selected and expanded to create a master iPSC bank. Edited cells from the iPSC master bank can then be differentiated into CD56+ CISH/TGFβRII DKO iNK cells.

[0316] Figure 8B and 8C depict two exemplary schematics of the process of differentiating iPSCs into iNK cells. As shown in Figure 8B and 8C, edited cells (or unedited control cells) were differentiated using a two-phase process. First, in the "hematopoietic differentiation phase," hiPSCs (edited and unedited) were cultured in StemDiff™ APEL2™ medium (StemCell Technologies) with SCF (40 ng/mL), BMP4 (20 ng/mL), and VEGF (20 ng/mL) from days 0-10, to produce spin embryoid bodies (SEBs). As shown in Figure 8B, SEBs were then cultured from days 11-39 in StemDiff™ APEL2™ medium comprising IL-3 (5 ng/mL, only present for the first week of culture), IL-7 (20 ng/mL), IL-15 (10 ng/mL), SCF (20 ng/mL), and Flt3L (10 ng/mL) in an NK cell differentiation phase. CISH KO iPSCs, TGFβRII KO iPSCs, CISH/TGFβRII DKO iPSCs, and unedited wild-type iPSC lines (PCS), were differentiated into iNKs according to the schematic in Figure 8B, and then characterized to assess whether they exhibited a phenotype congruent with NK cells (see Figures 9, 10, and 11A). CISH KO iPSCs, TGFβRII KO iPSCs, CISH/TGFβRII DKO iPSCs, and unedited wild-type iPSC lines, described in Figures 11B, 11C, 12B, 12C, and 13 were also

differentiated into iNKs utilizing the alternative method shown in Figure 8C, and then characterized to assess whether they exhibited a phenotype congruent with NK cells (see Figures 11B, 11C, 12B, 12C, and 13).

[0317] Specifically, the CISH KO iNKs, TGFβRII KO iNKs, CISH/TGFβRII DKO iNKs were assessed for exemplary phenotypic markers of (i) stem cells (CD34); and (ii) hematopoietic cells (CD43 and CD45) by flow cytometry. Briefly, two rows of embryoid bodies from a 96-well plate for each genotype were harvested for staining. Once a single cell solution was generated using Trypsin and mechanical disruption, the cells were stained for the human markers CD34, CD45, CD31, CD43, CD235a and CD41. As shown in Figure 9, CISH KO iNKs, TGFβRII KO iNKs, CISH/TGFβRII DKO iNKs, and iNKs derived from wild-type parental clones (PCS) exhibited lower levels of CD34 relative to control cells, which were purified CD34+ HSCs. CD34 expression levels were similar across these iNK cell clones indicating that editing of the iPSCs did not affect differentiation to the CD34+ stage. Figure 10 shows that CISH KO iNKs, TGFβRII KO iNKs, CISH/TGFβRII DKO iNKs, and iNKs derived from wild-type parental clones (PCS) exhibited similar surface expression profiles for CD43 and CD45. Thus, iNKS differentiated from edited and unedited iPSCs exhibited similar levels of markers for stem cells and hematopoietic cells, and both differentiated edited and unedited cells exhibited certain NK cell phenotypes based on marker expression profiles.

[0318] CISH KO iNKs, TGFβRII KO iNKs, CISH/TGFβRII DKO iNKs, iNKs derived from wild-type parental clones (WT), and NK cells derived from peripheral blood (PBNKs) were further assayed to determine their surface expression of CD56, a marker for NK cells. Briefly, cells were harvested on day 39 of differentiation, washed and resuspended in a flow staining buffer containing antibodies that recognize human CD56, CD16, NKp80, NKG2A, NKG2D, CD335, CD336, CD337, CD94, CD158. Cells events were recorded on a NovoCyte Quanteon Flow Cytometer (Agilent) and analyzed using FlowJo (FlowJo, LLC). Figure 11A shows that iNK cells derived from edited iPSCs exhibited similar CD56+ surface expression relative to iNKs derived from unedited iPSC parental clones and PBNK cells (at day 39 in culture). Figure 11B shows that iNK cells derived from edited iPSCs exhibited similar CD56+ and CD16+ surface expression relative to iNKs derived from unedited iPSC parental clones (at day 39 in culture). Figure 11C shows that iNK cells derived from edited

iPSCs exhibited similar CD56+ , CD54+, KIR+, CD16+, CD94+, NKG2A+, NKG2D+, NCR1+, NCR2+, and NCR3+ surface expression relative to iNKs derived from unedited iPSC parental clones and PBNK cells (at day 39 in culture)

[0319] To confirm cell functionality, cells were assessed using a tumor cell cytotoxicity assay on the xCelligence platform. Briefly, tumor targets, SK-OV-3 tumor cells, were plated and grown to an optimal cell density in 96-well xCelligence plates. iNKs were then added to the tumor targets at different E:T ratios (1:4, 1:2, 1:1, 2:1. 4:1 and 8:1) in the presence of TGFβ. Figure 12C shows that TGFβRII KO and CISH/TGFβRII DKO cells more effectively killed SK-OV-3 cells, as measured by percent cytolysis, relative to unedited iNK cells either in the presence or absence of TGF-β (at E:T ratios of 1:4, 1:2, 1:1, and 2:1).

[0320] While iNK cells generated using the alternative method described in Figure 8B were CD56+ and capable of killing tumor targets in an in vitro cytotoxicity assay, the iNKs did not express many of the canonical markers associated with mature NK cells such as CD16, NKG2A, and KIRs. A K562 feeder cell line is typically used to expand and mature iNKs that are generated by similar differentiation methodologies. After expansion on feeders, the iNKs often express CD16, KIRs and other surface markers indicative of a more mature phenotype. In order to identify a feeder free approach to achieve more mature iNKs with enhanced functionality, an alternative media composition was tested for the stage of differentiation between day 11 and day 39. Instead of culturing cells between day 11 and day 39 in APEL2 (as shown in Figure 8B), the spin embryoid bodies (SEBs) were cultured in NK MACS® media (MACS Miltenyi Biotec) with 15% human AB serum in the presence of the same cytokines as mentioned above. This protocol is depicted in Figure 8C. In order to compare the two media compositions, Day 11 SEBs from WT PCS, TGFβRII KO iPSCs, CISH KO iPSCs, and DKO iPSCs were split into two conditions for the second half of the differentiation process, one with APEL2 base and the other with the NKMACS + serum base. At day 39, the cell yield, marker expression, and cytotoxicity levels were assessed. In all cases, the NKMACS + serum condition (depicted in Figure 8C) outperformed the APEL2 condition (depicted in Figure 8B). Figure 8D shows that the NKMACS + serum condition yielded a greater fold expansion at the end of the 39 day process (nearly 300 fold expansion vs 100 fold expansion). When NK marker expression was analyzed by flow cytometry as described above, the iNKs cultured in NKMACS + serum were 34% CD16 positive and

exhibited 20% KIR expression while the APEL2 conditions yielded cells that were essentially negative for both markers. This was the case for all genotypes tested. In order to visualize the markers relative to time or condition, flow cytometry data was gated and analyzed in FlowJo and heat maps were constructed (Figures 8E and 8F). Samples were first cleaned by gating for live cells (FSC-H vs. LIVE/DEAD™ Fixable Yellow) followed by immune cells (SSC-A vs. FSC-A), singlets (FSC-H vs. FSC-A) and the natural killer cell population (CD56 vs. CD45). The NK population, defined as CD45+56+ cells, was gated and each marker was analyzed along the X-axis in an analysis synonymous to a histogram/count plot (CD16+, CD94+, NKG2A+, NKG2D+, CD335+, CD336+, CD337+, NKp80+, panKIR+). Statistics for the aforementioned markers are visualized with a double-gradient heat map (GraphPad Prism 8) with the key set to the following parameters: black=0, medium intensity 30<x<50, maximum intensity=100. Based on this analysis, the expression kinetics and magnitude across all genotypes were improved by the NKMACS + serum condition. The cells were also assessed in a tumor cell cytotoxicity assay as described previously. The iNKs generated in the NKMACS + serum conditions were capable of killing at a lower E:T ratio than the cells differentiated in APEL2, indicating that the improved NK maturation had a positive impact on the functionality of the cells (Figure 8G).

[0321] Analysis of additional differentiation markers in NKMACS + serum confirmed the presence of CD16 expression. Figure 11B shows analysis of specific subpopulations (CD45 vs CD56 and CD56 vs CD16) derived from unedited or DKO iPSCs. Additionally, the cell surface marker profile of unedited iNK cells and CISH/TGFβRII DKO iNKs in Figure 11C confirmed that the NK cell marker profile of the edited iNK cells was similar to that of unedited iNK cells. Taken together, these data show that Cas12a-edited single and double KO iPSC clones differentiate into iNK cells in a similar fashion as unedited iPSC clones, as defined by NK cell markers.

[0322] Additionally, certain edited iNK clonal cells (CISH single knockout "CISH_C2, C4, C5, and C8", TGFβRII single knockout "TGFβRII-C7", and TGFβRII/CISH double knockout "DKO-C1"), and parental clone iNK cells ("WT") were cultured in the presence of 1 ng/mL or 10 ng/mL IL-15, and differentiation markers were assessed at day 25, day 32, and day 39 post-hiPSC differentiation. As shown in Figure 14, surface expression phenotypes (measured as a percentage of the population) culturing in 10 ng/mL IL-15

resulted in a higher proportion of surface expression in the single knockouts, double knockouts, and the parental clonal line..

[0323] The edited iNK cells differentiated in NK MACS® medium + serum conditions were assessed for effector function *in vitro* using a range of molecular and functional analyses. First, a phosphoflow cytometry assay was performed to determine the phosphorylated state of STAT3 (pSTAT3) and SMAD2/3 (pSMAD2/3) in the day 39 iNK cells. CISH KO iNKs exhibited increased pSTAT3 upon IL-15 stimulation (Figure 11D), and CISH/TGFβRII DKO iNKs exhibited decreased pSMAD2/3 levels upon TGF-β stimulation as compared to unedited iNK cells (Figure 11E). These data suggest that CISH/TGFβRII DKO iNKs have enhanced sensitivity to IL-15 and resistance to TGF-β mediated immunosuppression. In addition, CISH/TGFβRII DKO iNKs were characterized for IFNγ and TNFα production using a phorbol myristate acetate and Ionomycin (PMA/IMN) stimulation assay. Briefly, cells were treated with 2 ng/ml of PMA and 0.125 μM of Ionomycin along with a protein transport inhibitor for 4 hours. The cells were harvested and stained using a standard intracellular staining protocol. The CISH/TGFβRII DKO iNKs produced significantly higher amounts of IFNγ and TFNα when stimulated with PMA/IMN (Figures 11F and 11G), providing evidence of enhanced cytokine production following stimulation relative to unedited control iNKs.

[0324] To test iNK tumor cell killing activity, a 3D solid tumor cell killing assay (depicted schematically in Figure 12A) was utilized. In brief, spheroids were formed by seeding 5,000 NucLight Red labeled SK-OV-3 cells in 96 well ultra-low attachment plates. Spheroids were incubated at 37°C before addition of effector cells (at different E:T ratios) and 10 ng/mL TGF-β, spheroids were subsequently imaged every 2 hours using the Incucyte S3 system for up to 120 hours. Data shown are normalized to the red object intensity at time of effector addition. Normalization of spheroid curves maintains the same efficacy patterns observed in non-normalized data. Using this assay, the cytotoxicity of iNKs differentiated from four CISH KO iPSC clones, two TGFβRII KO iPSC clones and one CISH/TGFβRII DKO iPSC clone were compared to control iNKs derived from the unedited parental iPSCs. As shown in Figure 12B, edited iNK cells were capable of reducing the size of SK-OV-3 spheroids more effectively than unedited iNK control cells (averaged data from 6 assays). In particular the CISH/TGFβRII DKO iNK cells reduced the size of SK-OV-3 spheroids to a

greater extent than unedited iNK cells at all E:T ratios greater than 0.01, and significantly at E:T ratios of 1 or higher. The TGFβRII KO clone 7 iNKs also exhibited significantly enhanced killing when compared to unedited iNK cells. While a number of single CISH KO clones did not show significant enhancement of killing at the 10:1 E:T ratio, the majority of clones did display a trend towards increased SK-OV-3 spheroid cell killing, with the greatest differential at the highest E:T ratio. To further elucidate the functionality of the edited iNKs, the cells were pushed to kill tumor targets repeatedly over a multiday period, herein described as an *in vitro* serial killing assay. At day 0 of the assay, 10 x 10^6 Nalm6 tumor cells (a B cell leukemia cell line) and 2 x 10^5 iNKs were plated in each well of a 96-well plate in the presence of IL-15 (10 ng/ml) and TGF-β (10ng/ml). At 48 hour intervals, a bolus of 5 x 10^3 Nalm6 tumor cells (a B cell leukemia cell line) was added to re-challenge the iNK population. As shown in Figure 13, the edited iNK cells (CISH/TGFβRII DKO iNK cells) exhibited continued killing of Nalm6 cells after multiple challenges with Nalm6 tumor cells, whereas unedited iNK cells were limited in their serial killing effect. The data supports the conclusion that the CISH and TGFβRII edits result in prolonged enhancement of cell killing.

[0325] Finally, edited iNK cells (CISH/TGFβRII DKO iNK cells) were assayed for their ability to kill tumor targets in an in vivo model. To this end, an established NOD scid gamma (NSG) xenograft model was utilized in an assay as depicted in Figure 15A. Briefly, 1 x 10^6 SK-OV-3 cells engineered to express luciferase were injected intraperitoneally (IP) at day 0. On day 3, the inoculated mice were imaged using an In vivo imaging system (IVIS) and randomized into 3 groups. The next day (day 4), 20 x 10^6 unedited iNKs or CISH/TGFβRII DKO iNKs were administered by IP injection, while a third group was injected with vehicle as a control. Following inoculation of the animals with tumor cells, animals were imaged once a week to measure tumor burden over time. Figure 15B depicts the bioluminescence of the tumors in the individual mice in the 3 different groups (n=9 in each group), vehicle, unedited iNKs, and CISH/TGFβRII DKO iNKs. The average tumor burden over time for these same animals is depicted in Figure 15C. A two way anova analysis was performed on the data, and CISH/TGFβRII DKO iNK treated animals had significantly less tumor burden as measured by bioluminescence when compared to animals treated with unedited iNKs (p value: 0.0004). By 10 days post-tumor implantation, mice injected with the CISH/TGFβRII DKO iNKs exhibited a significant reduction in the size of their tumors relative to mice injected with the vehicle controls or the unedited iNKs. The overall reduction

in tumor size is seen for several days, and at least until 35 days post-tumor implantation. These data show that the edited DKO iNKs were actively killing tumor cells in this in vivo model.

[0326] Overall, these results demonstrate that unedited and CISH/TGFβRII DKO iPSCs can be differentiated into iNK cells exhibiting canonical NK cell markers. Additionally, CISH/TGFβRII DKO iNK cells demonstrated enhanced anti-tumor activity against tumor cell lines derived from both solid and hematological malignancies.

Example 3: ADORA2A edited iPSCs give rise to edited iNKs with enhanced function

[0327] ADORA2A is another target gene of interest, the loss of which is hypothesized to affect NK cell function in a tumor microenvironment (TME). The ADORA2A gene encodes a receptor that responds to adenosine in the TME, resulting in the production of cAMP which functions to drive a number of inhibitory effects on NK cells. We hypothesized that knocking out the function of ADORA2A could enhance iNK cell function. Utilizing a similar approach to the one described in Examples 1 and 2, the PCS iPSC line was edited using a RNP having an engineered Cas12a with three amino acid substitutions (M537R, F870L, and H800A (SEQ ID NO: 1148)) and a gRNA specific to ADORA2A (except that 4 μM RNP was delivered to cells rather than 2 μM RNP). As described in Example 1, the gRNA was generated with a targeting domain consisting of RNA, an AsCpf1 scaffold of the sequence UAAUUUCUACUCUUGUAGAU (SEQ ID NO: 1153) located 5' of the targeting domain, and a 25-mer DNA extension of the sequence ATGTGTTTTTGTCAAAAGACCTTTT (SEQ ID NO: 1154) at the 5' terminus of the scaffold sequence. The ADORA2A gRNA sequence is shown in Table 11.

Table 11. Guide RNA sequence

Target	gRNA Targeting Domain Sequence	Full Length gRNA Sequence
ADORA2A 4113	CCAUCGGCCUGACUCCCAUG (SEQ ID NO: 1159)	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrCrCrArUrCrGrGrCrCrUrG

		rArCrUrCrCrCrArUrG (SEQ ID NO: 1160)

[0328] The bulk editing rate by the Cas12a RNP prior to clonal selection was 49% as determined by next-generation sequencing (NGS). Nonetheless, several clones that had both ADORA2A alleles edited were identified, expanded and differentiated. To determine whether an ADORA2A edited iPSC could yield CD45+CD56+ iNKs, both bulk and singled ADORA2A KO clones were differentiated using the NKMACS + serum protocol as described in Example 2 (Figure 8C). As shown in Figure 16A, edited iPSCs differentiated to iNKs with similar NK cell marker expression compared to unedited control iPSCs.

[0329] To confirm that Cas12a-mediated ADORA2A editing resulted in a functional deletion of the gene, cAMP accumulation in response to treatment with 5′-N-ethylcarboxamide adenosine ("NECA", a more stable adenosine analog that acts as an ADORA2A agonist) was assessed in both the edited and unedited control iNKs. Edited cells with a functional knockout of ADORA2A would not be expected to accumulate as much cAMP in the cells in response to NECA relative to cells with functional ADORA2A. Briefly, iNK cells were treated with varying concentrations of NECA for 15 minutes. The iNK cells were then lysed, and the cAMP in the lysate was then measured using a CisBio cAMP kit. As shown in Figure 16B, unedited iNKs had increased levels of cAMP accumulation as the concentration of NECA was increased (n=2). Conversely, the ADORA2A ("A2A KOs") KO iNKs showed minimal production of cAMP at increasing concentrations of NECA, indicating that the Cas12a-induced edits functionally knocked out ADORA2A function. The bulk iNKs (top two A2A KO iNK lines in Figure 16B) exhibited slightly higher levels of cAMP than the selected ADORA2A KO clones (lower four A2A KO iNK lines in Figure 16B), as would be expected from the lower editing rates in the bulk population. Based on this molecular evidence of functional ablation of ADORA2A, the iNKs would be expected to be resistant to the inhibitory effects of adenosine in a tumor microenvironment.

[0330] The ADORA2A KO iNKs were also tested in an in vitro NALM6 serial killing assay as described in Example 2, with one main difference: 100μM of NECA was added in place of TGFβ. The ADORA2A KO iNKs exhibited enhanced serial killing relative to the wild type iNKs in the presence of NECA, indicating that the ADORA2A KO iNKs were resistant to NECA inhibition (Figure 16C). As a result, the ADORA2A KO iNK cells

would be expected to have improved cytotoxicity against tumor cells in the presence of adenosine in the TME relative to unedited iNK cells.

Example 4: Generation of CISH/ TGFβRII /ADORA2A triple edited (TKO) iPSCs and the characterization of differentiated TKO iNKs

[0331] In order to generate CISH, TGFβRII, and ADORA2A triple edited (TKO) iPSCs, two approaches were taken; 1) two step editing in which the CISH/ TGFβRII DKO (CR) iPSC clone described in Examples 1 and 2 was edited at the ADORA2A locus via electroporation with an ADORA2A targeting RNP (as described in Example 3), and 2) simultaneous editing of PCS iPS cells with all 3 RNPs, one for each target gene. Both strategies utilized the editing protocol briefly described in Example 1. In the case of simultaneous editing, the total RNP concentration was 8 μM (Cish:2 μM+ TGFβRII:2 μM+ADORA2A:4μM). Regardless of the approach, cells were plated, expanded and colonies were picked as described above. Using NGS to analyze gDNA harvested from the iPSCs, it was determined that the bulk editing rates were 96.70%, 97.17%, and 90.16% for CISH, TGFβRII and ADORA2A, respectively, when all target genes were edited simultaneously. Picked colonies that had Insertions and/or Deletions (InDels) at all 6 alleles were selected for further analysis.

[0332] Similar to the analysis described in Example 1, unedited iPSCs and the edited iPSCs were differentiated to iNKs using the NK MACS + Serum condition (described in Figure 8C) and assessed by flow cytometry at different time points, including at day 25, day 32, and day 39 in culture. As shown in Figure 17A, analysis of the different NK surface markers revealed no major differences between clones that were generated by the two-step editing method (CR+A 8) or the simultaneous editing method (CRA 6). Both TKO clones (CR+A 8 and CRA 6) showed similar expression profiles to the unedited iNKs (Wt) at each time point. When the TKO iNK cells were analyzed for their responsiveness to NECA (as described in Example 3), both TKO iNKs had little to no cAMP accumulation (Figure 17B), demonstrating that ADORA2A was functionally knocked out. By contrast, the unedited iNKs demonstrated a NECA dose dependent increase in cAMP (Figure 17B). These results indicate that the TKO iNKs would be expected to be resistant to the inhibitory effects of adenosine in the TME. Finally, the CISH/TGFβRII/ADORA2A TKO iNKs were assessed alongside

CISH/ TGFβRII DKO iNKs, ADORA2A single KO (SKO) iNKs, and unedited iNKs in a 3D tumor cell killing assay. This assay was performed as described in Example 2 with IL-15 and TGFβ but without NECA. Interestingly, both the TKO (CRA6) and DKO (CR) iNKs outperformed the unedited iNKs in killing the tumor cells, indicating that both multiplex edited iNKs have enhanced function over unedited control cells (Figure 17C). These results show that knocking out ADORA2A does not negatively affect the ability of iNKs having CISH and TGFBRII KOs to kill tumor spheroid cells.

Example 5: Selection of CISH, TGFβRII, ADORA2A, TIGIT, and NKG2A targeting gRNAs.

[0333] The cutting efficiency of CISH, TGFBRII, ADORA2A, TIGIT, and NKG2A Cas12a guide RNAs were further tested. Guide RNAs were screened by complexing commercially synthesized gRNAs with Cas12a in vitro and delivering gRNA/Cas12a ribonucleoprotein (RNP) to IPSCs via electroporation. The iPSCs were edited using a RNP having an engineered Cas12a with three amino acid substitutions (M537R, F870L, and H800A (SEQ ID NO: 1148)). The gRNAs were generated with a targeting domain consisting of RNA, an AsCpf1 scaffold of the sequence UAAUUUCUACUCUUGUAGAU (SEQ ID NO: 1153) located 5' of the targeting domain, and a 25-mer DNA extension of the sequence ATGTGTTTTTGTCAAAAGACCTTTT (SEQ ID NO: 1154) at the 5' terminus of the scaffold sequence. Table 12 provides the targeting domains of the guide RNAs that were tested for editing activity.

Table 12: guide RNA sequences

Target	**gRNA Targeting Domain Sequence**
TGFβRII	UGAUGUGAGAUUUUCCACCUG (SEQ ID NO: 1161)
CISH	ACUGACAGCGUGAACAGGUAG (SEQ ID NO: 1162)
ADORA2A	CCAUCGGCCUGACUCCCAUGC (SEQ ID NO: 1163)

ADORA2A	CCAUCACCAUCAGCACCGGGU (SEQ ID NO: 1164)
ADORA2A	CCUGUGUGCUGGUGCCCCUGC (SEQ ID NO: 1165)
TIGIT	UGCAGAGAAAGGUGGCUCUAU (SEQ ID NO: 1166)
TIGIT	UCUGCAGAAAUGUUCCCCGUU (SEQ ID NO: 1167)
TIGIT	UAGGACCUCCAGGAAGAUUCU (SEQ ID NO: 1168)
NKG2A	GCAACUGAACAGGAAAUAACC (SEQ ID NO: 1169)
NKG2A	GUUGCUGCCUCUUUGGGUUUG (SEQ ID NO: 1170)
NKG2A	AAGGGAAUGACAAAACCUAUC (SEQ ID NO: 1171)

[0334] In brief, 100,000 iPSCs/well were transfected with the RNP of interest, cells were incubated at 37°C for 72 hours, and then harvested for DNA characterization. iPSCs were transfected with gRNA/Cas12a RNPs at various concentrations. The percentage editing events were determined for eight different RNP concentrations ranging from negative control (0 mM), to 8 mM.

[0335] As shown in Figure 18 panel 1, the TGFβRII gRNA (SEQ ID NO: 1161) exhibited an EC50 of ~79nM RNP. As shown in Figure 18 panel 2, the CISH gRNA (SEQ ID NO: 1162) exhibited an EC50 of ~50 nM RNP. As shown in Figure 18 panel 3, an ADORA2A gRNA (SEQ ID NO: 1163) included in RNP2960 exhibited an EC50 of ~63 nM RNP, while an ADORA2A gRNA (SEQ ID NO: 1164) included in RNP3109, or gRNA (SEQ ID NO: 1165) included in RNP3108 exhibited EC50 values of ~493 nM and ~280nM RNP respectively. As shown in Figure 18 panel 4, a TIGIT gRNA (SEQ ID NO: 1166) included in RNP2892 exhibited an EC50 of ~29 nM RNP, while a TIGIT gRNA (SEQ ID NO: 1167) included in RNP3106, or gRNA (SEQ ID NO: 167) included in RNP3107 exhibited EC50 values of ~1146 nM and ~40 nM RNP respectively. As shown in Figure 18 panel 5, a NKG2A gRNA (SEQ ID NO: 1169) included in RNP19142 exhibited an EC50 of

~8 nM RNP, while a NKG2A gRNA (SEQ ID NO: 1170) included in RNP3069, or gRNA (SEQ ID NO: 1171) included in RNP2891 exhibited EC50 values of ~12 nM and ~13 nM RNP respectively.

EQUIVALENTS

It is to be understood that while the disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the present disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

CLAIMS

1. A pluripotent human stem cell, wherein the stem cell comprises:

(i) a genomic edit that results in loss of function of Cytokine Inducible SH2 Containing Protein (CISH) and

(ii) a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway, or a genomic edit that results in a loss of function of adenosine A2a receptor (ADORA2A).

2. The pluripotent human stem cell of claim 1, wherein the stem cell comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway and a genomic edit that results in a loss of function of ADORA2A.

3. The pluripotent human stem cell of claim 1 or 2, wherein the stem cell comprises a genomic edit that results in a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor.

4. The pluripotent human stem cell of claim 3, wherein the TGF beta receptor is a TGF beta receptor II (TGFβRII).

5. The pluripotent human stem cell of any one of the preceding claims, wherein the stem cell expresses one or more pluripotency markers selected from the group consisting of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin, UTF-1, Oct4, Rex1, and Nanog.

6. A differentiated cell, wherein the differentiated cell is a daughter cell of the pluripotent human stem cell of any one of the preceding claims.

7. The differentiated cell of claim 6, wherein the differentiated cell is an immune cell.

8. The differentiated cell of claim 6, wherein the differentiated cell is a lymphocyte.

9. The differentiated daughter cell of claim 6, wherein the differentiated cell is a natural killer cell.

10. The differentiated cell of claim 6, wherein the stem cell is a human induced pluripotent stem cell (iPSC), and wherein the differentiated daughter cell is an iNK cell.

11. The differentiated cell of claim 6, wherein the cell:

(a) does not express endogenous CD3, CD4, and/or CD8; and

(b) expresses at least one endogenous gene encoding:

(i) CD56 (NCAM), CD49, CD43, and/or CD45, or any combination thereof;

(ii) NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 (CD16));

(iii) natural killer group-2 member D (NKG2D);

(iv) CD69;

(v) a natural cytotoxicity receptor;

or any combination of two or more thereof.

12. The cell of any of the preceding claims, wherein the cell comprises one or more additional genomic edits.

13. The cell of claim 12, wherein the cell:

(1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding:

(i) a chimeric antigen receptor (CAR);

(ii) a FcγRIII (CD16) or a variant of FcγRIII (CD16);

(iii) interleukin 15 (IL-15);

(iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor;

(v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vii) human leukocyte antigen G (HLA-G);

(viii) human leukocyte antigen E (HLA-E);

(ix) leukocyte surface antigen cluster of differentiation CD47 (CD47);

or any combination of two or more thereof;

and/or

(2) comprises at least one genomic edit that results in a loss of function of at least one of:

(i) ADORA2A;

(ii) T cell immunoreceptor with Ig and ITIM domains (TIGIT);

(iii) β-2 microglobulin (B2M);

(iv) programmed cell death protein 1 (PD-1);

(v) class II, major histocompatibility complex, transactivator (CIITA);

(vi) natural killer cell receptor NKG2A (natural killer group 2A);

(vii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;

(viii) cluster of differentiation 32B (CD32B, FCGR2B);

(ix) T cell receptor alpha constant (TRAC);

or any combination of two or more thereof.

14. A human induced pluripotent stem cell (iPSC), wherein the iPSC comprises a genomic edit that results in a loss of function of adenosine A2a receptor (ADORA2A).

15. The human iPSC of claim 14, wherein the iPSC comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway or a genomic edit that results in loss of function of Cytokine Inducible SH2 Containing Protein (CISH).

16. The human iPSC of claim 15, wherein the iPSC comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway and a genomic edit that results in loss of function of CISH.

17. The human iPSC of claim 15 or 16, wherein the iPSC comprises a genomic edit that results in a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor.

18. The human iPSC of claim 17, wherein the TGF beta receptor is a TGF beta receptor II (TGFβRII).

19. The human iPSC of any one of claims 14-18, wherein the iPSC expresses one or more pluripotency markers selected from the group consisting of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and Nanog.

20. A differentiated cell, wherein the differentiated cell is a daughter cell of the human iPSC of any one of claims 14-19.

21. The differentiated cell of claim 20, wherein the differentiated cell is an immune cell.

22. The differentiated cell of claim 20, wherein the differentiated cell is a lymphocyte.

23. The differentiated daughter cell of claim 20, wherein the differentiated cell is a natural killer cell.

24. The differentiated cell of claim 20, wherein the differentiated daughter cell is an iNK cell.

25. The differentiated cell of claim 20, wherein the cell:

(a) does not express endogenous CD3, CD4, and/or CD8; and

(b) expresses at least one endogenous gene encoding:

(i) CD56 (NCAM), CD49, CD43, and/or CD45, or any combination thereof;

(ii) NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 (CD16));

(iii) natural killer group-2 member D (NKG2D);

(iv) CD69;

(v) a natural cytotoxicity receptor;

or any combination of two or more thereof.

26. The cell of any of claims 14-25, wherein the cell comprises one or more additional genomic edits.

27. The cell of claim 26, wherein the cell:

(1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding:

(i) a chimeric antigen receptor (CAR);

(ii) a FcγRIII (CD16) or a variant of FcγRIII (CD16);

(iii) interleukin 15 (IL-15);

(iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor;

(v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vii) human leukocyte antigen G (HLA-G);

(viii) human leukocyte antigen E (HLA-E);

(ix) leukocyte surface antigen cluster of differentiation CD47 (CD47);

or any combination of two or more thereof;

and/or

(2) comprises at least one genomic edit that results in a loss of function of at least one of:

(i) cytokine inducible SH2 containing protein (CISH);

(ii) T cell immunoreceptor with Ig and ITIM domains (TIGIT);

(iii) β-2 microglobulin (B2M);

(iv) programmed cell death protein 1 (PD-1);

(v) class II, major histocompatibility complex, transactivator (CIITA);

(vi) natural killer cell receptor NKG2A (natural killer group 2A);

(vii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;

(viii) cluster of differentiation 32B (CD32B, FCGR2B);

(ix) T cell receptor alpha constant (TRAC);

or any combination of two or more thereof.

28. The cell of any one of claims 1-27, wherein:

the genomic edit resulting in loss of function of CISH was produced using a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 258-364, 1155, and 1162;

the genomic edit resulting in loss of function of TGFβRII was produced using a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 29-257, 1157, and 1161; and/or

the genomic edit resulting in loss of function of ADORA2A was produced using a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 827-1143, 1159, and 1163.

29. The cell of any one of claims 1-28, wherein:

the genomic edit resulting in loss of function of CISH was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease and (ii) a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 258-364, 1155, and 1162;

the genomic edit resulting in loss of function of TGFβRII was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease and (ii) a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 29-257, 1157, and 1161; and/or

the genomic edit resulting in loss of function of ADORA2A was produced using a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease and (ii) a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 827-1143, 1159, and 1163.

30. A method of making the cell of any one of claims 1-29, the method comprising contacting the cell with one or more of:

an RNA-guided nuclease and a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 258-364, 1155, and 1162;

an RNA-guided nuclease and a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 29-257, 1157, and 1161; and/or

an RNA-guided nuclease and a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 827-1143, 1159, and 1163.

31. A method of making the cell of any one of claims 1-30, the method comprising contacting the cell with one or more of:

a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease and (ii) a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 258-364, 1155, and 1162;

a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease and (ii) a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 29-257, 1157, and 1161; and/or

a ribonucleoprotein (RNP) complex comprising (i) an RNA-guided nuclease and (ii) a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence according to any one of SEQ ID NO: 827-1143, 1159, and 1163.

32. The method of any one of claims 29-31, wherein the RNA-guided nuclease is a Cas12a variant.

33. The method of claim 32, wherein the Cas12a variant comprises one or more amino acid substitutions selected from M537R, F870L, and H800A.

34. The method of claim 32, wherein the Cas12a variant comprises amino acid substitutions M537R, F870L, and H800A.

35. The method of claim 32, wherein the Cas12a variant comprises the amino acid sequence of SEQ ID NO:1148.

36. The method of any one of claims 30-35, comprising contacting the cell with:

(i) a guide RNA comprising a targeting domain sequence comprising the nucleotide sequence of SEQ ID NO: 1155 or 1162; a guide RNA comprises a targeting domain sequence comprising the nucleotide sequence of SEQ ID NO: 1157 or 1161; and a guide RNA comprises a targeting domain sequence comprising the nucleotide sequence of SEQ ID NO: 1159 or 1163; and

(ii) an RNA-guided nuclease comprising the amino acid sequence of one of SEQ ID NO:1144-1151 (or a portion thereof).

37. A pluripotent human stem cell, wherein the stem cell comprises a disruption in the transforming growth factor beta (TGF beta) signaling pathway.

38. The pluripotent human stem cell of claim 34, wherein the stem cell comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway.

39. The pluripotent human stem cell of claim 37 or 38, comprising a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor.

40. The pluripotent human stem cell of claim 39, wherein the TGF beta receptor is a TGF beta receptor II (TGFβRII).

41. The pluripotent human stem cell of any one of claims 37-40, further comprising a loss of function of an antagonist of interleukin signaling.

42. The pluripotent human stem cell of any one of claims 37-41, wherein the stem cell further comprises a genomic modification that results in the loss of function of an antagonist of interleukin signaling.

43. The pluripotent human stem cell of claim 41 or 42, wherein the antagonist of interleukin signaling is an antagonist of the IL-15 signaling pathway and/or of the IL-2 signaling pathway.

44. The pluripotent human stem cell of any one of claims 37-43, comprising a loss of function of Cytokine Inducible SH2 Containing Protein (CISH).

45. The pluripotent human stem cell of claim 44, wherein the stem cell comprises a genomic modification that results in the loss of function of CISH.

46. The pluripotent human stem cell of any one of claims 37-45, wherein the stem cell expresses one or more pluripotency markers selected from the group consisting of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and Nanog.

47. A differentiated cell, wherein the differentiated cell is a daughter cell of the pluripotent human stem cell of any one of claims 37-46.

48. The differentiated cell of claim 47, wherein the differentiated cell is an immune cell.

49. The differentiated cell of claim 47, wherein the differentiated cell is a lymphocyte.

50. The differentiated daughter cell of claim 47, wherein the differentiated cell is a natural killer cell.

51. The differentiated cell of claim 47, wherein the stem cell is a human induced pluripotent stem cell (iPSC), and wherein the differentiated daughter cell is an iNK cell.

52. The differentiated cell of claim 47, wherein the cell:

(a) does not express endogenous CD3, CD4, and/or CD8; and

(b) expresses at least one endogenous gene encoding:

(i) CD56 (NCAM), CD49, CD43, and/or CD45, or any combination thereof;

(ii) NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 (CD16));

(iii) natural killer group-2 member D (NKG2D);

(iv) CD69;

(v) a natural cytotoxicity receptor;

or any combination of two or more thereof.

53. The cell of any of claims 37-52, wherein the cell comprises one or more additional genomic edits.

54. The cell of claim 53, wherein the cell:

(1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding:

(i) a chimeric antigen receptor (CAR);

(ii) a FcγRIII (CD16) or a variant of FcγRIII (CD16);

(iii) interleukin 15 (IL-15);

(iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor;

(v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vii) human leukocyte antigen G (HLA-G);

(viii) human leukocyte antigen E (HLA-E);

(ix) leukocyte surface antigen cluster of differentiation CD47 (CD47);

or any combination of two or more thereof;

and/or

(2) comprises at least one genomic edit that results in a loss of function of at least one of:

(i) cytokine inducible SH2 containing protein (CISH);

(ii) adenosine A2a receptor (ADORA2A);

(iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT);

(iv) β-2 microglobulin (B2M);

(v) programmed cell death protein 1 (PD-1);

(vi) class II, major histocompatibility complex, transactivator (CIITA);

(vii) natural killer cell receptor NKG2A (natural killer group 2A);

(viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;

(ix) cluster of differentiation 32B (CD32B, FCGR2B);

(x) T cell receptor alpha constant (TRAC);

or any combination of two or more thereof.

55. A method of culturing a pluripotent human stem cell, comprising culturing the stem cell in a medium comprising activin.

56. The method of claim 55, wherein the pluripotent human stem cell is an embryonic stem cell or an induced pluripotent stem cell.

57. The method of claim 55 or 56, wherein the pluripotent human stem cell does not express TGFβRII.

58. The method of any one of claims 55-57, wherein the pluripotent human stem cell is genetically engineered not to express TGFβRII.

59. The method of any one of claims 55-57, wherein the pluripotent human stem cell is genetically engineered to knock out a gene encoding TGFβRII.

60. The method of any one of claims 55-59, wherein the activin is activin A.

61. The method of any one of claims 55-60, wherein the medium does not comprise TGFβ.

62. The method of any one of claims 55-61, wherein the culturing is performed for a defined period of time (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 days, or more).

63. The method of any one of claims 55-62, wherein at one or more times during or following the culturing step, the pluripotent human stem cell maintains pluripotency (e.g., exhibits one or more pluripotency markers).

64. The method of claim 63, wherein at one or more times during or following the culturing step, the pluripotent human stem cell expresses a detectable level of one or more of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, ALP, Sox2, E-cadherin,UTF-1, Oct4, Rex1, and Nanog.

65. The method of claim 63, wherein at a time during or following the culturing step, the pluripotent human stem cell is differentiated into cells of endoderm, mesoderm, and/or ectoderm lineage.

66. The method of claim 65, wherein the pluripotent human stem cell, or its progeny, is further differentiated into a natural killer (NK) cell.

67. The method of any one of claims 55-66, wherein the pluripotent human stem cell:

(1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding:

(i) a chimeric antigen receptor (CAR);

(ii) a FcγRIII (CD16) or a variant of FcγRIII (CD16);

(iii) interleukin 15 (IL-15);

(iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor;

(v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vii) human leukocyte antigen G (HLA-G);

(viii) human leukocyte antigen E (HLA-E);

(ix) leukocyte surface antigen cluster of differentiation CD47 (CD47);

or any combination of two or more thereof;

and/or

(2) comprises at least one genomic edit that results in a loss of function of at least one of:

(i) cytokine inducible SH2 containing protein (CISH);

(ii) adenosine A2a receptor (ADORA2A);

(iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT);

(iv) β-2 microglobulin (B2M);

(v) programmed cell death protein 1 (PD-1);

(vi) class II, major histocompatibility complex, transactivator (CIITA);

(vii) natural killer cell receptor NKG2A (natural killer group 2A);

(viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;

(ix) cluster of differentiation 32B (CD32B, FCGR2B);

(x) T cell receptor alpha constant (TRAC);

or any combination of two or more thereof.

68. A cell culture comprising (i) a pluripotent human stem cell and (ii) a cell culture medium comprising activin, wherein the pluripotent human stem cell comprises a disruption in the transforming growth factor beta (TGF beta) signaling pathway.

69. The cell culture of claim 68, wherein the stem cell comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway.

70. The cell culture of claim 69, wherein the genomic edit is a genomic edit.

71. The cell culture of any one of claims 68-70, wherein the stem cell comprises a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor.

72. The cell culture of claim 71, wherein the TGF beta receptor is a TGF beta receptor II (TGFβRII).

73. The cell culture of any one of claims 68-72, wherein the pluripotent human stem cell:

(1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding:

(i) a chimeric antigen receptor (CAR);

(ii) a FcγRIII (CD16) or a variant of FcγRIII (CD16);

(iii) interleukin 15 (IL-15);

(iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor;

(v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vii) human leukocyte antigen G (HLA-G);

(viii) human leukocyte antigen E (HLA-E);

(ix) leukocyte surface antigen cluster of differentiation CD47 (CD47);

or any combination of two or more thereof;

and/or

(2) comprises at least one genomic edit that results in a loss of function of at least one of:

(i) cytokine inducible SH2 containing protein (CISH);

(ii) adenosine A2a receptor (ADORA2A);

(iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT);

(iv) β-2 microglobulin (B2M);

(v) programmed cell death protein 1 (PD-1);

(vi) class II, major histocompatibility complex, transactivator (CIITA);

(vii) natural killer cell receptor NKG2A (natural killer group 2A);

(viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;

(ix) cluster of differentiation 32B (CD32B, FCGR2B);

(x) T cell receptor alpha constant (TRAC);

or any combination of two or more thereof.

74. A method of increasing a level of iNK cell activity comprising:

(i) providing a pluripotent human stem cell comprising a disruption in the transforming growth factor beta (TGF beta) signaling pathway; and

(ii) differentiating the pluripotent human stem cell into an iNK cell,

wherein the iNK cell has a higher level of cell activity as compared to an iNK cell not comprising a disruption of the TGF beta signaling pathway.

75. The method of claim 74, wherein the iNK is differentiated from a pluripotent human stem cell cultured in a medium comprising activin.

76. The method of claim 74 or 75, further comprising culturing the pluripotent human stem cell in a medium comprising activin before and/or during the differentiating step.

77. The method of any one of claims 74-76, further comprising disrupting the transforming growth factor beta (TGF beta) signaling pathway in the pluripotent human stem cell.

78. The method of any one of claims 73-77, wherein the stem cell comprises a genomic edit that results in a loss of function of an agonist of the TGF beta signaling pathway.

79. The method of any one of claims 73-78, wherein the stem cell comprises a loss of function of a TGF beta receptor or a dominant-negative variant of a TGF beta receptor.

80. The method of claim 79, wherein the TGF beta receptor is a TGF beta receptor II (TGFβRII).

81. The method of any one of claims 73-80, wherein the pluripotent human stem cell:

(1) comprises at least one genomic edit characterized by an exogenous nucleic acid expression construct that comprises a nucleic acid sequence encoding:

(i) a chimeric antigen receptor (CAR);

(ii) a FcγRIII (CD16) or a variant of FcγRIII (CD16);

(iii) interleukin 15 (IL-15);

(iv) an IL-15 receptor (IL-15R) agonist, or a constitutively active variant of an IL-15 receptor;

(v) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vi) an IL-12 receptor (IL-12R) agonist, or a constitutively active variant of an IL-12 receptor;

(vii) human leukocyte antigen G (HLA-G);

(viii) human leukocyte antigen E (HLA-E);

(ix) leukocyte surface antigen cluster of differentiation CD47 (CD47);

or any combination of two or more thereof;

and/or

(2) comprises at least one genomic edit that results in a loss of function of at least one of:

(i) cytokine inducible SH2 containing protein (CISH);

(ii) adenosine A2a receptor (ADORA2A);

(iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT);

(iv) β-2 microglobulin (B2M);

(v) programmed cell death protein 1 (PD-1);

(vi) class II, major histocompatibility complex, transactivator (CIITA);

(vii) natural killer cell receptor NKG2A (natural killer group 2A);

(viii) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;

(ix) cluster of differentiation 32B (CD32B, FCGR2B);

(x) T cell receptor alpha constant (TRAC);

or any combination of two or more thereof.

82. A method of treating a subject having or at risk of cancer, the method comprising administering to the subject the cell of any one of claims 6-13, 20-29, or 47-54, thereby treating the cancer in the subject.

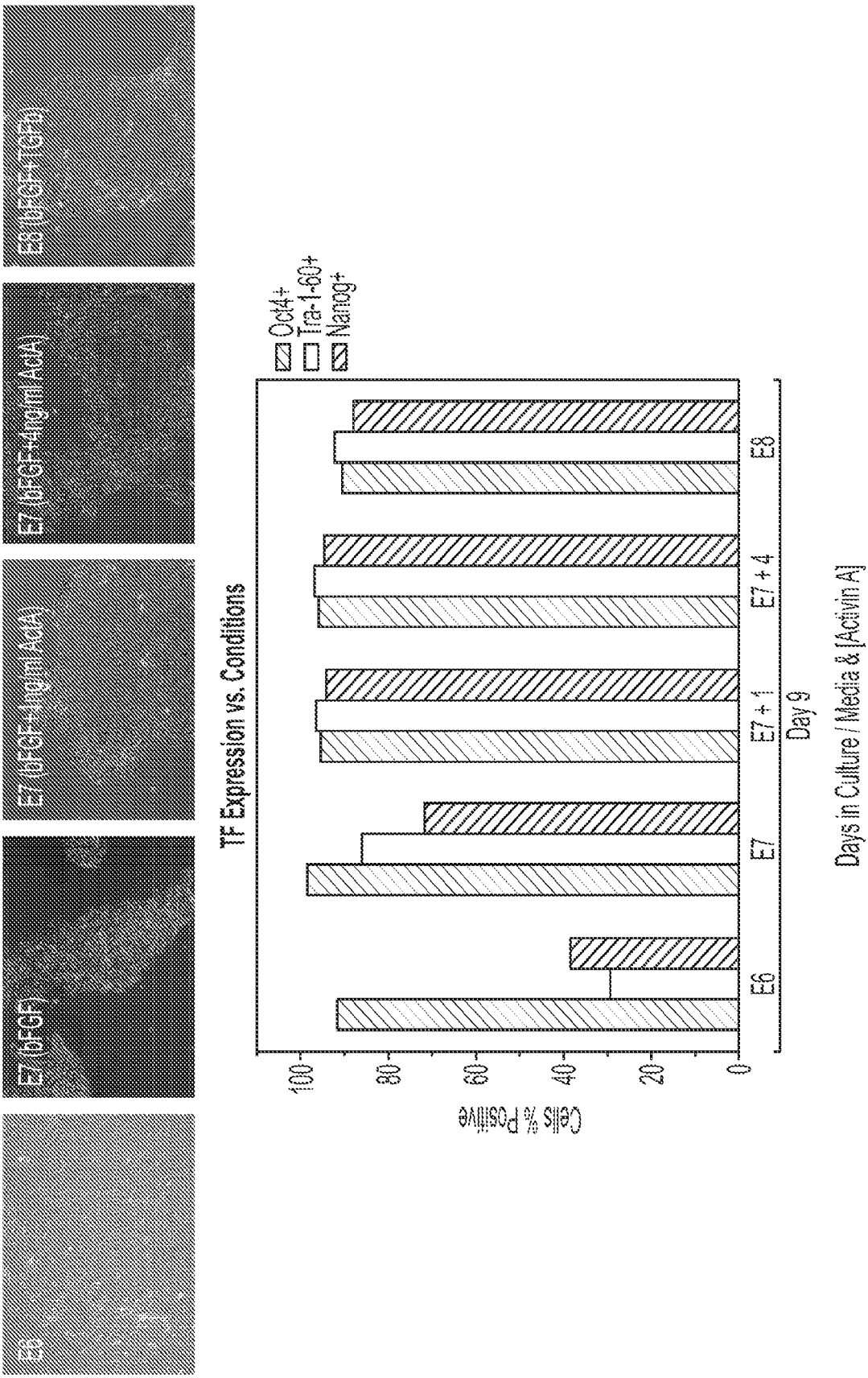

E6
E7 (bFGF)
E7 (bFGF+1ng/mlActA)
E7 (bFGF+4ng/mlActA)
E8 (bFGF+TGFb)
TF Expression vs. Conditions
Oct4+
Tra-1-60+
Nanog+
Cells % Positive
100
80
60
40
20
0
E6
E7
E7 + 1
E7 + 4
E8
Day 9
Days in Culture / Media & [Activin A]

FIG. 1

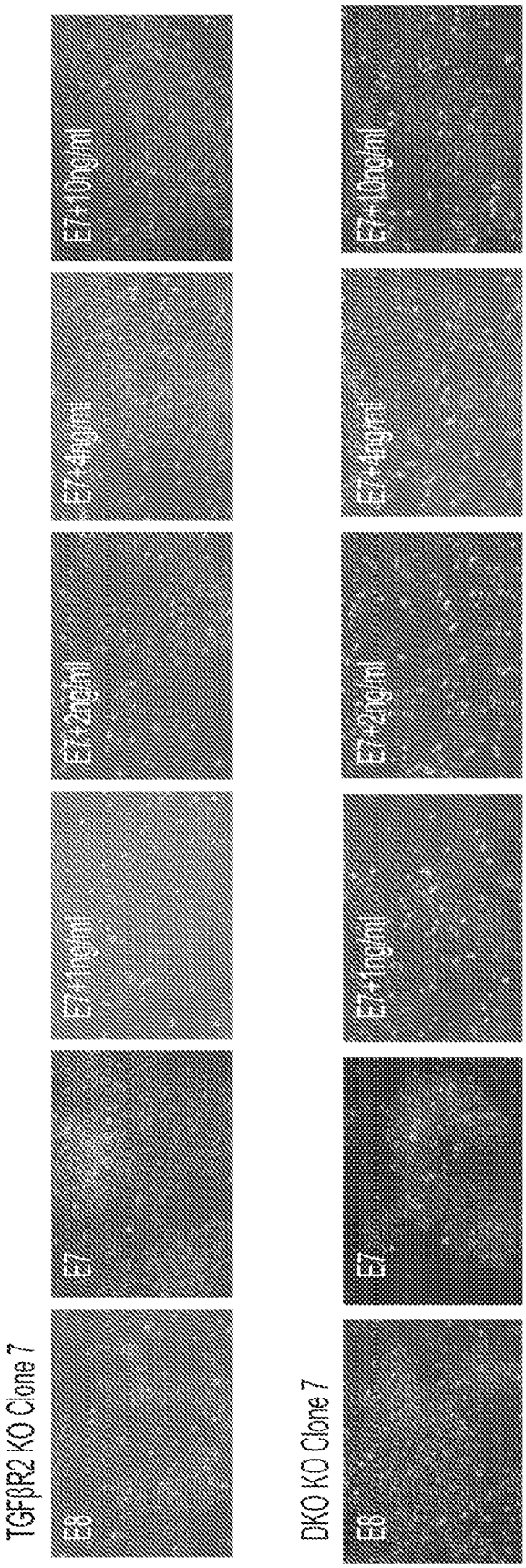

TGFβR2 KO Clone 7
E6
E7
E7+1ng/ml
E7+2ng/ml
E7+4ng/ml
E7+10ng/ml
DKO KO Clone 7
E6
E7
E7+1ng/ml
E7+2ng/ml
E7+4ng/ml
E7+10ng/ml

FIG. 2

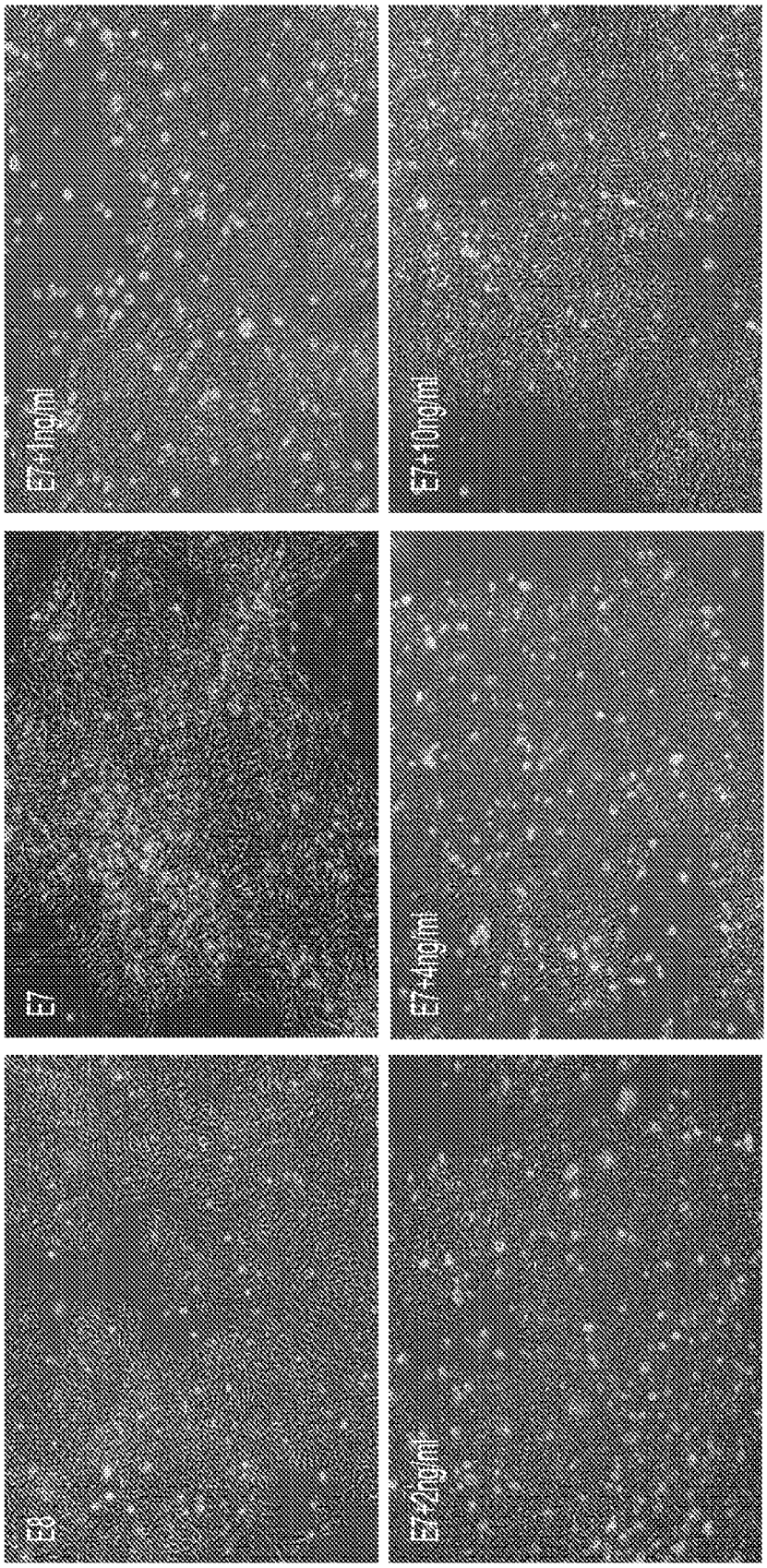


FIG. 3

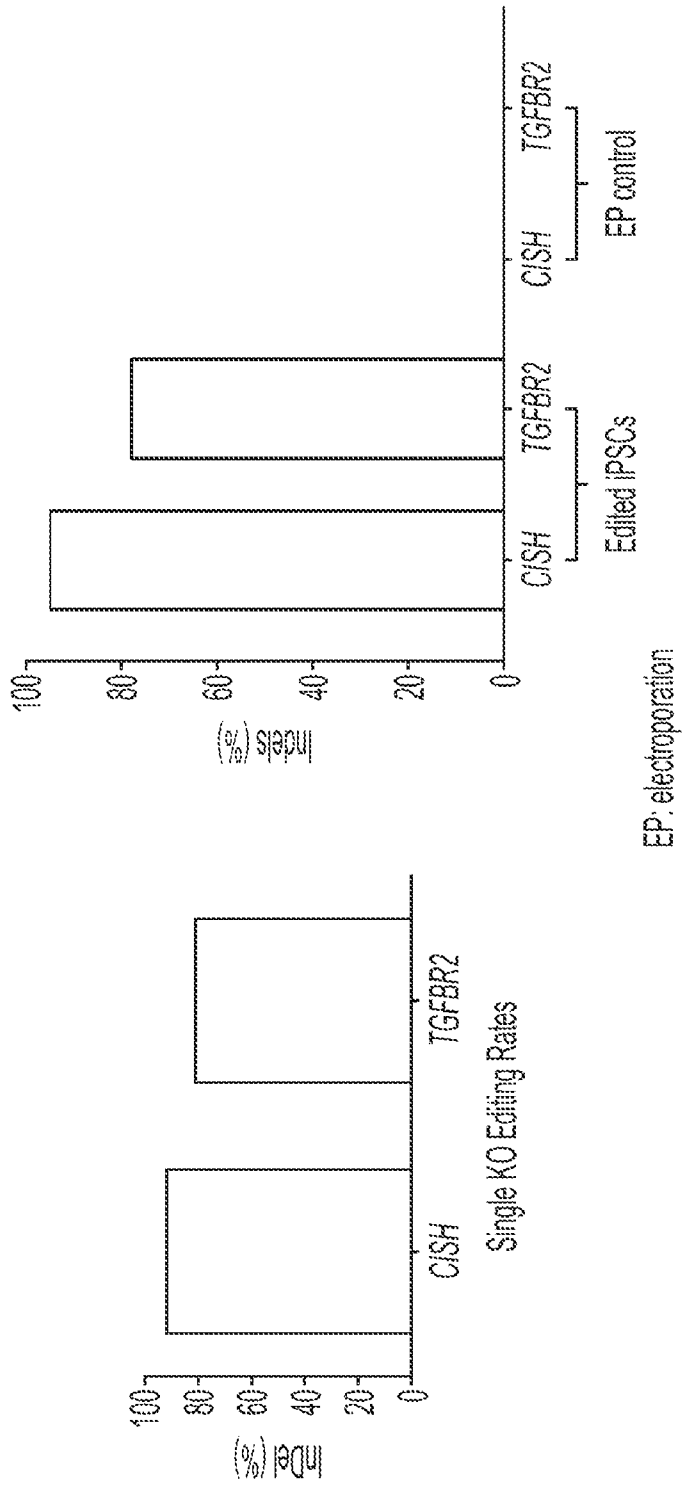

Indels (%)
100
80
60
40
20
0
CISH
TGFBR2
Edited iPSCs
CISH
TGFBR2
EP control
EP: electroporation
InDel (%)
100
80
60
40
20
0
CISH
TGFBR2
Single KO Editing Rates

FIG. 4A

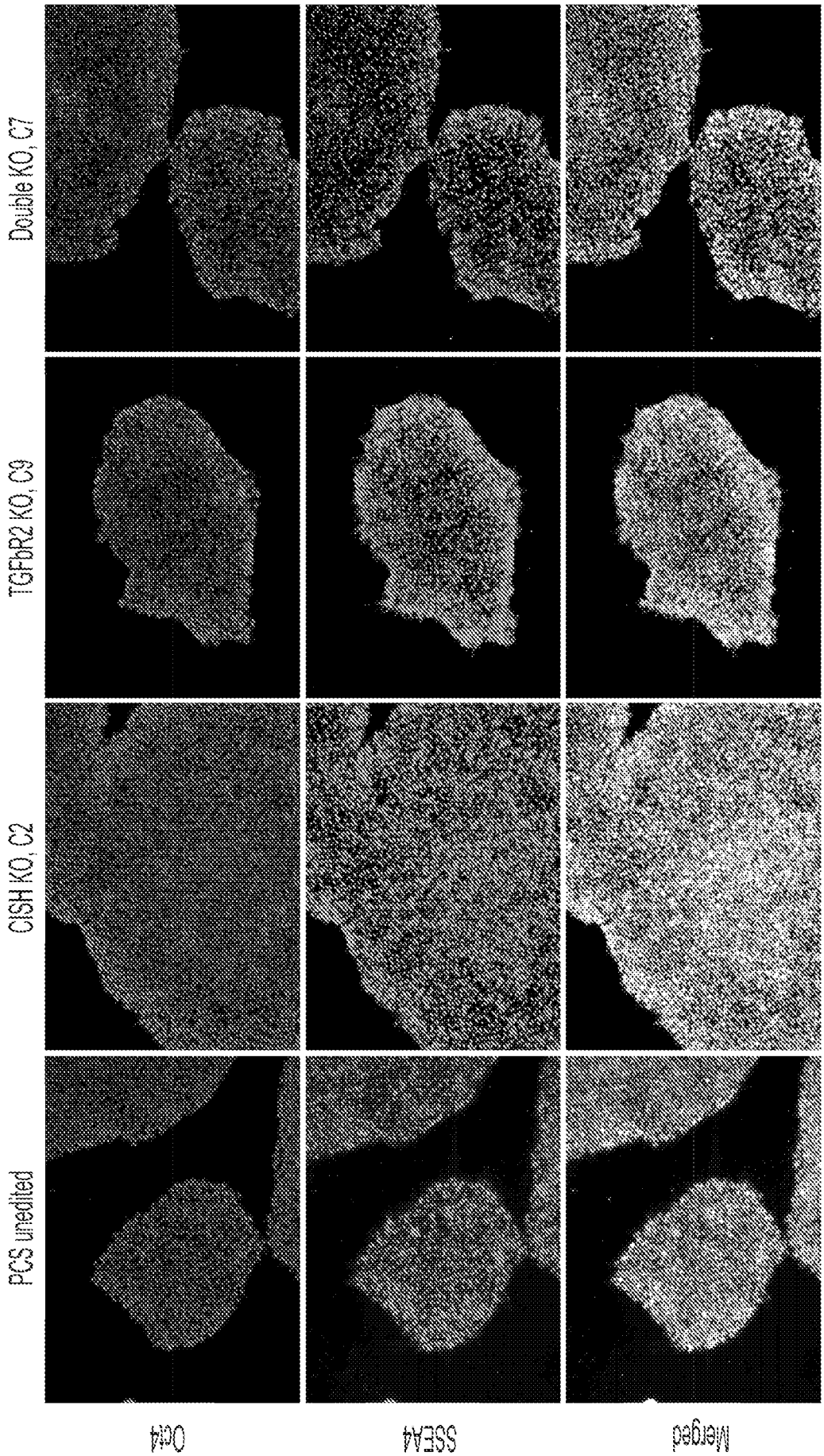

Double KO, C7
TGFbR2 KO, C9
CISH KO, C2
PCS unedited
Oct4
SSEA4
Merged

FIG. 4B

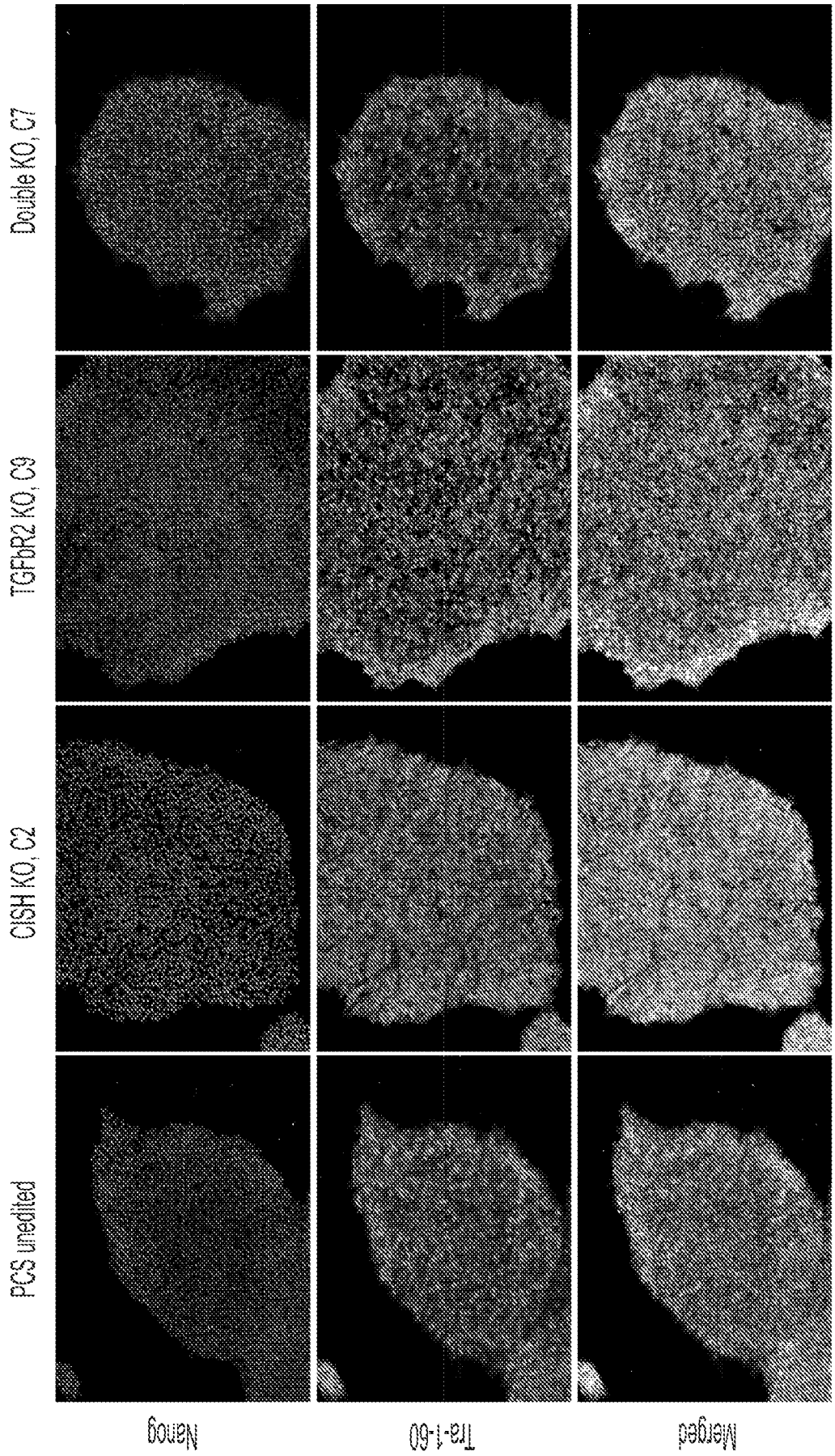

PCS unedited
CISH KO, C2
TGFbR2 KO, C9
Double KO, C7
Nanog
Tra-1-60
Merged

FIG. 5

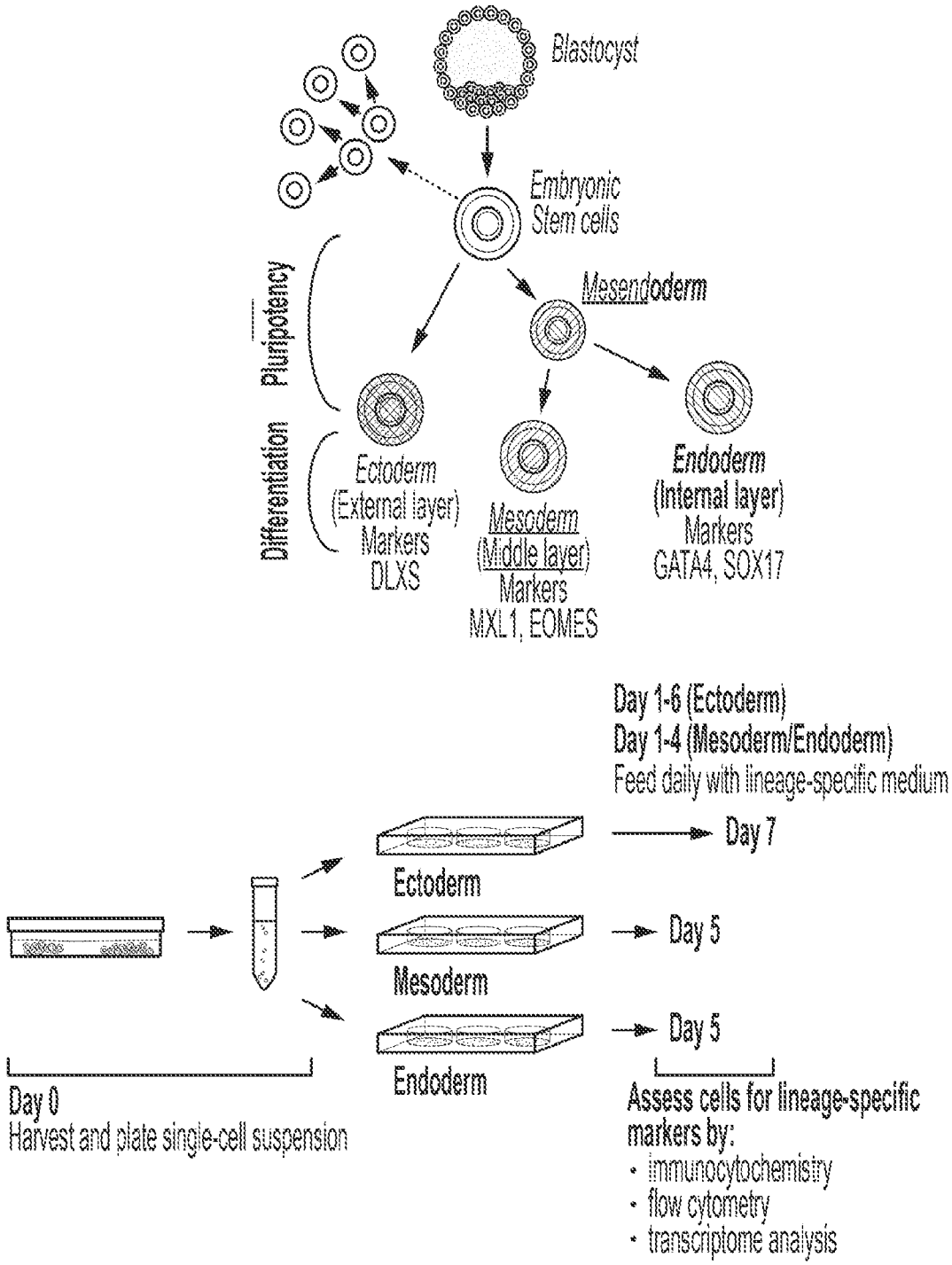

Blastocyst
Embryonic Stem cells
Pluripotency
Differentiation
Mesendoderm
Ectoderm (External layer) Markers DLXS
Mesoderm (Middle layer) Markers MXL1, EOMES
Endoderm (Internal layer) Markers GATA4, SOX17
Day 1-6 (Ectoderm)
Day 1-4 (Mesoderm/Endoderm)
Feed daily with lineage-specific medium
Ectoderm
Mesoderm
Endoderm
Day 7
Day 5
Day 5
Day 0
Harvest and plate single-cell suspension
Assess cells for lineage-specific markers by:
• immunocytochemistry
• flow cytometry
• transcriptome analysis

FIG. 6

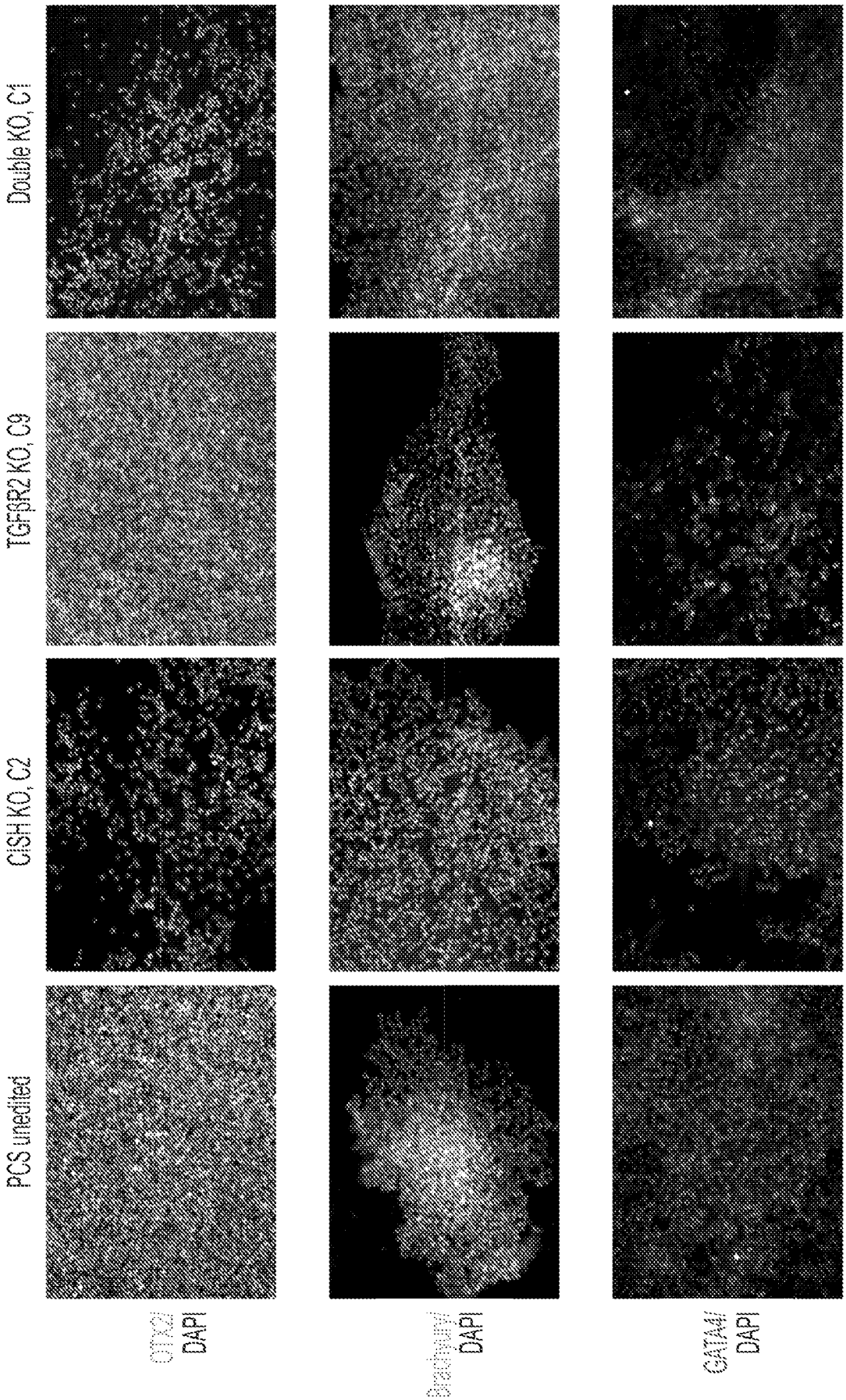

PCS unedited
CISH KO, C2
TGFβR2 KO, C9
Double KO, C1
OTX2/ DAPI
Brachyury/ DAPI
GATA4/ DAPI

FIG. 7A

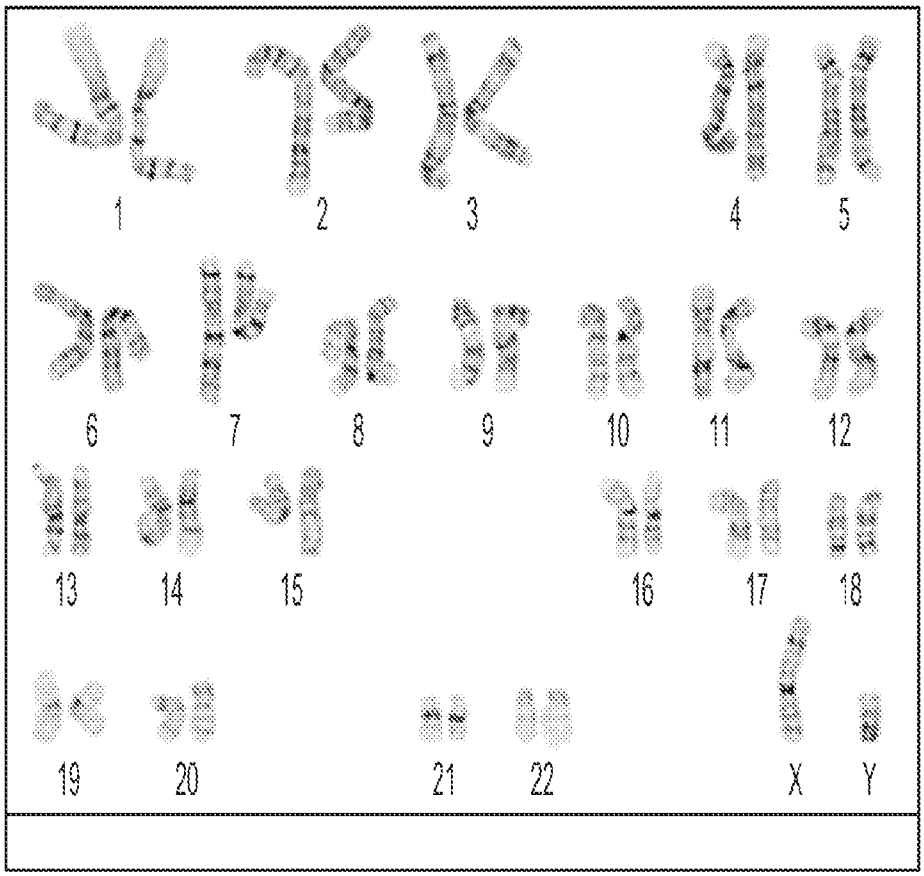


FIG. 7B

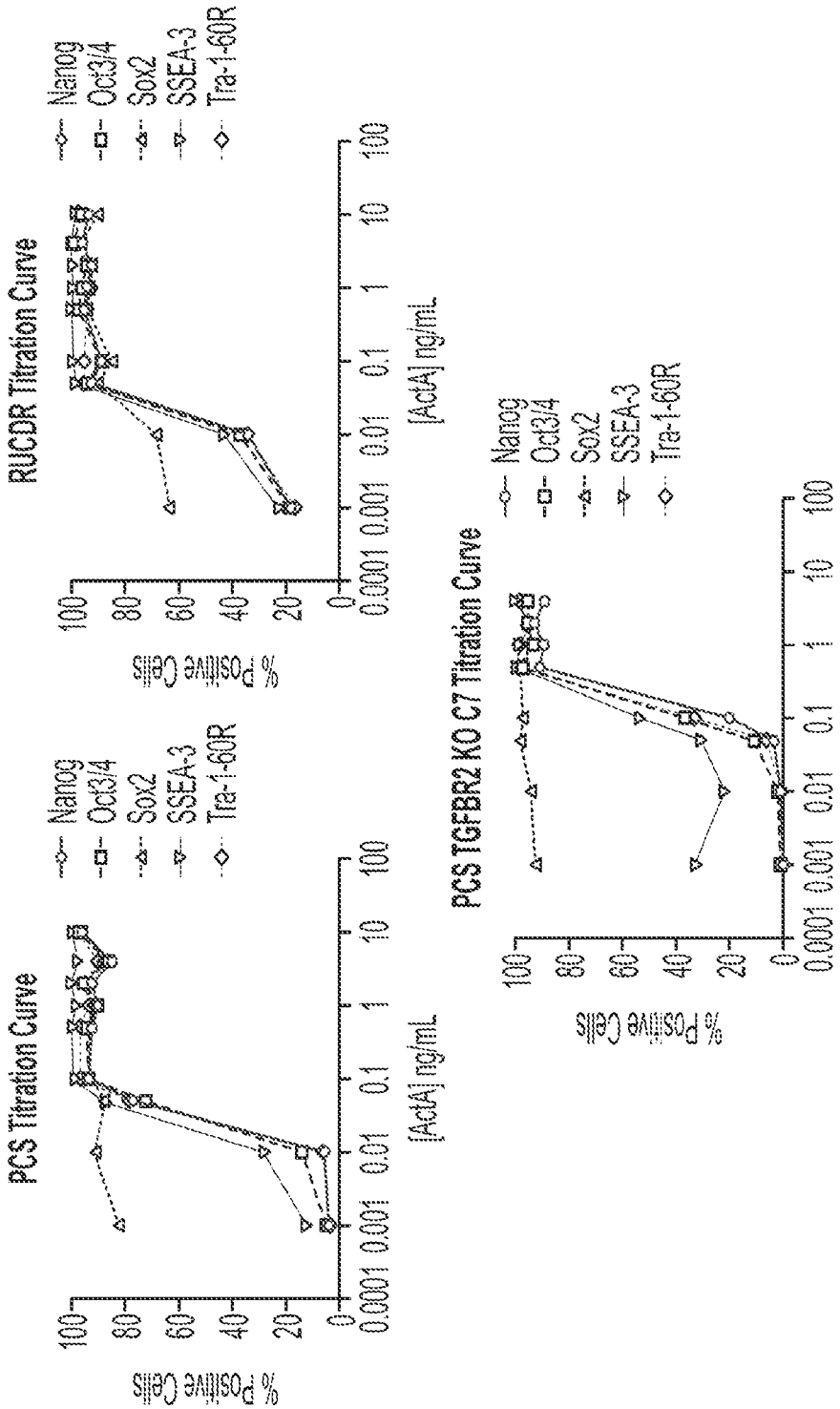

PCS Titration Curve
RUCDR Titration Curve
PCS TGFBR2 KO C7 Titration Curve
Nanog
Oct3/4
Sox2
SSEA-3
Tra-1-60R
% Positive Cells
[ActA] ng/mL
0
20
40
60
80
100
0.0001
0.001
0.01
0.1
1
10
100

FIG. 7C

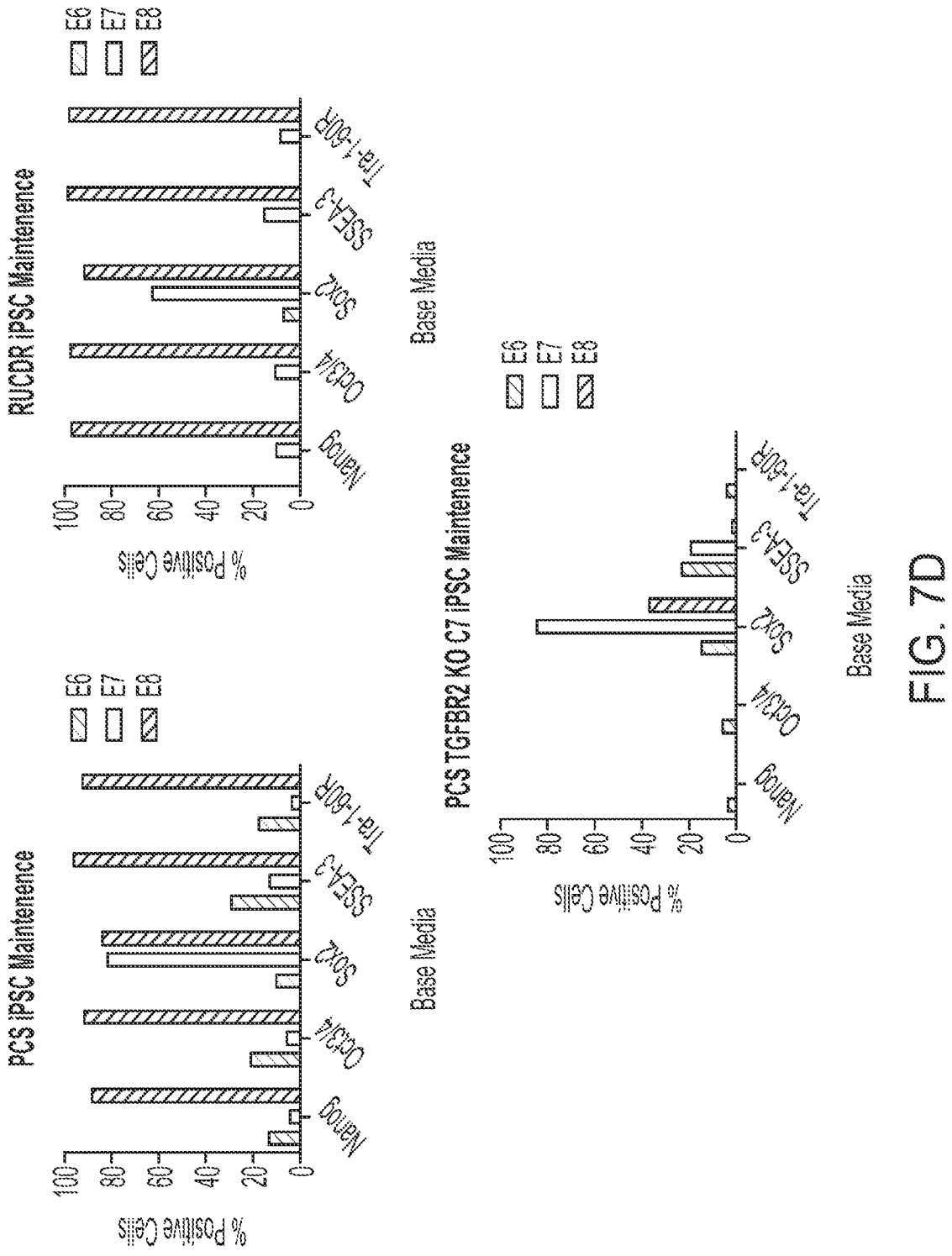

RUCDR IPSC Maintenance
PCS IPSC Maintenance
PCS TGFBR2 KO C7 IPSC Maintenance
% Positive Cells
0
20
40
60
80
100
E6
E7
E8
Nanog
Oct3/4
Sox2
SSEA-3
Tra-1-60R
Base Media

FIG. 7D

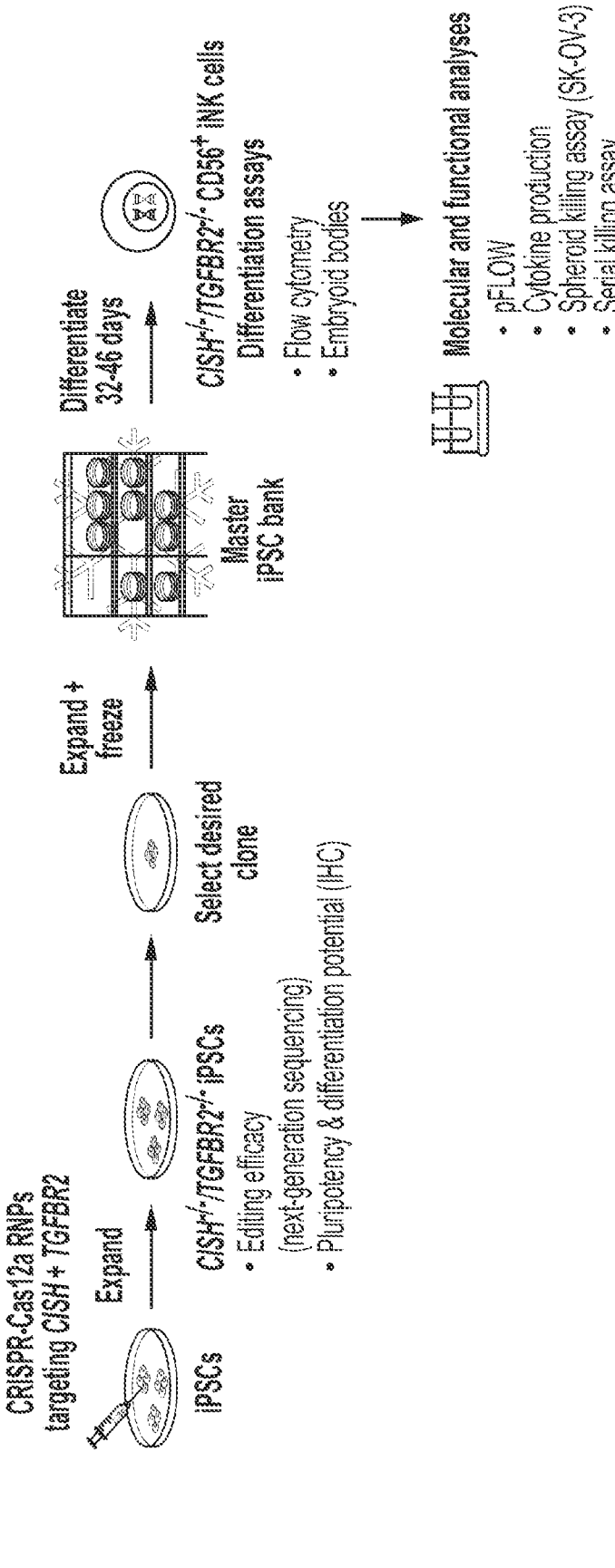

CRISPR-Cas12a RNPs targeting CISH + TGFBR2
iPSCs
Expand
CISH-/-/TGFBR2-/- iPSCs
• Editing efficacy (next-generation sequencing)
• Pluripotency & differentiation potential (IHC)
Select desired clone
Expand + freeze
Master iPSC bank
Differentiate 32-46 days
CISH-/-/TGFBR2-/- CD56+ iNK cells
Differentiation assays
• Flow cytometry
• Embryoid bodies
Molecular and functional analyses
• pFLOW
• Cytokine production
• Spheroid killing assay (SK-OV-3)
• Serial killing assay

FIG. 8A

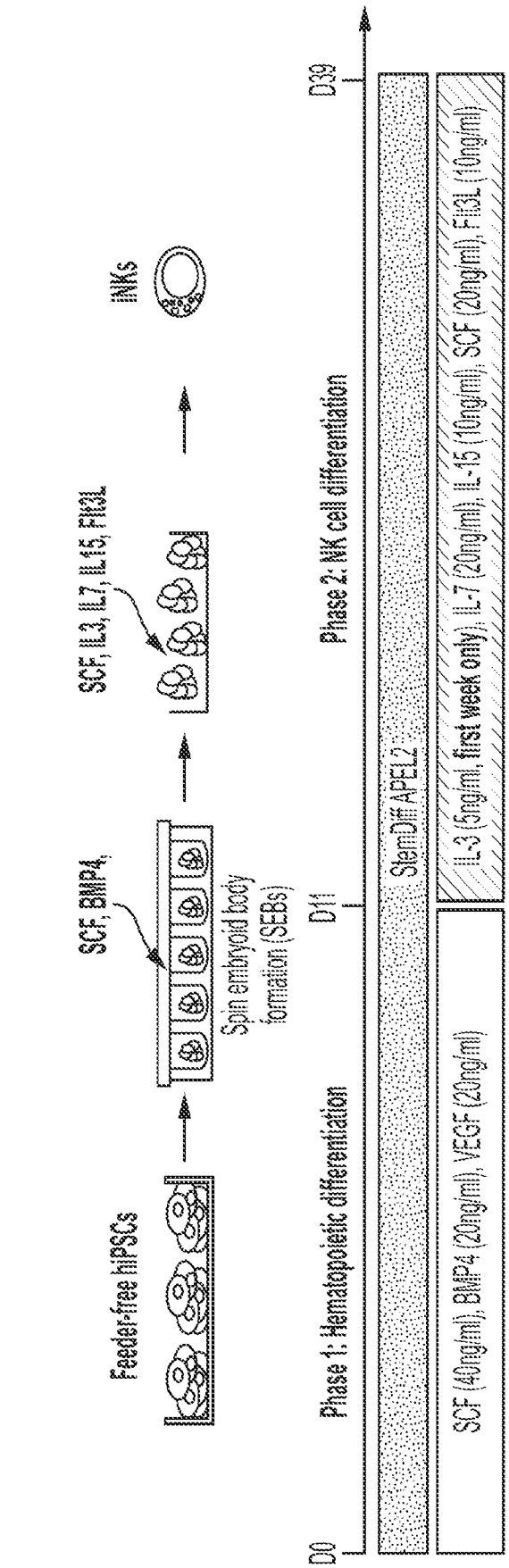

Feeder-free hiPSCs
SCF, BMP4,
Spin embryoid body formation (SEBs)
SCF, IL3, IL7, IL15, Flt3L
iNKs
D0
Phase 1: Hematopoietic differentiation
D11
Phase 2: NK cell differentiation
D39
StemDiff APEL2
SCF (40ng/ml), BMP4 (20ng/ml), VEGF (20ng/ml)
IL-3 (5ng/ml, first week only), IL-7 (20ng/ml), IL-15 (10ng/ml), SCF (20ng/ml), Flt3L (10ng/ml)

FIG. 8B

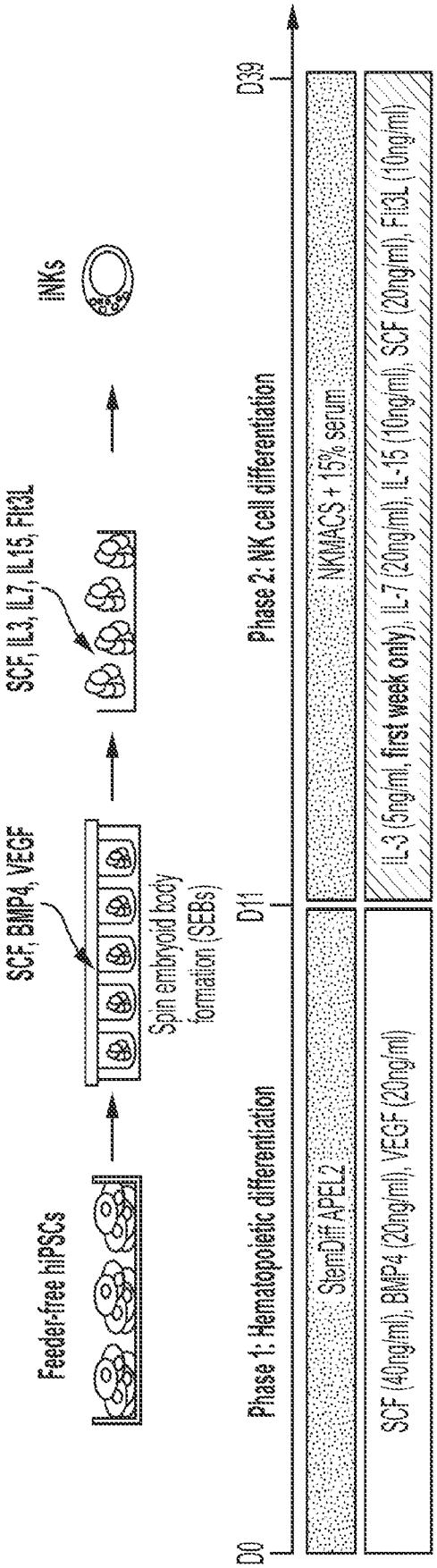

Feeder-free hiPSCs
SCF, BMP4, VEGF
Spin embryoid body formation (SEBs)
SCF, IL3, IL7, IL15, Flt3L
iNKs
D0
Phase 1: Hematopoietic differentiation
D11
Phase 2: NK cell differentiation
D39
StemDiff APEL2
NKMACS + 15% serum
SCF (40ng/ml), BMP4 (20ng/ml), VEGF (20ng/ml)
IL-3 (5ng/ml, first week only), IL-7 (20ng/ml), IL-15 (10ng/ml), SCF (20ng/ml), Flt3L (10ng/ml)

FIG. 8C

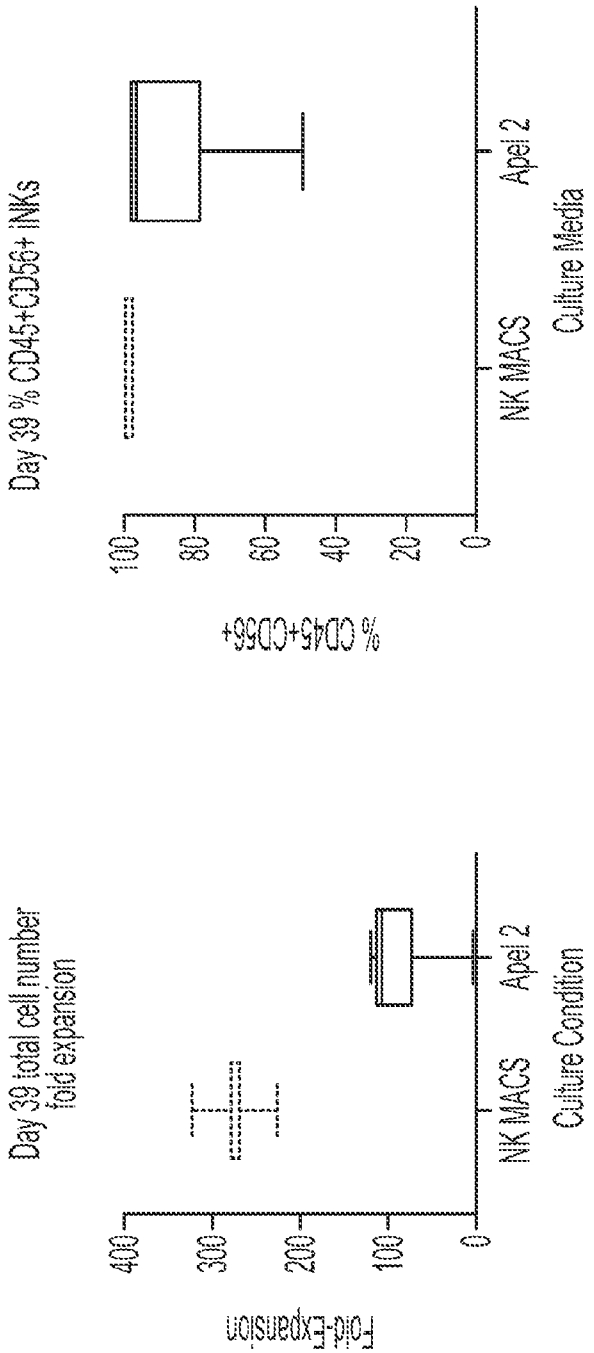

Day 39 % CD45+CD56+ iNKs
% CD45+CD56+
100
80
60
40
20
0
NK MACS
Apel 2
Culture Media
Day 39 total cell number
fold expansion
Fold-Expansion
400
300
200
100
0
NK MACS
Apel 2
Culture Condition

FIG. 8D

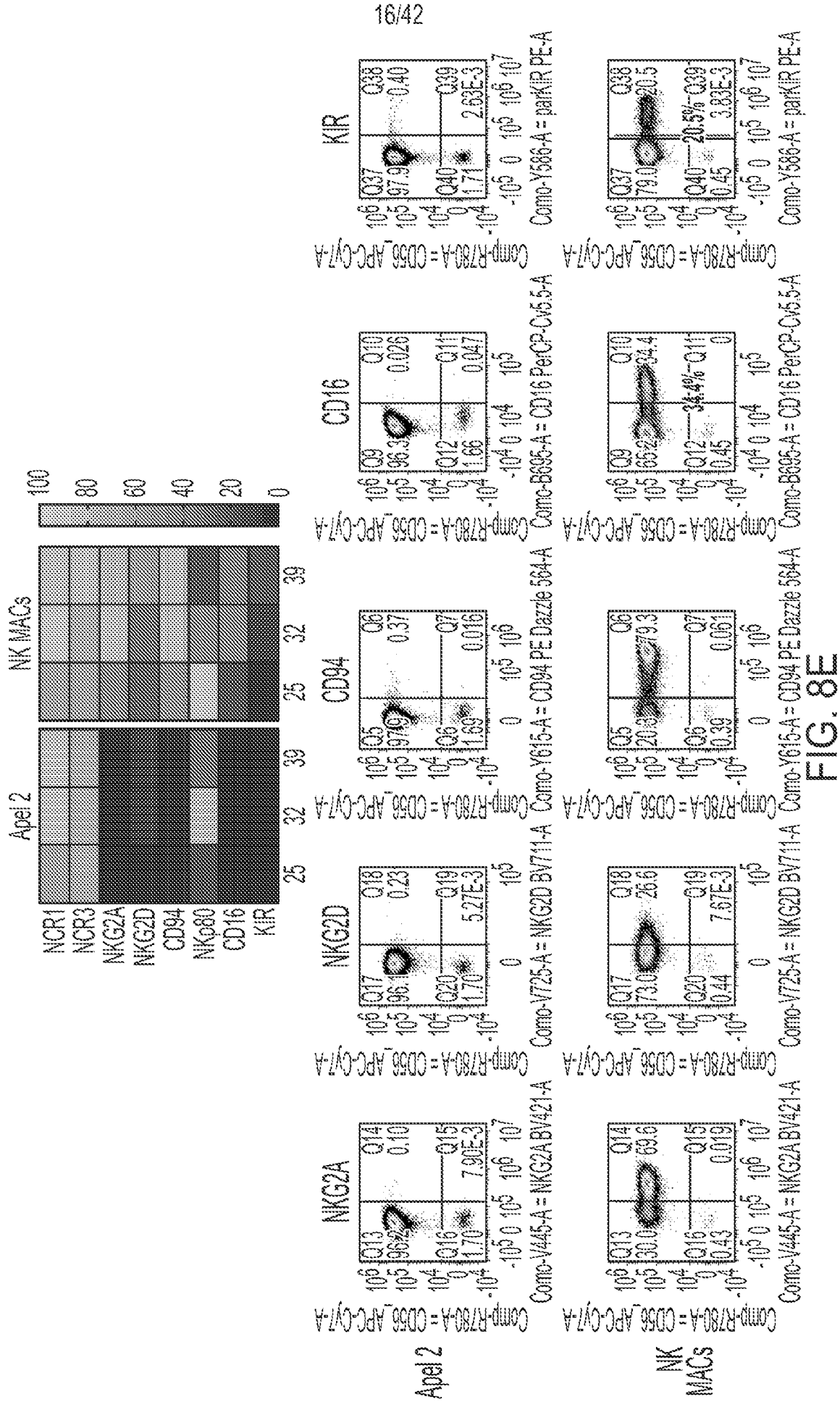

Apel 2
NK MACs
NCR1
NCR3
NKG2A
NKG2D
CD94
NKp80
CD16
KIR
100
80
60
40
20
0
25
32
39
NKG2A
NKG2D
CD94
CD16
KIR
Comp-R780-A = CD56_APC-Cy7-A
Como-V445-A = NKG2A BV421-A
Como-V725-A = NKG2D BV711-A
Como-V615-A = CD94 PE Dazzle 564-A
Como-B695-A = CD16 PerCP-Cy5.5-A
Como-Y586-A = panKIR PE-A
Q13 96.2
Q14 0.10
Q16 1.70
Q15 7.90E-3
Q17 96.1
Q18 0.23
Q20 1.70
Q19 5.27E-3
Q5 97.9
Q6 0.37
Q6 1.69
Q7 0.016
Q9 96.3
Q10 0.026
Q12 1.66
Q11 0.047
Q37 97.9
Q38 0.40
Q40 1.71
Q39 2.63E-3
Q13 30.0
Q14 69.6
Q16 0.43
Q15 0.019
Q17 73.0
Q18 26.6
Q20 0.44
Q19 7.67E-3
Q5 20.8
Q6 79.3
Q6 0.39
Q7 0.061
Q9 65.2
Q10 34.4
Q12 0.45
Q11 0
Q37 79.0
Q38 20.5
Q40 0.45
Q39 3.83E-3

FIG. 8E

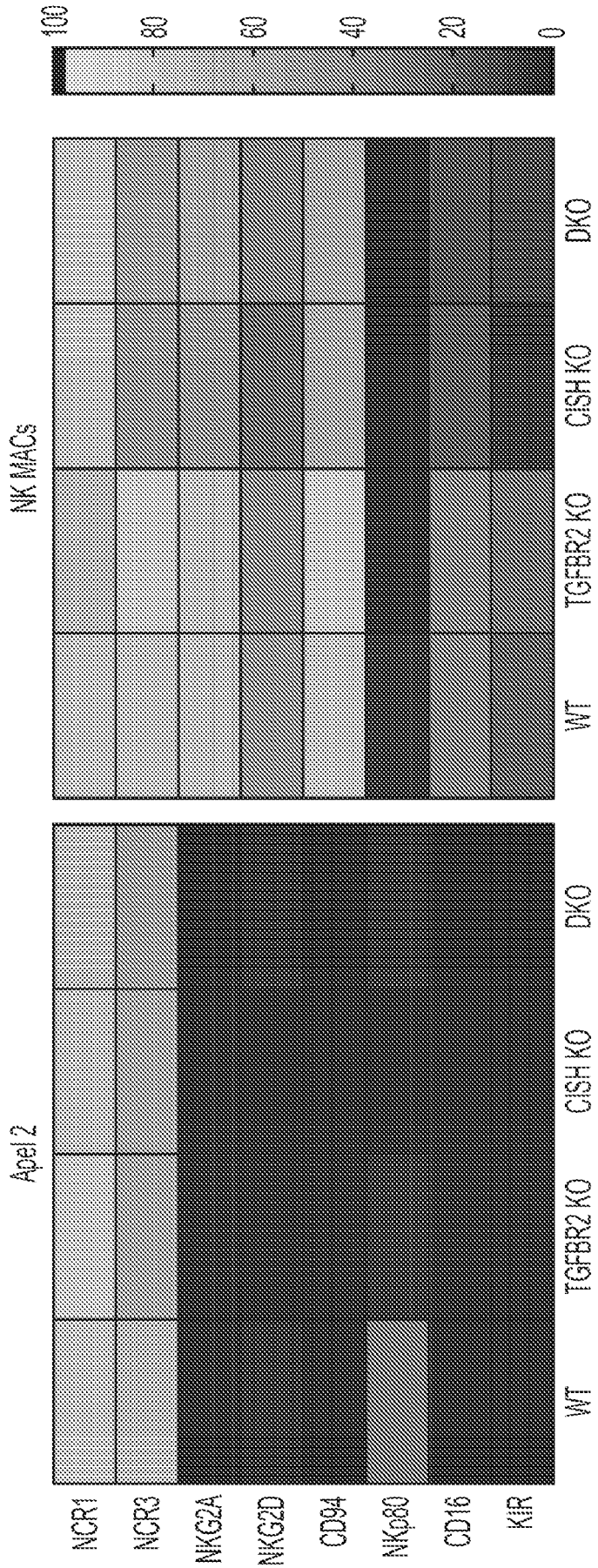

100
80
60
40
20
0
NK MACs
WT
TGFBR2 KO
CISH KO
DKO
Apel 2
WT
TGFBR2 KO
CISH KO
DKO
NCR1
NCR3
NKG2A
NKG2D
CD94
NKp80
CD16
KIR

FIG. 8F

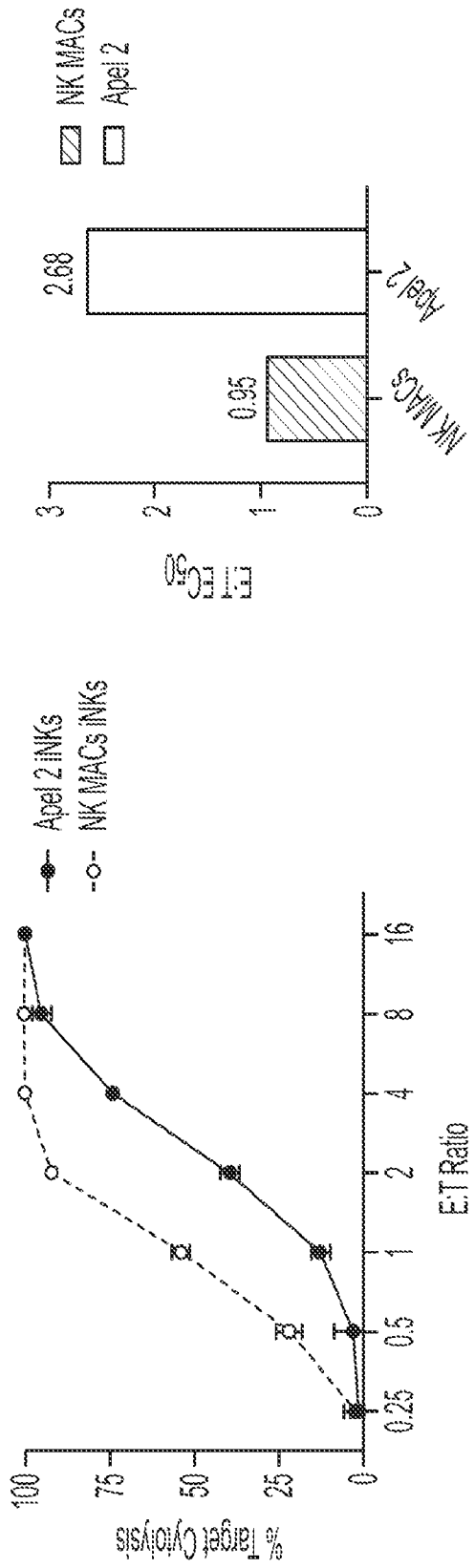

NK MACs
Apel 2
2.68
0.95
Apel 2
NK MACs
E:T EC50
3
2
1
0
Apel 2 iNKs
NK MACs iNKs
% Target Cytolysis
100
75
50
25
0
0.25
0.5
1
2
4
8
16
E:T Ratio

FIG. 8G

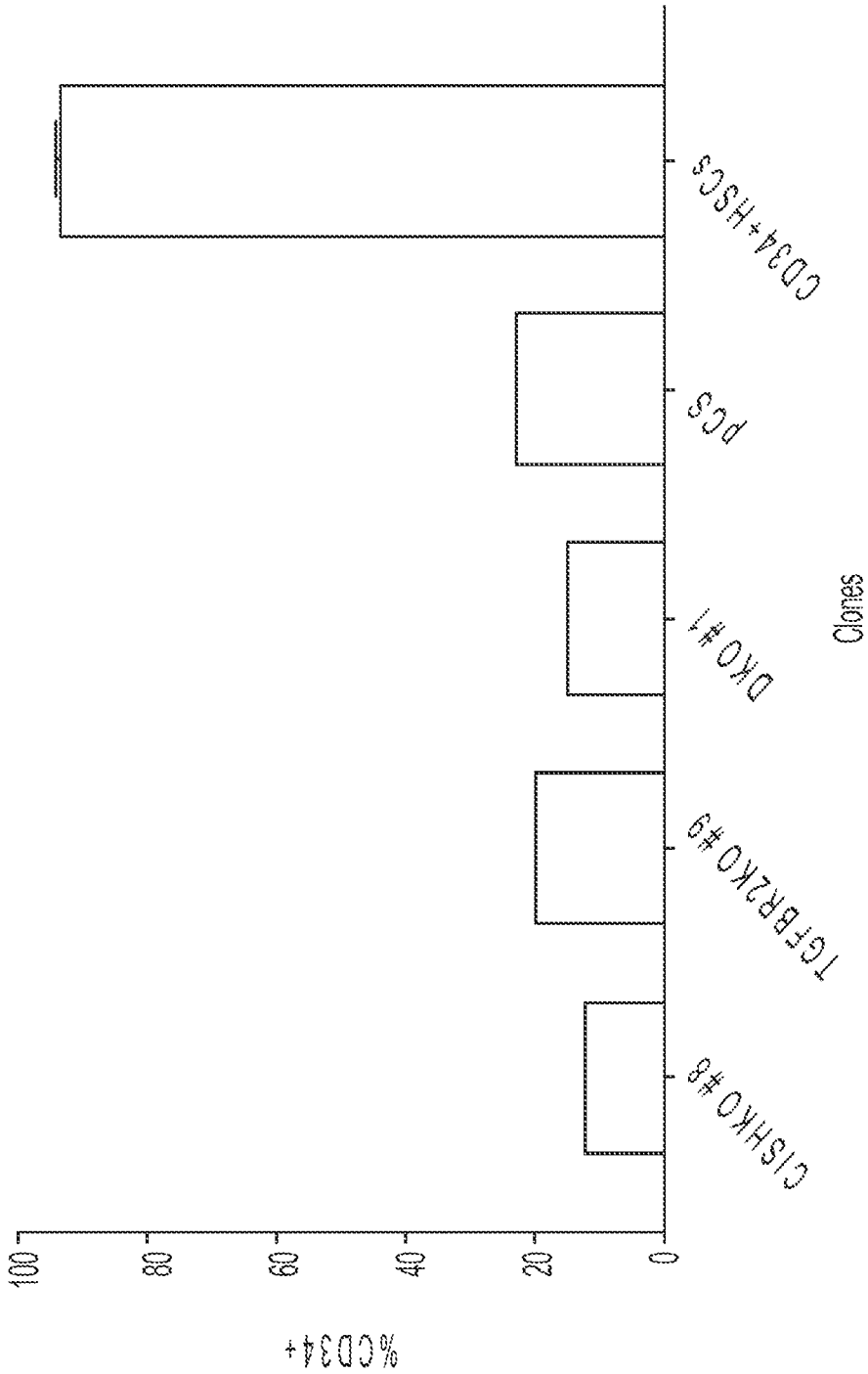

CD34+HSCs
PCS
DKO#1
TGFBR2KO#9
CISHKO#8
Clones
100
80
60
40
20
0
%CD34+

FIG. 9

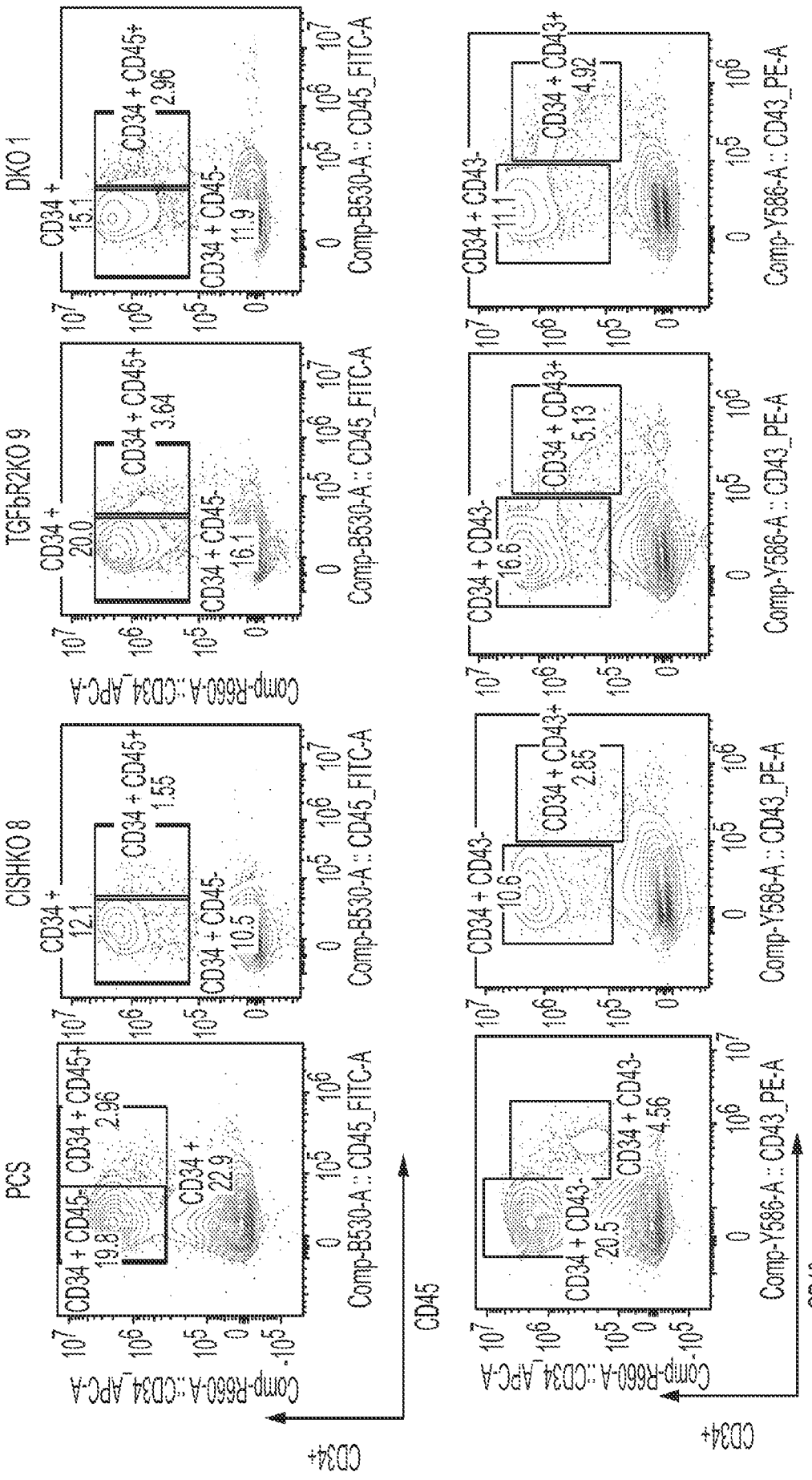

PCS
CISHKO 8
TGFbR2KO 9
DKO 1
CD34 + CD45-
19.8
CD34 + CD45+
2.96
CD34 +
22.9
CD34 +
12.1
CD34 + CD45+
1.55
CD34 + CD45-
10.5
CD34 +
20.0
CD34 + CD45+
3.64
CD34 + CD45-
16.1
CD34 +
15.1
CD34 + CD45+
2.96
CD34 + CD45-
11.9
Comp-R660-A :: CD34_APC-A
Comp-B530-A :: CD45_FITC-A
CD45
CD34+
CD34 + CD43-
20.5
CD34 + CD43+
4.56
CD34 + CD43-
10.6
CD34 + CD43+
2.85
CD34 + CD43-
16.6
CD34 + CD43+
5.13
CD34 + CD43-
11.1
CD34 + CD43+
4.92
Comp-Y586-A :: CD43_PE-A
CD43

FIG. 10

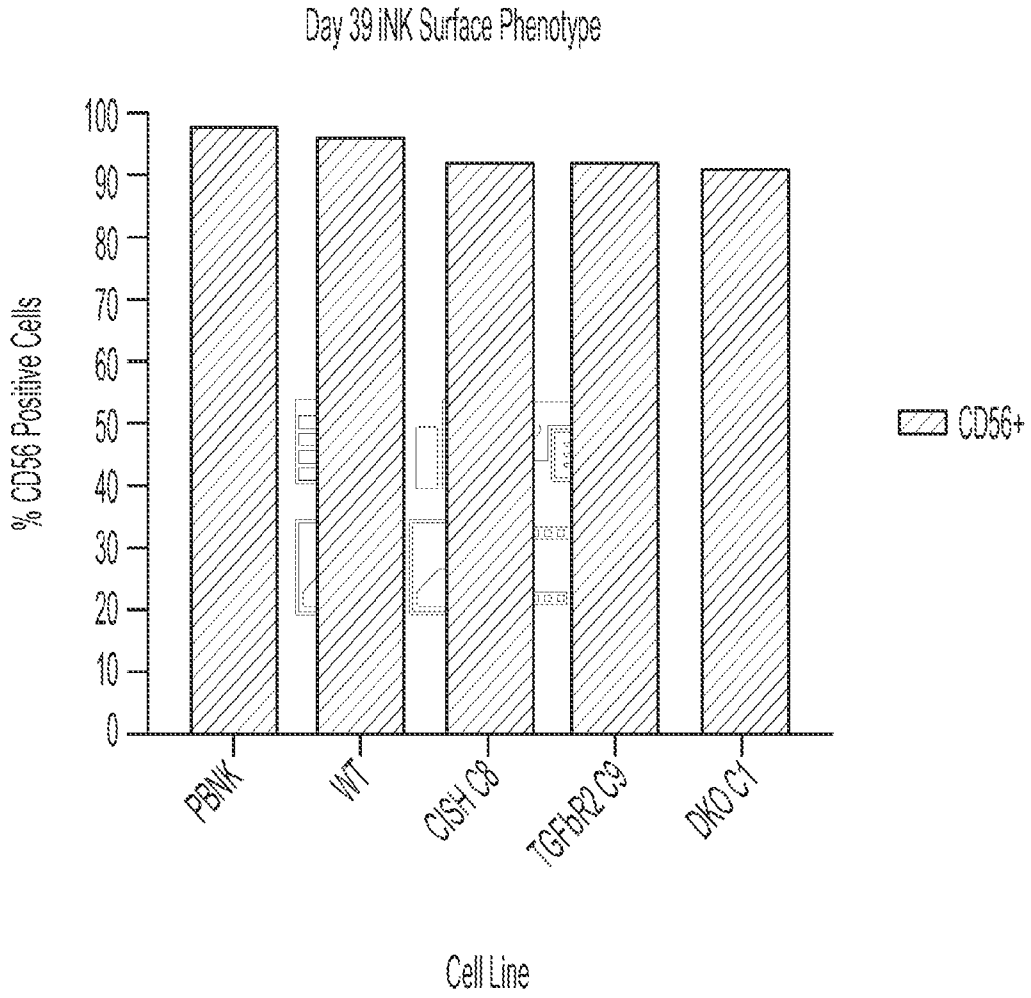

Day 39 iNK Surface Phenotype
% CD56 Positive Cells
100
90
80
70
60
50
40
30
20
10
0
PBNK
WT
CISH C8
TGFbR2 C9
DKO C1
Cell Line
CD56+

FIG. 11A

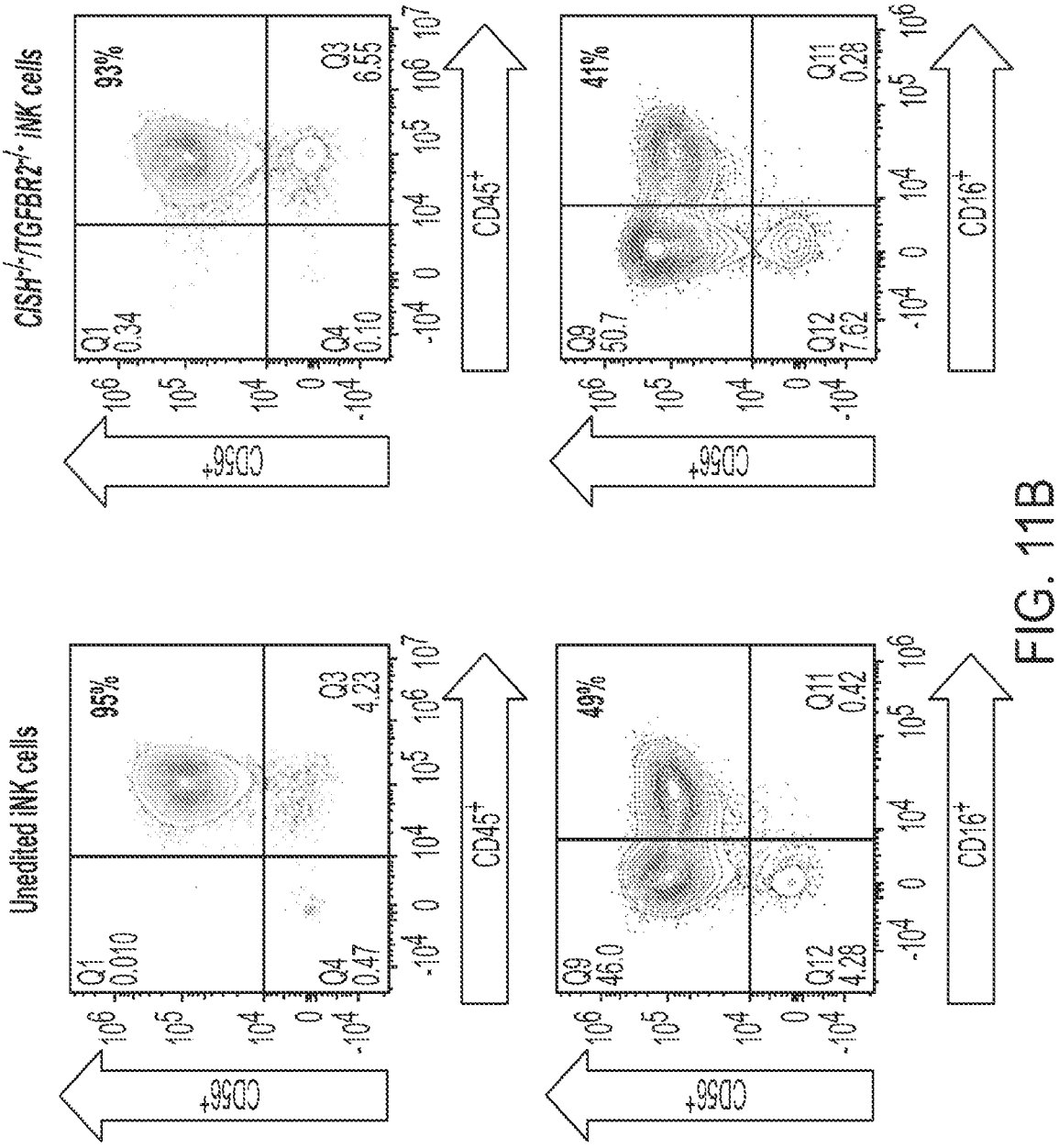

CISH-/-TGFBR2-/- iNK cells
Unedited iNK cells
93%
Q1 0.34
Q3 6.55
Q4 0.10
95%
Q1 0.010
Q3 4.23
Q4 0.47
41%
Q9 50.7
Q11 0.28
Q12 7.62
49%
Q9 46.0
Q11 0.42
Q12 4.28
CD56+
CD45+
CD16+

FIG. 11B

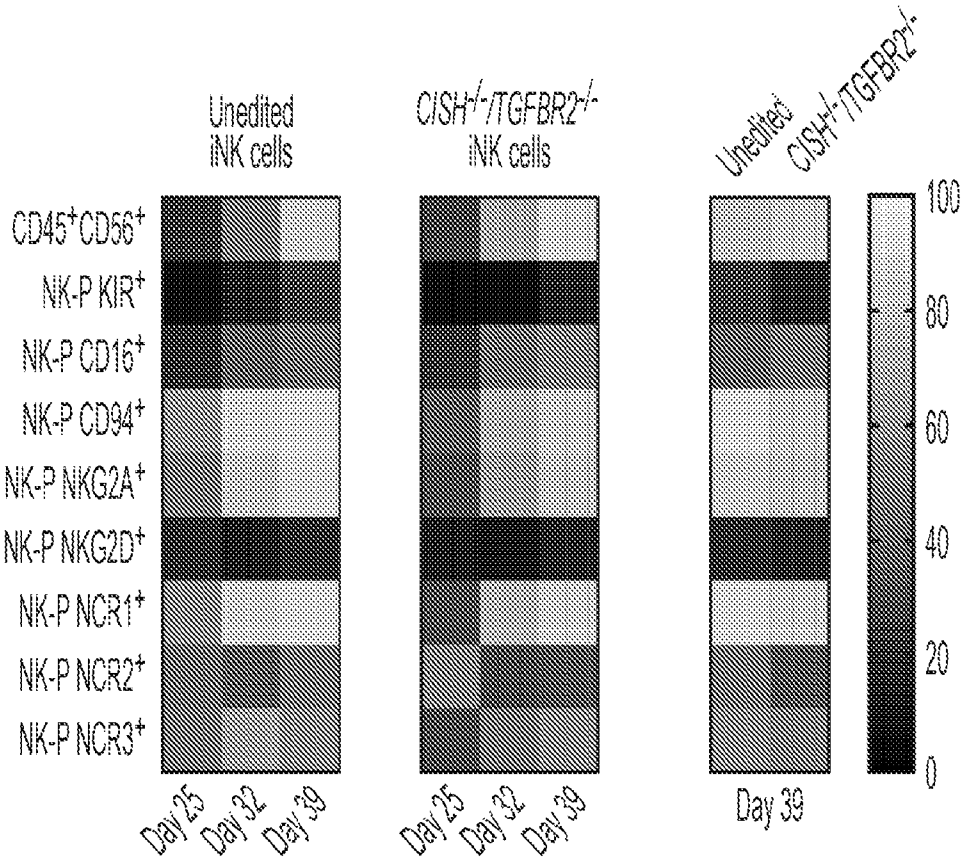

Unedited iNK cells
CISH-/-/TGFBR2-/- iNK cells
Unedited
CISH-/-/TGFBR2-/-
CD45+CD56+
NK-P KIR+
NK-P CD16+
NK-P CD94+
NK-P NKG2A+
NK-P NKG2D+
NK-P NCR1+
NK-P NCR2+
NK-P NCR3+
Day 25
Day 32
Day 39
Day 25
Day 32
Day 39
Day 39
100
80
60
40
20
0

FIG. 11C

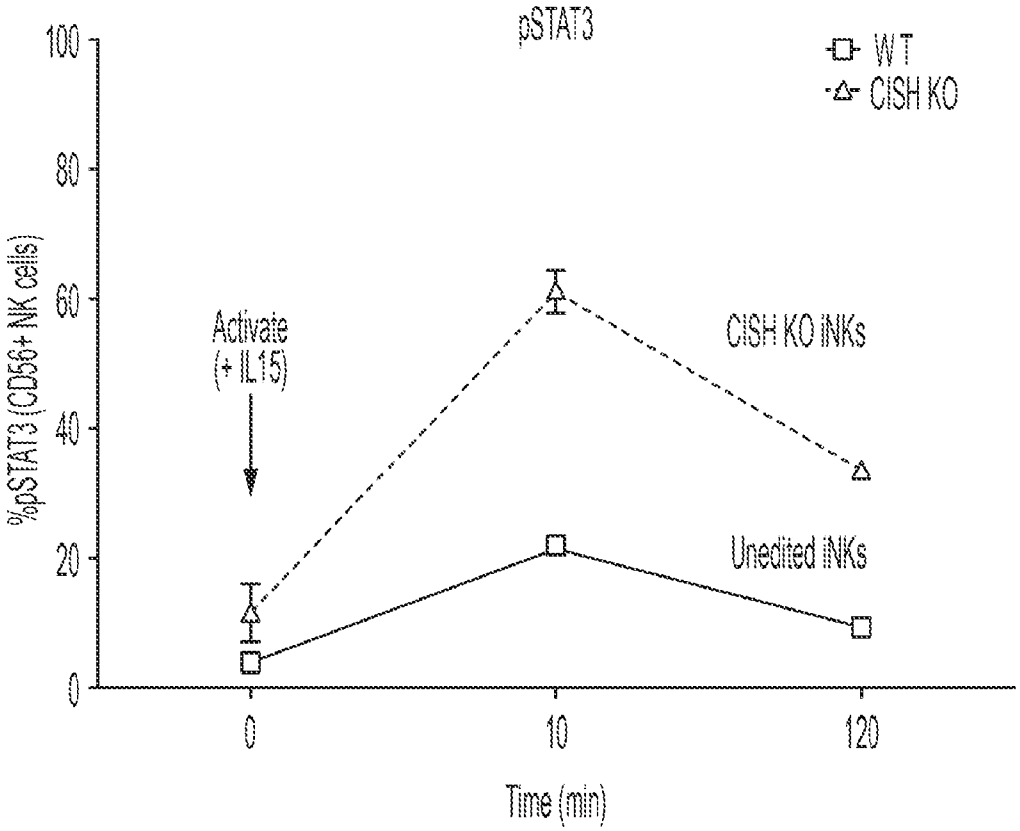

pSTAT3
WT
CISH KO
%pSTAT3 (CD56+ NK cells)
100
80
60
40
20
0
Activate
(+ IL15)
CISH KO iNKs
Unedited iNKs
0
10
120
Time (min)

FIG. 11D

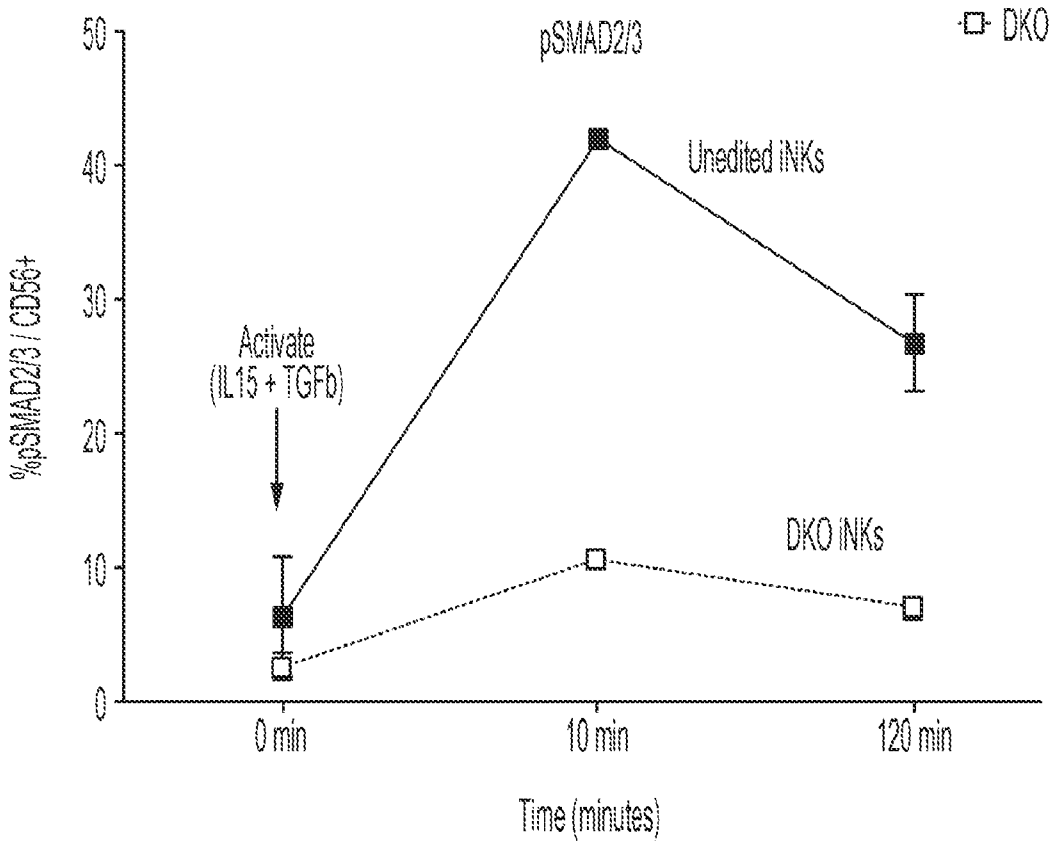

pSMAD2/3
DKO
Unedited iNKs
DKO iNKs
Activate
(IL15 + TGFb)
%pSMAD2/3 / CD56+
50
40
30
20
10
0
0 min
10 min
120 min
Time (minutes)

FIG. 11E

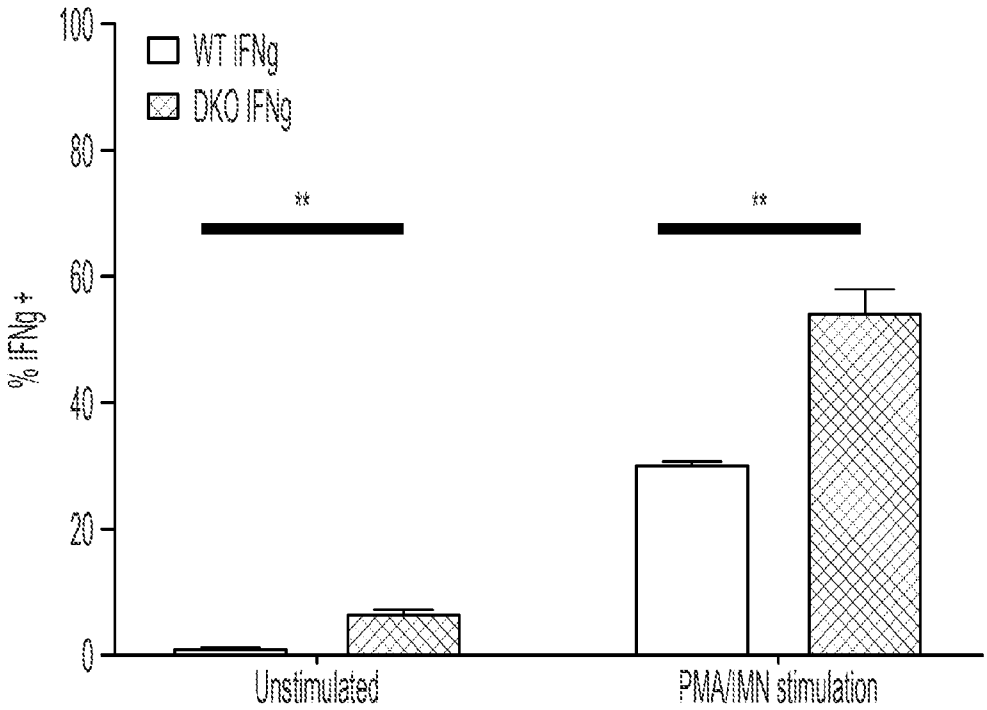

100
80
60
40
20
0
% IFNg+
WT IFNg
DKO IFNg
**
**
Unstimulated
PMA/IMN stimulation

FIG. 11F

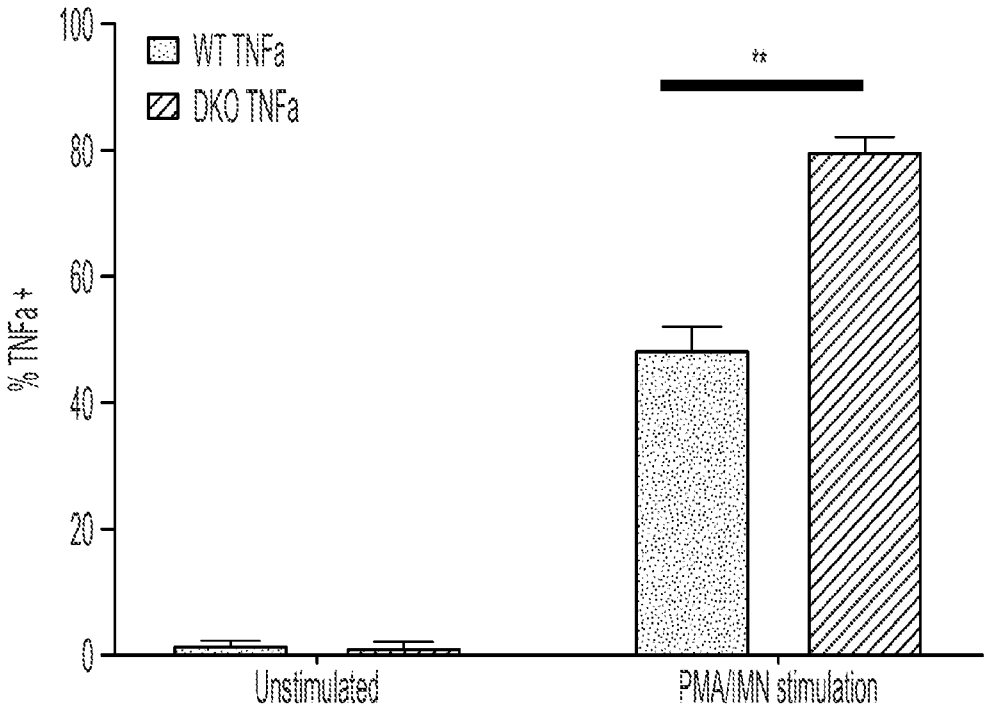

100
80
60
40
20
0
% TNFa +
WT TNFa
DKO TNFa
**
Unstimulated
PMA/IMN stimulation

FIG. 11G

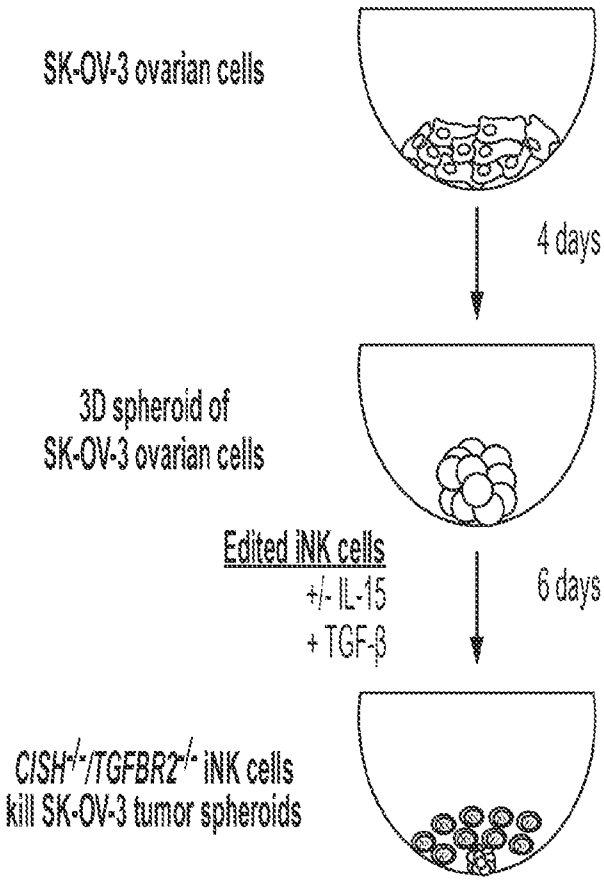

SK-OV-3 ovarian cells
4 days
3D spheroid of
SK-OV-3 ovarian cells
Edited iNK cells
+/- IL-15
+ TGF-β
6 days
CISH-/-/TGFBR2-/- iNK cells
kill SK-OV-3 tumor spheroids

FIG. 12A

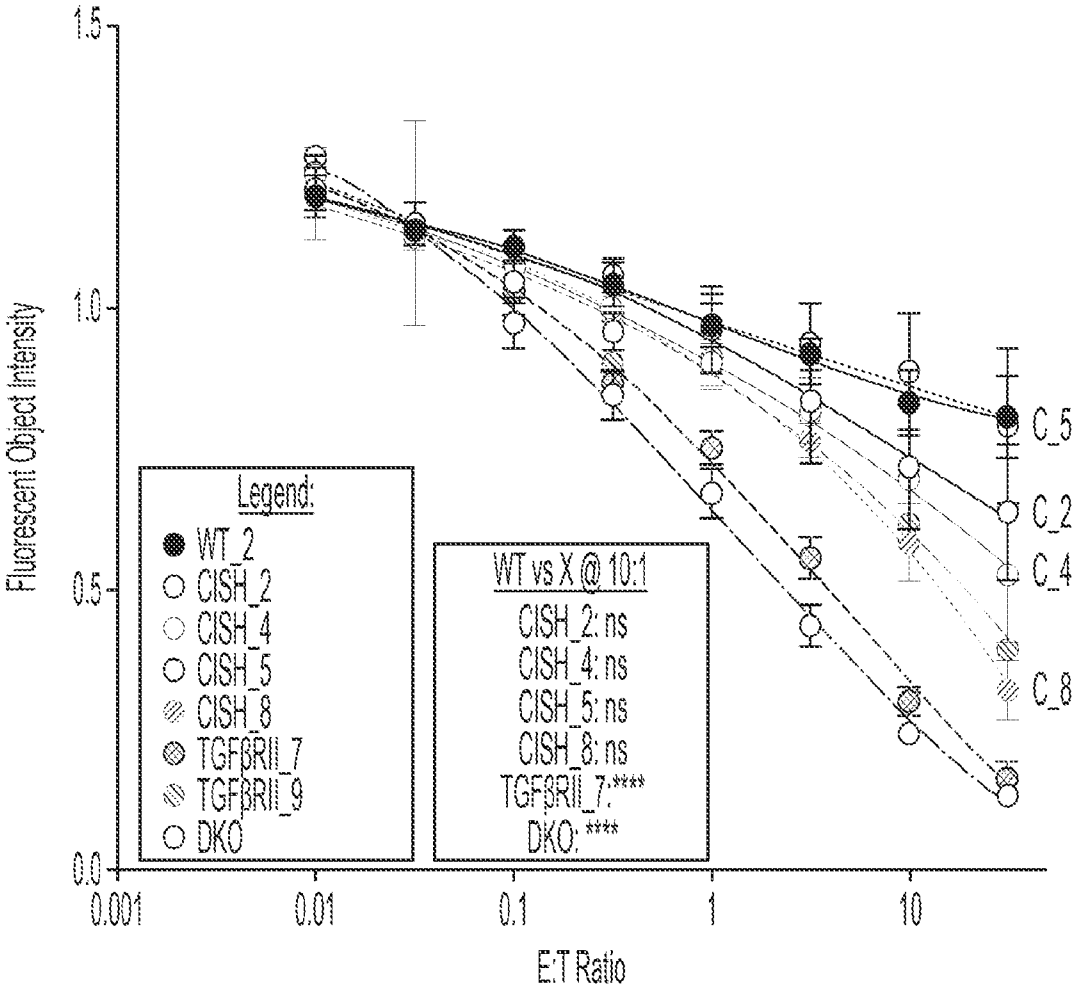

Fluorescent Object Intensity
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
E:T Ratio
Legend:
WT_2
CISH_2
CISH_4
CISH_5
CISH_8
TGFβRII_7
TGFβRII_9
DKO
WT vs X @ 10:1
CISH_2: ns
CISH_4: ns
CISH_5: ns
CISH_8: ns
TGFβRII_7: ****
DKO: ****
C_5
C_2
C_4
C_8

FIG. 12B

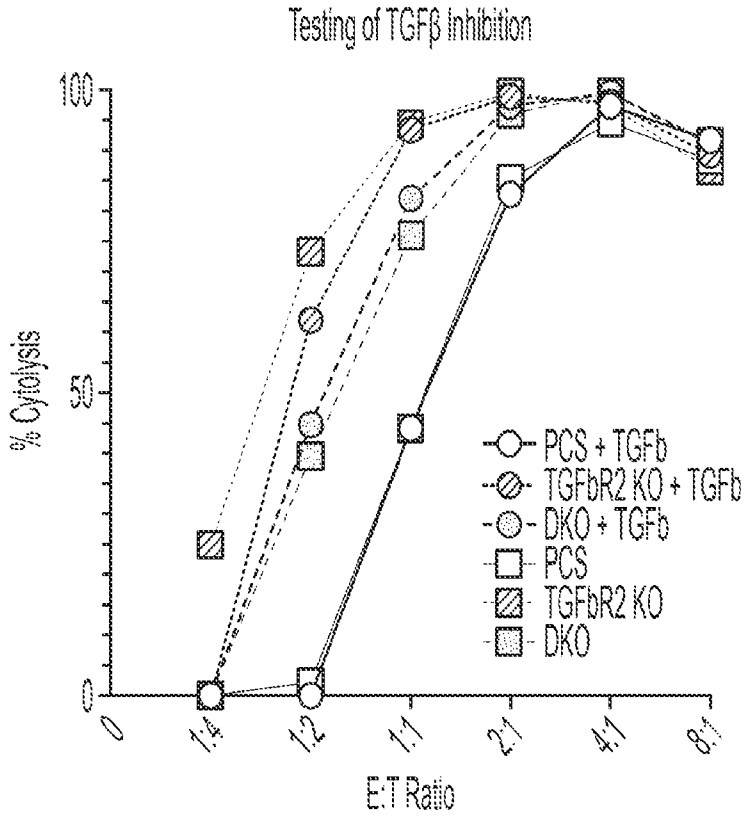

Testing of TGFβ Inhibition
% Cytolysis
100
50
0
0
1:4
1:2
1:1
2:1
4:1
8:1
E:T Ratio
PCS + TGFb
TGFbR2 KO + TGFb
DKO + TGFb
PCS
TGFbR2 KO
DKO

FIG. 12C

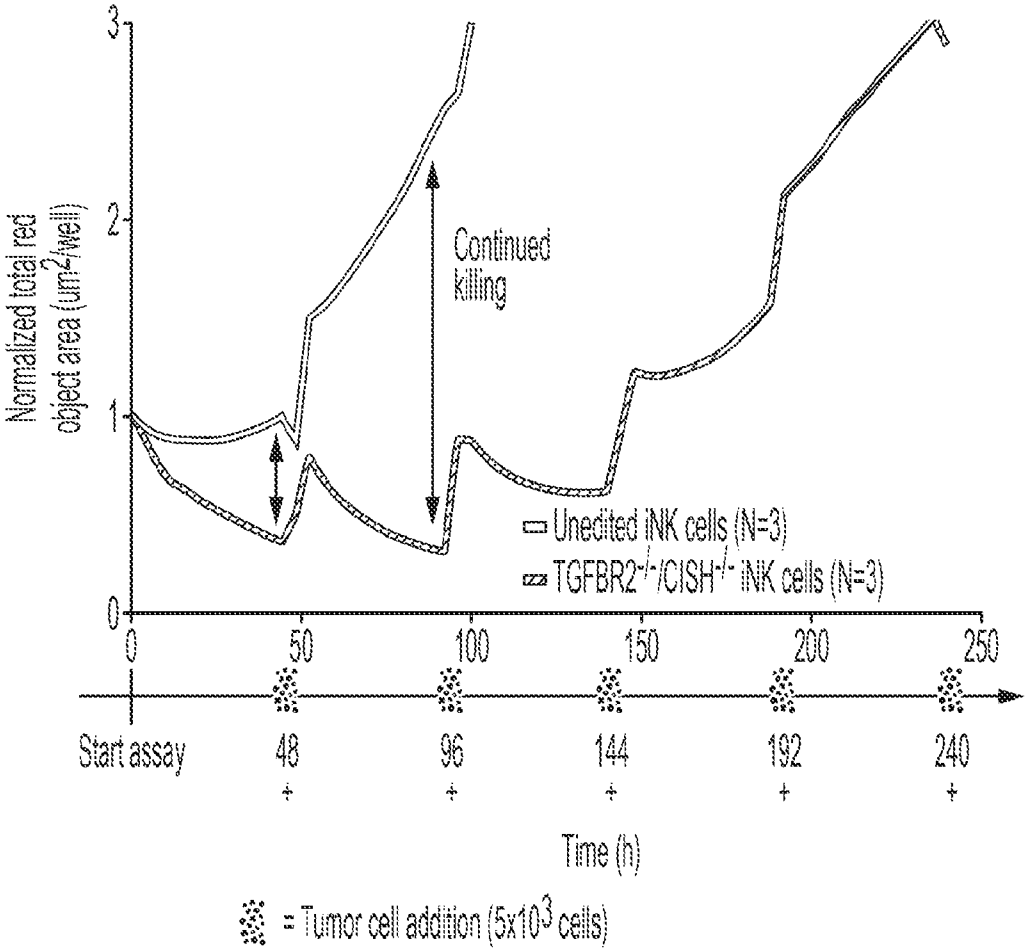

Normalized total red object area (um²/well)
3
2
1
0
Continued killing
Unedited iNK cells (N=3)
TGFBR2-/-/CISH-/- iNK cells (N=3)
0
50
100
150
200
250
Start assay
48
96
144
192
240
Time (h)
= Tumor cell addition (5x10³ cells)

FIG. 13

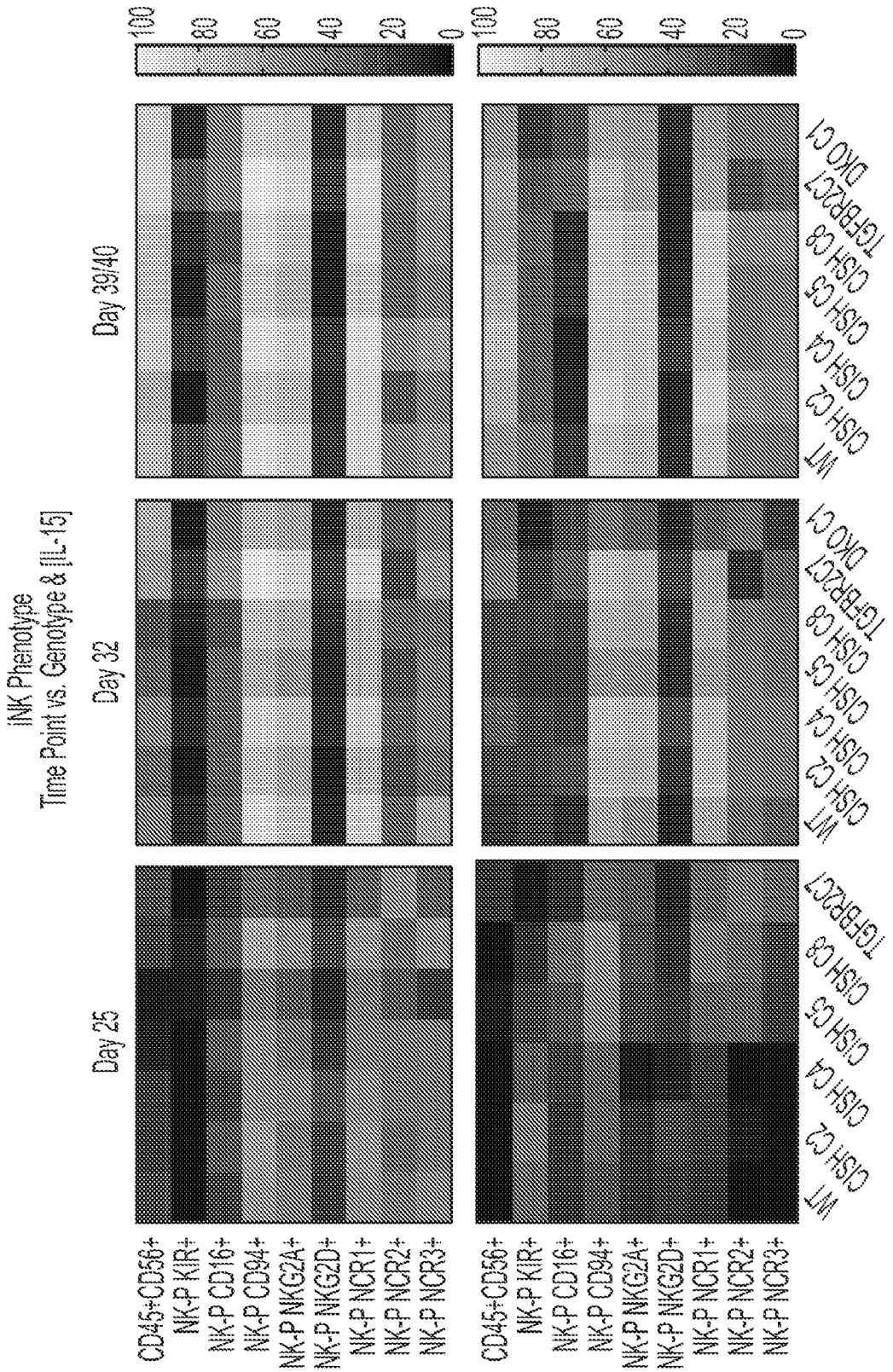

iNK Phenotype
Time Point vs. Genotype & [IL-15]
Day 25
Day 32
Day 39/40
[IL-15]
10 ng/mL
1 ng/mL
CD45+CD56+
NK-P KIR+
NK-P CD16+
NK-P CD94+
NK-P NKG2A+
NK-P NKG2D+
NK-P NCR1+
NK-P NCR2+
NK-P NCR3+
WT
CISH C2
CISH C4
CISH C5
CISH C8
TGFBR2CT
DKO CI
100
80
60
40
20
0

FIG. 14

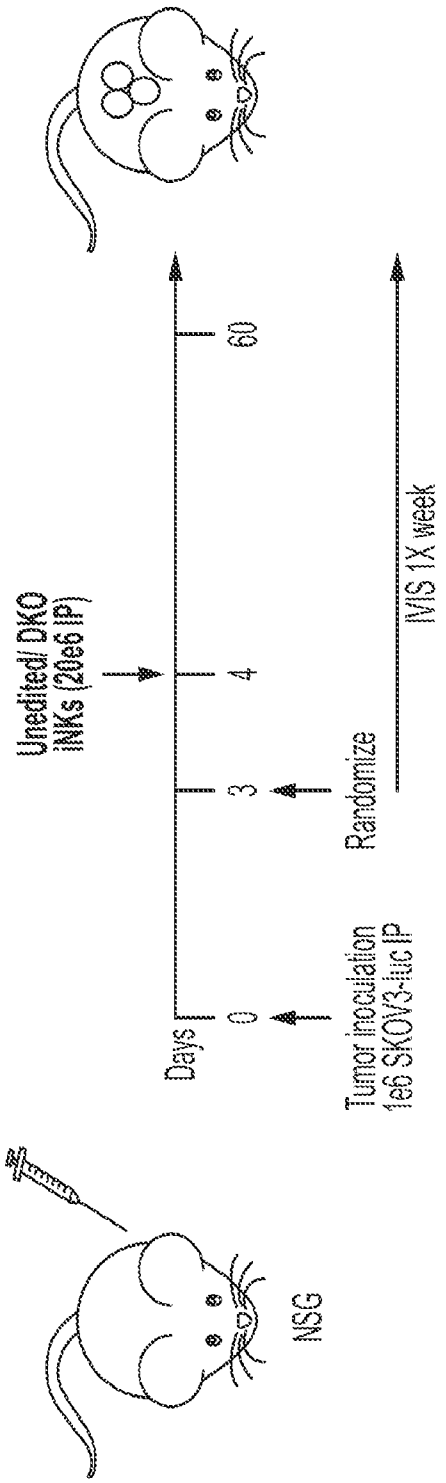

NSG
Tumor inoculation
1e6 SKOV3-luc IP
Days
0
3
4
60
Randomize
IVIS 1X week
Unedited/ DKO
iNKs (20e6 IP)

FIG. 15A

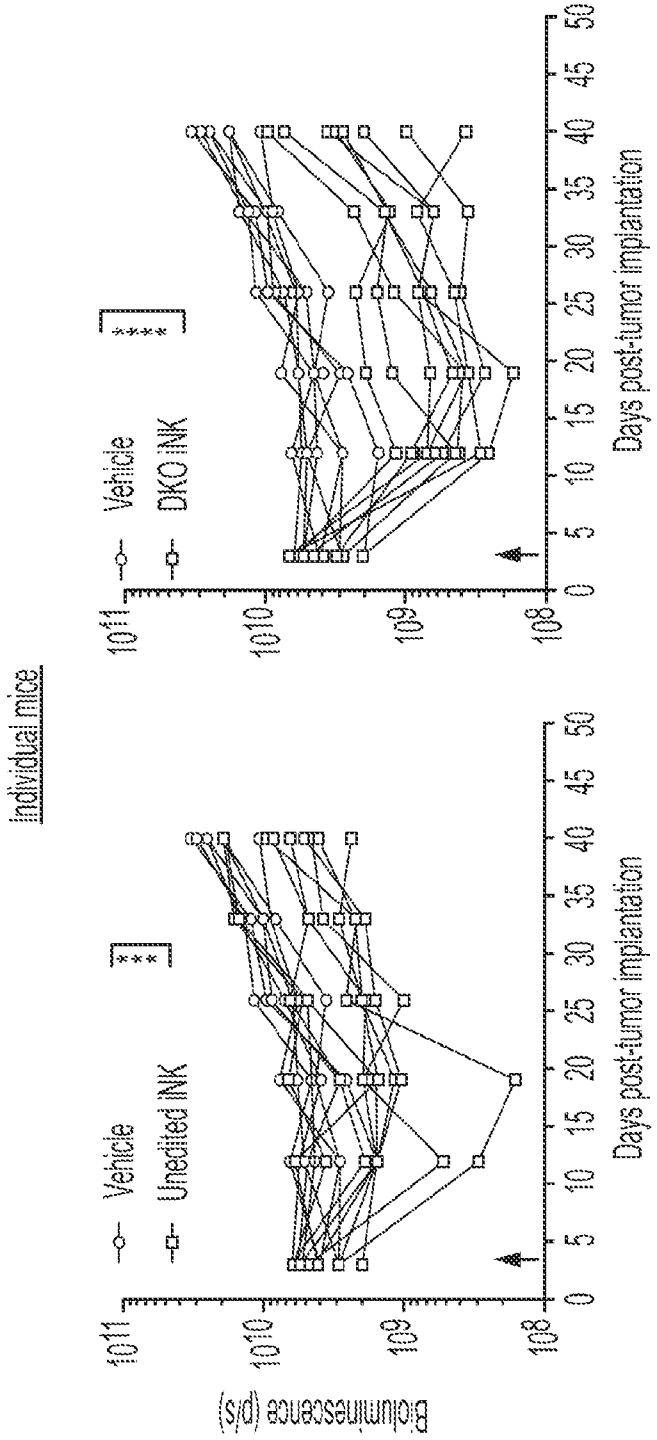

individual mice
Bioluminescence (p/s)
Vehicle
Unedited iNK
DKO iNK
****
Days post-tumor implantation

FIG. 15B

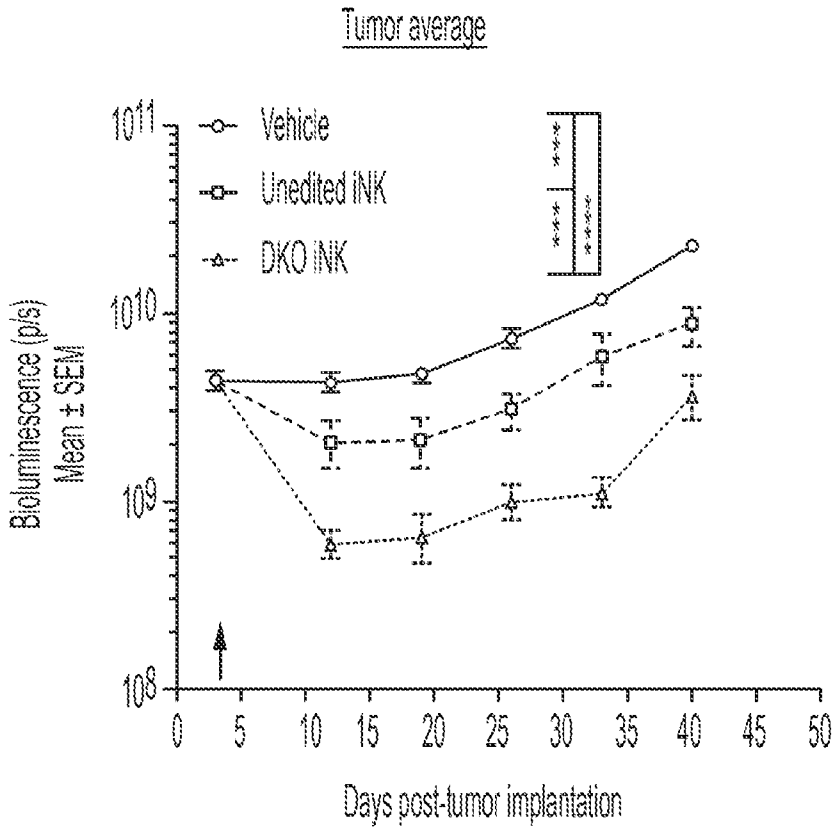

Tumor average
Vehicle
Unedited iNK
DKO iNK
****
****
Bioluminescence (p/s)
Mean ± SEM
10^{11}
10^{10}
10^{9}
10^{8}
0 5 10 15 20 25 30 35 40 45 50
Days post-tumor implantation

FIG. 15C

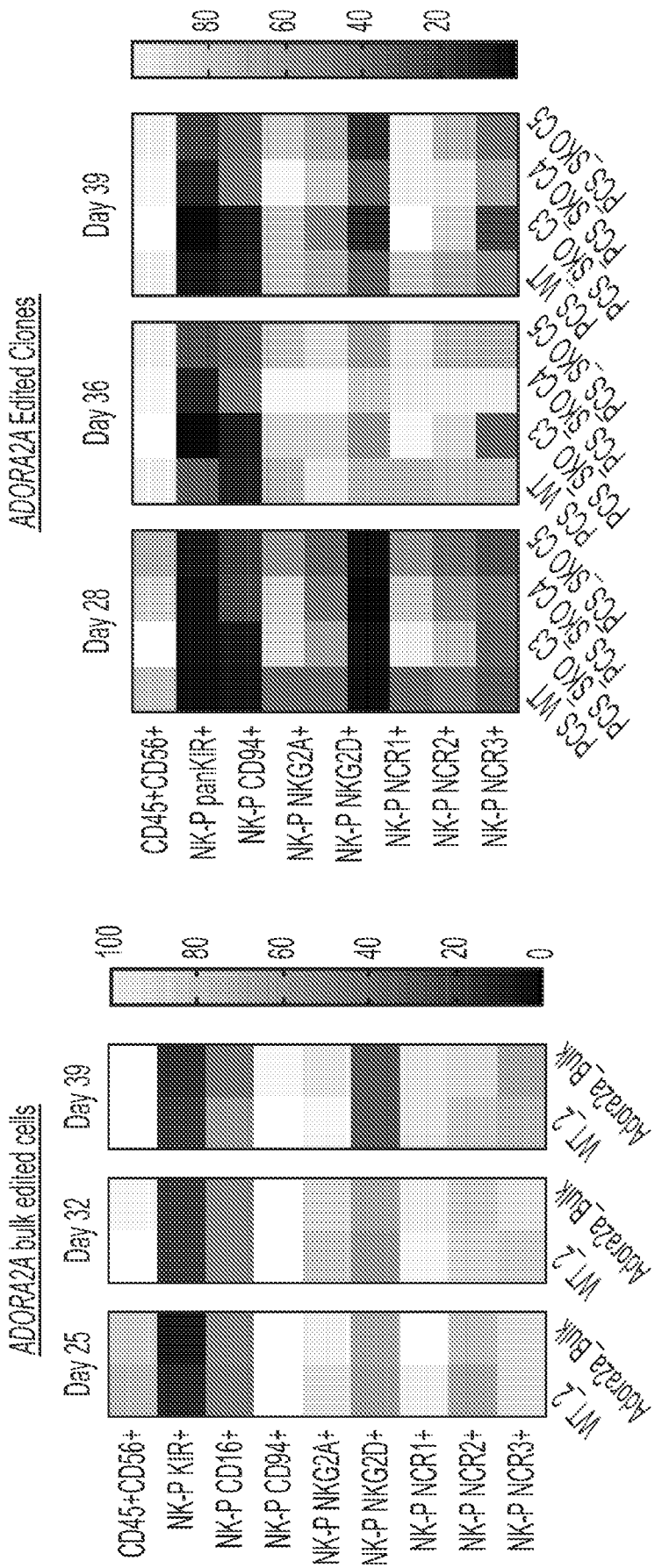

ADORA2A bulk edited cells
Day 25
Day 32
Day 39
WT_2
Adora2a_Bulk
CD45+CD56+
NK-P KIR+
NK-P CD16+
NK-P CD94+
NK-P NKG2A+
NK-P NKG2D+
NK-P NCR1+
NK-P NCR2+
NK-P NCR3+
100
80
60
40
20
0
ADORA2A Edited Clones
Day 28
Day 36
Day 39
PCS_WT
PCS_SKO_C3
PCS_SKO_C4
CD45+CD56+
NK-P panKIR+
NK-P CD94+
NK-P NKG2A+
NK-P NKG2D+
NK-P NCR1+
NK-P NCR2+
NK-P NCR3+
80
60
40
20

FIG. 16A

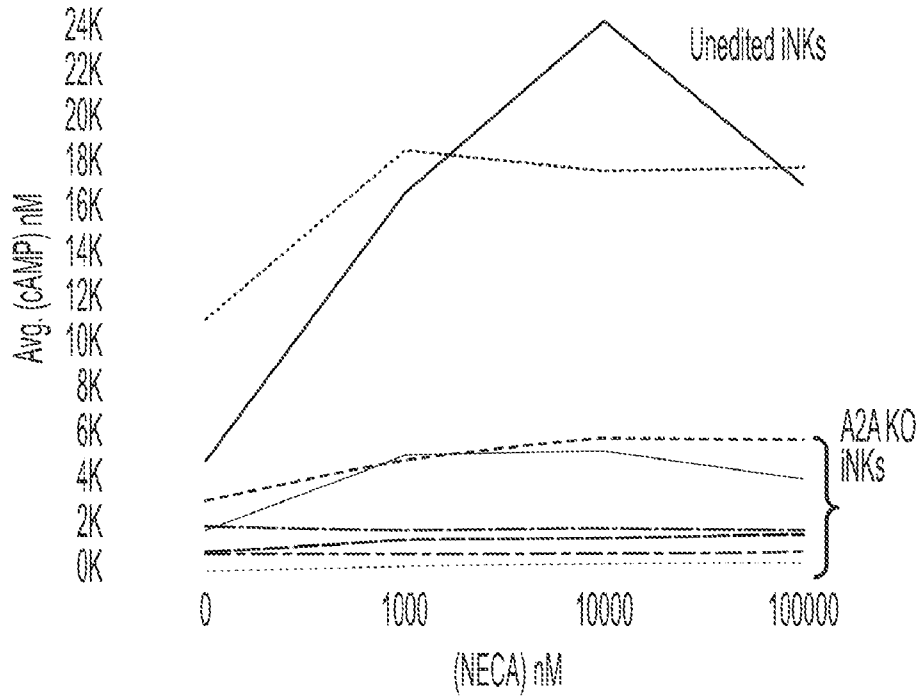

24K
22K
20K
18K
16K
14K
12K
10K
8K
6K
4K
2K
0K
Avg. (cAMP) nM
Unedited iNKs
A2A KO
iNKs
0
1000
10000
100000
(NECA) nM

FIG. 16B

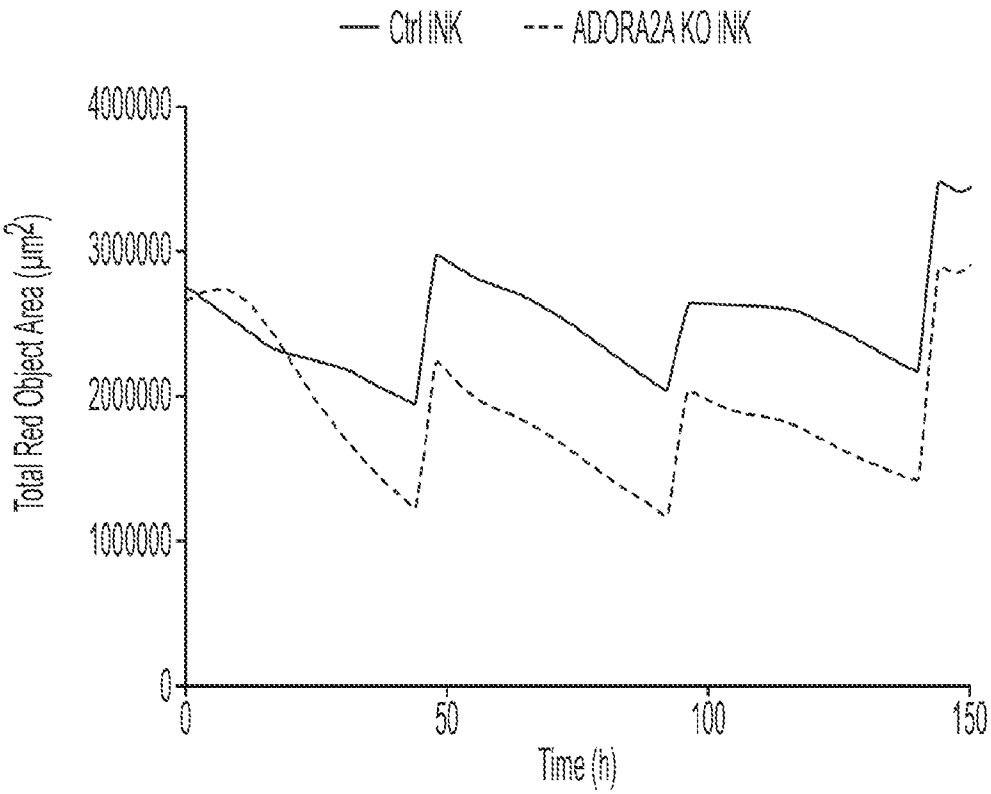

Ctrl INK
ADORA2A KO INK
Total Red Object Area (µm²)
4000000
3000000
2000000
1000000
0
0
50
100
150
Time (h)

FIG. 16C

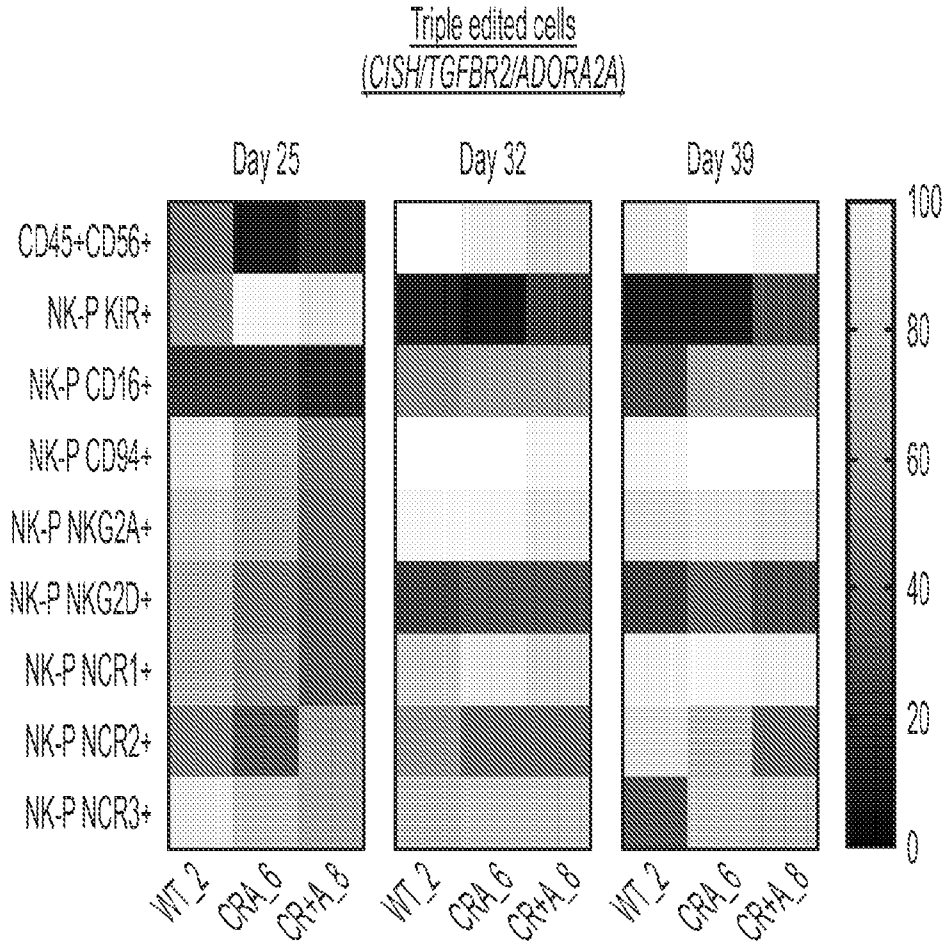

Triple edited cells
(CISH/TGFBR2/ADORA2A)
Day 25
Day 32
Day 39
CD45+CD56+
NK-P KIR+
NK-P CD16+
NK-P CD94+
NK-P NKG2A+
NK-P NKG2D+
NK-P NCR1+
NK-P NCR2+
NK-P NCR3+
WT_2
CRA_6
CR+A_8
100
80
60
40
20
0

FIG. 17A

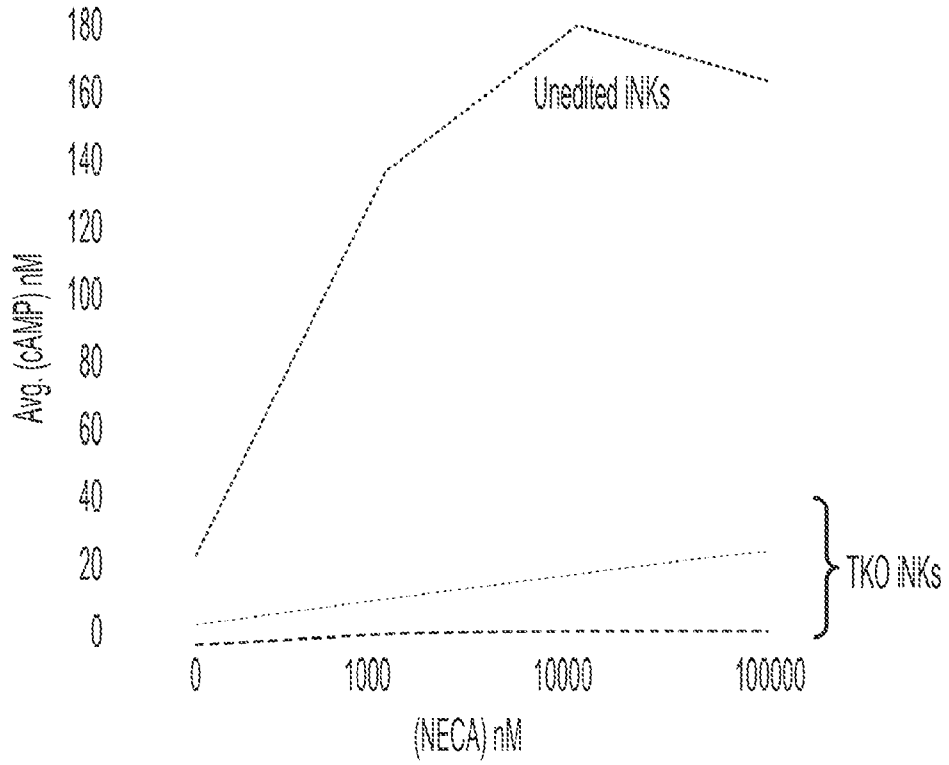

180
160
140
120
100
80
60
40
20
0
Avg. (cAMP) nM
Unedited iNKs
TKO iNKs
0
1000
10000
100000
(NECA) nM

FIG. 17B

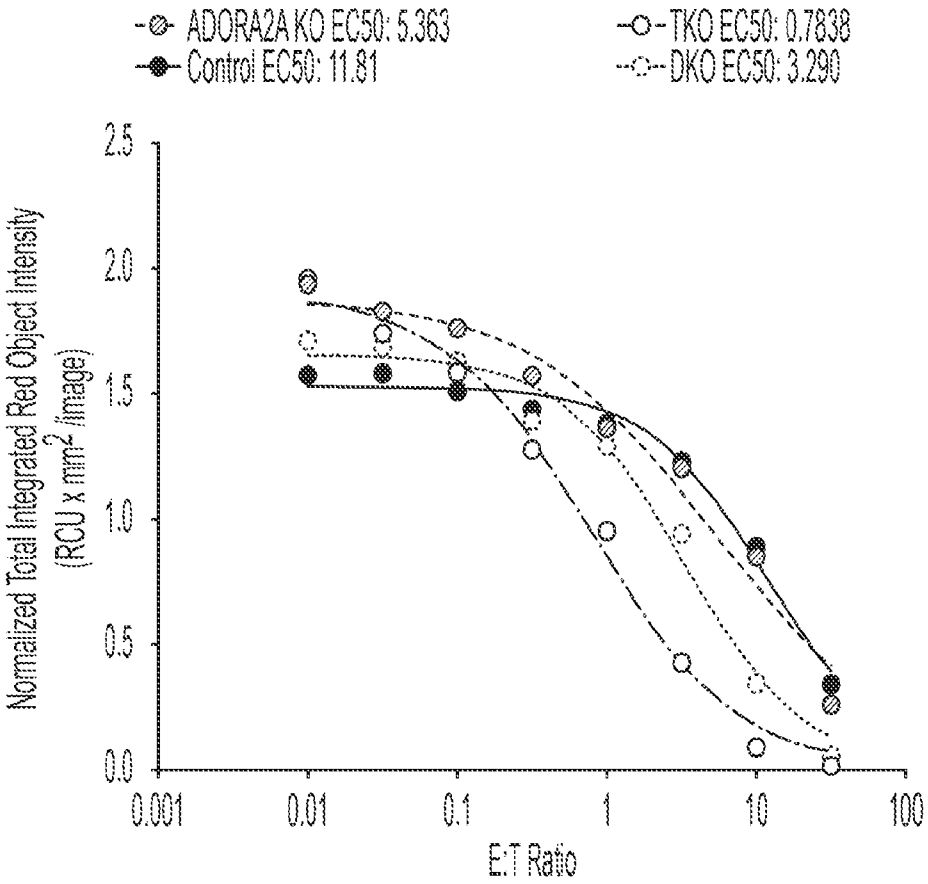

ADORA2A KO EC50: 5.363
Control EC50: 11.81
TKO EC50: 0.7838
DKO EC50: 3.290
Normalized Total Integrated Red Object Intensity
(RCU x mm2 /image)
2.5
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
100
E:T Ratio

FIG. 17C

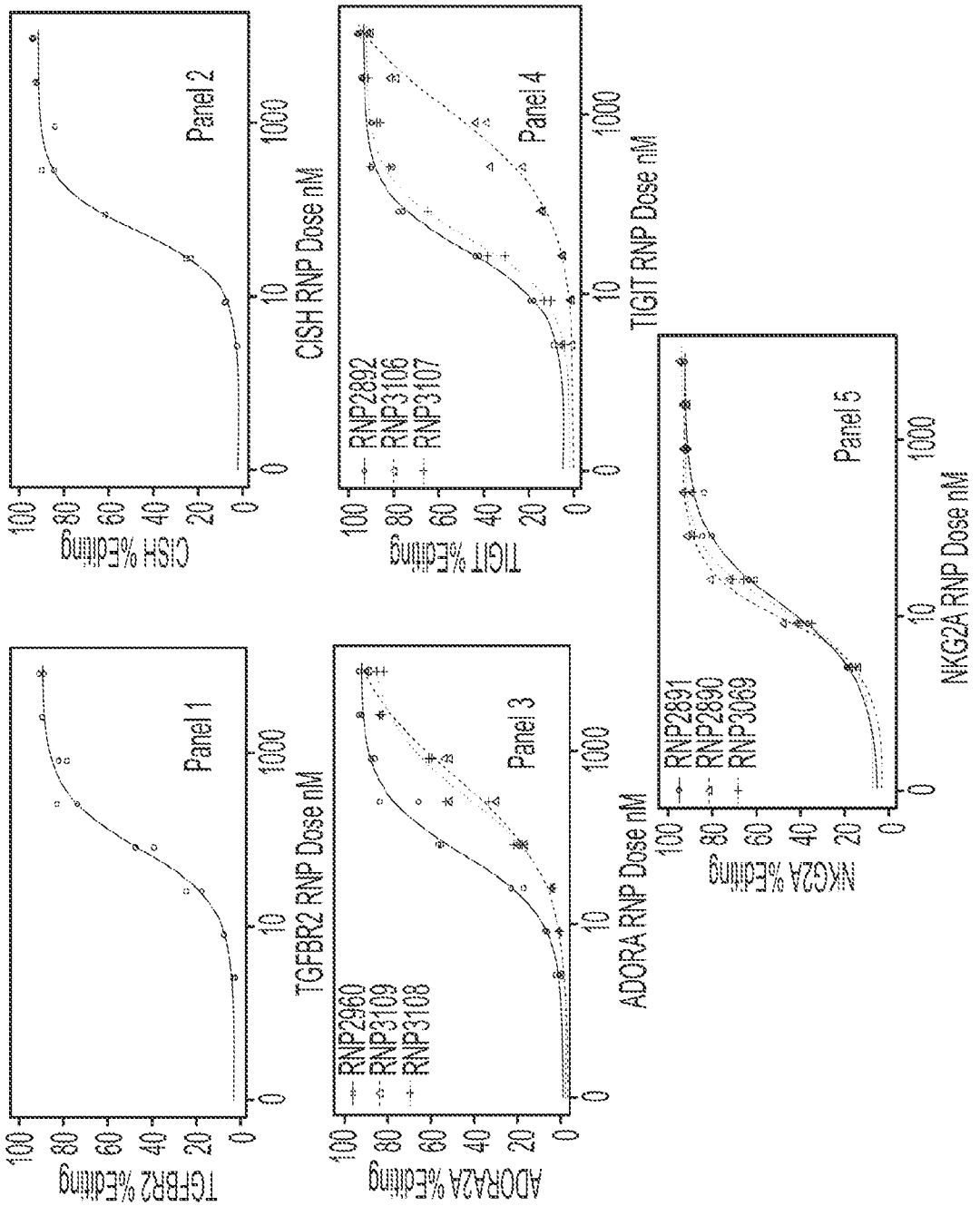

Panel 1
TGFBR2 %Editing
TGFBR2 RNP Dose nM
Panel 2
CISH %Editing
CISH RNP Dose nM
Panel 3
ADORA2A %Editing
ADORA RNP Dose nM
RNP2960
RNP3109
RNP3108
Panel 4
TIGIT %Editing
TIGIT RNP Dose nM
RNP2892
RNP3106
RNP3107
Panel 5
NKG2A %Editing
NKG2A RNP Dose nM
RNP2891
RNP2890
RNP3069

FIG. 18