MODIFIED NATURAL KILLER (NK) CELLS FOR IMMUNOTHERAPY

RELATED APPLICATIONS

[1] This application claims priority to U.S. Provisional Application No. 62/806,457, filed on February 15, 2019; U.S. Provisional Application No. 62/841,066, filed on April 30, 2019; U.S. Provisional Application No. 62/841,684, filed on May 1, 2019; and U.S. Provisional Application No. 62/943,649, filed on December 4, 2019, the entire contents of each of which are expressly incorporated herein by reference.

BACKGROUND

[2] NK cells are useful for immunotherapy approaches, for example, in the context of immuno-oncology. NK cells are a type of cytotoxic innate lymphocyte. NK cells play an important role in tumor immunity, and the cytotoxic activity of NK cells is tightly regulated by a network of activating and inhibitory pathways (see, *e.g.*, Gras Navarro A, Bjorklund AT and Chekenya M (2015) *Front. Immunol.* 6:202; incorporated in its entirety herein by reference).

[3] The use of naturally occurring or modified NK cells in immunotherapy approaches, *e.g.*, via autologous or allogeneic NK cell transfer, has been reported, and while some success has been achieved, such approaches are typically characterized by a suboptimal NK cell response. In the context of immune-oncology, it is believed that this suboptimal response is, at least in part, to tumors harnessing NK cell inhibitory pathways to suppress cytotoxic NK cell activity, limit NK cell invasion, and/or inhibit NK cell proliferation and survival. Thus, application of NK cells in the therapy of solid tumors has seen limited success.

[4] Initial work has been performed in trying to focus NK cell response on specific cells, *e.g.*, by expressing a chimeric antigen receptor in NK cells that targets the NK cells to tumor cells, or by modulating activating or inhibitory NK cell pathways to achieve a stronger and/or more sustained NK cell response. See, *e.g.*, Jing Y, *et al.* (2015) *PLoS ONE* 10(3):e0121788; and Oberschmidt 0, Kloess S and Koehl U (2017) *Front. Immunol.* 8:654; incorporated in their entireties herein by reference.

[5] In pursuit of an off-the shelf allogeneic NK cell therapy that could be used in combination with a therapeutic antibody, an induced pluripotent stem cell line has been developed in which cells express an enhanced version of CD16 (hnCD16), and NK cells have been derived from this iPSC line. See, *e.g.*, Li et al., *Cell Stem Cell.* 2018 Aug 2;23(2):181-192.e5; incorporated in its entirety herein by reference.

[6] However, to date all of these approaches have seen limited success. Therefore, there remains a need for the development of better therapeutic approaches for immunotherapy.

SUMMARY

[7] Some aspects of the present disclosure provide compositions, cells, cell populations, methods, strategies, and treatment modalities that are useful in the context of immunotherapeutic approaches, *e.g.*, immunooncology therapeutic approaches. In some embodiments, the present disclosure provides modified NK cells (or other lymphocytes) that are useful in NK cell therapy, *e.g.*, in the context of immunotherapeutic approaches. In some embodiments, the cells and cell populations provided herein are characterized by one or more modifications that enhance their efficacy in immunotherapeutic approaches. For example, in some embodiments, NK cells are provided that comprise one or more modifications that effect a loss-of-function in a gene or protein associated with inhibition of NK cell function in a therapeutic context, and/or one or more modifications that effect an expression of an exogenous nucleic acid or protein associated with an enhanced NK cell function in a therapeutic context. In some embodiments, the present disclosure provides modified NK cells that are derived from an induced pluripotent cell (iPSC). IPSC-derived NK cells are also referred to herein as iNK cells. In some embodiments, modified iNK cells are provided that are derived from a somatic cell, for example, and without limitation, from a fibroblast, a peripheral blood cell, or a developmentally mature T cell (T cell that have undergone thymic selection). In some embodiments, the NK or iNK cells provided herein comprise one or more genomic edits, *e.g.*, indels or insertions of exogenous nucleic acid constructs resulting from cutting a genomic locus with an RNA-guided nuclease. The use of RNA-guided nuclease technology in the context of the generation of modified NK and iNK cells allows for the engineering of complex alterations with enhanced characteristics relevant for clinical applications.

[8] Some aspects of the present disclosure provide complex editing strategies, and resulting NK cells having complex genomic alterations, that allow for the generation of advanced NK cell products for clinical applications, *e.g.*, for immunooncology therapeutic approaches. In some embodiments, the modified NK cells provided herein can serve as an off-the-shelf clinical solution for patients having, or having been diagnosed with, a hyperproliferative disease, such as, for example, a cancer. In some embodiments, the modified NK cells exhibit an enhanced survival, proliferation, NK cell response level, NK cell response duration, resistance against NK cell exhaustion, and/or target recognition as

compared to non-modified NK cells. For example, the modified NK cells provided herein may comprise genomic edits that result in: expression of a chimeric antigen receptor (CAR) of interest, *e.g.,* a CAR targeting mesothelin, EGFR, HER2 and/or MICA/B; expression of a CD16 variant, *e.g.*, a non-naturally occurring CD16 variant such as, for example, hnCD16 (see, e.g., Zhu et al., Blood 2017, 130:4452, the contents of which are incorporated herein in their entirety by reference); expression of an IL15/IL15RA fusion; a loss-of-function in TGF beta receptor 2 (TGFbetaR2); and/or expression of a dominant-negative variant of TGFbetaR2; a loss-of-function of ADORA2A; a loss-of-function of B2M; expression of HLA-G: a loss-of-function of a CIITA ; a loss-of-function of a PD1 ; a loss-of-function of TIGIT; and/or a loss-of-function of CISH; or any combination of two or more thereof in the modified NK cell. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of CISH. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of TIGIT. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH and a loss-of-function of TIGIT. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TIGIT and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TIGIT and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of ADORA2A and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of TIGIT. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of

ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TIGIT, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A.

[9] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[10] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, expression of a soluble MICA and/or MICB, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[11] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, expression of a soluble MICA and/or MICB, expression of an exogenous IL-12, expression of an exogenous IL-18, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[12] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, expression of an exogenous IL-12, expression of an exogenous

IL-18, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[13] In one aspect the disclosure features a modified lymphocyte, wherein the modified lymphocyte does not express endogenous CD3, CD4, and/or CD8; and expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, and/or CD45; (ii) NK cell receptor (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; wherein the modified lymphocyte further: (1) comprises at least one exogenous nucleic acid construct encoding: (i) a chimeric antigen receptor (CAR); (ii) a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) interleukin-12 receptor (IL-12R), or a variant thereof; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) a nucleic acid sequence encoding leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) exhibits a loss of function of at least one of: (i) transforming growth factor beta receptor 2 (TGFβR2); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microgobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) cytokine inducible SH2 containing protein (CISH); (vii) class II, major histocompatibility complex, transactivator (CIITA); (viii) natural killer cell receptor NKG2A (natural killer group 2A); (ix) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (x) cluster of differentiation 32B (CD32B, FCGR2B); (xi) T cell receptor alpha constant (TRAC); or any combination of two or more thereof. In one embodiment, the modified lymphocyte exhibits a loss of function of TGFβR2 and a loss-of-function of CISH. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of TIGIT. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH and a loss-of-function of TIGIT. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-

function of TIGIT and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TIGIT and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of ADORA2A and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of TIGIT. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TIGIT, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A.

[14] In one embodiment, the modified lymphocyte does not express endogenous CD3, CD4, and/or CD8; and expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, and/or CD45; (ii) NK cell receptor (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; wherein the modified lymphocyte further: (1) comprises at least one exogenous nucleic acid construct encoding: (i) a chimeric antigen receptor (CAR); (ii) a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) interleukin-12 receptor (IL-12R), or a variant thereof; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) a nucleic acid sequence encoding leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) exhibits a loss of function of

transforming growth factor beta receptor 2 (TGFβR2), cytokine inducible SH2 containing protein (CISH), or a combination thereof.

[15] In some embodiments, the nucleic acid construct is an expression construct comprising a nucleic acid sequence encoding the gene product listed under (1)(i)-(1(ix), or any combination thereof, operably linked to a promoter driving expression of the nucleic acid sequence in a target cell, e.g., in a modified lymphocyte, for example, a modified NK cell provided herein. In some embodiments, the promoter is specifically expressed in the target cell, e.g., the promoter is a lymphocyte- or NK-cell-specific promoter. In some embodiments, the promoter is a CD56 (NCAM) promoter. In some embodiments, the promoter is a CD49 promoter. In some embodiments, the promoter is a CD45 promoter. In some embodiments, the promoter is an FcγRIII promoter. In some embodiments, the promoter is an NKG2D promoter. In some embodiments, the promoter is a CD69 promoter.

[16] In some embodiments, the exogenous nucleic acid construct encoding a gene product listed under (1) is knocked into a genomic locus encoding a gene product listed under (2), resulting in a loss-of-function of the gene product listed under (2) and expression of a gene product encoded by the exogenous nucleic acid construct, either driven by a heterologous promoter, or driven by the endogenous promoter of the genomic locus that the exogenous nucleic acid construct is knocked into.

[17] In some embodiments, the exogenous nucleic acid construct encoding a gene product listed under (1) is knocked into a "safe harbor" locus, e.g., a ROSA26 locus, a collagen locus, or an AAVSI genomic locus.

[18] In some embodiments, the two or more HLA class II histocompatibility antigen alpha chain genes are selected from HLA-DQA1, HLA-DRA, HLA-DPA1, HLA-DMA, HLA-DQA2, and HLA-DOA. In some embodiments, the two or more HLA class II histocompatibility antigen beta chain genes are selected from HLA-DMB, HLA-DOB, HLA-DPB1, HLA-DQB1, HLA-DQB3, HLA-DQB2, HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5.

[19] In some embodiments, the modified lymphocyte comprises a rearranged endogenous T-cell receptor (TCR) locus. In some embodiments, the rearranged TCR comprises TCRα VJ and/or TCRβ V(D)J section rearrangements and complete V-domain exons.

[20] In some embodiments, the natural cytotoxicity receptor is NKp30, NKp44, NKp46, and/or CD158b.

[21] 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. Additional suitable variants will be apparent to those of ordinary skill in the art based on the present disclosure and the knowledge in the art. The disclosure is not limited in this respect.

[22] In some embodiments, the TGFβR2 is a dominant-negative variant of TGFβ receptor II (DN-TGFβR2).

[23] In some embodiments, the CAR is capable of binding mesothelin, EGFR, HER2, MICA/B, BCMA, CD19, CD22, CD20, CD33, CD123, androgen receptor, PSMA, PSCA, Muc1, HPV viral peptides (ie. E7), EBV viral peptides, CD70, WT1, CEA, EGFRvIII, IL13Rα2, GD2, CA125, CD7, EpCAM, Muc16, and/or CD30,

[24] In some embodiments, the modified lymphocyte is derived from a pluripotent or multipotent stem cell. In some embodiments, the multipotent stem cell is a hematopoietic stem cell (HSC). In some embodiments, the pluripotent stem cell is an induced pluripotent stem cell (iPSC). In some embodiments, the pluripotent stem cell is an embryonic stem cell (ESC).

[25] In some embodiments, the modified lymphocyte is derived from a pluripotent or multipotent stem cell that comprises at least one or more exogenous nucleic acid constructs encoding any of (1)(i)-(1)(ix), or any combination thereof; and/or at least one genomic alteration that effects the loss-of-function of any of (2)(i)-(2)(xi), or any combination thereof, in the lymphocyte.

[26] In some embodiments, the modified lymphocyte is derived from a pluripotent or multipotent stem cell that comprises at least one genomic alteration that effects the loss-of-function of any of (2)(i)-(2)(xi), or any combination thereof, in the lymphocyte.

[27] In some embodiments, the at least one genomic alteration that effects the loss-of-function of one or more (2)(i)-(2)(xi) in the lymphocyte comprises an insertion of an exogenous nucleic acid construct.

[28] In some embodiments, the exogenous nucleic acid construct encodes any of (1)(i)-(1)(ix), or any combination thereof.

[29] In some embodiments, the modified lymphocyte exhibits a loss-of-function in two or more of the genes/proteins listed under (2).

[30] In some embodiments, the modified lymphocyte comprises an indel or an insertion of an exogenous nucleotide construct into a genomic locus harboring a gene or encoding a protein under (2).

[31] In some embodiments, the modified lymphocyte comprises an indel or an insertion of an exogenous nucleotide construct into two or more genomic loci harboring a gene or encoding a protein under (2).

[32] In some embodiments, the modified lymphocyte was obtained by editing a genomic locus with an RNA-guided nuclease. In some embodiments, the RNA-guided nuclease is a CRISPR/Cas nuclease. In some embodiments, the RNA-guided nuclease is selected from the group consisting of SpCas9, SaCas9, (KKH) SaCas9, AsCpf1 (AsCas12a), LbCpf1, (LbCas12a), CasX, CasY, Cas12h1, Cas12i1, Cas12c1, Cas12c2, eSpCas9, Cas9-HF1, HypaCas9, dCas9-Fokl, Sniper-Cas9, xCas9, AaCas12b, evoCas9, SpCas9-NG, VRQR, VRER, NmeCas9, CjCas9, BhCas12b, and BhCas12b V4.

[33] In some embodiments, the modified lymphocyte is obtained by editing two or more genomic loci harboring genes encoding any of the proteins under (2). In some embodiments, at least two of the two or more genomic loci harboring genes encoding any of the proteins under (2) have been edited by a different RNA-guided nuclease. In some embodiments, at least one of the two or more genomic loci harboring genes encoding any of the proteins under (2) has been edited by Cas9, and wherein at least one of the loci has been edited by Cpf1.

[34] In some embodiments, the modified lymphocyte expresses endogenous CD56, CD49, and CD45.

[35] In some embodiments, the modified lymphocyte is a natural killer (NK) cell.

[36] In another aspect the disclosure features a modified cell, wherein the modified cell (1) comprises at least one exogenous nucleic acid construct encoding: (i) a chimeric antigen receptor (CAR); (ii) a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 ((CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) IL-12 receptor (IL-12R), or a variant thereof; (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) exhibits a loss of function of

at least one of: (i) transforming growth factor beta receptor 2 (TGFβR2); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microgobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) cytokine inducible SH2 containing protein (CISH); (vii) class II, major histocompatibility complex, transactivator (CIITA); (viii) natural killer cell receptor NKG2A (natural killer group 2A); (ix) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (x) cluster of differentiation 32B (CD32B, FCGR2B); (xi) T cell receptor alpha constant (TRAC); or any combination of two or more thereof. In one embodiment, the modified cell exhibits a loss of function of TGFβR2 and a loss-of-function of CISH. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of TIGIT. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of ADORA2A. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of CISH and a loss-of-function of TIGIT. In one embodiment, the modified cell exhibits a loss-of-function of CISH and a loss-of-function of ADORA2A. In one embodiment, the modified cell exhibits a loss-of-function of CISH and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of TIGIT and a loss-of-function of ADORA2A. In one embodiment, the modified cell exhibits a loss-of-function of TIGIT and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of ADORA2A and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of TIGIT. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of ADORA2A. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified cell exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function

of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of CISH, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified cell exhibits a loss-of-function of TIGIT, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A.

[37] In one embodiment, the modified cell (1) comprises at least one exogenous nucleic acid construct encoding: (i) a chimeric antigen receptor (CAR); (ii) a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, cluster of differentiation 16 ((CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) IL-12 receptor (IL-12R), or a variant thereof; (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) exhibits a loss of function of transforming growth factor beta receptor 2 (TGFβR2), cytokine inducible SH2 containing protein (CISH), or a combination thereof.

[38] In some embodiments of modified cells comprising an exogenous nucleic acid construct, e.g., modified lymphocytes provided herein, the exogenous nucleic acid construct is an expression construct comprising a nucleic acid sequence encoding the gene product listed under (1)(i)-(1(x), or any combination thereof, operably linked to a promoter driving expression of the nucleic acid sequence in a target cell, e.g., in a modified lymphocyte, for example, a modified NK cell provided herein. In some embodiments, the promoter is specifically expressed in the target cell, e.g., the promoter is a lymphocyte- or NK-cell-specific promoter. In some embodiments, the promoter is a CD56 (NCAM) promoter. In some embodiments, the promoter is a CD49 promoter. In some embodiments, the promoter is a CD45 promoter. In some embodiments, the promoter is an FcγRIII promoter. In some embodiments, the promoter is an NKG2D promoter. In some embodiments, the promoter is a CD69 promoter.

[39] In some embodiments of modified cells, e.g., modified lymphocytes provided herein, the exogenous nucleic acid construct encoding a gene product listed under (1) is knocked into a genomic locus encoding a gene product listed under (2), resulting in a loss-of-function of the gene product listed under (2) and expression of a gene product encoded by the exogenous nucleic acid construct, either driven by a heterologous promoter, or driven by the endogenous promoter of the genomic locus that the exogenous nucleic acid construct is knocked into.

[40] In some embodiments of modified cells, e.g., modified lymphocytes provided herein, comprising a loss of function in two or more HLA class II histocompatibility antigen alpha

chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes, the two or more HLA class II histocompatibility antigen alpha chain genes are selected from HLA-DQA1, HLA-DRA, HLA-DPA1, HLA-DMA, HLA-DQA2, and HLA-DOA. In some embodiments, the two or more HLA class II histocompatibility antigen beta chain genes are selected from HLA-DMB, HLA-DOB, HLA-DPB1, HLA-DQB1, HLA-DQB3, HLA-DQB2, HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5.

[41] In some embodiments, the modified cell is an immune cell. In some embodiments, the immune cell is a lymphocyte. In some embodiments, the lymphocyte is an NK cell. In some embodiments, the lymphocyte is an iNK cell.

[42] In some embodiments, the modified cell is a multipotent or pluripotent stem cell, e.g., an iPS cell, or a hematopoietic stem cell, or a differentiated cell derived from such a multipotent or pluripotent stem cell, e.g., an iNK cell.

[43] In some embodiments, the modified cell does not express an endogenous T-cell co-receptor.

[44] In some embodiments, the lymphocyte is a T cell.

[45] In some embodiments, the modified cell comprises a rearranged endogenous TCR locus, wherein the rearranged TCR comprises TCRα VJ and/or TCRβ V(D)J section rearrangements and complete V-domain exons.

[46] In some embodiments, the modified cell expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, and/or CD45; (ii) NK cell receptor (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.

[47] In some embodiments, the natural cytotoxicity receptor is NKp30, NKp44, NKp46, and/or CD158b.

[48] In some embodiments, the modified cell expresses at least one NK cell biomarker. In some embodiments, the NK cell biomarker is CD56, CD49, and/or CD45.

[49] In one aspect, disclosed herein is a population of cells comprising the modified lymphocyte described herein, or the modified cell described herein.

[50] In one aspect, disclosed herein is a pharmaceutical composition comprising the population of cells disclosed herein.

[51] In another aspect, the disclosure provides an isolated population of lymphocytes, wherein the population comprises at least $1x10^3$, at least $1x10^4$, at least $1x10^5$, at least $2x10^5$, at least $3x10^5$, at least $4x10^5$, at least $5x10^5$,at least $1x10^6$, at least $2x10^6$, at least $3x10^6$, at

least $4x10^6$, at least $5x10^6$,at least $1x10^7$, at least $1x10^7$, at least $2x10^7$, at least $3x10^7$, at least $4x10^7$, at least $5x10^7$,at least $1x10^8$, at least $2x10^8$, at least $3x10^8$, at least $4x10^8$, at least $5x10^8$,at least $1x10^9$, at least $1x10^9$, at least $2x10^9$, at least $3x10^9$, at least $4x10^9$, at least $5x10^9$,at least $1x10^{10}$, at least $2x10^{10}$, at least $3x10^{10}$, at least $4x10^{10}$, at least $5x10^{10}$, at least $1x10^{11}$, or at least $1x10^{12}$ cells, and wherein at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, at least 99.9%, at least 99.99%, at least 99.999%, or virtually 100% of the lymphocytes in the population: (a) comprise a rearranged T-cell receptor (TCR) locus; (b) do not express endogenous CD3; (c) express endogenous CD56 (NCAM), CD49, and/or CD45; and (d) expresses at least endogenous gene encoding: (i) NK cell receptor (cluster of differentiation 16 (CD16)); (ii) natural killer group-2 member D (NKG2D); (iii) CD69; (iv) a natural cytotoxicity receptor; or any combination of two or more thereof; and wherein the modified lymphocyte further: (1) comprises at least one exogenous nucleic acid construct encoding: (i) chimeric antigen receptor (CAR); (ii) non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) IL-12 receptor (IL-12R), or a variant thereof; (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) exhibits a loss of function of at least one of: (i) transforming growth factor beta receptor 2 (TGFβR2); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microgobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) cytokine inducible SH2 containing protein (CISH); (vii) class II, major histocompatibility complex, transactivator (CIITA); (viii) natural killer cell receptor NKG2A (natural killer group 2A); (ix) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (x) cluster of differentiation 32B (CD32B, FCGR2B); (xi) T cell receptor alpha constant (TRAC); or any combination of two or more thereof. In one embodiment, the modified lymphocyte exhibits a loss of function of TGFβR2 and a loss-of-function of CISH. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of TIGIT. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-

function of CISH and a loss-of-function of TIGIT. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TIGIT and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TIGIT and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of ADORA2A and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of TIGIT. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TGFbetaR2, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of CISH, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified lymphocyte exhibits a loss-of-function of TIGIT, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A.

[52] In one embodiment, the isolated population of myphocytes comprises at least $1x10^3$, at least $1x10^4$, at least $1x10^5$, at least $2x10^5$, at least $3x10^5$, at least $4x10^5$, at least $5x10^5$,at least $1x10^6$, at least $2x10^6$, at least $3x10^6$, at least $4x10^6$, at least $5x10^6$,at least $1x10^7$, at least $1x10^7$, at least $2x10^7$, at least $3x10^7$, at least $4x10^7$, at least $5x10^7$,at least $1x10^8$, at least $2x10^8$, at least $3x10^8$, at least $4x10^8$, at least $5x10^8$,at least $1x10^9$, at least $1x10^9$, at least $2x10^9$, at least $3x10^9$, at least $4x10^9$, at least $5x10^9$,at least $1x10^{10}$, at least $2x10^{10}$, at least $3x10^{10}$, at least $4x10^{10}$, at least $5x10^{10}$, at least $1x10^{11}$, or at least $1x10^{12}$ cells, and wherein at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, at least 99.9%, at least 99.99%, at least 99.999%, or virtually 100% of the lymphocytes in the

population: (a) comprise a rearranged T-cell receptor (TCR) locus; (b) do not express endogenous CD3; (c) express endogenous CD56 (NCAM), CD49, and/or CD45; and (d) expresses at least endogenous gene encoding: (i) NK cell receptor (cluster of differentiation 16 (CD16)); (ii) natural killer group-2 member D (NKG2D); (iii) CD69; (iv) a natural cytotoxicity receptor; or any combination of two or more thereof; and wherein the modified lymphocyte further: (1) comprises at least one exogenous nucleic acid construct encoding: (i) chimeric antigen receptor (CAR); (ii) non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) IL-12 receptor (IL-12R), or a variant thereof; (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) exhibits a loss of function of transforming growth factor beta receptor 2 (TGFβR2), cytokine inducible SH2 containing protein (CISH), or a combination thereof.

[53] In some embodiments, the rearranged TCR locus comprises of TCRα VJ and/or TCRβ V(D)J section rearrangements and complete V-domain exons. In some embodiments, the rearranged endogenous TCR locus consists of no more than two rearranged alleles.

[54] In some embodiments, the natural cytotoxicity receptor is NKp30, NKp44, NKp46, and/or CD158b.

[55] In some embodiments, the *in vitro* population of lymphocytes does not comprise more than 1%, more than 0.1%, more than 0.001%, more than 0.0001%, more than 0.00001%, more than 0.000001%, more than 0.0000001%, more than 0.00000001%, more than 0.000000001%, more than 0.0000000001%, or more than more than 0.00000000001% of cells expressing a reprogramming factor from an exogenous nucleic acid construct.

[56] In some embodiments, the *in vitro* population of lymphocytes does not comprise a cell expressing a reprogramming factor from an exogenous nucleic acid construct. In some embodiments, the reprogramming factor is Oct-4 and/or Sox-2.

[57] In some embodiments, the *in vitro* population of lymphocytes does not comprise cells harboring episomal expression constructs encoding a reprogramming factor.

[58] In some embodiments, each cell in *in vitro* population of lymphocytes comprises the same combination of an exogenous nucleic acid construct listed under (1) and a loss of function listed (2).

[59] In some embodiments, the *in vitro* population of lymphocytes comprises less than 0.001%, less than 0.002%, less than 0.003%, less than 0.004%, less than 0.005%, less than 0.006%, less than 0.007%, less than 0.008%, less than 0.009%, less than 0.01%, less than 0.02%, less than 0.03%, less than 0.04%, less than 0.05%, less than 0.06%, less than 0.07%, less than 0.08%, less than 0.09%, less than 0.1%, less than 0.2%, less than 0.3%, less than 0.4%, less than 0.5%, less than 0.6%, less than 0.7%, less than 0.8%, less than 0.9%, less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 6%, less than 7%, less than 8%, less than 9%, or less than 10% cell that harbor a chromosomal translocation.

[60] In another aspect, the disclosure provides a method of treating a subject, the method comprising administering any modified lymphocyte, any modified cell, any pharmaceutical composition, or the isolated *in vitro* population of cells as described in the disclosure, to a subject in need thereof. In some embodiments, the subject has, or is diagnosed with, a proliferative disease. In some embodiments, the proliferative disease is cancer. In some embodiments, the cancer is breast cancer, colorectal cancer, gastric cancer, renal cell carcinoma (RCC), or non-small cell lung cancer (NSCLC), solid tumors, 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, soft tissue sarcomas, and hematological cancers like ALL, CLL, NHL, DLBCL, AML, CML, multiple myeloma (MM).

[61] In some embodiments, the method of generating the modified lymphocyte, the modified cell, the population of cells, or the isolated *in vitro* population of lymphocytes of the disclosure comprises: (a) obtaining an induced pluripotent stem cell (iPSC); (b) modifying the iPSC, or an undifferentiated or differentiated daughter cell thereof, to comprise express at least one exogenous gene of (1) and/or to comprise a loss of function in at least one gene of (2); (c) directing differentiation of the iPSC to hematopoietic lineage cells, wherein the hematopoietic lineage cells retain the edited genetic loci comprised in the iPSCs.

[62] In some embodiments, directing differentiation comprises: (i) contacting iPSCs with a composition comprising a BMP pathway activator, and optionally bFGF, to obtain mesodermal cells; and (ii) contacting the mesodermal cells with a composition comprising a BMP pathway activator, bFGF, and a WNT pathway activator, to obtain mesodermal cells having definitive hemogenic endothelium (HE) potential, wherein the mesodermal cells having definitive hemogenic endothelium (HE) potential are capable of providing hematopoietic lineage cells; wherein mesodermal cells and mesodermal cells having

definitive HE potential are obtained in steps (i) and (ii) without the step of forming embryoid bodies; wherein the hematopoietic lineage cells comprise definitive hemogenic endothelium cells, hematopoietic stem and progenitor cells (HSC), hematopoietic multipotent progenitor cell (MPP), pre-T cell progenitor cells, pre-NK cell progenitor cells, T cell progenitor cells, NK cell progenitor cells, T cells, NK cells, NKT cells, or B cells.

[63] In some embodiments, the method of directing differentiation of iPSCs to hematopoietic lineage cells further comprises: contacting the mesodermal cells having definitive HE potential with a composition comprising bFGF and a ROCK inhibitor to obtain definitive HE cells.

[64] In some embodiments, the method of directing differentiation further comprises: contacting the definitive HE cells with a composition comprising a BMP activator, and optionally a ROCK inhibitor, and one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GMCSF, EPO, bFGF, VEGF, SCF, IL6, Flt3L and IL11 to obtain hematopoietic multipotent progenitor cells (MPP).

[65] In some embodiments, the method of directing differentiation further comprises: contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7; and optionally one or more of a BMP activator, a ROCK inhibitor, TPO, VEGF and bFGF to obtain pre-T cell progenitors, T cell progenitors, and/or T cells.

[66] In some embodiments, the method of directing differentiation further comprises: contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, TPO, IL7 and IL15, and optionally one or more of a BMP activator, a ROCK inhibitor, VEGF and bFGF to obtain pre-NK cell progenitors, NK cell progenitors, and/or NK cells.

[67] In some embodiments, the method of generating the modified lymphocyte, the modified cell, the population of cells, or the isolated *in vitro* population of lymphocytes of the disclosure further comprises: prior to step c), contacting the pluripotent stem cells with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, to seed and expand the cells.

[68] In some embodiments, the method of generating the modified lymphocyte, the modified cell, the population of cells, or the isolated *in vitro* population of lymphocytes of the disclosure further comprises: detecting a rearranged T-cell receptor (TCR) locus in the hematopoietic lineage cells. In some embodiments, the method further comprises selecting the hematopoietic lineage cells comprising the rearranged TCR locus based on the TCR

encoded by the rearranged TCR locus binding an antigen of interest. In some embodiments, the antigen of interest is a tumor antigen.

[69] In another aspect, the disclosure provides a method, the method comprising: reprogramming a donor cell to a pluripotent state; editing a target locus in the donor cell genome; and differentiating the reprogrammed donor cell into a lymphocyte. In some embodiments, the editing is performed before or during the step of reprogramming of the donor cell to a pluripotent state. In some embodiments, the donor cell is a fibroblast, a peripheral blood cell, a lymphocyte, or a T cell.

[70] In another aspect, the disclosure provides a method, the method comprising: differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises: (1) an exogenous nucleic acid comprising: (i) a nucleic acid encoding a chimeric antigen receptor (CAR); (ii) a nucleic acid encoding a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16); (iii) a nucleic acid encoding interleukin 15 (IL-15); (iv) a nucleic acid encoding IL-15R, or a variant thereof;(v) a nucleic acid encoding interleukin 12 (IL-12); (vi) a nucleic acid encoding IL-12R, or a variant thereof; (vii) a nucleic acid encoding 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 (2) an indel, or an insertion of an exogenous nucleic acid in one or more of the following genetic loci:(i) transforming growth factor beta receptor 2 (TGFβR2); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β-2 microgobulin (B2M); (v) programmed cell death protein 1 (PD-1, CD279); (vi) cytokine inducible SH2 containing protein (CISH); (vii) class II, major histocompatibility complex, transactivator (CIITA); (viii) natural killer cell receptor NKG2A (natural killer group 2A); (ix) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (x) cluster of differentiation 32B (CD32B, FCGR2B); (xi) T cell receptor alpha constant (TRAC); or any combination of two or more thereof, wherein the indel or insertion results in a loss-of-function of a gene product encoded by the respective genetic locus or loci. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2 and CISH, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2 and/or CISH.

In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2 and TIGIT, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2 and/or TIGIT. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2 and ADORA2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2 and/or ADORA2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2 and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2 and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in CISH and TIGIT, wherein the indel or insertion results in a loss-of-function of a gene product encoded by CISH and/or TIGIT. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in CISH and ADORA2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by CISH and/or ADORA2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in CISH and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by CISH and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TIGIT and ADORA2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TIGIT and/or ADORA2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TIGIT and NKG2A, wherein the indel or insertion results in a loss-of-function

of a gene product encoded by TIGIT and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in ADORA2A and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by ADORA2A and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2, CISH and TIGIT, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2, CISH and/or TIGIT. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2, CISH and ADORA2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2, CISH and/or ADORA2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2, CISH and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2, CISH and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2, TIGIT and ADORA2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2, TIGIT and/or ADORA2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2, TIGIT and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2, TIGIT and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TGFβR2, ADORA2A and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TGFβR2, ADORA2A and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the

genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in CISH, TIGIT and ADORA2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by CISH, TIGIT and/or ADORA2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in CISH, TIGIT and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by CISH, TIGIT and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in CISH, ADORA2A and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by CISH, ADORA2A and/or NKG2A. In one embodiment, the method comprises differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises an indel, or an insertion of an exogenous nucleic acid in TIGIT, ADORA2A and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by TIGIT, ADORA2A and/or NKG2A.

[71] In some embodiments, the exogenous nucleic acid of (2) is the exogenous nucleic acid of (1). In some embodiments, the pluripotent stem cell is an iPS cell. In some embodiments, the differentiating comprises contacting the pluripotent stem cell with a differentiation medium or a sequence of differentiation media.

BRIEF DESCRIPTION OF THE DRAWINGS

[72] **Fig. 1A** and **1B** depict that robust single and double-gene editing of TGFBR2 and CISH was achieved in NK cells. Both single and simultaneous targeting of TGFBR2 and CISH in NK cells using CRISPR-Cpf1 produced in/dels at both targets in greater than 80% of NK cells, with greater than 90% of edited NK cells viable at 72 hours post-editing.

[73] **Fig. 2A** and **2B** depict that normalization of spheroid curves maintains the same efficacy patterns as observed in the non-normalized data, as analyzed across 3 unique donors and 5 independent experiments. Each single knockout (SKO) NK group was significantly more efficient at reducing the SK-OV-3 spheroid size than the control NK, and the double knockout (DKO) NK group was significantly more efficient at reducing the SK-OV-3 spheroid size than the SKO NK groups. **Fig. 2A** depicts SK-OV-3 spheriod analysis at 10:1 E:T with 10ng/mL TGFbeta (3 donors, 5 independent experiments). **Fig. 2B** has error bars

that are SEM. Statistical significance is a result of 2-way ANOVA analysis. 2-way ANOVA analysis excludes time points greater than 104 hours due to missing time points in some experiments. Mixed model analysis yields same or improved statistical significance between groups when all time points are considered.

[74] **Fig. 3A** and **3B** depict that the CISH/TGFBR2 double knockout NK cells demonstrate superior effector function over the single knockout NK cells or the control NK cells in the SK-OV-3 spheroid assay, even at lower NK effector cell to target cell (E:T) ratios. **Fig. 3A** depicts the SK-OV-3 spheroid analysis at 20:1 E:T, with 10 ng/mL TGF-β, as analyzed across 3 unique donors and 5 independent experiments. **Fig. 3B** depicts the SK-OV-3 spheroid analysis at 10:1 E:T, with 10 ng/mL TGF-β, as analyzed across 4 unique donors and 7 independent experiments. These marginal differences between the different E:T ratios at all conditions suggests that the effector cell phenotype is driven by the knockout rather than NK cell to target ratio.

[75] **Fig. 4A** and **4B** depict that the CISH/TGFBR2 double knockout NK cells demonstrate superior effector function over the single knockout NK cells or the control NK cells in the PC-3 spheroid assay, even at lower NK effector cell to target cell (E:T) ratios. **Fig. 4A** depicts the PC-3 spheroid analysis at 20:1 E:T, with 10 ng/mL TGF-β, as analyzed across 3 unique donors and 5 independent experiments. **Fig. 4B** depicts the PC-3 spheroid analysis at 10:1 E:T, with 10 ng/mL TGF-β, as analyzed across 4 unique donors and 7 independent experiments. These marginal differences between the different E:T ratios at all conditions suggests that the effector cell phenotype is driven by the knockout rather than NK cell to target ratio.

[76] **Fig. 5A** and **5B** depict that CISH/TGFBR2 double knockout NK cells demonstrate superior effector function over the single knockout NK cells or the control NK cells in the SK-OV-3 and PC-3 spheroid assays, in the absence of any exogenous cytokine. **Fig. 5A** depicts the SK-OV-3 spheroid analysis at 10:1 E:T, in the absence of any exogenous cytokine, as analyzed across 4 unique donors and 7 independent experiments. **Fig. 5B** depicts the PC-3 spheroid analysis at 10:1 E:T, in the absence of any exogenous cytokine, as analyzed across 4 unique donors and 7 independent experiments.

[77] **Fig. 6A** depicts that IFN-γ concentrations correlate with NK cell efficacy in the spheroid assay. The SK-OV-3 spheroid analysis was performed across different E:Ts with 10 ng/mL TGF-β and 5 ng/mL IL-15. The analysis for 5:1 and 10:1 E:T was performed across 4 unique donors and 7 independent experiments. The analysis for 20:1 E:T was performed across 3 unique donors, and 5 independent experiments.

[78] **Fig. 6B** depicts that TNF-α concentrations correlate with NK cell efficacy in the spheroid assay. The SK-OV-3 spheroid analysis was performed across different E:Ts with 10 ng/mL TGF-β and 5 ng/mL IL-15. The analysis for 5:1 and 10:1 E:T was performed across 4 unique donors and 7 independent experiments. The analysis for 20:1 E:T was performed across 3 unique donors, and 5 independent experiments.

[79] **Fig. 6C** depicts marker expression in CISH/ TGFBR2 double knockout (DKO) NK cells. Control (non-edited) and double knockout NK cells were harvested for staining at 72 hours post editing. Expression of the NK activation markers CD25 and CD69 were quantified. Double KO NK cells expressed significantly higher levels of activation markers CD25 and CD69 as compared to control NK cells.

[80] **Fig 6D** depicts anti-tumor activity of NK cells was measured in an in vivo model. NSG mice received an intraperitoneal injection of 500,000 SKOV3 tumor cells labeled with luciferase. Seven days post-tumor implantation, 10 million edited (CISH/TGFBR2 double-knockout) or unedited (control) NK cells were injected into the peritoneal cavity of the tumor-bearing mice. Tumor burden was monitored weekly by IP administration of luciferin and IVIS imaging. Two-way ANOVA analysis was performed at day 34 to determine statistical significance between control and DKO NK cell groups (****, $P<0.0001$)

[81] **Fig. 7A** depicts the robust single gene editing of TIGIT achieved in NK cells, across 2 independent experiments and 3 unique donors.

[82] **Fig. 7B** depicts the robust single gene editing of NKG2A achieved in NK cells, across 2 independent experiments and 3 unique donors.

[83] **Fig. 7C** depicts the robust single gene editing of ADORA2A achieved in NK cells, across 3 independent experiments and 3 unique donors.

[84] **Fig. 8A** and **8B** depict that TIGIT single knockout NK cells demonstrate superior effector function over the unedited control NK cells in the *in vitro* spheroid assay, at different effector cell to target cell (E:T) ratios. **Fig. 8A** depicts the tumor spheroid analysis at 20:1 E:T, as analyzed across 2 unique donors and 2 independent experiments. Red object intensity was measured every two hours for 6 days on an Incucyte imaging system. **Fig. 8B** depicts the tumor spheroid analysis at 1.25:1, 2.5:1. 5:1. 10:1 and 20:1 effector to target ratios, as analyzed across 2 unique donors and 2 independent experiments. Red object intensity is shown at 100 hours following NK cell addition.

[85] **Fig. 9A** and **9B** depict that NKG2A single knockout NK cells demonstrate superior effector function over the unedited control NK cells in the *in vitro* spheroid assay, at different effector cell to target cell (E:T) ratios. **Fig. 9A** depicts the tumor spheroid analysis at 20:1

E:T, as analyzed across 2 unique donors and 2 independent experiments. Red object intensity was measured every two hours for 6 days on an Incucyte imaging system. **Fig. 9B** depicts the tumor spheroid analysis at 1.25:1, 2.5:1. 5:1. 10:1 and 20:1 E:T, as analyzed across 2 unique donors and 2 independent experiments. Red object intensity is shown at 100 hours following NK cell addition.

[86] **Fig. 10A** and **10B** depict that ADORA2A single knockout NK cells demonstrate superior effector function over the unedited control NK cells in the *in vitro* spheroid assay, at different effector cell to target cell (E:T) ratios. **Fig. 10A** depicts the tumor spheroid analysis at 20:1 E:T, as analyzed across 2 unique donors and 2 independent experiments. Red object intensity was measured every two hours for 6 days on an Incucyte imaging system. **Fig. 10B** depicts the tumor spheroid analysis at 1.25:1, 2.5:1. 5:1. 10:1 and 20:1 E:T, as analyzed across 2 unique donors and 2 independent experiments. Red object intensity is shown at 100 hours following NK cell addition.

[87] **Fig. 11** depicts triple gene editing of TGFbR2/CISH/TIGIT achieved in NK cells.

[88] **Fig. 12A** and **12B** depict that TGFbR2/CISH/TIGIT triple knockout NK cells demonstrate superior effector function over the unedited control NK cells in the *in vitro* spheroid assay, at different effector cell to target cell (E:T) ratios. **Fig. 12A** depicts the tumor spheroid analysis at 20:1 E:T. Red object intensity was measured every two hours for 6 days on an Incucyte imaging system. **Fig. 12B** depicts the tumor spheroid analysis at 5:1. 10:1 and 20:1 E:T. Red object intensity is shown at 100 hours following NK cell addition.

DETAILED DESCRIPTION

[89] Some aspects of the present disclosure provide strategies, compositions, and methods useful for engineering "off the shelf" allogeneic cells that can be used in clinical applications. Some aspects of the present disclosure provide strategies, compositions, and methods useful for engineering pluripotent or multipotent stem cells (e.g., induced pluripotent stem cells (iPSCs) or hematopoietic stem cells (HSCs) that can be used to derive differentiated daughter cells, e.g., modified lymphocytes, such as iNK cells. Immunoreactivity, both graft-versus-host and host-versus-graft, is a major challenge for clinical applications of allogeneic cells. Some aspects of the present disclosure provide strategies, compositions, and methods for engineering cells that address various aspect of immunoreactivity typically encountered by non-modified cell grafts in allogeneic settings.

[90] Some aspects of this disclosure provide strategies, compositions, and methods useful for overcoming "nonself" host-versus-graft immunoreactivity, e.g., by removing MHC Class

I and II functionality in target cells for allogeneic clinical applications. For example, in some embodiments, MHC Class I and II functionality is achieved by effecting a loss-of-function of B2M (Class I) and of CIITA (Class II) and/or two or more MHC Class II alpha and/or beta chains, as described in more detail elsewhere herein.

[91] Some aspects of the present disclosure provide strategies, compositions, and methods useful for overcoming "missing self" host-versus-graft immunoreactivity, e.g., by introducing an exogenous expression construct comprising a nucleic acid sequence encoding an NK inhibitory modality into target cells for allogeneic clinical applications. For example, in some embodiments, such "missing self" immunoreactivity is addressed by effecting transgenic expression of HLA-G, HLA-E, and/or CD47 in target cells for allogeneic clinical applications.

[92] Some aspects of the present disclosure provide strategies, compositions, and methods useful for overcoming graft-versus-host T-cell receptor (TCR) alloreactivity by removing endogenous TCR functionality. For example, in some embodiments, strategies, compositions, and methods useful for the generation of modified cells for allogeneic clinical applications from multipotent or pluripotent stem cells are provided herein that include engineering the stem cells to comprise the immunomodulatory modifications described herein, and then differentiating the stem cells into a cell type for administration to a patient in need thereof, e.g., into lymphocytes, such as, e.g., iNK cells, for immunotherapy. In some embodiments, the pluripotent or multipotent stem cells are derived from a cell expressing a TCR or comprising a rearranged TCR locus, e.g., from a T-cell, and in some such embodiments, a differentiated lymphocyte derived from such engineered stem cells may express the TCR and be the target of TCR alloreactivity. . In some such embodiments, it is advantageous to effect a loss-of-function of the endogenous TCR expression products, and the present disclosure provides strategies, compositions, and methods useful for achieving such a loss-of-function in the respective cells, e.g., by effecting a loss-of-function of TRAC as described in more detail elsewhere herein.

[93] Some aspects of the present disclosure relate to the generation of modified NK cells (or other lymphocytes) that are useful as therapeutic agents, *e.g.*, in the context of immunooncology. For example, at least some of the modified NK cells provided herein exhibit enhanced NK cell response characteristics as compared to non-modified NK cells, *e.g.*, enhanced target recognition, enhanced NK cell response level and/or duration, improved NK cell survival, delayed NK cell exhaustion, enhanced target recognition, and/or recognition of a target not typically recognized by non-modified NK cells.

[94] Some aspects of the present disclosure provide compositions, methods, and strategies for the generation of modified NK cells. In some embodiments, such modified NK cells are generated by editing the genome of mature NK cells. In some embodiments, modified NK cells are generated by editing the genome of a cell from which an NK cell is derived, either in vitro or in vivo. In some embodiments, the cell from which and NK cell is derived is a stem cell, for example, a hematopoietic stem cell (HSC), or a pluripotent stem cells, such as, *e.g.*, an embryonic stem cell (ES cell) or an induced pluripotent stem cell (iPS cell). For example, in some embodiments, modified NK cells are generated by editing the genome of an ES cell, an iPS cell, or a hematopoietic stem cell, and subsequently differentiating the edited stem cell into an NK cell. In some embodiments, where the generation of modified NK cells involves differentiation of the modified NK cell from an iPS cell, the editing of the genome may take place at any suitable time during the generation, maintenance, or differentiation of the iPS cell. For example, where a donor cell is reprogrammed into an iPS cell, the donor cell, *e.g.*, a somatic cell such as, for example, a fibroblast cell or a T lymphocyte, may be subjected to the gene editing approaches described herein before reprogramming to an iPS cell, during the reprogramming procedure, or after the donor cell has been reprogrammed to an iPS cell.

[95] NK cells derived from iPS cells are also referred to herein as iNK cells. In some embodiments, the present disclosure provides compositions, methods, and strategies for generating iNK cells that have been derived from developmentally mature cells, also referred to as somatic cells, such as, for example, fibroblasts or peripheral blood cells.

[96] In some embodiments, the present disclosure provides compositions, methods, and strategies for generating iNK cells that have been derived from developmentally mature T cells (T cells that have 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 induced pluripotent stem (iPS) cell, and throughout differentiation of the resulting iPS cell to a somatic cell.

[97] One advantage of using T cells for the generation of iPS cells is that T cells can be edited with relative ease, *e.g.*, by CRISPR-based methods or other gene-editing methods.

[98] Another advantage of using T cells for the generation of iPS cells is that the rearranged TCR locus allows for genetic tracking of individual cells and their daughter cells. 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.

[99] A third 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 pose a concern when editing cells by CRISPR technology, and in particular when generating cells carrying multiple edits.

[100] A fourth advantage of using T cell derived iPS cells as a starting point for the derivation of therapeutic lymphocytes is that it allows 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 iPS cells, and then deriving lymphocytes from these iPS cells that express the TCR (*e.g.*, T cells). This strategy would also allow for activating the TCR in other cell types, *e.g.*, by genetic or epigenetic strategies.

[101] A fifth advantage of using T cell derived iPS cells as a starting point for iNK differentiation is that the 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 will 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.

Definitions and Abbreviations

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

[103] 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.”

[104] The conjunctions “or” and “and/or” are used interchangeably as non-exclusive disjunctions.

[105] “Subject” means a human or non-human animal. A human subject can be any age (*e.g.,* an infant, child, young adult, or adult), and may suffer from a disease, or may be in need of alteration of a gene or a combination of specific genes. Alternatively, the subject may be an animal, which term includes, but is not limited to, a mammal, and, more particularly, a non-human primate, a rodent (*e.g.*, a mouse, rat, hamster, *etc.*), a rabbit, a guinea pig, a dog, a cat, and so on. In certain embodiments of this disclosure, the subject is

livestock, *e.g.,* a cow, a horse, a sheep, or a goat. In certain embodiments, the subject is poultry.

[106] The terms "treatment," "treat," and "treating," refer to a clinical intervention aimed to reverse, alleviate, delay the onset of, or inhibit the progress, and/or prevent or delay the recurrence of a disease or disorder, or one or more symptoms thereof, as described herein. 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, treatment may be administered to a susceptible individual prior to the onset of symptoms (*e.g.*, in light of genetic or other susceptibility factors). Treatment may also be continued after symptoms have resolved, for example to prevent or delay their recurrence.

[107] "Prevent," "preventing," and "prevention" 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.

[108] The terms "polynucleotide", "nucleotide sequence", "nucleic acid", "nucleic acid molecule", "nucleic acid sequence", and "oligonucleotide" refer to a series of nucleotide bases (also called "nucleotides") in DNA and RNA, and mean any chain of two or more nucleotides. The polynucleotides, nucleotide sequences, nucleic acids *etc.* can be chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. They can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, its hybridization parameters, *etc.* A nucleotide sequence typically carries genetic information, including, but not limited to, the information used by cellular machinery to make proteins and enzymes. These terms include double- or single-stranded genomic DNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and antisense polynucleotides. These terms also include nucleic acids containing modified bases.

[109] 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

[110] The terms "protein," "peptide" and "polypeptide" 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. 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.

[111] The term "variant" 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.

[112] 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. In contrast, 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.

[113] The terms "RNA-guided nuclease" and "RNA-guided nuclease molecule" are used interexchangably 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.

[114] 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 embodiment, 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. A nuclease variant 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. Suitable nucleases and nuclease variants may also include purification tags (e.g., polyhistidine tags) and 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.

[115] In some embodiments, the RNA-guided nuclease is an *Acidaminococcus sp.* Cpf1 variant (AsCpf1 variant). 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).

[116] The term "hematopoietic stem cell," or "definitive hematopoietic stem cell" as used herein, refers to CD34+ stem cells capable of giving rise to both mature myeloid and lymphoid cell types including T cells, natural killer cells and B cells.

[117] As used herein, the terms "reprogramming" or "dedifferentiation" or "increasing cell potency" or "increasing developmental potency" refers to a method of increasing the potency of a cell or dedifferentiating the cell to a less differentiated state. For example, 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. In other words, a reprogrammed cell is one that is in a less differentiated state than the same cell in a non-reprogrammed state. In some embodiments, the term “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 iPS cell. Suitable methods for the generation of iPS cells from somatic or multipotent stem cells are well known to those of skill in the art.

[118] As used herein, the term "differentiation" 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. 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 iPS cell 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. 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. The term "committed", when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it 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 or revert to a less differentiated cell type.

[119] As used herein, the terms “differentiation marker,” "differentiation marker gene," or "differentiation gene," refers to genes or proteins whose expression are indicative of cell differentiation occurring within a cell, such as a pluripotent cell. 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.

[120] As used herein, the term "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" refers to the expression or levels of expression of a plurality of differentiation marker genes.

[121] As used herein in the context of cellular developmental potential, the term "potency" or “developmental potency” refers to the sum of all developmental options accessible to the cell (*i.e.*, the developmental potency). 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.

[122] As used herein, the term "pluripotent" refers to the ability of a cell to form all lineages of the body or soma (*i.e.,* the embryo proper). 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. Pluripotency is a continuum of developmental potencies ranging from the 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).

[123] As used herein, the term "induced pluripotent stem cell" or, iPS cell refers to a stem cell obtained from a differentiated somatic, *e.g.*, adult, neonatal, or fetal cell by a process

referred to as reprogramming into cells capable of differentiating into tissues of all three germ or dermal layers: mesoderm, endoderm, and ectoderm. IPS cells are not found in nature.

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

[125] As used herein, the term "multipotent stem cell" 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. Thus, a multipotent cell can 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. "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, the term "multipotency" refers to a state of a cell with a degree of developmental potential that is less than totipotent and pluripotent.

[126] Pluripotency can be determined, in part, by assessing pluripotency characteristics of the cells. 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; (ıv) 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.

[127] As used herein, the term "pluripotent stem cell morphology" refers to the classical morphological features of an embryonic stem cell. Normal embryonic stem cell morphology is characterized by being round and small in shape, with a high nucleus-to-cytoplasm ratio, the notable presence of nucleoli, and typical intercell spacing.

Genome Editing Systems

[128] The present disclosure relates to the generation of modified NK cells, *e.g.*, NK cells the genome of which has been modified, or that are derived from a multipotent or pluripotent

stem cell, *e.g.*, an HSC, ES cell, or iPS cell, the genome of which has been modified. The NK cells and stem cells provided herein can be modified using any gene-editing technology known to those of ordinary skill in the art, including, for example, by using genome editing systems, *e.g.,* CRISPR.

[129] The term "genome editing system" refers to any system having RNA-guided DNA editing activity. 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.

[130] 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), incorporated by reference herein), 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.

[131] 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.

[132] 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), which is incorporated by reference herein, 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.

[133] As another example, WO 2016/073990 by Cotta-Ramusino, *et al.* ("Cotta-Ramusino"), incorporated by reference herein, 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", incorporated by reference herein) 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.

[134] 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).

[135] 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.

[136] In certain embodiments, genome editing systems modify a target sequence, or modify expression of a 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"), which is incorporated by reference. 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

[137] 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 to a target sequence such as a genomic or episomal sequence in a cell. gRNAs can 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), which is incorporated by reference), and in Cotta-Ramusino.

[138] 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"), all of which are incorporated by reference herein.)

[139] 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"), incorporated by reference herein), "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.

[140] In addition to the targeting domains, gRNAs typically (but not necessarily, *e.g.*, 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), both incorporated by reference herein). 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.

[141] 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.

[142] 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), incorporated by reference herein). 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).

[143] Those of skill in the art will appreciate 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.

[144] 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.

[145] In some embodiments, the guide RNA used comprises a modification as compared to the standard gRNA scaffold. Such modifications may comprise, for example, chemical modifications of a part of the gRNA, e.g., of a nucleobase or backbone moiety. In some embodiments, such a modification may also include the presence of a DNA nucleotide within the gRNA, e.g., within or outside of the targeting domain. In some embodiments, the modification may include an extension of the gRNA scaffold, e.g., by addition of 1-100 nucleotides, including RNA and/or DNA nucleotides at the 3' or the 5' terminus of the guide RNA, e.g., at the terminus distal to the targeting domain.

[146] 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.

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

[148] 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.

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

[150] 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. In certain embodiments, a gRNA used herein includes a DNA extension as well as one or more phosphorothioate linkage modifications, one or more phosphorodithioate (PS2) linkage modifications, one or more 2'-O-methyl modifications, 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 DNA extension may comprise a sequence set forth in Table 3 that includes a DNA extension.

Without wishing to be bound by theory, it is contemplated that any DNA extension may be used 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.

[151] 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, an RNA extension may comprise a sequence set forth in Table 3. 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, 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 in Table 3 that includes an RNA extension. gRNAs including an RNA extension at the 5' end of the gRNA may comprise a sequence disclosed herein. gRNAs including an RNA extension at the 3' end of the gRNA may comprise a sequence disclosed herein.

[152] 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.

[153] In some embodiments, a gRNA which includes a modification, e.g., a DNA extension at the 5' end, 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., an NK cell.

[154] Exemplary suitable 5' extensions for Cpf1 guide RNAs are provided in the table below:

[155] Table 3: gRNA 5' Extensions

5' extension Sequence ID No:	5' extension sequence	5' modification
	rCrUrUrUrU	+5 RNA
	rArArGrArCrCrUrUrUrU	+10 RNA
	rArUrGrUrGrUrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrUrU	+25 RNA
	rArGrGrCrCrArGrCrUrUrGrCrCrGrGrUrUrUrUrUrUrArGrUrCrGrUrGrCrUrGrCrUrUrCrArUrGrUrGrUrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrUrU	+60 RNA
	CTTTT	+5 DNA
	AAGACCTTTT	+10 DNA
	ATGTGTTTTTGTCAAAAGACCTTTT	+25 DNA
	AGGCCAGCTTGCCGGTTTTTTAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAGACCTTTT	+60 DNA
	TTTTTGTCAAAAGACCTTTT	+20 DNA
	GCTTCATGTGTTTTTGTCAAAAGACCTTTT	+30 DNA
	GCCGGTTTTTTAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAGACCTTTT	+50 DNA
	TAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAGACCTTTT	+40 DNA
	C*C*GAAGTTTTCTTCGGTTTT	+20 DNA + 2xPS
	T*T*TTTCCGAAGTTTTCTTCGGTTTT	+25 DNA + 2xPS
	A*A*CGCTTTTTCCGAAGTTTTCTTCGGTTTT	+30 DNA + 2xPS
	G*C*GTTGTTTTCAACGCTTTTTCCGAAGTTTTCTTCGGTTTT	+41 DNA + 2xPS
	G*G*CTTCTTTTGAAGCCTTTTTGCGTTGTTTTCAACGCTTTTTCCGAAGTTTTCTTCGGTTTT	+62 DNA + 2xPS
	A*T*GTGTTTTTGTCAAAAGACCTTTT	+25 DNA + 2xPS
	AAAAAAAAAAAAAAAAAAAAAAAAA	+25 A
	TTTTTTTTTTTTTTTTTTTTTTTTT	+25 T
	mA*mU*rGrUrGrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrU rU	+25 RNA + 2xPS
	mA*mA*rArArArArArArArArArArArArArArArArArArArArArArA rA	PolyA RNA + 2xPS
	mU*mU*rUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrU rU	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

[156] 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.

gRNA design

[157] 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. Each of these references is incorporated by reference herein. 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.

[158] In certain embodiments, one or more or all of the nucleotides in a gRNA are modified. Strategies for modifying a gRNA are described in WO2019/152519, published August 8, 2019, the entire contents of which are expressly incorporated herein by reference.

[159] 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 specifc 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:__) would have a targeting domain of the corresponding RNA sequence UCUGCAGAAAUGUUCCCCGU (SEQ ID NO:__) . 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/tracerRNA 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:__) , 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:__) . 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:__) . 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.

[160] 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 4.

Table 4: TIGIT gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme
TIGIT4170	TCTGCAGAAATGTTCCCCGT	20	AsCpf1
TIGIT4171	TGCAGAGAAAGGTGGCTCTA	20	AsCpf1
TIGIT4172	TAATGCTGACTTGGGGTGGC	20	AsCpf1
TIGIT4173	TAGGACCTCCAGGAAGATTC	20	AsCpf1

TIGIT4174	TAGTCAACGCGACCACCACG	20	AsCpf1
TIGIT4175	TCCTGAGGTCACCTTCCACA	20	AsCpf1
TIGIT4176	TATTGTGCCTGTCATCATTC	20	AsCpf1
TIGIT4177	TGACAGGCACAATAGAAACAA	21	SauCas9
TIGIT4178	GACAGGCACAATAGAAACAAC	21	SauCas9
TIGIT4179	AAACAACGGGGAACATTTCTG	21	SauCas9
TIGIT4180	ACAACGGGGAACATTTCTGCA	21	SauCas9
TIGIT4181	TGATAGAGCCACCTTTCTCTG	21	SauCas9
TIGIT4182	GGGTCACTTGTGCCGTGGTGG	21	SauCas9
TIGIT4183	GGCACAAGTGACCCAGGTCAA	21	SauCas9
TIGIT4184	GTCCTGCTGCTCCCAGTTGAC	21	SauCas9
TIGIT4185	TGGCCATTTGTAATGCTGACT	21	SauCas9
TIGIT4186	TGGCACATCTCCCCATCCTTC	21	SauCas9
TIGIT4187	CATCTCCCCATCCTTCAAGGA	21	SauCas9
TIGIT4188	CCACTCGATCCTTGAAGGATG	21	SauCas9
TIGIT4189	GGCCACTCGATCCTTGAAGGA	21	SauCas9
TIGIT4190	CCTGGGGCCACTCGATCCTTG	21	SauCas9
TIGIT4191	GACTGGAGGGTGAGGCCCAGG	21	SauCas9
TIGIT4192	ATCGTTCACGGTCAGCGACTG	21	SauCas9
TIGIT4193	GTCGCTGACCGTGAACGATAC	21	SauCas9
TIGIT4194	CGCTGACCGTGAACGATACAG	21	SauCas9
TIGIT4195	GCATCTATCACACCTACCCTG	21	SauCas9
TIGIT4196	CCTACCCTGATGGGACGTACA	21	SauCas9
TIGIT4197	TACCCTGATGGGACGTACACT	21	SauCas9
TIGIT4198	CCCTGATGGGACGTACACTGG	21	SauCas9
TIGIT4199	TTCTCCCAGTGTACGTCCCAT	21	SauCas9
TIGIT4200	GGAGAATCTTCCTGGAGGTCC	21	SauCas9

TIGIT4201	CATGGCTCCAAGCAATGGAAT	21	SauCas9
TIGIT4202	CGCGGCCATGGCTCCAAGCAA	21	SauCas9
TIGIT4203	TCGCGGCCATGGCTCCAAGCA	21	SauCas9
TIGIT4204	CATCGTGGTGGTCGCGTTGAC	21	SauCas9
TIGIT4205	AAAGCCCTCAGAATCCATTCT	21	SauCas9
TIGIT4206	CATTCTGTGGAAGGTGACCTC	21	SauCas9
TIGIT4207	TTCTGTGGAAGGTGACCTCAG	21	SauCas9
TIGIT4208	CCTGAGGTCACCTTCCACAGA	21	SauCas9
TIGIT4209	TTCTCCTGAGGTCACCTTCCA	21	SauCas9
TIGIT4210	AGGAGAAAATCAGCTGGACAG	21	SauCas9
TIGIT4211	GGAGAAAATCAGCTGGACAGG	21	SauCas9
TIGIT4212	GCCCCAGTGCTCCCTCACCCC	21	SauCas9
TIGIT4213	TGGACACAGCTTCCTGGGGGT	21	SauCas9
TIGIT4214	TCTGCCTGGACACAGCTTCCT	21	SauCas9
TIGIT4215	AGCTGCACCTGCTGGGCTCTG	21	SauCas9
TIGIT4216	GCTGGGCTCTGTGGAGAGCAG	21	SauCas9
TIGIT4217	TGGGCTCTGTGGAGAGCAGCG	21	SauCas9
TIGIT4218	CTGCATGACTACTTCAATGTC	21	SauCas9
TIGIT4219	AATGTCCTGAGTTACAGAAGC	21	SauCas9
TIGIT4220	TGGGTAACTGCAGCTTCTTCA	21	SauCas9
TIGIT4221	GACAGGCACAATAGAAACAA	20	SpyCas9
TIGIT4222	ACAGGCACAATAGAAACAAC	20	SpyCas9
TIGIT4223	CAGGCACAATAGAAACAACG	20	SpyCas9
TIGIT4224	GGGAACATTTCTGCAGAGAA	20	SpyCas9
TIGIT4225	AACATTTCTGCAGAGAAAGG	20	SpyCas9
TIGIT4226	ATGTCACCTCTCCTCCACCA	20	SpyCas9
TIGIT4227	CTTGTGCCGTGGTGGAGGAG	20	SpyCas9

TIGIT4228	GGTCACTTGTGCCGTGGTGG	20	SpyCas9
TIGIT4229	CACCACGGCACAAGTGACCC	20	SpyCas9
TIGIT4230	CTGGGTCACTTGTGCCGTGG	20	SpyCas9
TIGIT4231	GACCTGGGTCACTTGTGCCG	20	SpyCas9
TIGIT4232	CACAAGTGACCCAGGTCAAC	20	SpyCas9
TIGIT4233	ACAAGTGACCCAGGTCAACT	20	SpyCas9
TIGIT4234	CCAGGTCAACTGGGAGCAGC	20	SpyCas9
TIGIT4235	CTGCTGCTCCCAGTTGACCT	20	SpyCas9
TIGIT4236	CCTGCTGCTCCCAGTTGACC	20	SpyCas9
TIGIT4237	GGAGCAGCAGGACCAGCTTC	20	SpyCas9
TIGIT4238	CATTACAAATGGCCAGAAGC	20	SpyCas9
TIGIT4239	GGCCATTTGTAATGCTGACT	20	SpyCas9
TIGIT4240	GCCATTTGTAATGCTGACTT	20	SpyCas9
TIGIT4241	CCATTTGTAATGCTGACTTG	20	SpyCas9
TIGIT4242	TTTGTAATGCTGACTTGGGG	20	SpyCas9
TIGIT4243	CCCCAAGTCAGCATTACAAA	20	SpyCas9
TIGIT4244	GCACATCTCCCCATCCTTCA	20	SpyCas9
TIGIT4245	CCCATCCTTCAAGGATCGAG	20	SpyCas9
TIGIT4246	CACTCGATCCTTGAAGGATG	20	SpyCas9
TIGIT4247	CCACTCGATCCTTGAAGGAT	20	SpyCas9
TIGIT4248	GCCACTCGATCCTTGAAGGA	20	SpyCas9
TIGIT4249	TTCAAGGATCGAGTGGCCCC	20	SpyCas9
TIGIT4250	TGGGGCCACTCGATCCTTGA	20	SpyCas9
TIGIT4251	GATCGAGTGGCCCCAGGTCC	20	SpyCas9
TIGIT4252	AGTGGCCCCAGGTCCCGGCC	20	SpyCas9
TIGIT4253	GTGGCCCCAGGTCCCGGCCT	20	SpyCas9
TIGIT4254	GAGGCCCAGGCCGGGACCTG	20	SpyCas9

TIGIT4255	TGAGGCCCAGGCCGGGACCT	20	SpyCas9
TIGIT4256	GTGAGGCCCAGGCCGGGACC	20	SpyCas9
TIGIT4257	TGGAGGGTGAGGCCCAGGCC	20	SpyCas9
TIGIT4258	CTGGAGGGTGAGGCCCAGGC	20	SpyCas9
TIGIT4259	GCGACTGGAGGGTGAGGCCC	20	SpyCas9
TIGIT4260	CGGTCAGCGACTGGAGGGTG	20	SpyCas9
TIGIT4261	GTTCACGGTCAGCGACTGGA	20	SpyCas9
TIGIT4262	CGTTCACGGTCAGCGACTGG	20	SpyCas9
TIGIT4263	TATCGTTCACGGTCAGCGAC	20	SpyCas9
TIGIT4264	TCGCTGACCGTGAACGATAC	20	SpyCas9
TIGIT4265	CGCTGACCGTGAACGATACA	20	SpyCas9
TIGIT4266	GCTGACCGTGAACGATACAG	20	SpyCas9
TIGIT4267	GTACTCCCCTGTATCGTTCA	20	SpyCas9
TIGIT4268	ATCTATCACACCTACCCTGA	20	SpyCas9
TIGIT4269	TCTATCACACCTACCCTGAT	20	SpyCas9
TIGIT4270	TACCCTGATGGGACGTACAC	20	SpyCas9
TIGIT4271	ACCCTGATGGGACGTACACT	20	SpyCas9
TIGIT4272	AGTGTACGTCCCATCAGGGT	20	SpyCas9
TIGIT4273	TCCCAGTGTACGTCCCATCA	20	SpyCas9
TIGIT4274	CTCCCAGTGTACGTCCCATC	20	SpyCas9
TIGIT4275	GTACACTGGGAGAATCTTCC	20	SpyCas9
TIGIT4276	CACTGGGAGAATCTTCCTGG	20	SpyCas9
TIGIT4277	CTGAGCTTTCTAGGACCTCC	20	SpyCas9
TIGIT4278	AGGTTCCAGATTCCATTGCT	20	SpyCas9
TIGIT4279	AAGCAATGGAATCTGGAACC	20	SpyCas9
TIGIT4280	GATTCCATTGCTTGGAGCCA	20	SpyCas9
TIGIT4281	TGGCTCCAAGCAATGGAATC	20	SpyCas9

TIGIT4282	GCGGCCATGGCTCCAAGCAA	20	SpyCas9
TIGIT4283	TGGAGCCATGGCCGCGACGC	20	SpyCas9
TIGIT4284	AGCCATGGCCGCGACGCTGG	20	SpyCas9
TIGIT4285	GACCACCAGCGTCGCGGCCA	20	SpyCas9
TIGIT4286	GCAGATGACCACCAGCGTCG	20	SpyCas9
TIGIT4287	CATCTGCACAGCAGTCATCG	20	SpyCas9
TIGIT4288	CTGCACAGCAGTCATCGTGG	20	SpyCas9
TIGIT4289	AGCCCTCAGAATCCATTCTG	20	SpyCas9
TIGIT4290	CTCAGAATCCATTCTGTGGA	20	SpyCas9
TIGIT4291	TTCCACAGAATGGATTCTGA	20	SpyCas9
TIGIT4292	CTTCCACAGAATGGATTCTG	20	SpyCas9
TIGIT4293	ATTCTGTGGAAGGTGACCTC	20	SpyCas9
TIGIT4294	TGAGGTCACCTTCCACAGAA	20	SpyCas9
TIGIT4295	GACCTCAGGAGAAAATCAGC	20	SpyCas9
TIGIT4296	CAGGAGAAAATCAGCTGGAC	20	SpyCas9
TIGIT4297	GTCCAGCTGATTTTCTCCTG	20	SpyCas9
TIGIT4298	GAGAAAATCAGCTGGACAGG	20	SpyCas9
TIGIT4299	AATCAGCTGGACAGGAGGAA	20	SpyCas9
TIGIT4300	CCCAGTGCTCCCTCACCCCC	20	SpyCas9
TIGIT4301	CTGGGGGTGAGGGAGCACTG	20	SpyCas9
TIGIT4302	CCTGGGGGTGAGGGAGCACT	20	SpyCas9
TIGIT4303	TCCTGGGGGTGAGGGAGCAC	20	SpyCas9
TIGIT4304	ACACAGCTTCCTGGGGGTGA	20	SpyCas9
TIGIT4305	GACACAGCTTCCTGGGGGTG	20	SpyCas9
TIGIT4306	ACCCCCAGGAAGCTGTGTCC	20	SpyCas9
TIGIT4307	GCCTGGACACAGCTTCCTGG	20	SpyCas9
TIGIT4308	TGCCTGGACACAGCTTCCTG	20	SpyCas9

TIGIT4309	CTGCCTGGACACAGCTTCCT	20	SpyCas9
TIGIT4310	TCTGCCTGGACACAGCTTCC	20	SpyCas9
TIGIT4311	CAGGCAGAAGCTGCACCTGC	20	SpyCas9
TIGIT4312	AGGCAGAAGCTGCACCTGCT	20	SpyCas9
TIGIT4313	CAGCAGGTGCAGCTTCTGCC	20	SpyCas9
TIGIT4314	GCTGCACCTGCTGGGCTCTG	20	SpyCas9
TIGIT4315	TGCTCTCCACAGAGCCCAGC	20	SpyCas9
TIGIT4316	CTGGGCTCTGTGGAGAGCAG	20	SpyCas9
TIGIT4317	TGGGCTCTGTGGAGAGCAGC	20	SpyCas9
TIGIT4318	GGGCTCTGTGGAGAGCAGCG	20	SpyCas9
TIGIT4319	CTGTGGAGAGCAGCGGGGAG	20	SpyCas9
TIGIT4320	ATTGAAGTAGTCATGCAGCT	20	SpyCas9
TIGIT4321	TGTCCTGAGTTACAGAAGCC	20	SpyCas9
TIGIT4322	GTCCTGAGTTACAGAAGCCT	20	SpyCas9
TIGIT4323	TACCCAGGCTTCTGTAACTC	20	SpyCas9
TIGIT4324	TGAAGAAGCTGCAGTTACCC	20	SpyCas9
TIGIT4325	TGCAGCTTCTTCACAGAGAC	20	SpyCas9
TIGIT5053	GTTGTTTCTATTGTGCCTGT	20	AsCpf1 RR
TIGIT5054	CGTTGTTTCTATTGTGCCTG	20	AsCpf1 RR
TIGIT5055	CCGTTGTTTCTATTGTGCCT	20	AsCpf1 RR
TIGIT5056	CCACGGCACAAGTGACCCAG	20	AsCpf1 RR
TIGIT5057	AGTTGACCTGGGTCACTTGT	20	AsCpf1 RR
TIGIT5058	AAGTCAGCATTACAAATGGC	20	AsCpf1 RR
TIGIT5059	CATCCTTCAAGGATCGAGTG	20	AsCpf1 RR
TIGIT5060	ATCCTTCAAGGATCGAGTGG	20	AsCpf1 RR
TIGIT5061	AGGATCGAGTGGCCCCAGGT	20	AsCpf1 RR
TIGIT5062	AGGTCCCGGCCTGGGCCTCA	20	AsCpf1 RR

TIGIT5063	GGCCTGGGCCTCACCCTCCA	20	AsCpf1 RR
TIGIT5064	CGGTCAGCGACTGGAGGGTG	20	AsCpf1 RR
TIGIT5065	GTCGCTGACCGTGAACGATA	20	AsCpf1 RR
TIGIT5066	TGTATCGTTCACGGTCAGCG	20	AsCpf1 RR
TIGIT5067	CTGTATCGTTCACGGTCAGC	20	AsCpf1 RR
TIGIT5068	ATCAGGGTAGGTGTGATAGA	20	AsCpf1 RR
TIGIT5069	AGTGTACGTCCCATCAGGGT	20	AsCpf1 RR
TIGIT5070	GGAAGATTCTCCCAGTGTAC	20	AsCpf1 RR
TIGIT5071	TGGAGGTCCTAGAAAGCTCA	20	AsCpf1 RR
TIGIT5072	AGCAATGGAATCTGGAACCT	20	AsCpf1 RR
TIGIT5073	AGATTCCATTGCTTGGAGCC	20	AsCpf1 RR
TIGIT5074	GATTCCATTGCTTGGAGCCA	20	AsCpf1 RR
TIGIT5075	ATTGCTTGGAGCCATGGCCG	20	AsCpf1 RR
TIGIT5076	TTGCTTGGAGCCATGGCCGC	20	AsCpf1 RR
TIGIT5077	CAGAATGGATTCTGAGGGCT	20	AsCpf1 RR
TIGIT5078	ACAGAATGGATTCTGAGGGC	20	AsCpf1 RR
TIGIT5079	TTCTGTGGAAGGTGACCTCA	20	AsCpf1 RR
TIGIT5080	GCTGATTTTCTCCTGAGGTC	20	AsCpf1 RR
TIGIT5081	TCCTGTCCAGCTGATTTTCT	20	AsCpf1 RR
TIGIT5082	TTCCTCCTGTCCAGCTGATT	20	AsCpf1 RR
TIGIT5083	TGGGGGTGAGGGAGCACTGG	20	AsCpf1 RR
TIGIT5084	AGTGCTCCCTCACCCCCAGG	20	AsCpf1 RR
TIGIT5085	TCACCCCCAGGAAGCTGTGT	20	AsCpf1 RR
TIGIT5086	CAGGAAGCTGTGTCCAGGCA	20	AsCpf1 RR
TIGIT5087	AGGAAGCTGTGTCCAGGCAG	20	AsCpf1 RR
TIGIT5088	GGCAGAAGCTGCACCTGCTG	20	AsCpf1 RR
TIGIT5089	CAGAGCCCAGCAGGTGCAGC	20	AsCpf1 RR

TIGIT5090	GCTGCTCTCCACAGAGCCCA	20	AsCpf1 RR
TIGIT5091	CGCTGCTCTCCACAGAGCCC	20	AsCpf1 RR
TIGIT5092	ATGTCCTGAGTTACAGAAGC	20	AsCpf1 RR

[161] 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 5.

Table 5. ADORA2a gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme
ADORA2A337	GAGCACACCCACTGCGATGT	20	SpyCas9
ADORA2A338	GATGGCCAGGAGACTGAAGA	20	SpyCas9
ADORA2A339	CTGCTCACCGGAGCGGGATG	20	SpyCas9
ADORA2A340	GTCTGTGGCCATGCCCATCA	20	SpyCas9
ADORA2A341	TCACCGGAGCGGGATGCGGA	20	SpyCas9
ADORA2A342	GTGGCAGGCAGCGCAGAACC	20	SpyCas9
ADORA2A343	AGCACACCAGCACATTGCCC	20	SpyCas9
ADORA2A344	CAGGTTGCTGTTGAGCCACA	20	SpyCas9
ADORA2A345	CTTCATTGCCTGCTTCGTCC	20	SpyCas9
ADORA2A346	GTACACCGAGGAGCCCATGA	20	SpyCas9
ADORA2A347	GATGGCAATGTAGCGGTCAA	20	SpyCas9
ADORA2A348	CTCCTCGGTGTACATCACGG	20	SpyCas9
ADORA2A349	CGAGGAGCCCATGATGGGCA	20	SpyCas9
ADORA2A350	GGGCTCCTCGGTGTACATCA	20	SpyCas9
ADORA2A351	CTTTGTGGTGTCACTGGCGG	20	SpyCas9
ADORA2A352	CCGCTCCGGTGAGCAGGGCC	20	SpyCas9
ADORA2A353	GGGTTCTGCGCTGCCTGCCA	20	SpyCas9
ADORA2A354	GGACGAAGCAGGCAATGAAG	20	SpyCas9
ADORA2A355	GTGCTGATGGTGATGGCAAA	20	SpyCas9
ADORA2A356	AGCGCAGAACCCGGTGCTGA	20	SpyCas9
ADORA2A357	GAGCTCCATCTTCAGTCTCC	20	SpyCas9
ADORA2A358	TGCTGATGGTGATGGCAAAG	20	SpyCas9
ADORA2A359	GGCGGCGGCCGACATCGCAG	20	SpyCas9
ADORA2A360	AATGAAGAGGCAGCCGTGGC	20	SpyCas9
ADORA2A361	GGGCAATGTGCTGGTGTGCT	20	SpyCas9
ADORA2A362	CATGCCCATCATGGGCTCCT	20	SpyCas9
ADORA2A363	AATGTAGCGGTCAATGGCGA	20	SpyCas9
ADORA2A364	AGTAGTTGGTGACGTTCTGC	20	SpyCas9
ADORA2A365	AGCGGTCAATGGCGATGGCC	20	SpyCas9
ADORA2A366	CGCATCCCGCTCCGGTGAGC	20	SpyCas9

ADORA2A367	GCATCCCGCTCCGGTGAGCA	20	SpyCas9
ADORA2A368	TGGGCAATGTGCTGGTGTGC	20	SpyCas9
ADORA2A369	CAACTACTTTGTGGTGTCAC	20	SpyCas9
ADORA2A370	CGCTCCGGTGAGCAGGGCCG	20	SpyCas9
ADORA2A371	GATGGTGATGGCAAAGGGGA	20	SpyCas9
ADORA2A372	GGTGTACATCACGGTGGAGC	20	SpyCas9
ADORA2A373	GAACGTCACCAACTACTTTG	20	SpyCas9
ADORA2A374	CAGTGACACCACAAAGTAGT	20	SpyCas9
ADORA2A375	GGCCATCCTGGGCAATGTGC	20	SpyCas9
ADORA2A376	CCCGGCCCTGCTCACCGGAG	20	SpyCas9
ADORA2A377	CACCAGCACATTGCCCAGGA	20	SpyCas9
ADORA2A378	TTTGCCATCACCATCAGCAC	20	SpyCas9
ADORA2A379	CTCCACCGTGATGTACACCG	20	SpyCas9
ADORA2A380	GGAGCTGGCCATTGCTGTGC	20	SpyCas9
ADORA2A381	CAGGATGGCCAGCACAGCAA	20	SpyCas9
ADORA2A382	GAACCCGGTGCTGATGGTGA	20	SpyCas9
ADORA2A383	TGGAGCTCTGCGTGAGGACC	20	SpyCas9
ADORA2A384	CCCGCTCCGGTGAGCAGGGC	20	SpyCas9
ADORA2A385	AGGCAATGAAGAGGCAGCCG	20	SpyCas9
ADORA2A386	CCGGCCCTGCTCACCGGAGC	20	SpyCas9
ADORA2A387	GCGGCGGCCGACATCGCAGT	20	SpyCas9
ADORA2A388	GGTGCTGATGGTGATGGCAA	20	SpyCas9
ADORA2A389	CTACTTTGTGGTGTCACTGG	20	SpyCas9
ADORA2A390	TACACCGAGGAGCCCATGAT	20	SpyCas9
ADORA2A391	TCTGTGGCCATGCCCATCAT	20	SpyCas9
ADORA2A392	ATTGCTGTGCTGGCCATCCT	20	SpyCas9
ADORA2A393	CGTGAGGACCAGGACGAAGC	20	SpyCas9
ADORA2A394	TTGCCATCACCATCAGCACC	20	SpyCas9
ADORA2A395	GGATGCGGATGGCAATGTAG	20	SpyCas9
ADORA2A396	TTGCCATCCGCATCCCGCTC	20	SpyCas9
ADORA2A397	TGAAGATGGAGCTCTGCGTG	20	SpyCas9
ADORA2A398	CATTGCTGTGCTGGCCATCC	20	SpyCas9
ADORA2A399	TGCTGGTGTGCTGGGCCGTG	20	SpyCas9
ADORA2A820	GGCTCCTCGGTGTACATCACG	21	SauCas9
ADORA2A821	GAGCTCTGCGTGAGGACCAGG	21	SauCas9
ADORA2A822	GATGGAGCTCTGCGTGAGGAC	21	SauCas9
ADORA2A823	CCAGCACACCAGCACATTGCC	21	SauCas9
ADORA2A824	AGGACCAGGACGAAGCAGGCA	21	SauCas9
ADORA2A825	TGCCATCCGCATCCCGCTCCG	21	SauCas9
ADORA2A826	GTGTGGCTCAACAGCAACCTG	21	SauCas9
ADORA2A827	AGCTCCACCGTGATGTACACC	21	SauCas9
ADORA2A828	GTAGCGGTCAATGGCGATGGC	21	SauCas9
ADORA2A829	CGGTGCTGATGGTGATGGCAA	21	SauCas9
ADORA2A830	CCCTGCTCACCGGAGCGGGAT	21	SauCas9
ADORA2A831	GTGACGTTCTGCAGGTTGCTG	21	SauCas9

ADORA2A832	GCTCCACCGTGATGTACACCG	21	SauCas9
ADORA2A833	ACTGAAGATGGAGCTCTGCGT	21	SauCas9
ADORA2A834	CCAGCTCCACCGTGATGTACA	21	SauCas9
ADORA2A835	CCTTTGCCATCACCATCAGCA	21	SauCas9
ADORA2A836	CCGGTGCTGATGGTGATGGCA	21	SauCas9
ADORA2A837	CCTGGGCAATGTGCTGGTGTG	21	SauCas9
ADORA2A838	AGGCAGCCGTGGCAGGCAGCG	21	SauCas9
ADORA2A839	GCGATGGCCAGGAGACTGAAG	21	SauCas9
ADORA2A840	CGATGGCCAGGAGACTGAAGA	21	SauCas9
ADORA2A841	TCCCGCTCCGGTGAGCAGGGC	21	SauCas9
ADORA2A842	TGCTTCGTCCTGGTCCTCACG	21	SauCas9
ADORA2A843	ACCAGGACGAAGCAGGCAATG	21	SauCas9
ADORA2A844	ATGTACACCGAGGAGCCCATG	21	SauCas9
ADORA2A845	TCGTCTGTGGCCATGCCCATC	21	SauCas9
ADORA2A846	TCAATGGCGATGGCCAGGAGA	21	SauCas9
ADORA2A847	GGTGCTGATGGTGATGGCAAA	21	SauCas9
ADORA2A848	TAGCGGTCAATGGCGATGGCC	21	SauCas9
ADORA2A849	TCCGCATCCCGCTCCGGTGAG	21	SauCas9
ADORA2A850	CTGGCGGCGGCCGACATCGCA	21	SauCas9
ADORA2A851	GCCATTGCTGTGCTGGCCATC	21	SauCas9
ADORA2A852	ATCCCGCTCCGGTGAGCAGGG	21	SauCas9
ADORA2A853	AGACTGAAGATGGAGCTCTGC	21	SauCas9
ADORA2A854	CCCCGGCCCTGCTCACCGGAG	21	SauCas9
ADORA2A855	ATGGTGATGGCAAAGGGGATG	21	SauCas9
ADORA2A856	GCTCCTCGGTGTACATCACGG	21	SauCas9
ADORA2A248	TGTCGATGGCAATAGCCAAG	20	SpyCas9
ADORA2A249	AGAAGTTGGTGACGTTCTGC	20	SpyCas9
ADORA2A250	TTCGCCATCACCATCAGCAC	20	SpyCas9
ADORA2A251	GAAGAAGAGGCAGCCATGGC	20	SpyCas9
ADORA2A252	CACAAGCACGTTACCCAGGA	20	SpyCas9
ADORA2A253	CAACTTCTTCGTGGTATCTC	20	SpyCas9
ADORA2A254	CAGGATGGCCAGCACAGCAA	20	SpyCas9
ADORA2A255	AATTCCACTCCGGTGAGCCA	20	SpyCas9
ADORA2A256	AGCGCAGAAGCCAGTGCTGA	20	SpyCas9
ADORA2A257	GTGCTGATGGTGATGGCGAA	20	SpyCas9
ADORA2A258	GGAGCTGGCCATTGCTGTGC	20	SpyCas9
ADORA2A259	AATAGCCAAGAGGCTGAAGA	20	SpyCas9
ADORA2A260	CTCCTCGGTGTACATCATGG	20	SpyCas9
ADORA2A261	GGACAAAGCAGGCGAAGAAG	20	SpyCas9
ADORA2A262	TCTGGCGGCGGCTGACATCG	20	SpyCas9
ADORA2A263	TGGGTAACGTGCTTGTGTGC	20	SpyCas9
ADORA2A264	GATGTACACCGAGGAGCCCA	20	SpyCas9
ADORA2A265	TAACCCCTGGCTCACCGGAG	20	SpyCas9
ADORA2A266	TCACCGGAGTGGAATTCGGA	20	SpyCas9
ADORA2A267	GCGGCGGCTGACATCGCGGT	20	SpyCas9

ADORA2A268	GATGGTGATGGCGAATGGGA	20	SpyCas9
ADORA2A269	GGCTTCTGCGCTGCCTGCCA	20	SpyCas9
ADORA2A270	ATTCCACTCCGGTGAGCCAG	20	SpyCas9
ADORA2A271	GGTGTACATCATGGTGGAGC	20	SpyCas9
ADORA2A272	ATTGCTGTGCTGGCCATCCT	20	SpyCas9
ADORA2A273	CTCCACCATGATGTACACCG	20	SpyCas9
ADORA2A274	GGCGGCGGCTGACATCGCGG	20	SpyCas9
ADORA2A275	TACACCGAGGAGCCCATGGC	20	SpyCas9
ADORA2A276	GGGTAACGTGCTTGTGTGCT	20	SpyCas9
ADORA2A277	CAGGTTGCTGTTGATCCACA	20	SpyCas9
ADORA2A278	TGAAGATGGAACTCTGCGTG	20	SpyCas9
ADORA2A279	GATGGCGATGTATCTGTCGA	20	SpyCas9
ADORA2A280	CTTCTTCGCCTGCTTTGTCC	20	SpyCas9
ADORA2A281	AGGCGAAGAAGAGGCAGCCA	20	SpyCas9
ADORA2A282	TGCTTGTGTGCTGGGCCGTG	20	SpyCas9
ADORA2A283	GAAGCCAGTGCTGATGGTGA	20	SpyCas9
ADORA2A284	CGTGAGGACCAGGACAAAGC	20	SpyCas9
ADORA2A285	TGGAACTCTGCGTGAGGACC	20	SpyCas9
ADORA2A286	CATTGCTGTGCTGGCCATCC	20	SpyCas9
ADORA2A287	TTCTCCCGCCATGGGCTCCT	20	SpyCas9
ADORA2A288	TGGCTCACCGGAGTGGAATT	20	SpyCas9
ADORA2A289	TGCTGATGGTGATGGCGAAT	20	SpyCas9
ADORA2A290	CTTCGTGGTATCTCTGGCGG	20	SpyCas9
ADORA2A291	AGCACACAAGCACGTTACCC	20	SpyCas9
ADORA2A292	GGGCTCCTCGGTGTACATCA	20	SpyCas9
ADORA2A293	GTACACCGAGGAGCCCATGG	20	SpyCas9
ADORA2A294	GAACGTCACCAACTTCTTCG	20	SpyCas9
ADORA2A295	TCGCCATCCGAATTCCACTC	20	SpyCas9
ADORA2A296	GAGTTCCATCTTCAGCCTCT	20	SpyCas9
ADORA2A297	GAATTCCACTCCGGTGAGCC	20	SpyCas9
ADORA2A298	CAGAGATACCACGAAGAAGT	20	SpyCas9
ADORA2A299	CTTCTTCGTGGTATCTCTGG	20	SpyCas9
ADORA2A695	CAGTGCTGATGGTGATGGCGA	21	SauCas9
ADORA2A696	CGAATTCCACTCCGGTGAGCC	21	SauCas9
ADORA2A697	CCGAATTCCACTCCGGTGAGC	21	SauCas9
ADORA2A698	GCTGAAGATGGAACTCTGCGT	21	SauCas9
ADORA2A699	CGTGCTTGTGTGCTGGGCCGT	21	SauCas9
ADORA2A700	GTGAGGACCAGGACAAAGCAG	21	SauCas9
ADORA2A701	TCGATGGCAATAGCCAAGAGG	21	SauCas9
ADORA2A702	CATCGACAGATACATCGCCAT	21	SauCas9
ADORA2A703	GTACACCGAGGAGCCCATGGC	21	SauCas9
ADORA2A704	GCTCCACCATGATGTACACCG	21	SauCas9
ADORA2A705	AAGCCAGTGCTGATGGTGATG	21	SauCas9
ADORA2A706	CACCGCGATGTCAGCCGCCGC	21	SauCas9
ADORA2A707	AGGCTGAAGATGGAACTCTGC	21	SauCas9

ADORA2A708	GCCGCCGCCAGAGATACCACG	21	SauCas9
ADORA2A709	AGCTCCACCATGATGTACACC	21	SauCas9
ADORA2A710	AGGCAGCCATGGCAGGCAGCG	21	SauCas9
ADORA2A711	CCTGGCTCACCGGAGTGGAAT	21	SauCas9
ADORA2A712	CCAGCTCCACCATGATGTACA	21	SauCas9
ADORA2A713	ACCAGGACAAAGCAGGCGAAG	21	SauCas9
ADORA2A714	CCTGGGTAACGTGCTTGTGTG	21	SauCas9
ADORA2A715	AGGACCAGGACAAAGCAGGCG	21	SauCas9
ADORA2A716	TCAGCCGCCGCCAGAGATACC	21	SauCas9
ADORA2A717	GGCTCCTCGGTGTACATCATG	21	SauCas9
ADORA2A718	CTGGCGGCGGCTGACATCGCG	21	SauCas9
ADORA2A719	GATGGAACTCTGCGTGAGGAC	21	SauCas9
ADORA2A720	GCTCCTCGGTGTACATCATGG	21	SauCas9
ADORA2A721	TGTACACCGAGGAGCCCATGG	21	SauCas9
ADORA2A722	GCCATTGCTGTGCTGGCCATC	21	SauCas9
ADORA2A723	CAATAGCCAAGAGGCTGAAGA	21	SauCas9
ADORA2A724	ATGGTGATGGCGAATGGGATG	21	SauCas9
ADORA2A725	ATGTACACCGAGGAGCCCATG	21	SauCas9
ADORA2A726	GTGTGGATCAACAGCAACCTG	21	SauCas9
ADORA2A727	TGCTTTGTCCTGGTCCTCACG	21	SauCas9
ADORA2A728	GTAACCCCTGGCTCACCGGAG	21	SauCas9
ADORA2A729	CCAGCACACAAGCACGTTACC	21	SauCas9
ADORA2A730	TATCTGTCGATGGCAATAGCC	21	SauCas9
ADORA2A731	GCAATAGCCAAGAGGCTGAAG	21	SauCas9
ADORA2A732	AGTGCTGATGGTGATGGCGAA	21	SauCas9
ADORA2A733	ACACCGAGGAGCCCATGGCGG	21	SauCas9
ADORA2A734	CGCCATCCGAATTCCACTCCG	21	SauCas9
ADORA2A4111	TGGTGTCACTGGCGGCGGCC	20	AsCpf1
ADORA2A4112	CCATCACCATCAGCACCGGG	20	AsCpf1
ADORA2A4113	CCATCGGCCTGACTCCCATG	20	AsCpf1
ADORA2A4114	GCTGACCGCAGTTGTTCCAA	20	AsCpf1
ADORA2A4115	AGGATGTGGTCCCCATGAAC	20	AsCpf1
ADORA2A4116	CCTGTGTGCTGGTGCCCCTG	20	AsCpf1
ADORA2A4117	CGGATCTTCCTGGCGGCGCG	20	AsCpf1
ADORA2A4118	CCCTCTGCTGGCTGCCCCTA	20	AsCpf1
ADORA2A4119	TTCTGCCCCGACTGCAGCCA	20	AsCpf1
ADORA2A4120	AAGGCAGCTGGCACCAGTGC	20	AsCpf1
ADORA2A4121	TAAGGGCATCATTGCCATCTG	21	SauCas9
ADORA2A4122	CGGCCTGACTCCCATGCTAGG	21	SauCas9
ADORA2A4123	GCAGTTGTTCCAACCTAGCAT	21	SauCas9
ADORA2A4124	CCGCAGTTGTTCCAACCTAGC	21	SauCas9
ADORA2A4125	CAAGAACCACTCCCAGGGCTG	21	SauCas9
ADORA2A4126	CTTGGCCCTCCCCGCAGCCCT	21	SauCas9
ADORA2A4127	CACTTGGCCCTCCCCGCAGCC	21	SauCas9
ADORA2A4128	GGCCAAGTGGCCTGTCTCTTT	21	SauCas9

ADORA2A4129	TTCATGGGGACCACATCCTCA	21	SauCas9
ADORA2A4130	TGAAGTACACCATGTAGTTCA	21	SauCas9
ADORA2A4131	CTGGTGCCCCTGCTGCTCATG	21	SauCas9
ADORA2A4132	GCTCATGCTGGGTGTCTATTT	21	SauCas9
ADORA2A4133	CTTCAGCTGTCGTCGCGCCGC	21	SauCas9
ADORA2A4134	CGCGACGACAGCTGAAGCAGA	21	SauCas9
ADORA2A4135	GATGGAGAGCCAGCCTCTGCC	21	SauCas9
ADORA2A4136	GCGTGGCTGCAGTCGGGGCAG	21	SauCas9
ADORA2A4137	ACGATGGCCAGGTACATGAGC	21	SauCas9
ADORA2A4138	CTCTCCCACACCAATTCGGTT	21	SauCas9
ADORA2A4139	GATTCACAACCGAATTGGTGT	21	SauCas9
ADORA2A4140	GGGATTCACAACCGAATTGGT	21	SauCas9
ADORA2A4141	CGTAGATGAAGGGATTCACAA	21	SauCas9
ADORA2A4142	GGATACGGTAGGCGTAGATGA	21	SauCas9
ADORA2A4143	TCATCTACGCCTACCGTATCC	21	SauCas9
ADORA2A4144	CGGATACGGTAGGCGTAGATG	21	SauCas9
ADORA2A4145	GCGGAAGGTCTGGCGGAACTC	21	SauCas9
ADORA2A4146	AATGATCTTGCGGAAGGTCTG	21	SauCas9
ADORA2A4147	GACGTGGCTGCGAATGATCTT	21	SauCas9
ADORA2A4148	TTGCTGCCTCAGGACGTGGCT	21	SauCas9
ADORA2A4149	CAAGGCAGCTGGCACCAGTGC	21	SauCas9
ADORA2A4150	CGGGCACTGGTGCCAGCTGCC	21	SauCas9
ADORA2A4151	CTTGGCAGCTCATGGCAGTGA	21	SauCas9
ADORA2A4152	CCGTCTCAACGGCCACCCGCC	21	SauCas9
ADORA2A4153	CACACTCCTGGCGGGTGGCCG	21	SauCas9
ADORA2A4154	TGCCGTTGGCCCACACTCCTG	21	SauCas9
ADORA2A4155	CCATTGGGCCTCCGCTCAGGG	21	SauCas9
ADORA2A4156	CATAGCCATTGGGCCTCCGCT	21	SauCas9
ADORA2A4157	AATGGCTATGCCCTGGGGCTG	21	SauCas9
ADORA2A4158	ATGCCCTGGGGCTGGTGAGTG	21	SauCas9
ADORA2A4159	GCCCTGGGGCTGGTGAGTGGA	21	SauCas9
ADORA2A4160	TGGTGAGTGGAGGGAGTGCCC	21	SauCas9
ADORA2A4161	GAGGGAGTGCCCAAGAGTCCC	21	SauCas9
ADORA2A4162	AGGGAGTGCCCAAGAGTCCCA	21	SauCas9
ADORA2A4163	GTCTGGGAGGCCCGTGTTCCC	21	SauCas9
ADORA2A4164	CATGGCTAAGGAGCTCCACGT	21	SauCas9
ADORA2A4165	GAGCTCCTTAGCCATGAGCTC	21	SauCas9
ADORA2A4166	GCTCCTTAGCCATGAGCTCAA	21	SauCas9
ADORA2A4167	GGCCTAGATGACCCCCTGGCC	21	SauCas9
ADORA2A4168	CCCCCTGGCCCAGGATGGAGC	21	SauCas9
ADORA2A4169	CTCCTGCTCCATCCTGGGCCA	21	SauCas9
ADORA2A4416	CCGTGATGTACACCGAGGAG	20	AsCpf1 RR
ADORA2A4417	CTTTGCCATCACCATCAGCA	20	AsCpf1 RR
ADORA2A4418	TTTGCCATCACCATCAGCAC	20	AsCpf1 RR
ADORA2A4419	TTGCCTGCTTCGTCCTGGTC	20	AsCpf1 RR

ADORA2A4420	TCCTGGTCCTCACGCAGAGC	20	AsCpf1 RR
ADORA2A4421	TCTTCAGTCTCCTGGCCATC	20	AsCpf1 RR
ADORA2A4422	GTCTCCTGGCCATCGCCATT	20	AsCpf1 RR
ADORA2A4423	ACCTAGCATGGGAGTCAGGC	20	AsCpf1 RR
ADORA2A4424	AACCTAGCATGGGAGTCAGG	20	AsCpf1 RR
ADORA2A4425	ATGCTAGGTTGGAACAACTG	20	AsCpf1 RR
ADORA2A4426	GCAGCCCTGGGAGTGGTTCT	20	AsCpf1 RR
ADORA2A4427	CGCAGCCCTGGGAGTGGTTC	20	AsCpf1 RR
ADORA2A4428	AGGGCTGCGGGGAGGGCCAA	20	AsCpf1 RR
ADORA2A4429	TGGGGACCACATCCTCAAAG	20	AsCpf1 RR
ADORA2A4430	CATGAACTACATGGTGTACT	20	AsCpf1 RR
ADORA2A4431	ATGAACTACATGGTGTACTT	20	AsCpf1 RR
ADORA2A4432	ACTTCTTTGCCTGTGTGCTG	20	AsCpf1 RR
ADORA2A4433	TGCTGCTCATGCTGGGTGTC	20	AsCpf1 RR
ADORA2A4434	CAAATAGACACCCAGCATGA	20	AsCpf1 RR
ADORA2A4435	GCTGTCGTCGCGCCGCCAGG	20	AsCpf1 RR
ADORA2A4436	TGGCGGCGCGACGACAGCTG	20	AsCpf1 RR
ADORA2A4437	TCTGCTTCAGCTGTCGTCGC	20	AsCpf1 RR
ADORA2A4438	GGCAGAGGCTGGCTCTCCAT	20	AsCpf1 RR
ADORA2A4439	CGGCAGAGGCTGGCTCTCCA	20	AsCpf1 RR
ADORA2A4440	CCGGCAGAGGCTGGCTCTCC	20	AsCpf1 RR
ADORA2A4441	CACTGCAGAAGGAGGTCCAT	20	AsCpf1 RR
ADORA2A4442	TGCTGCCAAGTCACTGGCCA	20	AsCpf1 RR
ADORA2A4443	ACAATGATGGCCAGTGACTT	20	AsCpf1 RR
ADORA2A4444	TACACATCATCAACTGCTTC	20	AsCpf1 RR
ADORA2A4445	CTTTCTTCTGCCCCGACTGC	20	AsCpf1 RR
ADORA2A4446	GACTGCAGCCACGCCCCTCT	20	AsCpf1 RR
ADORA2A4447	TCTCTGGCTCATGTACCTGG	20	AsCpf1 RR
ADORA2A4448	CAACCGAATTGGTGTGGGAG	20	AsCpf1 RR
ADORA2A4449	ACACCAATTCGGTTGTGAAT	20	AsCpf1 RR
ADORA2A4450	GTTGTGAATCCCTTCATCTA	20	AsCpf1 RR
ADORA2A4451	TTCATCTACGCCTACCGTAT	20	AsCpf1 RR
ADORA2A4452	TCTACGCCTACCGTATCCGC	20	AsCpf1 RR
ADORA2A4453	CGAGTTCCGCCAGACCTTCC	20	AsCpf1 RR
ADORA2A4454	GCCAGACCTTCCGCAAGATC	20	AsCpf1 RR
ADORA2A4455	CCAGACCTTCCGCAAGATCA	20	AsCpf1 RR
ADORA2A4456	GCAAGATCATTCGCAGCCAC	20	AsCpf1 RR
ADORA2A4457	CAAGATCATTCGCAGCCACG	20	AsCpf1 RR
ADORA2A4458	CAGCCACGTCCTGAGGCAGC	20	AsCpf1 RR
ADORA2A4459	AGGCAGCTGGCACCAGTGCC	20	AsCpf1 RR
ADORA2A4460	TCACTGCCATGAGCTGCCAA	20	AsCpf1 RR
ADORA2A4461	TCTCAACGGCCACCCGCCAG	20	AsCpf1 RR
ADORA2A4462	CTCAGGGTGGGGAGCACTGC	20	AsCpf1 RR
ADORA2A4463	CACCCTGAGCGGAGGCCCAA	20	AsCpf1 RR
ADORA2A4464	ACCCTGAGCGGAGGCCCAAT	20	AsCpf1 RR

ADORA2A4465	AGGGCATAGCCATTGGGCCT	20	AsCpf1 RR
ADORA2A4466	CTCACCAGCCCCAGGGCATA	20	AsCpf1 RR
ADORA2A4467	TCCACTCACCAGCCCCAGGG	20	AsCpf1 RR
ADORA2A4468	TGGGACTCTTGGGCACTCCC	20	AsCpf1 RR
ADORA2A4469	CTGGGACTCTTGGGCACTCC	20	AsCpf1 RR
ADORA2A4470	CCTGGGACTCTTGGGCACTC	20	AsCpf1 RR
ADORA2A4471	AGGGGAACACGGGCCTCCCA	20	AsCpf1 RR
ADORA2A4472	CGTCTGGGAGGCCCGTGTTC	20	AsCpf1 RR
ADORA2A4473	AGACGTGGAGCTCCTTAGCC	20	AsCpf1 RR
ADORA2A4474	TTGAGCTCATGGCTAAGGAG	20	AsCpf1 RR
ADORA2A4475	CTGGCCTAGATGACCCCCTG	20	AsCpf1 RR
ADORA2A4476	TGGCCTAGATGACCCCCTGG	20	AsCpf1 RR
ADORA2A4477	TCCTGGGCCAGGGGGTCATC	20	AsCpf1 RR
ADORA2A4478	CTGGCCCAGGATGGAGCAGG	20	AsCpf1 RR
ADORA2A4479	TGGCCCAGGATGGAGCAGGA	20	AsCpf1 RR
ADORA2A4480	CGCGAGTTCCGCCAGACCTT	20	AsCpf1 RVR
ADORA2A4481	CCCTGGGGCTGGTGAGTGGA	20	AsCpf1 RVR

[162] In some embodiments the gRNA for use in the disclosure is a gRNA targeting TGFbetaR2 (TGFbetaR2 gRNA). In some embodiments, the gRNA targeting TGFbetaR2 is one or more of the gRNAs described in Table 6.

Table 6. TGFbetaR2 gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme
TGFBR24326	CAGGACGATGTGCAGCGGCC	20	AsCpf1 RR
TGFBR24327	ACCGCACGTTCAGAAGTCGG	20	AsCpf1 RR
TGFBR24328	ACAACTGTGTAAATTTTGTG	20	AsCpf1 RR
TGFBR24329	CAACTGTGTAAATTTTGTGA	20	AsCpf1 RR
TGFBR24330	ACCTGTGACAACCAGAAATC	20	AsCpf1 RR
TGFBR24331	CCTGTGACAACCAGAAATCC	20	AsCpf1 RR
TGFBR24332	TGTGGCTTCTCACAGATGGA	20	AsCpf1 RR
TGFBR24333	TCTGTGAGAAGCCACAGGAA	20	AsCpf1 RR
TGFBR24334	AAGCTCCCCTACCATGACTT	20	AsCpf1 RR
TGFBR24335	GAATAAAGTCATGGTAGGGG	20	AsCpf1 RR
TGFBR24336	AGAATAAAGTCATGGTAGGG	20	AsCpf1 RR
TGFBR24337	CTACCATGACTTTATTCTGG	20	AsCpf1 RR
TGFBR24338	TACCATGACTTTATTCTGGA	20	AsCpf1 RR
TGFBR24339	TAATGCACTTTGGAGAAGCA	20	AsCpf1 RR
TGFBR24340	TTCATAATGCACTTTGGAGA	20	AsCpf1 RR
TGFBR24341	AAGTGCATTATGAAGGAAAA	20	AsCpf1 RR
TGFBR24342	TGTGTTCCTGTAGCTCTGAT	20	AsCpf1 RR
TGFBR24343	TGTAGCTCTGATGAGTGCAA	20	AsCpf1 RR

TGFBR24344	AGTGACAGGCATCAGCCTCC	20	AsCpf1 RR
TGFBR24345	AGTGGTGGCAGGAGGCTGAT	20	AsCpf1 RR
TGFBR24346	AGGTTGAACTCAGCTTCTGC	20	AsCpf1 RR
TGFBR24347	CAGGTTGAACTCAGCTTCTG	20	AsCpf1 RR
TGFBR24348	ACCTGGGAAACCGGCAAGAC	20	AsCpf1 RR
TGFBR24349	CGTCTTGCCGGTTTCCCAGG	20	AsCpf1 RR
TGFBR24350	GCGTCTTGCCGGTTTCCCAG	20	AsCpf1 RR
TGFBR24351	TGAGCTTCCGCGTCTTGCCG	20	AsCpf1 RR
TGFBR24352	GCGAGCACTGTGCCATCATC	20	AsCpf1 RR
TGFBR24353	GGATGATGGCACAGTGCTCG	20	AsCpf1 RR
TGFBR24354	AGGATGATGGCACAGTGCTC	20	AsCpf1 RR
TGFBR24355	CGTGTGCCAACAACATCAAC	20	AsCpf1 RR
TGFBR24356	GCTCAATGGGCAGCAGCTCT	20	AsCpf1 RR
TGFBR24357	ACCAGGGTGTCCAGCTCAAT	20	AsCpf1 RR
TGFBR24358	CACCAGGGTGTCCAGCTCAA	20	AsCpf1 RR
TGFBR24359	CCACCAGGGTGTCCAGCTCA	20	AsCpf1 RR
TGFBR24360	GCTTGGCCTTATAGACCTCA	20	AsCpf1 RR
TGFBR24361	GAGCAGTTTGAGACAGTGGC	20	AsCpf1 RR
TGFBR24362	AGAGGCATACTCCTCATAGG	20	AsCpf1 RR
TGFBR24363	CTATGAGGAGTATGCCTCTT	20	AsCpf1 RR
TGFBR24364	AAGAGGCATACTCCTCATAG	20	AsCpf1 RR
TGFBR24365	TATGAGGAGTATGCCTCTTG	20	AsCpf1 RR
TGFBR24366	GATTGATGTCTGAGAAGATG	20	AsCpf1 RR
TGFBR24367	CTCCTCAGCCGTCAGGAACT	20	AsCpf1 RR
TGFBR24368	GTTCCTGACGGCTGAGGAGC	20	AsCpf1 RR
TGFBR24369	GCTCCTCAGCCGTCAGGAAC	20	AsCpf1 RR
TGFBR24370	TGACGGCTGAGGAGCGGAAG	20	AsCpf1 RR
TGFBR24371	TCTTCCGCTCCTCAGCCGTC	20	AsCpf1 RR
TGFBR24372	AACTCCGTCTTCCGCTCCTC	20	AsCpf1 RR
TGFBR24373	CAACTCCGTCTTCCGCTCCT	20	AsCpf1 RR
TGFBR24374	CCAACTCCGTCTTCCGCTCC	20	AsCpf1 RR
TGFBR24375	ACGCCAAGGGCAACCTACAG	20	AsCpf1 RR
TGFBR24376	CGCCAAGGGCAACCTACAGG	20	AsCpf1 RR
TGFBR24377	AGCTGATGACATGCCGCGTC	20	AsCpf1 RR
TGFBR24378	GGGCGAGGGAGCTGCCCAGC	20	AsCpf1 RR
TGFBR24379	CGGGCGAGGGAGCTGCCCAG	20	AsCpf1 RR
TGFBR24380	CCGGGCGAGGGAGCTGCCCA	20	AsCpf1 RR
TGFBR24381	TCGCCCGGGGGATTGCTCAC	20	AsCpf1 RR
TGFBR24382	ACATGGAGTGTGATCACTGT	20	AsCpf1 RR
TGFBR24383	CAGTGATCACACTCCATGTG	20	AsCpf1 RR
TGFBR24384	TGTGGGAGGCCCAAGATGCC	20	AsCpf1 RR
TGFBR24385	TGTGCACGATGGGCATCTTG	20	AsCpf1 RR
TGFBR24386	CGAGGATATTGGAGCTCTTG	20	AsCpf1 RR
TGFBR24387	ATATCCTCGTGAAGAACGAC	20	AsCpf1 RR
TGFBR24388	GACGCAGGGAAAGCCCAAAG	20	AsCpf1 RR

TGFBR24389	CTGCGTCTGGACCCTACTCT	20	AsCpf1 RR
TGFBR24390	TGCGTCTGGACCCTACTCTG	20	AsCpf1 RR
TGFBR24391	CAGACAGAGTAGGGTCCAGA	20	AsCpf1 RR
TGFBR24392	GCCAGCACGATCCCACCGCA	20	AsCpf1 RVR
TGFBR24393	AAGGAAAAAAAAAAGCCTGG	20	AsCpf1 RVR
TGFBR24394	ACACCAGCAATCCTGACTTG	20	AsCpf1 RVR
TGFBR24395	ACTAGCAACAAGTCAGGATT	20	AsCpf1 RVR
TGFBR24396	GCAACTCCCAGTGGTGGCAG	20	AsCpf1 RVR
TGFBR24397	TGTCATCATCATCTTCTACT	20	AsCpf1 RVR
TGFBR24398	GACCTCAGCAAAGCGACCTT	20	AsCpf1 RVR
TGFBR24399	AGGCCAAGCTGAAGCAGAAC	20	AsCpf1 RVR
TGFBR24400	AGGAGTATGCCTCTTGGAAG	20	AsCpf1 RVR
TGFBR24401	CCTCTTGGAAGACAGAGAAG	20	AsCpf1 RVR
TGFBR24402	TTCTCATGCTTCAGATTGAT	20	AsCpf1 RVR
TGFBR24403	CTCGTGAAGAACGACCTAAC	20	AsCpf1 RVR
TGFbR2036	GGCCGCTGCACATCGTCCTG	20	SpyCas9
TGFbR2037	GCGGGGTCTGCCATGGGTCG	20	SpyCas9
TGFbR2038	AGTTGCTCATGCAGGATTTC	20	SpyCas9
TGFbR2039	CCAGAATAAAGTCATGGTAG	20	SpyCas9
TGFbR2040	CCCCTACCATGACTTTATTC	20	SpyCas9
TGFbR2041	AAGTCATGGTAGGGGAGCTT	20	SpyCas9
TGFbR2042	AGTCATGGTAGGGGAGCTTG	20	SpyCas9
TGFbR2043	ATTGCACTCATCAGAGCTAC	20	SpyCas9
TGFbR2044	CCTAGAGTGAAGAGATTCAT	20	SpyCas9
TGFbR2045	CCAATGAATCTCTTCACTCT	20	SpyCas9
TGFbR2046	AAAGTCATGGTAGGGGAGCT	20	SpyCas9
TGFbR2047	GTGAGCAATCCCCCGGGCGA	20	SpyCas9
TGFbR2048	GTCGTTCTTCACGAGGATAT	20	SpyCas9
TGFbR2049	GCCGCGTCAGGTACTCCTGT	20	SpyCas9
TGFbR2050	GACGCGGCATGTCATCAGCT	20	SpyCas9
TGFbR2051	GCTTCTGCTGCCGGTTAACG	20	SpyCas9
TGFbR2052	GTGGATGACCTGGCTAACAG	20	SpyCas9
TGFbR2053	GTGATCACACTCCATGTGGG	20	SpyCas9
TGFbR2054	GCCCATTGAGCTGGACACCC	20	SpyCas9
TGFbR2055	GCGGTCATCTTCCAGGATGA	20	SpyCas9
TGFbR2056	GGGAGCTGCCCAGCTTGCGC	20	SpyCas9
TGFbR2057	GTTGATGTTGTTGGCACACG	20	SpyCas9
TGFbR2058	GGCATCTTGGGCCTCCCACA	20	SpyCas9
TGFbR2059	GCGGCATGTCATCAGCTGGG	20	SpyCas9
TGFbR2060	GCTCCTCAGCCGTCAGGAAC	20	SpyCas9
TGFbR2061	GCTGGTGTTATATTCTGATG	20	SpyCas9
TGFbR2062	CCGACTTCTGAACGTGCGGT	20	SpyCas9
TGFbR2063	TGCTGGCGATACGCGTCCAC	20	SpyCas9
TGFbR2064	CCCGACTTCTGAACGTGCGG	20	SpyCas9
TGFbR2065	CCACCGCACGTTCAGAAGTC	20	SpyCas9

TGFbR2066	TCACCCGACTTCTGAACGTG	20	SpyCas9
TGFbR2067	CCCACCGCACGTTCAGAAGT	20	SpyCas9
TGFbR2068	CGAGCAGCGGGGTCTGCCAT	20	SpyCas9
TGFbR2069	ACGAGCAGCGGGGTCTGCCA	20	SpyCas9
TGFbR2070	AGCGGGGTCTGCCATGGGTC	20	SpyCas9
TGFbR2071	CCTGAGCAGCCCCCGACCCA	20	SpyCas9
TGFbR2072	CCATGGGTCGGGGGCTGCTC	20	SpyCas9
TGFbR2073	AACGTGCGGTGGGATCGTGC	20	SpyCas9
TGFbR2074	GGACGATGTGCAGCGGCCAC	20	SpyCas9
TGFbR2075	GTCCACAGGACGATGTGCAG	20	SpyCas9
TGFbR2076	CATGGGTCGGGGGCTGCTCA	20	SpyCas9
TGFbR2077	CAGCGGGGTCTGCCATGGGT	20	SpyCas9
TGFbR2078	ATGGGTCGGGGGCTGCTCAG	20	SpyCas9
TGFbR2079	CGGGGTCTGCCATGGGTCGG	20	SpyCas9
TGFbR2080	AGGAAGTCTGTGTGGCTGTA	20	SpyCas9
TGFbR2081	CTCCATCTGTGAGAAGCCAC	20	SpyCas9
TGFbR2082	ATGATAGTCACTGACAACAA	20	SpyCas9
TGFbR2083	GATGCTGCAGTTGCTCATGC	20	SpyCas9
TGFbR2084	ACAGCCACACAGACTTCCTG	20	SpyCas9
TGFbR2085	GAAGCCACAGGAAGTCTGTG	20	SpyCas9
TGFbR2086	TTCCTGTGGCTTCTCACAGA	20	SpyCas9
TGFbR2087	CTGTGGCTTCTCACAGATGG	20	SpyCas9
TGFbR2088	TCACAAAATTTACACAGTTG	20	SpyCas9
TGFbR2089	GACAACATCATCTTCTCAGA	20	SpyCas9
TGFbR2090	TCCAGAATAAAGTCATGGTA	20	SpyCas9
TGFbR2091	GGTAGGGGAGCTTGGGGTCA	20	SpyCas9
TGFbR2092	TTCTCCAAAGTGCATTATGA	20	SpyCas9
TGFbR2093	CATCTTCCAGAATAAAGTCA	20	SpyCas9
TGFbR2094	CACATGAAGAAAGTCTCACC	20	SpyCas9
TGFbR2095	TTCCAGAATAAAGTCATGGT	20	SpyCas9
TGFbR2096	TTTTCCTTCATAATGCACTT	20	SpyCas9
TGFBR24024	CACAGTTGTGGAAACTTGAC	20	AsCpf1
TGFBR24039	CCCAACTCCGTCTTCCGCTC	20	AsCpf1
TGFBR24040	GGCTTTCCCTGCGTCTGGAC	20	AsCpf1
TGFBR24036	CTGAGGTCTATAAGGCCAAG	20	AsCpf1
TGFBR24026	TGATGTGAGATTTTCCACCT	20	AsCpf1
TGFBR24038	CCTATGAGGAGTATGCCTCT	20	AsCpf1
TGFBR24033	AAGTGACAGGCATCAGCCTC	20	AsCpf1
TGFBR24028	CCATGACCCCAAGCTCCCCT	20	AsCpf1
TGFBR24031	CTTCATAATGCACTTTGGAG	20	AsCpf1
TGFBR24032	TTCATGTGTTCCTGTAGCTC	20	AsCpf1
TGFBR24029	TTCTGGAAGATGCTGCTTCT	20	AsCpf1
TGFBR24035	CCCACCAGGGTGTCCAGCTC	20	AsCpf1
TGFBR24037	AGACAGTGGCAGTCAAGATC	20	AsCpf1
TGFBR24041	CCTGCGTCTGGACCCTACTC	20	AsCpf1

TGFBR24025	CACAACTGTGTAAATTTTGT	20	AsCpf1
TGFBR24030	GAGAAGCAGCATCTTCCAGA	20	AsCpf1
TGFBR24027	TGGTTGTCACAGGTGGAAAA	20	AsCpf1
TGFBR24034	CCAGGTTGAACTCAGCTTCT	20	AsCpf1
TGFBR24043	ATCACAAAATTTACACAGTTG	21	SauCas9
TGFBR24065	GGCATCAGCCTCCTGCCACCA	21	SauCas9
TGFBR24110	GTTAGCCAGGTCATCCACAGA	21	SauCas9
TGFBR24099	GCTGGGCAGCTCCCTCGCCCG	21	SauCas9
TGFBR24064	CAGGAGGCTGATGCCTGTCAC	21	SauCas9
TGFBR24094	GAGGAGCGGAAGACGGAGTTG	21	SauCas9
TGFBR24108	CGTCTGGACCCTACTCTGTCT	21	SauCas9
TGFBR24058	TTTTTCCTTCATAATGCACTT	21	SauCas9
TGFBR24075	CCATTGAGCTGGACACCCTGG	21	SauCas9
TGFBR24057	CTTCTCCAAAGTGCATTATGA	21	SauCas9
TGFBR24103	GCCCAAGATGCCCATCGTGCA	21	SauCas9
TGFBR24060	TCATGTGTTCCTGTAGCTCTG	21	SauCas9
TGFBR24048	GTGATGCTGCAGTTGCTCATG	21	SauCas9
TGFBR24087	TCTCATGCTTCAGATTGATGT	21	SauCas9
TGFBR24081	TCCCTATGAGGAGTATGCCTC	21	SauCas9
TGFBR24044	CATCACAAAATTTACACAGTT	21	SauCas9
TGFBR24077	ATTGAGCTGGACACCCTGGTG	21	SauCas9
TGFBR24080	CAGTCAAGATCTTTCCCTATG	21	SauCas9
TGFBR24046	AGGATTTCTGGTTGTCACAGG	21	SauCas9
TGFBR24101	TCCACAGTGATCACACTCCAT	21	SauCas9
TGFBR24079	AGCAGAACACTTCAGAGCAGT	21	SauCas9
TGFBR24072	CCGGCAAGACGCGGAAGCTCA	21	SauCas9
TGFBR24074	GATGTCAGAGCGGTCATCTTC	21	SauCas9
TGFBR24062	TCATTGCACTCATCAGAGCTA	21	SauCas9
TGFBR24054	CTTCCAGAATAAAGTCATGGT	21	SauCas9
TGFBR24045	AGATTTTCCACCTGTGACAAC	21	SauCas9
TGFBR24049	ACTGCAGCATCACCTCCATCT	21	SauCas9
TGFBR24098	AGCTGGGCAGCTCCCTCGCCC	21	SauCas9
TGFBR24090	TGACGGCTGAGGAGCGGAAGA	21	SauCas9
TGFBR24076	CATTGAGCTGGACACCCTGGT	21	SauCas9
TGFBR24078	AGCAAAGCGACCTTTCCCCAC	21	SauCas9
TGFBR24067	CGCGTTAACCGGCAGCAGAAG	21	SauCas9
TGFBR24063	GAAATATGACTAGCAACAAGT	21	SauCas9
TGFBR24107	AGACAGAGTAGGGTCCAGACG	21	SauCas9
TGFBR24047	CAGGATTTCTGGTTGTCACAG	21	SauCas9
TGFBR24096	CTCCTGTAGGTTGCCCTTGGC	21	SauCas9
TGFBR24105	ACAGAGTAGGGTCCAGACGCA	21	SauCas9
TGFBR24056	GCTTCTCCAAAGTGCATTATG	21	SauCas9
TGFBR24068	GCAGCAGAAGCTGAGTTCAAC	21	SauCas9
TGFBR24093	TGAGGAGCGGAAGACGGAGTT	21	SauCas9
TGFBR24055	CTTTGGAGAAGCAGCATCTTC	21	SauCas9

TGFBR24053	CTCCCCTACCATGACTTTATT	21	SauCas9
TGFBR24106	GACAGAGTAGGGTCCAGACGC	21	SauCas9
TGFBR24092	CTGAGGAGCGGAAGACGGAGT	21	SauCas9
TGFBR24102	GGGCATCTTGGGCCTCCCACA	21	SauCas9
TGFBR24082	CCAAGAGGCATACTCCTCATA	21	SauCas9
TGFBR24051	AGAATGACGAGAACATAACAC	21	SauCas9
TGFBR24097	CCTGACGCGGCATGTCATCAG	21	SauCas9
TGFBR24073	AGCGAGCACTGTGCCATCATC	21	SauCas9
TGFBR24104	GCAGGTTAGGTCGTTCTTCAC	21	SauCas9
TGFBR24050	ACCTCCATCTGTGAGAAGCCA	21	SauCas9
TGFBR24052	TAAAGTCATGGTAGGGGAGCT	21	SauCas9
TGFBR24061	TCAGAGCTACAGGAACACATG	21	SauCas9
TGFBR24086	TCTCAGACATCAATCTGAAGC	21	SauCas9
TGFBR24066	CATCAGCCTCCTGCCACCACT	21	SauCas9
TGFBR24089	CGCTCCTCAGCCGTCAGGAAC	21	SauCas9
TGFBR24071	AACCTGGGAAACCGGCAAGAC	21	SauCas9
TGFBR24095	TCCACGCCAAGGGCAACCTAC	21	SauCas9
TGFBR24100	GAGGTGAGCAATCCCCCGGGC	21	SauCas9
TGFBR24069	CAGCAGAAGCTGAGTTCAACC	21	SauCas9
TGFBR24083	TCCAAGAGGCATACTCCTCAT	21	SauCas9
TGFBR24070	AGCAGAAGCTGAGTTCAACCT	21	SauCas9
TGFBR24088	CCAGTTCCTGACGGCTGAGGA	21	SauCas9
TGFBR24085	AGGAGTATGCCTCTTGGAAGA	21	SauCas9
TGFBR24084	TTCCAAGAGGCATACTCCTCA	21	SauCas9
TGFBR24042	CAACTGTGTAAATTTTGTGAT	21	SauCas9
TGFBR24059	TGAAGGAAAAAAAAAAAGCCTG	21	SauCas9
TGFBR24091	CGTCTTCCGCTCCTCAGCCGT	21	SauCas9
TGFBR24109	CCAGGTCATCCACAGACAGAG	21	SauCas9
TGFBR2736	GCCTAGAGTGAAGAGATTCAT	21	SpyCas9
TGFBR2737	GTTCTCCAAAGTGCATTATGA	21	SpyCas9
TGFBR2738	GCATCTTCCAGAATAAAGTCA	21	SpyCas9

[163] 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 7.

Table 7. CISH gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme
CISH0873	CAACCGTCTGGTGGCCGACG	20	SpyCas9
CISH0874	CAGGATCGGGGCTGTCGCTT	20	SpyCas9
CISH0875	TCGGGCCTCGCTGGCCGTAA	20	SpyCas9
CISH0876	GAGGTAGTCGGCCATGCGCC	20	SpyCas9

CISH0877	CAGGTGTTGTCGGGCCTCGC	20	SpyCas9
CISH0878	GGAGGTAGTCGGCCATGCGC	20	SpyCas9
CISH0879	GGCATACTCAATGCGTACAT	20	SpyCas9
CISH0880	CCGCCTTGTCATCAACCGTC	20	SpyCas9
CISH0881	AGGATCGGGGCTGTCGCTTC	20	SpyCas9
CISH0882	CCTTGTCATCAACCGTCTGG	20	SpyCas9
CISH0883	TACTCAATGCGTACATTGGT	20	SpyCas9
CISH0884	GGGTTCCATTACGGCCAGCG	20	SpyCas9
CISH0885	GGCACTGCTTCTGCGTACAA	20	SpyCas9
CISH0886	GGTTGATGACAAGGCGGCAC	20	SpyCas9
CISH0887	TGCTGGGGCCTTCCTCGAGG	20	SpyCas9
CISH0888	TTGCTGGCTGTGGAGCGGAC	20	SpyCas9
CISH0889	TTCTCCTACCTTCGGGAATC	20	SpyCas9
CISH0890	GACTGGCTTGGGCAGTTCCA	20	SpyCas9
CISH0891	CATGCAGCCCTTGCCTGCTG	20	SpyCas9
CISH0892	AGCAAAGGACGAGGTCTAGA	20	SpyCas9
CISH0893	GCCTGCTGGGGCCTTCCTCG	20	SpyCas9
CISH0894	CAGACTCACCAGATTCCCGA	20	SpyCas9
CISH0895	ACCTCGTCCTTTGCTGGCTG	20	SpyCas9
CISH0896	CTCACCAGATTCCCGAAGGT	20	SpyCas9
CISH7048	TACGCAGAAGCAGTGCCCGC	20	AsCpf1
CISH7049	AGGTGTACAGCAGTGGCTGG	20	AsCpf1
CISH7050	GGTGTACAGCAGTGGCTGGT	20	AsCpf1
CISH7051	CGGATGTGGTCAGCCTTGTG	20	AsCpf1
CISH7052	CACTGACAGCGTGAACAGGT	20	AsCpf1
CISH7053	ACTGACAGCGTGAACAGGTA	20	AsCpf1
CISH7054	GCTCACTCTCTGTCTGGGCT	20	AsCpf1
CISH7055	CTGGCTGTGGAGCGGACTGG	20	AsCpf1
CISH7056	GCTCTGACTGTACGGGGCAA	20	AsCpf1 RR
CISH7057	AGCTCTGACTGTACGGGGCA	20	AsCpf1 RR
CISH7058	ACAGTACCCCTTCCAGCTCT	20	AsCpf1 RR
CISH7059	CGTCGGCCACCAGACGGTTG	20	AsCpf1 RR
CISH7060	CCAGCCACTGCTGTACACCT	20	AsCpf1 RR
CISH7061	ACCCCGGCCCTGCCTATGCC	20	AsCpf1 RR
CISH7062	GGTATCAGCAGTGCAGGAGG	20	AsCpf1 RR
CISH7063	GATGTGGTCAGCCTTGTGCA	20	AsCpf1 RR
CISH7064	GGATGTGGTCAGCCTTGTGC	20	AsCpf1 RR
CISH7065	GGCCACGCATCCTGGCCTTT	20	AsCpf1 RR
CISH7066	GAAAGGCCAGGATGCGTGGC	20	AsCpf1 RR
CISH7067	ACTGCTTGTCCAGGCCACGC	20	AsCpf1 RR
CISH7068	TCTGGACTCCAACTGCTTGT	20	AsCpf1 RR
CISH7069	GTCTGGACTCCAACTGCTTG	20	AsCpf1 RR
CISH7070	GCTTCCGTCTGGACTCCAAC	20	AsCpf1 RR
CISH7071	GACGGAAGCTGGAGTCGGCA	20	AsCpf1 RR
CISH7072	CGCTGTCAGTGAAAACCACT	20	AsCpf1 RR

CISH7073	CTGACAGCGTGAACAGGTAG	20	AsCpf1 RR
CISH7074	TTACGGCCAGCGAGGCCCGA	20	AsCpf1 RR
CISH7075	ATTACGGCCAGCGAGGCCCG	20	AsCpf1 RR
CISH7076	GGAATCTGGTGAGTCTGAGG	20	AsCpf1 RR
CISH7077	CCCTCAGACTCACCAGATTC	20	AsCpf1 RR
CISH7078	CGAAGGTAGGAGAAGGTCTT	20	AsCpf1 RR
CISH7079	GAAGGTAGGAGAAGGTCTTG	20	AsCpf1 RR
CISH7080	GCACCTTTGGCTCACTCTCT	20	AsCpf1 RR
CISH7081	TCGAGGAGGTGGCAGAGGGT	20	AsCpf1 RR
CISH7082	TGGAACTGCCCAAGCCAGTC	20	AsCpf1 RR
CISH7083	AGGGACGGGGCCCACAGGGG	20	AsCpf1 RR
CISH7084	GGGACGGGGCCCACAGGGGC	20	AsCpf1 RR
CISH7085	CTCCACAGCCAGCAAAGGAC	20	AsCpf1 RR
CISH7086	CAGCCAGCAAAGGACGAGGT	20	AsCpf1 RR
CISH7087	CTGCCTTCTAGACCTCGTCC	20	AsCpf1 RR
CISH7088	CCTAAGGAGGATGCGCCTAG	20	AsCpf1 RVR
CISH7089	TGGCCTCCTGCACTGCTGAT	20	AsCpf1 RVR
CISH7090	AGCAGTGCAGGAGGCCACAT	20	AsCpf1 RVR
CISH7091	CCGACTCCAGCTTCCGTCTG	20	AsCpf1 RVR
CISH7092	GGGGTTCCATTACGGCCAGC	20	AsCpf1 RVR
CISH7093	CACAGCAGATCCTCCTCTGG	20	AsCpf1 RVR
CISH7094	ATTGCCCCGTACAGTCAGAG	21	SauCas9
CISH7095	CCCGTACAGTCAGAGCTGGA	21	SauCas9
CISH7096	TGGTGGAGGAGCAGGCAGTG	21	SauCas9
CISH7097	TCCTTAGGCATAGGCAGGGC	21	SauCas9
CISH7098	CGGCCCTGCCTATGCCTAAG	21	SauCas9
CISH7099	TAGGCATAGGCAGGGCCGGG	21	SauCas9
CISH7100	AGGCAGGGCCGGGGTGGGAG	21	SauCas9
CISH7101	GCAGGATCGGGGCTGTCGCT	21	SauCas9
CISH7102	CTGCACAAGGCTGACCACAT	21	SauCas9
CISH7103	TGCACAAGGCTGACCACATC	21	SauCas9
CISH7104	CTGACCACATCCGGAAAGGC	21	SauCas9
CISH7105	GGCCACGCATCCTGGCCTTT	21	SauCas9
CISH7106	GCGTGGCCTGGACAAGCAGT	21	SauCas9
CISH7107	GACAAGCAGTTGGAGTCCAG	21	SauCas9
CISH7108	GTTGGAGTCCAGACGGAAGC	21	SauCas9
CISH7109	ATGCGTACATTGGTGGGGCC	21	SauCas9
CISH7110	TGGCCCCACCAATGTACGCA	21	SauCas9
CISH7111	GCTACCTGTTCACGCTGTCA	21	SauCas9
CISH7112	TGACAGCGTGAACAGGTAGC	21	SauCas9
CISH7113	GTCGGGCCTCGCTGGCCGTA	21	SauCas9
CISH7114	GCACTTGCCTAGGCTGGTAT	21	SauCas9
CISH7115	GGGAATCTGGTGAGTCTGAG	21	SauCas9
CISH7116	CTCACCAGATTCCCGAAGGT	21	SauCas9
CISH7117	CTCCTACCTTCGGGAATCTG	21	SauCas9

CISH7118	CAAGACCTTCTCCTACCTTC	21	SauCas9
CISH7119	CCAAGACCTTCTCCTACCTT	21	SauCas9
CISH7120	GCCAAGACCTTCTCCTACCT	21	SauCas9
CISH7121	TATGCACAGCAGATCCTCCT	21	SauCas9
CISH7122	CAAAGGTGCTGGACCCAGAG	21	SauCas9
CISH7123	GGCTCACTCTCTGTCTGGGC	21	SauCas9
CISH7124	AGGGTACCCCAGCCCAGACA	21	SauCas9
CISH7125	AGAGGGTACCCCAGCCCAGA	21	SauCas9
CISH7126	GTACCCTCTGCCACCTCCTC	21	SauCas9
CISH7127	CCTTCCTCGAGGAGGTGGCA	21	SauCas9
CISH7128	ATGACTGGCTTGGGCAGTTC	21	SauCas9
CISH7129	GGCCCCTGTGGGCCCCGTCC	21	SauCas9
CISH7130	AGGACGAGGTCTAGAAGGCA	21	SauCas9

[164] 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 8.

Table 8: B2M gRNAs

gRNA name	gRNA Targeting Domain Target sequence (DNA)	Length	Enzyme
B2M1	TATAAGTGGAGGCGTCGCGC	20	SpyCas9
B2M2	GGGCACGCGTTTAATATAAG	20	SpyCas9
B2M3	ACTCACGCTGGATAGCCTCC	20	SpyCas9
B2M4	GGCCGAGATGTCTCGCTCCG	20	SpyCas9
B2M5	CACGCGTTTAATATAAGTGG	20	SpyCas9
B2M6	AAGTGGAGGCGTCGCGCTGG	20	SpyCas9
B2M7	GAGTAGCGCGAGCACAGCTA	20	SpyCas9
B2M8	AGTGGAGGCGTCGCGCTGGC	20	SpyCas9
B2M9	GCCCGAATGCTGTCAGCTTC	20	SpyCas9
B2M10	CGCGAGCACAGCTAAGGCCA	20	SpyCas9
B2M11	CTCGCGCTACTCTCTCTTTC	20	SpyCas9
B2M12	GGCCACGGAGCGAGACATCT	20	SpyCas9
B2M13	CGTGAGTAAACCTGAATCTT	20	SpyCas9
B2M14	AGTCACATGGTTCACACGGC	20	SpyCas9
B2M15	AAGTCAACTTCAATGTCGGA	20	SpyCas9
B2M16	CAGTAAGTCAACTTCAATGT	20	SpyCas9
B2M17	ACCCAGACACATAGCAATTC	20	SpyCas9
B2M18	GCATACTCATCTTTTTCAGT	20	SpyCas9
B2M19	ACAGCCCAAGATAGTTAAGT	20	SpyCas9
B2M20	GGCATACTCATCTTTTTCAG	20	SpyCas9

B2M21	TTCCTGAAGCTGACAGCATT	20	SpyCas9
B2M22	TCACGTCATCCAGCAGAGAA	20	SpyCas9
B2M23	CAGCCCAAGATAGTTAAGTG	20	SpyCas9
B2M-c1	AAUUCUCUCUCCAUUCUU	18	AsCpf1
B2M-c2	AAUUCUCUCUCCAUUCUUC	19	AsCpf1
B2M-c3	AAUUCUCUCUCCAUUCUUCA	20	AsCpf1
B2M-c4	AAUUCUCUCUCCAUUCUUCAG	21	AsCpf1
B2M-c5	AAUUCUCUCUCCAUUCUUCAGU	22	AsCpf1
B2M-c6	AAUUCUCUCUCCAUUCUUCAGUA	23	AsCpf1
B2M-c7	AAUUCUCUCUCCAUUCUUCAGUAA	24	AsCpf1
B2M-c8	ACUUUCCAUUCUCUGCUG	18	AsCpf1
B2M-c9	ACUUUCCAUUCUCUGCUGG	19	AsCpf1
B2M-c10	ACUUUCCAUUCUCUGCUGGA	20	AsCpf1
B2M-c11	ACUUUCCAUUCUCUGCUGGAU	21	AsCpf1
B2M-c12	ACUUUCCAUUCUCUGCUGGAUG	22	AsCpf1
B2M-c13	ACUUUCCAUUCUCUGCUGGAUGA	23	AsCpf1
B2M-c14	ACUUUCCAUUCUCUGCUGGAUGAC	24	AsCpf1
B2M-c15	AGCAAGGACUGGUCUUUC	18	AsCpf1
B2M-c16	AGCAAGGACUGGUCUUUCU	19	AsCpf1
B2M-c17	AGCAAGGACUGGUCUUUCUA	20	AsCpf1
B2M-c18	AGCAAGGACUGGUCUUUCUAU	21	AsCpf1
B2M-c19	AGCAAGGACUGGUCUUUCUAUC	22	AsCpf1
B2M-c20	AGCAAGGACUGGUCUUUCUAUCU	23	AsCpf1
B2M-c21	AGCAAGGACUGGUCUUUCUAUCUC	24	AsCpf1
B2M-c22	AGUGGGGGUGAAUUCAGU	18	AsCpf1
B2M-c23	AGUGGGGGUGAAUUCAGUG	19	AsCpf1
B2M-c24	AGUGGGGGUGAAUUCAGUGU	20	AsCpf1
B2M-c25	AGUGGGGGUGAAUUCAGUGUA	21	AsCpf1
B2M-c26	AGUGGGGGUGAAUUCAGUGUAG	22	AsCpf1
B2M-c27	AGUGGGGGUGAAUUCAGUGUAGU	23	AsCpf1
B2M-c28	AGUGGGGGUGAAUUCAGUGUAGUA	24	AsCpf1
B2M-c29	AUCCAUCCGACAUUGAAG	18	AsCpf1
B2M-c30	AUCCAUCCGACAUUGAAGU	19	AsCpf1
B2M-c31	AUCCAUCCGACAUUGAAGUU	20	AsCpf1
B2M-c32	AUCCAUCCGACAUUGAAGUUG	21	AsCpf1
B2M-c33	AUCCAUCCGACAUUGAAGUUGA	22	AsCpf1
B2M-c34	AUCCAUCCGACAUUGAAGUUGAC	23	AsCpf1
B2M-c35	AUCCAUCCGACAUUGAAGUUGACU	24	AsCpf1
B2M-c36	CAAUUCUCUCUCCAUUCU	18	AsCpf1
B2M-c37	CAAUUCUCUCUCCAUUCUU	19	AsCpf1
B2M-c38	CAAUUCUCUCUCCAUUCUUC	20	AsCpf1
B2M-c39	CAAUUCUCUCUCCAUUCUUCA	21	AsCpf1

B2M-c40	CAAUUCUCUCUCCAUUCUUCAG	22	AsCpf1
B2M-c41	CAAUUCUCUCUCCAUUCUUCAGU	23	AsCpf1
B2M-c42	CAAUUCUCUCUCCAUUCUUCAGUA	24	AsCpf1
B2M-c43	CAGUGGGGGUGAAUUCAG	18	AsCpf1
B2M-c44	CAGUGGGGGUGAAUUCAGU	19	AsCpf1
B2M-c45	CAGUGGGGGUGAAUUCAGUG	20	AsCpf1
B2M-c46	CAGUGGGGGUGAAUUCAGUGU	21	AsCpf1
B2M-c47	CAGUGGGGGUGAAUUCAGUGUA	22	AsCpf1
B2M-c48	CAGUGGGGGUGAAUUCAGUGUAG	23	AsCpf1
B2M-c49	CAGUGGGGGUGAAUUCAGUGUAGU	24	AsCpf1
B2M-c50	CAUUCUCUGCUGGAUGAC	18	AsCpf1
B2M-c51	CAUUCUCUGCUGGAUGACG	19	AsCpf1
B2M-c52	CAUUCUCUGCUGGAUGACGU	20	AsCpf1
B2M-c53	CAUUCUCUGCUGGAUGACGUG	21	AsCpf1
B2M-c54	CAUUCUCUGCUGGAUGACGUGA	22	AsCpf1
B2M-c55	CAUUCUCUGCUGGAUGACGUGAG	23	AsCpf1
B2M-c56	CAUUCUCUGCUGGAUGACGUGAGU	24	AsCpf1
B2M-c57	CCCGAUAUUCCUCAGGUA	18	AsCpf1
B2M-c58	CCCGAUAUUCCUCAGGUAC	19	AsCpf1
B2M-c59	CCCGAUAUUCCUCAGGUACU	20	AsCpf1
B2M-c60	CCCGAUAUUCCUCAGGUACUC	21	AsCpf1
B2M-c61	CCCGAUAUUCCUCAGGUACUCC	22	AsCpf1
B2M-c62	CCCGAUAUUCCUCAGGUACUCCA	23	AsCpf1
B2M-c63	CCCGAUAUUCCUCAGGUACUCCAA	24	AsCpf1
B2M-c64	CCGAUAUUCCUCAGGUAC	18	AsCpf1
B2M-c65	CCGAUAUUCCUCAGGUACU	19	AsCpf1
B2M-c66	CCGAUAUUCCUCAGGUACUC	20	AsCpf1
B2M-c67	CCGAUAUUCCUCAGGUACUCC	21	AsCpf1
B2M-c68	CCGAUAUUCCUCAGGUACUCCA	22	AsCpf1
B2M-c69	CCGAUAUUCCUCAGGUACUCCAA	23	AsCpf1
B2M-c70	CCGAUAUUCCUCAGGUACUCCAAA	24	AsCpf1
B2M-c71	CUCACGUCAUCCAGCAGA	18	AsCpf1
B2M-c72	CUCACGUCAUCCAGCAGAG	19	AsCpf1
B2M-c73	CUCACGUCAUCCAGCAGAGA	20	AsCpf1
B2M-c74	CUCACGUCAUCCAGCAGAGAA	21	AsCpf1
B2M-c75	CUCACGUCAUCCAGCAGAGAAU	22	AsCpf1
B2M-c76	CUCACGUCAUCCAGCAGAGAAUG	23	AsCpf1
B2M-c77	CUCACGUCAUCCAGCAGAGAAUGG	24	AsCpf1
B2M-c78	CUGAAUUGCUAUGUGUCU	18	AsCpf1
B2M-c79	CUGAAUUGCUAUGUGUCUG	19	AsCpf1
B2M-c80	CUGAAUUGCUAUGUGUCUGG	20	AsCpf1
B2M-c81	CUGAAUUGCUAUGUGUCUGGG	21	AsCpf1

B2M-c82	CUGAAUUGCUAUGUGUCUGGGU	22	AsCpf1
B2M-c83	CUGAAUUGCUAUGUGUCUGGGUU	23	AsCpf1
B2M-c84	CUGAAUUGCUAUGUGUCUGGGUUU	24	AsCpf1
B2M-c85	GAGUACCUGAGGAAUAUC	18	AsCpf1
B2M-c86	GAGUACCUGAGGAAUAUCG	19	AsCpf1
B2M-c87	GAGUACCUGAGGAAUAUCGG	20	AsCpf1
B2M-c88	GAGUACCUGAGGAAUAUCGGG	21	AsCpf1
B2M-c89	GAGUACCUGAGGAAUAUCGGGA	22	AsCpf1
B2M-c90	GAGUACCUGAGGAAUAUCGGGAA	23	AsCpf1
B2M-c91	GAGUACCUGAGGAAUAUCGGGAAA	24	AsCpf1
B2M-c92	UAUCUCUUGUACUACACU	18	AsCpf1
B2M-c93	UAUCUCUUGUACUACACUG	19	AsCpf1
B2M-c94	UAUCUCUUGUACUACACUGA	20	AsCpf1
B2M-c95	UAUCUCUUGUACUACACUGAA	21	AsCpf1
B2M-c96	UAUCUCUUGUACUACACUGAAU	22	AsCpf1
B2M-c97	UAUCUCUUGUACUACACUGAAUU	23	AsCpf1
B2M-c98	UAUCUCUUGUACUACACUGAAUUC	24	AsCpf1
B2M-c99	UCAAUUCUCUCUCCAUUC	18	AsCpf1
B2M-c100	UCAAUUCUCUCUCCAUUCU	19	AsCpf1
B2M-c101	UCAAUUCUCUCUCCAUUCUU	20	AsCpf1
B2M-c102	UCAAUUCUCUCUCCAUUCUUC	21	AsCpf1
B2M-c103	UCAAUUCUCUCUCCAUUCUUCA	22	AsCpf1
B2M-c104	UCAAUUCUCUCUCCAUUCUUCAG	23	AsCpf1
B2M-c105	UCAAUUCUCUCUCCAUUCUUCAGU	24	AsCpf1
B2M-c106	UCACAGCCCAAGAUAGUU	18	AsCpf1
B2M-c107	UCACAGCCCAAGAUAGUUA	19	AsCpf1
B2M-c108	UCACAGCCCAAGAUAGUUAA	20	AsCpf1
B2M-c109	UCACAGCCCAAGAUAGUUAAG	21	AsCpf1
B2M-c110	UCACAGCCCAAGAUAGUUAAGU	22	AsCpf1
B2M-c111	UCACAGCCCAAGAUAGUUAAGUG	23	AsCpf1
B2M-c112	UCACAGCCCAAGAUAGUUAAGUGG	24	AsCpf1
B2M-c113	UCAGUGGGGGUGAAUUCA	18	AsCpf1
B2M-c114	UCAGUGGGGGUGAAUUCAG	19	AsCpf1
B2M-c115	UCAGUGGGGGUGAAUUCAGU	20	AsCpf1
B2M-c116	UCAGUGGGGGUGAAUUCAGUG	21	AsCpf1
B2M-c117	UCAGUGGGGGUGAAUUCAGUGU	22	AsCpf1
B2M-c118	UCAGUGGGGGUGAAUUCAGUGUA	23	AsCpf1
B2M-c119	UCAGUGGGGGUGAAUUCAGUGUAG	24	AsCpf1
B2M-c120	UGGCCUGGAGGCUAUCCA	18	AsCpf1
B2M-c121	UGGCCUGGAGGCUAUCCAG	19	AsCpf1
B2M-c122	UGGCCUGGAGGCUAUCCAGC	20	AsCpf1
B2M-c123	UGGCCUGGAGGCUAUCCAGCG	21	AsCpf1

B2M-c124	UGGCCUGGAGGCUAUCCAGCGU	22	AsCpf1
B2M-c125	UGGCCUGGAGGCUAUCCAGCGUG	23	AsCpf1
B2M-c126	UGGCCUGGAGGCUAUCCAGCGUGA	24	AsCpf1
B2M-c127	AUAGAUCGAGACAUGUAA	18	AsCpf1
B2M-c128	AUAGAUCGAGACAUGUAAG	19	AsCpf1
B2M-c129	AUAGAUCGAGACAUGUAAGC	20	AsCpf1
B2M-c130	AUAGAUCGAGACAUGUAAGCA	21	AsCpf1
B2M-c131	AUAGAUCGAGACAUGUAAGCAG	22	AsCpf1
B2M-c132	AUAGAUCGAGACAUGUAAGCAGC	23	AsCpf1
B2M-c133	AUAGAUCGAGACAUGUAAGCAGCA	24	AsCpf1
B2M-c134	CAUAGAUCGAGACAUGUA	18	AsCpf1
B2M-c135	CAUAGAUCGAGACAUGUAA	19	AsCpf1
B2M-c136	CAUAGAUCGAGACAUGUAAG	20	AsCpf1
B2M-c137	CAUAGAUCGAGACAUGUAAGC	21	AsCpf1
B2M-c138	CAUAGAUCGAGACAUGUAAGCA	22	AsCpf1
B2M-c139	CAUAGAUCGAGACAUGUAAGCAG	23	AsCpf1
B2M-c140	CAUAGAUCGAGACAUGUAAGCAGC	24	AsCpf1
B2M-c141	CUCCACUGUCUUUUUCAU	18	AsCpf1
B2M-c142	CUCCACUGUCUUUUUCAUA	19	AsCpf1
B2M-c143	CUCCACUGUCUUUUUCAUAG	20	AsCpf1
B2M-c144	CUCCACUGUCUUUUUCAUAGA	21	AsCpf1
B2M-c145	CUCCACUGUCUUUUUCAUAGAU	22	AsCpf1
B2M-c146	CUCCACUGUCUUUUUCAUAGAUC	23	AsCpf1
B2M-c147	CUCCACUGUCUUUUUCAUAGAUCG	24	AsCpf1
B2M-c148	UCAUAGAUCGAGACAUGU	18	AsCpf1
B2M-c149	UCAUAGAUCGAGACAUGUA	19	AsCpf1
B2M-c150	UCAUAGAUCGAGACAUGUAA	20	AsCpf1
B2M-c151	UCAUAGAUCGAGACAUGUAAG	21	AsCpf1
B2M-c152	UCAUAGAUCGAGACAUGUAAGC	22	AsCpf1
B2M-c153	UCAUAGAUCGAGACAUGUAAGCA	23	AsCpf1
B2M-c154	UCAUAGAUCGAGACAUGUAAGCAG	24	AsCpf1
B2M-c155	UCCACUGUCUUUUUCAUA	18	AsCpf1
B2M-c156	UCCACUGUCUUUUUCAUAG	19	AsCpf1
B2M-c157	UCCACUGUCUUUUUCAUAGA	20	AsCpf1
B2M-c158	UCCACUGUCUUUUUCAUAGAU	21	AsCpf1
B2M-c159	UCCACUGUCUUUUUCAUAGAUC	22	AsCpf1
B2M-c160	UCCACUGUCUUUUUCAUAGAUCG	23	AsCpf1
B2M-c161	UCCACUGUCUUUUUCAUAGAUCGA	24	AsCpf1
B2M-c162	UCUCCACUGUCUUUUUCA	18	AsCpf1
B2M-c163	UCUCCACUGUCUUUUUCAU	19	AsCpf1
B2M-c164	UCUCCACUGUCUUUUUCAUA	20	AsCpf1
B2M-c165	UCUCCACUGUCUUUUUCAUAG	21	AsCpf1

B2M-c166	UCUCCACUGUCUUUUUCAUAGA	22	AsCpf1
B2M-c167	UCUCCACUGUCUUUUUCAUAGAU	23	AsCpf1
B2M-c168	UCUCCACUGUCUUUUUCAUAGAUC	24	AsCpf1
B2M-c169	UUCUCCACUGUCUUUUUC	18	AsCpf1
B2M-c170	UUCUCCACUGUCUUUUUCA	19	AsCpf1
B2M-c171	UUCUCCACUGUCUUUUUCAU	20	AsCpf1
B2M-c172	UUCUCCACUGUCUUUUUCAUA	21	AsCpf1
B2M-c173	UUCUCCACUGUCUUUUUCAUAG	22	AsCpf1
B2M-c174	UUCUCCACUGUCUUUUUCAUAGA	23	AsCpf1
B2M-c175	UUCUCCACUGUCUUUUUCAUAGAU	24	AsCpf1
B2M-c176	UUUCUCCACUGUCUUUUU	18	AsCpf1
B2M-c177	UUUCUCCACUGUCUUUUUC	19	AsCpf1
B2M-c178	UUUCUCCACUGUCUUUUUCA	20	AsCpf1
B2M-c179	UUUCUCCACUGUCUUUUUCAU	21	AsCpf1
B2M-c180	UUUCUCCACUGUCUUUUUCAUA	22	AsCpf1
B2M-c181	UUUCUCCACUGUCUUUUUCAUAG	23	AsCpf1
B2M-c182	UUUCUCCACUGUCUUUUUCAUAGA	24	AsCpf1
B2M-c183	UUUUCUCCACUGUCUUUU	18	AsCpf1
B2M-c184	UUUUCUCCACUGUCUUUUU	19	AsCpf1
B2M-c185	UUUUCUCCACUGUCUUUUUC	20	AsCpf1
B2M-c186	UUUUCUCCACUGUCUUUUUCA	21	AsCpf1
B2M-c187	UUUUCUCCACUGUCUUUUUCAU	22	AsCpf1
B2M-c188	UUUUCUCCACUGUCUUUUUCAUA	23	AsCpf1
B2M-c189	UUUUCUCCACUGUCUUUUUCAUAG	24	AsCpf1

[165] 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 9.

Table 9: NKG2A gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	Enzyme
NKG2A55	GAGGTAAAGCGTTTGCATTTG	21	AsCpf1
NKG2A56	CCTCTAAAGCTTATGCTTACA	21	AsCpf1
NKG2A57	AGTCGATTTACTTGTAGCACT	21	AsCpf1
NKG2A58	CTTGTAGCACTGCACAGTTAA	21	AsCpf1
NKG2A59	TCCATTACAGGATAAAAGACT	21	AsCpf1
NKG2A60	CTCCATTACAGGATAAAAGAC	21	AsCpf1
NKG2A61	TCTCCATTACAGGATAAAAGA	21	AsCpf1
NKG2A62	ATCCTGTAATGGAGAAAAATC	21	AsCpf1
NKG2A63	TCCTGTAATGGAGAAAAATCC	21	AsCpf1

NKG2A136	AAACATGAGTAAGTTGTTTTG	21	AsCpf1
NKG2A137	GCTTTCAAACATGAGTAAGTT	21	AsCpf1
NKG2A138	AAAGCCAAACCATTCATTGTC	21	AsCpf1
NKG2A139	GTAACAGCAGTCATCATCCAT	21	AsCpf1
NKG2A140	ACCATCCTCATGGATTGGTGT	21	AsCpf1
NKG2A141	TGTCCATCATTTCACCATCCT	21	AsCpf1
NKG2A142	GAAATTTCTGTCCATCATTTC	21	AsCpf1
NKG2A143	AGAAATTTCTGTCCATCATTT	21	AsCpf1
NKG2A144	TTTTAGAAATTTCTGTCCATC	21	AsCpf1
NKG2A145	CTTTTAGAAATTTCTGTCCAT	21	AsCpf1
NKG2A146	TTTTCTTTTAGAAATTTCTGT	21	AsCpf1
NKG2A147	TAAAAGAAAAGAAAGAATTTT	21	AsCpf1
NKG2A270	AAACATTTACATCTTACCATT	21	AsCpf1
NKG2A271	CATCTTACCATTTCTTCTTCA	21	AsCpf1
NKG2A272	TATAGATAATGAAGAAGAAAT	21	AsCpf1
NKG2A273	TTCTTCATTATCTATAGAAAG	21	AsCpf1
NKG2A274	CTGGCCTGTACTTCGAAGAAC	21	AsCpf1
NKG2A275	CTTACCAATGTAGTAACAACT	21	AsCpf1
NKG2A276	GCACGTCATTGTGGCCATTGT	21	AsCpf1
NKG2A277	TTTAGCACGTCATTGTGGCCA	21	AsCpf1
NKG2A414	CCATCAGCTCCAGAGAAGCTC	21	AsCpf1
NKG2A415	TCTCCCTGCAGATTTACCATC	21	AsCpf1
NKG2A437	AAATGCTTTACCTTTGCAGTG	21	AsCpf1
NKG2A438	AATGCTTTACCTTTGCAGTGA	21	AsCpf1
NKG2A439	CCTTTGCAGTGATAGGTTTTG	21	AsCpf1
NKG2A440	CAGTGATAGGTTTTGTCATTC	21	AsCpf1
NKG2A441	AAGGGAATGACAAAACCTATC	21	AsCpf1
NKG2A442	CAAGGGAATGACAAAACCTAT	21	AsCpf1
NKG2A443	GTCATTCCCTTGAAAATCCTG	21	AsCpf1
NKG2A444	TCATTCCCTTGAAAATCCTGA	21	AsCpf1
NKG2A445	TGAAGGTTTAATTCCGCATAG	21	AsCpf1
NKG2A446	GAAGGTTTAATTCCGCATAGG	21	AsCpf1
NKG2A447	AAGGTTTAATTCCGCATAGGT	21	AsCpf1
NKG2A448	ATTCCGCATAGGTTATTTCCT	21	AsCpf1
NKG2A449	GCAACTGAACAGGAAATAACC	21	AsCpf1
NKG2A450	AGCAACTGAACAGGAAATAAC	21	AsCpf1
NKG2A451	CTGTTCAGTTGCTAAAATGGA	21	AsCpf1
NKG2A452	TATTGCCTTTAGGTTTTCGTT	21	AsCpf1
NKG2A453	ATTGCCTTTAGGTTTTCGTTG	21	AsCpf1
NKG2A454	TTGCCTTTAGGTTTTCGTTGC	21	AsCpf1
NKG2A455	GGTTTTCGTTGCTGCCTCTTT	21	AsCpf1
NKG2A456	CGTTGCTGCCTCTTTGGGTTT	21	AsCpf1

NKG2A457	GTTGCTGCCTCTTTGGGTTTG	21	AsCpf1
NKG2A458	GGTTTGGGGGCAGATTCAGGT	21	AsCpf1
NKG2A459	GGGGCAGATTCAGGTCTGAGT	21	AsCpf1

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

RNA-guided nucleases

[167] 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.

[168] 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.

[169] Various RNA-guided nucleases may require different sequential relationships between PAMs and protospacers. For example, Cas9 nucleases recognize PAM sequences that are 3' of the protospacer, while

[170] Cpf1, on the other hand, generally recognizes PAM sequences that are 5' of the protospacer.

[171] 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. And 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).

[172] 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), incorporated by reference herein), or that that do not cut at all.

Cas9

[173] Crystal structures have been determined for *S. pyogenes* Cas9 (Jinek 2014), and for *S. aureus* Cas9 in complex with a unimolecular guide RNA and a target DNA (Nishimasu 2014; Anders 2014; and Nishimasu 2015).

[174] 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.

[175] 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.

[176] 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

[177] The crystal structure of *Acidaminococcus sp.* Cpf1 in complex with crRNA and a double-stranded (ds) DNA 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.

[178] 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.

Modifications of RNA-guided nucleases

[179] The RNA-guided nucleases described above 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.

[180] 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 and Yamano, as well as in Cotta-Ramusino. 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 .

[181] 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 (Kleinstiver I) and *S. aureus* (Kleinstiver *et al.*, Nat Biotechnol. 2015 Dec; 33(12): 1293–1298 (Klienstiver II)). Kleinstiver *et al.* have also described modifications that improve the targeting fidelity of Cas9 (Nature, 2016 January 28; 529, 490-495 (Kleinstiver III)). Each of these references is incorporated by reference herein.

[182] 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 (Zetsche II), incorporated by reference), and by Fine *et al.* (Sci Rep. 2015 Jul 1;5:10777 (Fine), incorporated by reference).

[183] 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 for all purposes herein.

[184] 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.

[185] 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.

[186] 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). 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.

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

[188] MGHHHHHHGSTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKAR
NDHYKELKPIIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNA
IHDYFIGRTDNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTT
YFSGFYENRKNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAI
GIFVSTSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDET
AHIIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALF
NELNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKH
EDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGL
YHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQMPT
LASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDA
AKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKT
GDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQR
IAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAEL
FYRPKSRMKRMAARLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNV
ITKEVSHEIIKDRRFTSDKFFFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGE
RNLIYITVIDSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLS
QVIHEIVDLMIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEK
VGGVLNPYQLTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHF
LEGFDFLHYDVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKR
IVPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRS
VLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKE
SKDLKLQNGISNQDWLAYIQELRNGSPKKKRKVGSPKKKRKV

[189] Cpf1 variant 1 amino acid sequence (SEQ ID NO:[XX])

[190] MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRI
YKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDNLTD
AINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENRKNVFS
AEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIEEVFSF
PFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPHRFIPL
FKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLTHIFIS
HKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEIISAAGK
ELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAVDESNE
VDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQRPTLASGWDVNKEKNNG
AILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCSTQLKAV
TAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREALCKWI
DFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGK
LYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHR
LGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRR
FTSDKFLFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGK
ILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQ
AVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQLTDQF
TSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHYDVKTG
DFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHRFTGRY
RDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNAATGED
YINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNGISNQD
WLAYIQELRNGRSSDDEATADSQHAAPPKKKRKVGGSGGSGGSGGSGGSGGSGGSGGSLEHH
HHHH

[191] Cpf1 variant 2 amino acid sequence (SEQ ID NO:[XX])

[192] MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKP
IIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRT
DNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENR
KNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIE
EVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPH
RFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLT
HIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEII
SAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAV
DESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQMPTLASGWDVNK
EKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCST
QLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREA
LCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMK
RMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
IKDRRFTSDKFFFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVI
DSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDL
MIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQ
LTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHY
DVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHR
FTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNA
ATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNG
ISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRKVGGSGGSGGSGGSGGSGGSGGSGG
SLEHHHHHH

[193] Cpf1 variant 3 amino acid sequence (SEQ ID NO:1096)

[194] MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKP
IIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRT
DNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENR
KNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIE
EVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPH
RFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLT
HIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEII
SAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAV
DESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQRPTLASGWDVNK
EKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCST
QLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREA
LCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMK
RMAARLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
IKDRRFTSDKFLFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVI
DSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDL
MIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQ
LTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHY
DVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHR
FTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNA
ATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNG
ISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRKVGGSGGSGGSGGSGGSGGSGGSGG
SLEHHHHHH

[195] Cpf1 variant 4 amino acid sequence (SEQ ID NO:1097)

[196] MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKP
IIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRT
DNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENR
KNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIE
EVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPH
RFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLT
HIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEII
SAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAV
DESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQRPTLASGWDVNK
EKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCST
QLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREA
LCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMK
RMAARLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
IKDRRFTSDKFLFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVI
DSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDL
MIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQ
LTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHY
DVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHR
FTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNA
ATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNG
ISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRKV

[197] Cpf1 variant 5 amino acid sequence (SEQ ID NO:1107)

[198] MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKP
IIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRT
DNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENR
KNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIE
EVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPH
RFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLT
HIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEII
SAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAV
DESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQRPTLASGWDVNK
EKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCST
QLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREA
LCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMK
RMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
IKDRRFTSDKFLFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVI
DSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDL
MIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQ
LTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHY
DVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHR
FTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNA
ATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNG
ISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRKV

[199] Cpf1 variant 6 amino acid sequence (SEQ ID NO:1108)

[200] MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKP
IIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRT
DNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENR
KNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIE
EVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPH
RFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLT
HIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEII
SAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAV
DESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQRPTLASGWDVNK
EKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCST
QLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREA
LCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMK
RMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
IKDRRFTSDKFLFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVI
DSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDL
MIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQ
LTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHY
DVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHR
FTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNA
ATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNG
ISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRKVGGSGGSGGSGGSGGSGGSGGSGG
SLEHHHHHH

[201] Cpf1 variant 7 amino acid sequence (SEQ ID NO:[[XX]])

[202] MGRDPGKPIPNPLLGLDSTAPKKKRKVGIHGVPAATQFEGFTNLYQVSKTLR
FELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYKTYADQCLQLVQLDWENLSAAI
DSYRKEKTEETRNALIEEQATYRNAIHDYFIGRTDNLTDAINKRHAEIYKGLFKAELFNGKV
LKQLGTVTTTEHENALLRSFDKFTTYFSGFYENRKNVFSAEDISTAIPHRIVQDNFPKFKEN
CHIFTRLITAVPSLREHFENVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQIDLYNQLLGGIS
REAGTEKIKGLNEVLNLAIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFILEEFKSDEE
VIQSFCKYKTLLRNENVLETAEALFNELNSIDLTHIFISHKKLETISSALCDHWDTLRNALY
ERRISELTGKITKSAKEKVQRSLKHEDINLQEIISAAGKELSEAFKQKTSEILSHAHAALDQ
PLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFY
NKARNYATKKPYSVEKFKLNFQMPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYK
ALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCSTQLKAVTAHFQTHTTPILLSNNFIEPLEI
TKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRP
SSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPNLHT
LYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQKTPIPDTLYQ
ELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFFFHVPITLNYQAANSPS
KFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSLNTIQQFDYQKKLDNRE
KERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVVLENLNFGFKSKRTGIAEKA
VYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQLTDQFTSFAKMGTQSGFLFYVPAPYTSK
IDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRGLPGFMP
AWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDG
SNILPKLLENDDSHAIDTMVALIRSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPEW
PMDADANGAYHIALKGQLLLNHLKESKDLKLQNGISNQDWLAYIQELRNPKKKRKVKLAAAL
EHHHHHH

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

[204] MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKP
IIDRIYKTYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNALIEEQATYRNAIHDYFIGRT
DNLTDAINKRHAEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENR
KNVFSAEDISTAIPHRIVQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIE
EVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPH
RFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLLRNENVLETAEALFNELNSIDLT
HIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKHEDINLQEII
SAAGKELSEAFKQKTSEILSHAHAALDQPLPTTLKKQEEKEILKSQLDSLLGLYHLLDWFAV
DESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQMPTLASGWDVNK
EKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTEKTSEGFDKMYYDYFPDAAKMIPKCST
QLKAVTAHFQTHTTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREA
LCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMK
RMAHRLGEKMLNKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
IKDRRFTSDKFFFHVPITLNYQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVI
DSTGKILEQRSLNTIQQFDYQKKLDNREKERVAARQAWSVVGTIKDLKQGYLSQVIHEIVDL
MIHYQAVVVLENLNFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQ
LTDQFTSFAKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDFLHY
DVKTGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPFIAGKRIVPVIENHR
FTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDDSHAIDTMVALIRSVLQMRNSNA
ATGEDYINSPVRDLNGVCFDSRFQNPEWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNG
ISNQDWLAYIQELRN

Nucleic acids encoding RNA-guided nucleases

[205] 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).

[206] 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.

[207] 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.

[208] 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.

Functional analysis of candidate molecules

[209] Candidate RNA-guided nucleases, gRNAs, and complexes thereof, can be evaluated by standard methods known in the art. See, *e.g.* Cotta-Ramusino. The stability of RNP complexes may be evaluated by differential scanning fluorimetry, as described below.

Differential Scanning Fluorimetry (DSF)

[210] The thermostability of ribonucleoprotein (RNP) complexes comprising gRNAs and RNA-guided nucleases can be measured via DSF. The DSF technique measures the thermostability of a protein, which can increase under favorable conditions such as the addition of a binding RNA molecule, *e.g.*, a gRNA.

[211] A DSF assay can be performed according to any suitable protocol, and can be employed in any suitable setting, including without limitation (a) testing different conditions (*e.g.* different stoichiometric ratios of gRNA: RNA-guided nuclease protein, different buffer solutions, *etc.*) to identify optimal conditions for RNP formation; and (b) testing modifications (*e.g.* chemical modifications, alterations of sequence, *etc.*) of an RNA-guided nuclease and/or a gRNA to identify those modifications that improve RNP formation or

stability. One readout of a DSF assay is a shift in melting temperature of the RNP complex; a relatively high shift suggests that the RNP complex is more stable (and may thus have greater activity or more favorable kinetics of formation, kinetics of degradation, or another functional characteristic) relative to a reference RNP complex characterized by a lower shift. When the DSF assay is deployed as a screening tool, a threshold melting temperature shift may be specified, so that the output is one or more RNPs having a melting temperature shift at or above the threshold. For instance, the threshold can be 5-10°C (*e.g.* 5°, 6°, 7°, 8°, 9°, 10°) or more, and the output may be one or more RNPs characterized by a melting temperature shift greater than or equal to the threshold.

[212] Two non-limiting examples of DSF assay conditions are set forth below:

[213] To determine the best solution to form RNP complexes, a fixed concentration (*e.g.* 2 μM) of Cas9 in water+10x SYPRO Orange® (Life Technologies cat#S-6650) is dispensed into a 384 well plate. An equimolar amount of gRNA diluted in solutions with varied pH and salt is then added. After incubating at room temperature for 10' and brief centrifugation to remove any bubbles, a Bio-Rad CFX384™ Real-Time System C1000 Touch™ Thermal Cycler with the Bio-Rad CFX Manager software is used to run a gradient from 20°C to 90°C with a 1°C increase in temperature every 10 seconds.

[214] The second assay consists of mixing various concentrations of gRNA with fixed concentration (*e.g.* 2 μM) Cas9 in optimal buffer from assay 1 above and incubating (*e.g.* at RT for 10') in a 384 well plate. An equal volume of optimal buffer + 10x SYPRO Orange® (Life Technologies cat#S-6650) is added and the plate sealed with Microseal® B adhesive (MSB-1001). Following brief centrifugation to remove any bubbles, a Bio-Rad CFX384™ Real-Time System C1000 Touch™ Thermal Cycler with the Bio-Rad CFX Manager software is used to run a gradient from 20°C to 90°C with a 1°C increase in temperature every 10 seconds.

Genome Editing Strategies

[215] The genome editing systems described above are used, in various embodiments of the present disclosure, to generate edits in (*i.e.* to alter) targeted regions of DNA within or obtained from a cell. Various strategies are described herein to generate particular edits, and these strategies are generally described in terms of the desired repair outcome, the number and positioning of individual edits (*e.g.* SSBs or DSBs), and the target sites of such edits.

[216] Genome editing strategies that involve the formation of SSBs or DSBs are characterized by repair outcomes including: (a) deletion of all or part of a targeted region; (b) insertion into or replacement of all or part of a targeted region; or (c) interruption of all or

part of a targeted region. This grouping is not intended to be limiting, or to be binding to any particular theory or model, and is offered solely for economy of presentation. Skilled artisans will appreciate that the listed outcomes are not mutually exclusive and that some repairs may result in other outcomes. The description of a particular editing strategy or method should not be understood to require a particular repair outcome unless otherwise specified.

[217] Replacement of a targeted region generally involves the replacement of all or part of the existing sequence within the targeted region with a homologous sequence, for instance through gene correction or gene conversion, two repair outcomes that are mediated by HDR pathways. HDR is promoted by the use of a donor template, which can be single-stranded or double stranded, as described in greater detail below. Single or double stranded templates can be exogenous, in which case they will promote gene correction, or they can be endogenous (*e.g.* a homologous sequence within the cellular genome), to promote gene conversion. Exogenous templates can have asymmetric overhangs (*i.e.* the portion of the template that is complementary to the site of the DSB may be offset in a 3' or 5' direction, rather than being centered within the donor template), for instance as described by Richardson *et al.* (Nature Biotechnology 34, 339–344 (2016), (Richardson), incorporated by reference). In instances where the template is single stranded, it can correspond to either the complementary (top) or non-complementary (bottom) strand of the targeted region.

Gene constructs

[218] In some aspects, the present disclosure provides complex editing strategies, and resulting modified cells having complex genomic alterations, that allow for the generation of advanced NK cell products for clinical applications, *e.g.*, for immunooncology therapeutic approaches.

[219] In some embodiments, the genomic alterations are introduced by use of one or more HDR expression constructs. In some embodiments, the genomic alterations are introduced by use of one or more HDR expression constructs. In some embodiments, the one or more HDR expression constructs comprise one or more donor HDR templates. In some embodiments, the one or more donor HDR templates comprise one or more expression cassettes encoding one or more cDNAs. In some embodiments, the donor HDR template comprises one expression cassette. In some embodiments, the donor HDR template comprises two expression cassettes. In some embodiments, the donor HDR template comprises three expression cassettes. In some embodiments, the donor HDR template comprises four expression cassettes. In some embodiments, the donor HDR template comprises five expression cassettes. In some embodiments, the donor HDR template comprises six

expression cassettes. In some embodiments, the donor HDR template comprises seven expression cassettes. In some embodiments, the donor HDR template comprises eight expression cassettes. In some embodiments, the donor HDR template comprises nine expression cassettes. In some embodiments, the donor HDR template comprises ten expression cassettes. In some embodiments, the one or more expression cassette is monocistronic. In some embodiments, the one or more expression cassette is bicistronic.

[220] In some embodiments, the one or more expression cassettes comprise one cDNA. In some embodiments, the one or more expression cassettes comprise two cDNAs. In some embodiments, the one or more expression cassettes comprise three cDNAs. In some embodiments, the one or more expression cassettes comprise four cDNAs. In some embodiments, the one or more expression cassettes comprise five cDNAs. In some embodiments, the one or more expression cassettes comprise six cDNAs. In some embodiments, the one or more expression cassettes comprise seven cDNAs. In some embodiments, the one or more expression cassettes comprise eight cDNAs. In some embodiments, the one or more expression cassettes comprise nine cDNAs. In some embodiments, the one or more expression cassettes comprise ten cDNAs. In some embodiments, the one or more expression cassettes comprise one or more cDNAs separated by a 2A sequence. In some embodiments, the one or more expression cassettes comprise two cDNAs separated by a 2A sequence. In some embodiments, the one or more expression cassettes comprise three cDNAs separated by a 2A sequence.

[221] In some embodiments, the HDR expression construct comprises one or more cDNAs driven by a heterologous promoter.

[222] In some embodiments, the one or more expression cassettes comprise a cDNA for the expression of one or more genes listed in Table 10.

[223] In some embodiments, the HDR expression construct comprises one or more donor templates for inserting an inactivating mutation in a target gene, wherein the gene product has less, or no, function (being partially or wholly inactivated). In some embodiments, the HDR expression construct comprises one or more donor templates for inserting an inactivating mutation in a target gene, wherein the gene product has no function (wholly inactivated).

[224] In some embodiments, the modified cell of the disclosure comprises at least one exogenous nucleic acid construct encoding a cDNA of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of two or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any

combination of three or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of four or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of five or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of six or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of seven or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of eight or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of nine or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of ten or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10.

[225] In some embodiments, the modified NK cell of the disclosure, comprises at least one exogenous nucleic acid construct encoding a cDNA of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of two or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of three or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of four or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of five or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of six or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of seven or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of eight or more exogenous nucleic acid constructs encoding cDNAs of one or

more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of nine or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10. In some embodiments, the modified cell of the disclosure, comprise any combination of ten or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10.

[226] In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least one or more genes listed in Table 11, or any combination of two or more thereof. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least two or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least three or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least four or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least five or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least six or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least seven or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least eight or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least nine or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least ten or more genes listed in Table 11.

[227] In some embodiments, the modified NK cell of the disclosure, exhibits a loss of function of at least one or more genes listed in Table 11, or any combination of two or more thereof. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least two or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least three or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least four or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least five or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least six or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least seven or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least eight or more genes listed in Table 11. In some embodiments, the modified cell of the

disclosure exhibits a loss of function of at least nine or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure exhibits a loss of function of at least ten or more genes listed in Table 11.

[228] In some embodiments, the modified cell of the disclosure comprises at least one exogenous nucleic acid construct encoding a cDNA of one or more genes listed in Table 10 and exhibits a loss of function of at least one gene listed in Table 11. In some embodiments, the modified cell of the disclosure, comprise any combination of two or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10 and at least one gene listed in Table 11. In some embodiments, the modified cell of the disclosure comprises at least one exogenous nucleic acid construct encoding cDNAs of one or more genes listed in Table 10 and a loss of function of two or more genes listed in Table 11. In some embodiments, the modified cell of the disclosure comprises two or more exogenous nucleic acid constructs encoding cDNAs of one or more genes listed in Table 10 and a loss of function of two or more genes listed in Table 11.

[229] Gene conversion and gene correction are facilitated, in some cases, by the formation of one or more nicks in or around the targeted region, as described in Ran and Cotta-Ramusino. In some cases, a dual-nickase strategy is used to form two offset SSBs that, in turn, form a single DSB having an overhang (*e.g.* a 5' overhang).

[230] Interruption and/or deletion of all or part of a targeted sequence can be achieved by a variety of repair outcomes. As one example, a sequence can be deleted by simultaneously generating two or more DSBs that flank a targeted region, which is then excised when the DSBs are repaired, as is described in Maeder for the LCA10 mutation. As another example, a sequence can be interrupted by a deletion generated by formation of a double strand break with single-stranded overhangs, followed by exonucleolytic processing of the overhangs prior to repair.

[231] One specific subset of target sequence interruptions is mediated by the formation of an indel within the targeted sequence, where the repair outcome is typically mediated by NHEJ pathways (including Alt-NHEJ). NHEJ is referred to as an "error prone" repair pathway because of its association with indel mutations. In some cases, however, a DSB is repaired by NHEJ without alteration of the sequence around it (a so-called "perfect" or "scarless" repair); this generally requires the two ends of the DSB to be perfectly ligated. Indels, meanwhile, are thought to arise from enzymatic processing of free DNA ends before they are ligated that adds and/or removes nucleotides from either or both strands of either or both free ends.

[232] Because the enzymatic processing of free DSB ends may be stochastic in nature, indel mutations tend to be variable, occurring along a distribution, and can be influenced by a variety of factors, including the specific target site, the cell type used, the genome editing strategy used, *etc.* Even so, it is possible to draw limited generalizations about indel formation: deletions formed by repair of a single DSB are most commonly in the 1-50 bp range, but can reach greater than 100-200 bp. Insertions formed by repair of a single DSB tend to be shorter and often include short duplications of the sequence immediately surrounding the break site. However, it is possible to obtain large insertions, and in these cases, the inserted sequence has often been traced to other regions of the genome or to plasmid DNA present in the cells.

[233] Indel mutations – and genome editing systems configured to produce indels – are useful for interrupting target sequences, for example, when the generation of a specific final sequence is not required and/or where a frameshift mutation would be tolerated. They can also be useful in settings where particular sequences are preferred, insofar as the certain sequences desired tend to occur preferentially from the repair of an SSB or DSB at a given site. Indel mutations are also a useful tool for evaluating or screening the activity of particular genome editing systems and their components. In these and other settings, indels can be characterized by (a) their relative and absolute frequencies in the genomes of cells contacted with genome editing systems and (b) the distribution of numerical differences relative to the unedited sequence, *e.g.* ±1, ±2, ±3, *etc.* As one example, in a lead-finding setting, multiple gRNAs can be screened to identify those gRNAs that most efficiently drive cutting at a target site based on an indel readout under controlled conditions. Guides that produce indels at or above a threshold frequency, or that produce a particular distribution of indels, can be selected for further study and development. Indel frequency and distribution can also be useful as a readout for evaluating different genome editing system implementations or formulations and delivery methods, for instance by keeping the gRNA constant and varying certain other reaction conditions or delivery methods.

Multiplex Strategies

[234] While exemplary strategies discussed above have focused on repair outcomes mediated by single DSBs, genome editing systems according to this disclosure may also be employed to generate two or more DSBs, either in the same locus or in different loci. Strategies for editing that involve the formation of multiple DSBs, or SSBs, are described in, for instance, Cotta-Ramusino. In some embodiments, where multiple edits are made in the genome of an NK cell, or a cell that an NK cell is derived from, the edits are made at the

same time or in close temporal proximity. In some such embodiments, two or more genomic edits are effected by two or more different RNA-guided nucleases. For example, one of the genomic edits may be effected by saCas9 (in connection with the respective saCas9 guide RNA), and a different genomic edit may be effected by Cpf1 (in connection with the respective Cpf1 guide RNA). In some embodiments, using different RNA-guided nucleases in the context of multiplex genomic editing approaches is advantageous as compared to using the same RNA-guided nuclease for two or more edits, *e.g.*, in that it allows to decrease the likelihood or frequency of undesirable effects, such as, for example, off-target cutting, and the occurrence of genomic translocations.

Donor template design

[235] Donor template design is described in detail in the literature, for instance in Cotta-Ramusino. DNA oligomer donor templates (oligodeoxynucleotides or ODNs), which can be single stranded (ssODNs) or double-stranded (dsODNs), can be used to facilitate HDR-based repair of DSBs, and are particularly useful for introducing alterations into a target DNA sequence, inserting a new sequence into the target sequence, or replacing the target sequence altogether.

[236] Whether single-stranded or double stranded, donor templates generally include regions that are homologous to regions of DNA within or near (*e.g.* flanking or adjoining) a target sequence to be cleaved. These homologous regoins are referred to here as "homology arms," and are illustrated schematically below:

[5' homology arm] — [replacement sequence] —- [3' homology arm].

[237] The homology arms can have any suitable length (including 0 nucleotides if only one homology arm is used), and 3' and 5' homology arms can have the same length, or can differ in length. The selection of appropriate homology arm lengths can be influenced by a variety of factors, such as the desire to avoid homologies or microhomologies with certain sequences such as Alu repeats or other very common elements. For example, a 5' homology arm can be shortened to avoid a sequence repeat element. In other embodiments, a 3' homology arm can be shortened to avoid a sequence repeat element. In some embodiments, both the 5' and the 3' homology arms can be shortened to avoid including certain sequence repeat elements. In addition, some homology arm designs can improve the efficiency of editing or increase the frequency of a desired repair outcome. For example, Richardson *et al.* Nature Biotechnology 34, 339–344 (2016) (Richardson), which is incorporated by reference, found that the relative asymmetry of 3' and 5' homology arms of single stranded donor templates influenced repair rates and/or outcomes.

[238] Replacement sequences in donor templates have been described elsewhere, including in Cotta-Ramusino *et al.* A replacement sequence can be any suitable length (including zero nucleotides, where the desired repair outcome is a deletion), and typically includes one, two, three or more sequence modifications relative to the naturally-occurring sequence within a cell in which editing is desired. One common sequence modification involves the alteration of the naturally-occurring sequence to repair a mutation that is related to a disease or condition of which treatment is desired. Another common sequence modification involves the alteration of one or more sequences that are complementary to, or code for, the PAM sequence of the RNA-guided nuclease or the targeting domain of the gRNA(s) being used to generate an SSB or DSB, to reduce or eliminate repeated cleavage of the target site after the replacement sequence has been incorporated into the target site.

[239] Where a linear ssODN is used, it can be configured to (i) anneal to the nicked strand of the target nucleic acid, (ii) anneal to the intact strand of the target nucleic acid, (iii) anneal to the plus strand of the target nucleic acid, and/or (iv) anneal to the minus strand of the target nucleic acid. An ssODN may have any suitable length, *e.g.*, about, at least, or no more than 150-200 nucleotides (*e.g.*, 150, 160, 170, 180, 190, or 200 nucleotides).

[240] It should be noted that a template nucleic acid can also be a nucleic acid vector, such as a viral genome or circular double stranded DNA, *e.g.*, a plasmid. Nucleic acid vectors comprising donor templates can include other coding or non-coding elements. For example, a template nucleic acid can be delivered as part of a viral genome (*e.g.* in an AAV or lentiviral genome) that includes certain genomic backbone elements (*e.g.* inverted terminal repeats, in the case of an AAV genome) and optionally includes additional sequences coding for a gRNA and/or an RNA-guided nuclease. In certain embodiments, the donor template can be adjacent to, or flanked by, target sites recognized by one or more gRNAs, to facilitate the formation of free DSBs on one or both ends of the donor template that can participate in repair of corresponding SSBs or DSBs formed in cellular DNA using the same gRNAs. Exemplary nucleic acid vectors suitable for use as donor templates are described in Cotta-Ramusino.

[241] Whatever format is used, a template nucleic acid can be designed to avoid undesirable sequences. In certain embodiments, one or both homology arms can be shortened to avoid overlap with certain sequence repeat elements, *e.g.*, Alu repeats, LINE elements, *etc.*

Quantitative measurement of on-target gene editing

[242] It should be noted that the genome editing systems of the present disclosure allow for the detection and quantitative measurement of on-target gene editing outcomes, including

targeted integration. The compositions and methods described herein can rely on the use of donor templates comprising a 5' homology arm, a cargo, a one or more priming sites, a 3' homology arm, and optionally stuffer sequence. For example, International Patent Publication No. WO2019/014564 by Ramusino *et al.* (Ramusino), which is incorporated by reference herein in its entirety, describes compositions and methods which allow for the quantitative analysis of on-target gene editing outcomes, including targeted integration events, by embedding one or more primer binding sites (*i.e.*, priming sites) into a donor template that are substantially identical to a priming site present at the targeted genomic DNA locus (*i.e.*, the target nucleic acid). The priming sites are embedded into the donor template such that, when homologous recombination of the donor template with a target nucleic acid occurs, successful targeted integration of the donor template integrates the priming sites from the donor template into the target nucleic acid such that at least one amplicon can be generated in order to quantitatively determine the on-target editing outcomes.

[243] In some embodiments, the target nucleic acid comprises a first priming site (P1) and a second priming site (P2), and the donor template comprises a cargo sequence, a first priming site (P1'), and a second priming site (P2'), wherein P2' is located 5' from the cargo sequence, wherein P1' is located 3' from the cargo sequence (*i.e.*, A1--P2'--N--P1'--A2), wherein P1' is substantially identical to P1, and wherein P2' is substantially identical to P2. After accurate homology-driven targeted integration, three amplicons are produced using a single PCR reaction with two oligonucleotide primers. The first amplicon, Amplicon X, is generated from the primer binding sites originally present in the genomic DNA (P1 and P2), and may be sequenced to analyze on-target editing events that do not result in targeted integration (*e.g.,* insertions, deletions, gene conversion). The remaining two amplicons are mapped to the 5' and 3' junctions after homology-driven targeted integration. The second amplicon, Amplicon Y, results from the amplification of the nucleic acid sequence between P1 and P2' following a targeted integration event at the target nucleic acid, thereby amplifying the 5' junction. The third amplicon, Amplicon Z, results from the amplification of the nucleic acid sequence between P1' and P2 following a targeted integration event at the target nucleic acid, thereby amplifying the 3' junction. Sequencing of these amplicons provides a quantitative assessment of targeted integration at the target nucleic acid, in addition to information about the fidelity of the targeted integration. To avoid any biases inherent to amplicon size, stuffer sequence may optionally be included in the donor template to keep all three expected amplicons the same length.

Implementation of genome editing systems: delivery, formulations, and routes of administration

[244] As discussed above, the genome editing systems of this disclosure can be implemented in any suitable manner, meaning that the components of such systems, including without limitation the RNA-guided nuclease, gRNA, and optional donor template nucleic acid, can be delivered, formulated, or administered in any suitable form or combination of forms that results in the transduction, expression or introduction of a genome editing system and/or causes a desired repair outcome in a cell, tissue or subject. The genome editing systems according to this disclosure can incorporate multiple gRNAs, multiple RNA-guided nucleases, and other components such as proteins, and a variety of implementations will be evident to the skilled artisan based on the principles illustrated in systems of the disclosure. In some embodiments the genome editing system of the disclosure are delivered into cells as an ribonucleoprotein (RNP) complex. In some embodiments, one or more RNP complexes are delivered to the cell sequentially in any order, or simultaneously.

[245] Nucleic acids encoding the various elements of a genome editing system according to the present disclosure can be administered to subjects or delivered into cells by art-known methods or as described herein. For example, RNA-guided nuclease-encoding and/or gRNA-encoding DNA, as well as donor template nucleic acids can be delivered by, *e.g.,* vectors (*e.g.,* viral or non-viral vectors), non-vector based methods (*e.g.,* using naked DNA or DNA complexes), or a combination thereof. In some embodiments the genome editing system of the disclosure are delivered by AAV.

[246] Nucleic acids encoding genome editing systems or components thereof can be delivered directly to cells as naked DNA or RNA, for instance by means of transfection or electroporation, or can be conjugated to molecules (*e.g.,* N-acetylgalactosamine) promoting uptake by the target cells (*e.g.,* erythrocytes, HSCs). In some embodiments the genome editing system of the disclosure are delivered into cells by electroporation.

[247] One promising solution to improve cell therapy processes consists on the direct delivery of active proteins into human cells. A protein delivery agent, the Feldan Shuttle, is a protein-based delivery agent, which is designed for cell therapy (Del'Guidice et al., PLoS One. 2018 Apr 4;13(4):e0195558; incorporated in its entirety herein by reference). In some embodiments the genome editing system of the disclosure are delivered into cells by the Feldan Shuttle.

[248] The modified cells of the disclosure can be administered by any known routes of administration known to a person of kill in the art, at the time of filing this application. In

some embodiments the modified cells of the disclosure are administered intravenously (IV). In some embodiments the modified NK cells of the disclosure are administered intravenously (IV).

[249] As used herein, "dose" refers to a specific quantity of a pharmacologically active material for administration to a subject for a given time. Unless otherwise specified, the doses recited refer to NK cells having complex genomic alterations, that allow for the generation of advanced NK cell products for clinical applications. In some embodiments, a dose of modified NK cells refers to an effective amount of modified NK cells. For example, in some embodiments a dose or effective amount of modified NK cells refers to about $1 \times 10^9 - 5 \times 10^9$ modified NK cells, or about $2 \times 10^9 - 5 \times 10^9$ modified NK cells per dose. In some embodiments a dose or effective amount of modified NK cells refers to about $3 \times 10^9 - 5 \times 10^9$ modified NK cells, or about $4 \times 10^9 - 5 \times 10^9$ modified NK cells per dose.

Generation of modified iNK cells

[250] Some aspects of this disclosure relate to the generation of genetically modified NK cells that are derived from stem cells, *e.g.*, from multipotent cells, such as, *e.g.*, HSCs, or from pluripotent stem cells, such as, *e.g.*, ES cells or iPS cells. In some embodiments, where genetically modified iNK cells are derived from iPS cells, the iPS cells are derived from a somatic donor cell. In some embodiments, where genetically modified iNK cells are derived from iPS cells, the iPS cells are derived from a multipotent donor cell, *e.g.*, from an HSC.

[251] The genomic edits present in the final iNK cell can be made at any stage of the process of reprogramming the donor cell to the iPS cell state, during the iPS cell state, and/or at any stage of the process of differentiating the iPS cell to an iNK state, *e.g.*, at an intermediary state, such as, for example, an iPS cell-derived HSC state, or even up to or at the final iNK cell state. In some embodiments, one or more genomic edits present in a modified iNK cell provided herein is made before reprogramming the donor cell to the iPS cell state. In some embodiments, all edits present in a 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 iPS cell stage, or during the differentiation process. In some embodiments, two or more edits present in a modified iNK cell provided herein are made at different times and/or at different cell stages of the reprogramming/differentiation process. For example, in some embodiments, an edit is made at the donor cell stage and an different edit is made at the iPS cell stage; in some embodiments, an edit is made at the

reprogramming stage and a different edit is made at the iPS cell stage. These examples are provided to illustrate some of the strategies provided herein, and are not meant to be limiting.

[252] A variety of cell types can be used as a donor cell that can be subjected to the reprogramming, differentiation, and genomic editing strategies provided herein for the derivation of modified iNK cells. The donor cell to be subjected to the reprogramming, differentiation, and genomic editing strategies provided herein can be any suitable cell type. 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.

[253] In some embodiments, the donor cell is a human cell. In some embodiments, the donor cell is a non-human primate cell. In some embodiments, the donor cell is a mammalian cell. In some embodiments, the donor cell is a somatic cell. In some embodiments, the donor cell is a stem or progenitor cell. In certain embodiments, the donor cell is not part of a human embryo and its derivation does not involve the destruction of a human embryo.

[254] In some embodiments, iNK cells, and methods of deriving such iNK cells, having one or more genomic alterations (*e.g.*, a knock-out of a gene undesirable for immunooncology therapeutic approaches, and/or a knock-in of an exogenous nucleic acid, *e.g.* an expression construct encoding a gene product desirable for immunooncology therapeutic approaches) are provided herein. In some embodiments, the iNK cells are derived from an iPS cell, which in turn is derived from a somatic donor cell. Any suitable somatic cell can be used in the generation of iPS cells, and in turn, the generation of iNK cells. Suitable strategies for deriving iPS cells from various somatic donor cell types have been described and are known in the art. In some embodiments, the somatic donor cell is a fibroblast cell. In some embodiments, the somatic donor cell is a mature T cell.

[255] For example, in some embodiments, the somatic donor cell, from which an iPS cell, 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 induced pluripotent stem (iPS) cell, and throughout differentiation of the resulting iPS cell to a somatic cell.

[256] In certain embodiments, the 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.

[257] One advantage of using T cells for the generation of iPS cells is that T cells can be edited with relative ease, *e.g.*, by CRISPR-based methods or other gene-editing methods. Another advantage of using T cells for the generation of iPS cells is that the rearranged TCR locus allows for genetic tracking of individual cells and their daughter cells. 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.

[258] A third 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 pose a concern when editing cells by CRISPR technology, and in particular when generating cells carrying multiple edits.

[259] A fourth advantage of using T cell derived iPS cells as a starting point for the derivation of therapeutic lymphocytes is that it allows 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 iPS cells, and then deriving lymphocytes from these iPS cells that express the TCR (*e.g.*, T cells). This strategy would also allow for activating the TCR in other cell types, *e.g.*, by genetic or epigenetic strategies.

[260] A fifth advantage of using T cell derived iPS cells as a starting point for iNK differentiation is that the 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 will 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.

[261] In certain embodiments, the donor cell being manipulated, *e.g.*, the cell being reprogrammed and/or the cell, the genome of which is being edited, is 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.

[262] In certain embodiments, the donor cell is 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 certain embodiments, the donor cell is 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 certain embodiments, the donor cell is a myeloid progenitor cell (*e.g.,* a common myeloid progenitor (CMP) cell or granulocyte macrophage progenitor (GMP) cell). In certain embodiments, the donor cell is a lymphoid progenitor cell, *e.g.,* a common lymphoid progenitor (CLP) cell. In certain embodiments, the donor cell is an erythroid progenitor cell (*e.g.,* an MEP cell). In certain embodiments, the donor cell is 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 certain embodiments, the donor cell is 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 certain embodiments, the donor cell is a mobilized peripheral blood hematopoietic $CD34^+$ cell (after the patient is treated with a mobilization agent, *e.g.,* G-CSF or Plerixafor). In certain embodiments, the donor cell is a peripheral blood endothelial cell.

[263] In some embodiments, the donor cell is a dividing cell. In other embodiments, the donor cell is a non-dividing cell.

[264] In some embodiments, the modified iNK cells resulting from the methods and strategies of reprogramming, differentiating, and editing provided herein, are administered to a subject in need thereof, *e.g.*, in the context of an immunooncology therapeutic approach. In some embodiments, donor cells, or any cells of any stage of the reprogramming, differentiating, and 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.

Cell Reprogramming

[265] 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. In other words, a reprogrammed cell is one that is in a less differentiated state than the same cell in a non-reprogrammed state.

[266] The reprogramming of the cells of the disclosure can be performed by utilizing several methods. Examples of some methods for reprogramming somatic cells of the disclosure are described in, but are not limited to, Valamehr *et al.* WO2017/078807 ("Valamehr") and Mendlein *et al.* WO2010/108126 ("Mendlein"), which are hereby incorporated by reference in their entireties.

[267] Briefly, a method for directing differentiation of pluripotent stem cells into cells of a definitive hematopoietic lineage, may comprise: (i) contacting pluripotent stem cells with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of mesodermal cells from the pluripotent stem cells; (ii) contacting the mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, wherein the composition is optionally free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of mesodermal cells having definitive HE potential from the mesodermal cells; (iii) contacting the mesodermal cells having definitive HE potential with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; and optionally, a Wnt pathway activator, wherein the composition is optionally free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of definitive hemogenic endothelium from pluripotent stem cell-derived mesodermal cells having definitive hemogenic endothelium potential; and optionally, subjecting pluripotent stem cells, pluripotent stem cell-derived mesodermal cells, mesodermal cells having hemogenic

endothelium, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%.

[268] In some embodiments of the method for directing differentiation of pluripotent stem cells into cells of a hematopoietic lineage, the method further comprises contacting pluripotent stem cells with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, wherein the composition is free of TGFβ receptor/ALK inhibitors, to seed and expand the pluripotent stem cells. In some embodiments, the pluripotent stem cells are iPSCs. In some embodiments, the iPSCs are naïve iPSCs. In some embodiments, the iPSC comprises one or more genetic imprints, and wherein the one or more genetic imprints comprised in the iPSC are retained in the pluripotent stem cell derived hematopoietic cells differentiated therefrom.

[269] In some embodiments of the method for directing differentiation of pluripotent stem cells into cells of a hematopoietic lineage, the differentiation of the pluripotent stem cells into cells of hematopoietic lineage is void of generation of embryoid bodies, and is in a monolayer culturing form.

[270] In some embodiments of the above method, the obtained pluripotent stem cell-derived definitive hemogenic endothelium cells are CD34+. In some embodiments, the obtained definitive hemogenic endothelium cells are CD34+CD43−. In some embodiments, the definitive hemogenic endothelium cells are CD34+CD43−CXCR4−CD73−. In some embodiments, the definitive hemogenic endothelium cells are CD34+ CXCR4−CD73−. In some embodiments, the definitive hemogenic endothelium cells are CD34+CD43−CD93−. In some embodiments, the definitive hemogenic endothelium cells are CD34+ CD93−.

[271] In some embodiments of the above method, the method further comprises (i) contacting pluripotent stem cell-derived definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L, TPO, and IL7; and optionally a BMP activator; to initiate the differentiation of the definitive hemogenic endothelium to pre-T cell progenitors; and optionally, (ii) contacting the pre-T cell progenitors with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7, but free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors, to initiate the differentiation of the pre-T cell progenitors to T cell progenitors or T cells. In some embodiments of the method, the pluripotent stem cell-derived T cell progenitors are CD34+CD45+CD7+. In some

embodiments of the method, the pluripotent stem cell-derived T cell progenitors are CD45+CD7+.

[272] In yet some embodiments of the above method for directing differentiation of pluripotent stem cells into cells of a hematopoietic lineage, the method further comprises: (i) contacting pluripotent stem cell-derived definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L, TPO, IL3, IL7, and IL15; and optionally, a BMP activator, to initiate differentiation of the definitive hemogenic endothelium to pre-NK cell progenitor; and optionally, (ii) contacting pluripotent stem cells-derived pre-NK cell progenitors with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, IL3, IL7, and IL15, wherein the medium is free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors, to initiate differentiation of the pre-NK cell progenitors to NK cell progenitors or NK cells. In some embodiments, the pluripotent stem cell-derived NK progenitors are CD3−CD45+CD56+CD7+. In some embodiments, the pluripotent stem cell-derived NK cells are CD3−CD45+CD56+, and optionally further defined by NKp46+, CD57+ and CD16+.

[273] In yet some embodiments of the above method for directing differentiation of pluripotent stem cells into NK cells, the method further comprises knocking out the gene Nrg1 in the pluripotent stem cells.

[274] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived T lineage cells, which comprises: (i) contacting pluripotent stem cells with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of mesodermal cells from pluripotent stem cells; (ii) contacting the mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, but free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of the mesodermal cells having definitive HE potential from the mesodermal cells; (iii) contacting mesodermal cells having definitive HE potential with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; and optionally, a Wnt pathway activator; wherein the composition is free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of definitive hemogenic endothelium from mesodermal cells having definitive HE potential; (iv) contacting definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L, TPO, and IL7; and

optionally a BMP activator; to initiate differentiation of the definitive hemogenic endothelium to pre-T cell progenitors; and (v) contacting the pre-T cell progenitors with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7, wherein the composition is free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors; to initiate differentiation of the pre-T cell progenitors to T cell progenitors or T cells; and optionally, the seeded pluripotent stem cells, mesodermal cells, mesodermal cells having definitive HE potential, and/or definitive hemogenic endothelium may be subject to low oxygen tension between about 2% to about 10%. In some embodiments, group II of the above method further comprises: contacting iPSCs with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGFβ receptor/ALK inhibitors, to seed and expand pluripotent stem cells; and/or wherein the pluripotent stem cells. In some embodiments, the pluripotent stem cells are iPSCs. In some embodiments, the iPSCs are naïve iPSC. In some embodiments of the method, the differentiation of the pluripotent stem cells into T cell lineages is void of generation of embryoid bodies, and is in a monolayer culturing format.

[275] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived NK lineage cells, which comprises: (i) contacting pluripotent stem cells with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of mesodermal cells from the pluripotent stem cells; (ii) contacting mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, and optionally free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of mesodermal cells having definitive HE potential from mesodermal cells; (iii) contacting mesodermal cells having definitive HE potential with a composition comprising one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; a ROCK inhibitor; optionally a Wnt pathway activator; and optionally free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of pluripotent stem cell-derived definitive hemogenic endothelium from the pluripotent stem cell-derived mesodermal cells having definitive HE potential; (iv) contacting pluripotent stem cell-derived definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L, TPO, IL3, IL7, and IL15, and optionally, a BMP activator, to initiate differentiation of the pluripotent stem cell-derived definitive hemogenic endothelium to pre-NK cell progenitors; and (v) contacting pluripotent stem cell-derived pre-NK cell progenitors with a composition comprising one or more growth

factors and cytokines selected from the group consisting of SCF, Flt3L, IL3, IL7, and IL15, but free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors, to initiate differentiation of the pluripotent stem cell-derived pre-NK cell progenitors to pluripotent stem cell-derived NK cell progenitors or NK cells; and optionally, subjecting seeded pluripotent stem cells, pluripotent stem cell-derived-mesodermal cells, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%. In some embodiments, the method for generating pluripotent stem cell-derived NK lineage cells of group II further comprises contacting iPSCs with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGFβ receptor/ALK inhibitors, to seed and expand the iPSCs. In some embodiments, the iPSCs are naïve iPSCs. In some embodiments, the method for generating pluripotent stem cell-derived NK lineage cells is void of generation of embryoid bodies, and is in a monolayer culturing format.

[276] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived definitive hemogenic endothelium, the method comprises: (i) contacting iPSCs with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of pluripotent stem cell-derived mesodermal cells from pluripotent stem cells; (ii) contacting pluripotent stem cell-derived mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, and optionally free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of pluripotent stem cell-derived mesodermal cells having definitive HE potential from pluripotent stem cell-derived mesodermal cells; (iii) contacting pluripotent stem cell-derived mesodermal cells having definitive HE potential with a composition comprising one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; a ROCK inhibitor; and optionally a Wnt pathway activator, and optionally free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of pluripotent stem cell-derived definitive hemogenic endothelium from the pluripotent stem cell-derived mesodermal cells having definitive HE potential; and optionally, subjecting seeded pluripotent stem cells, pluripotent stem cell-derived mesodermal cells, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%. In some embodiments, the above method for generating pluripotent stem cell-derived definitive hemogenic endothelium, further comprises: contacting iPSCs with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGFβ receptor/ALK inhibitors, to seed and expand the iPSCs; and/or wherein the iPSCs are naïve iPSCs. In some embodiments, the iPSC comprises one or more genetic imprints, and wherein the one or more

genetic imprints comprised in the iPSC are retained in the pluripotent stem cell derived definitive hemogenic endothelium cells differentiated therefrom. In some embodiments, the above method of differentiating iPSCs into cells of a definitive hemogenic endothelium is void of generation of embryoid bodies, and is in monolayer culturing format.

[277] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived multipotent progenitors of hematopoietic lineage, comprising: (i) contacting iPSCs with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of pluripotent stem cell-derived mesodermal cells from iPSCs; (ii) contacting pluripotent stem cell-derived mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, but free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of the mesodermal cells having definitive HE potential from the mesodermal cells; (iii) contacting mesodermal cells having definitive HE potential with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; and optionally, a Wnt pathway activator, wherein the composition is free of TGFβ receptor/ALK inhibitor, to initiate differentiation and expansion of definitive hemogenic endothelium from mesodermal cells having definitive HE potential; (iv) contacting definitive hemogenic endothelium with a composition comprising a BMP activator, a ROCK inhibitor, one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GMCSF, EPO, bFGF, VEGF, SCF, IL6, Flt3L and IL11, to initiate differentiation of definitive hemogenic endothelium to pre-HSC; and (v) contacting pre-HSC with a composition comprising a BMP activator, one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GMCSF, EPO, bFGF, VEGF, SCF, IL6, and IL11, but free of ROCK inhibitor, to initiate differentiation of the pre-HSC to hematopoietic multipotent progenitors; and optionally, subjecting seeded pluripotent stem cells, mesodermal cells, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%. In some embodiments, the above method for generating pluripotent stem cell-derived hematopoiesis multipotent progenitors further comprises contacting pluripotent stem cells with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGFβ receptor/ALK inhibitors, to seed and expand the pluripotent stem cells. In some embodiments, the pluripotent stem cells are iPSCs. In some embodiments, the iPSCs are naïve iPSCs. In some embodiments, the iPSC comprises one or more genetic imprints, and wherein the one or more genetic imprints comprised in the iPSC are retained in the pluripotent stem cell derived hematopoietic multipotent progenitor cells differentiated

therefrom. In some embodiments, the differentiation of the pluripotent stem cells into hematopoiesis multipotent progenitors using the above method is void of generation of embryoid bodies, and is in monolayer culturing format.

[278] In some embodiments, the disclosure provides a composition comprising: one or more cell populations generated from the culture platform disclosed herein: pluripotent stem cells-derived (i) CD34+ definitive hemogenic endothelium (iCD34), wherein the iCD34 cells have capacity to differentiate into multipotent progenitor cells, T cell progenitors, NK cell progenitors, T cells, NK cells, NKT cells and B cells, and wherein the iCD34 cells are CD34+CD43−; (ii) definitive hemogenic endothelium (iHE), wherein the iHE cells are CD34+, and at least one of CD43−, CD93−, CXCR4−, CD73−, and CXCR4−CD73−; (iii) pluripotent stem cell-derived definitive HSCs, wherein the iHSC is CD34+CD45+; (iv) hematopoietic multipotent progenitor cells, wherein the iMPP cells are CD34+CD45+; (v) T cell progenitors, wherein the T cell progenitors are CD34+CD45+CD7+ or CD34−CD45+CD7+; (vi) T cells, wherein the T cells are CD45+CD3+CD4+ or CD45+CD3+CD8+; (vii) NK cell progenitors, wherein the NK cell progenitors are CD45+CD56+CD7+; (viii) NK cells, wherein the NK cells are CD3−CD45+CD56+, and optionally further defined by NKp46+, CD57+, and CD16+; (ix) NKT cells, wherein the NKT cells are CD45+Vα24Jα18+CD3+; and (x) B cells, wherein the B cells are CD45+CD19+.

[279] In some embodiments, the disclosure provides one or more cell lines, or clonal cells generated using the methods disclosed herein: pluripotent stem cell-derived (i) CD34+ definitive hemogenic endothelium (iCD34), wherein the iCD34 cells have capacity to differentiate into multipotent progenitor cells, T cell progenitors, NK cell progenitors, T cells, NK cells, and NKT cells, and wherein the iCD34 cells are CD34+CD43−; (ii) definitive hemogenic endothelium (iHE), wherein the iHE cell line or clonal cells are CD34+, and at least one of CD43−, CD93−, CXCR4−, CD73−, and CXCR4−CD73−; (iii) definitive HSCs, wherein the iHSCs is CD34+CD45+; (iv) hematopoietic multipotent progenitor cells (iMPP), wherein the iMPP cells are CD34+CD45+; (v) T cell progenitors, wherein the T cell progenitors are CD34+CD45+CD7+ or CD34−CD45+CD7+; (vi) T cells, wherein the T cells are CD45+CD3+CD4+ or CD45+CD3+CD8+; (vii) NK cell progenitors, wherein the NK cell progenitors are CD45+CD56+CD7+; (viii) NK cells, wherein the NK cells are CD3−CD45+CD56+, and optionally further defined by NKp46+, CD57+, and CD16+; (ix) NKT cells, wherein the NKT cells are CD45+Vα24Jα18+CD3+; and (x) B cells, wherein the B cells are CD45+CD19+.

[280] In some embodiments, the present disclosure provides a method of promoting hematopoietic self-renewal, reconstitution or engraftment using one or more of cell populations, cell lines or clonal cells generated using methods as disclosed: pluripotent stem cell-derived (i) CD34+ definitive hemogenic endothelium (iCD34), wherein the iCD34 cells have capacity to differentiate into multipotent progenitor cells, T cell progenitors, NK cell progenitors, T cells NK cells and NKT cells, and wherein the iCD34 cells are CD34+CD43−; (ii) definitive hemogenic endothelium (iHE), wherein the iHE cell line or clonal cells are CD34+, and at least one of CD43−, CD93−, CXCR4−, CD73−, and CXCR4−CD73−; (iii) definitive HSCs, wherein the iHSCs are CD34+CD45+; (iv) hematopoietic multipotent progenitor cells, wherein the iMPP cells are CD34+CD45+; (v) T cell progenitors, wherein the T cell progenitors are CD34+CD45+CD7+ or CD34−CD45+CD7+; (vi) T cells, wherein the T cells are CD45+CD3+CD4+ or CD45+CD3+CD8+; (vii) NK cell progenitors, wherein the NK cell progenitors are CD45+CD56+CD7+; (viii) NK cells, wherein the NK cells are CD3−CD45+CD56+, and optionally further defined by NKp46+, CD57+, and CD16+; (ix) NKT cells, wherein the NKT cells are CD45+Vα24Jα18+CD3+; and (x) B cells, wherein the B cells are CD45+CD19+.

[281] In some embodiments, the present disclosure provides a method of generating hematopoietic lineage cells with enhanced therapeutic properties, and the method comprises: obtaining iPSCs comprising one or more genetic imprints; and directing differentiation of iPSCs to hematopoietic lineage cells. The step of directed differentiation further comprises: (i) contacting the pluripotent stem cells with a composition comprising a BMP pathway activator, and optionally bFGF, to obtain mesodermal cells; and (ii) contacting the mesodermal cells with a composition comprising a BMP pathway activator, bFGF, and a WNT pathway activator, to obtain mesodermal cells having definitive hemogenic endothelium (HE) potential, wherein the mesodermal cells having definitive hemogenic endothelium (HE) potential are capable of providing hematopoietic lineage cells. Preferably, the mesodermal cells and mesodermal cells having definitive HE potential are obtained in steps (i) and (ii) without the step of forming embryoid bodies, and the obtained hematopoietic lineage cells comprise definitive hemogenic endothelium cells, hematopoietic stem and progenitor cells (HSC), hematopoietic multipotent progenitor cell (MPP), pre-T cell progenitor cells, pre-NK cell progenitor cells, T cell progenitor cells, NK cell progenitor cells, T cells, NK cells, NKT cells, or B cells. Moreover, the hematopoietic lineage cells retain the genetic imprints comprised in the iPSCs for directed differentiation.

[282] In some embodiments, the step of directed differentiation of the above method further comprises: (i) contacting the mesodermal cells having definitive HE potential with a composition comprising bFGF and a ROCK inhibitor to obtain definitive HE cells; (ii) contacting the definitive HE cells with a composition comprising a BMP activator, and optionally a ROCK inhibitor, and one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GMCSF, EPO, bFGF, VEGF, SCF, IL6, Flt3L and IL11 to obtain hematopoietic multipotent progenitor cells (MPP); (iii) contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7; and optionally one or more of a BMP activator, a ROCK inhibitor, TPO, VEGF and bFGF to obtain pre-T cell progenitors, T cell progenitors, and/or T cells; or (iv) contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, TPO, IL7 and IL15, and optionally one or more of a BMP activator, a ROCK inhibitor, VEGF and bFGF to obtain pre-NK cell progenitors, NK cell progenitors, and/or NK cells.

[283] Briefly, the method may comprise reprogramming a mature source T or B cell to obtain induced pluripotent stem cells (iPSCs); and detecting the presence, in the iPSCs or the hematopoietic lineage cells derived therefrom, of a specific V(D)J recombination that is same as the one comprised in the mature T or B cell for generating the iPSC. In some embodiments, the above method further comprises isolating iPSCs or hematopoietic lineage cells comprising the same V(D)J recombination as that of the mature source T or B cell. In some embodiments, the above method comprises, prior to reprogramming the source cells, obtaining a mature source T or B cell for reprogramming; and determining V(D)J recombination comprised in immunoglobulins (Ig) or T cell receptors (TCR) that is specific to the mature source T or B cell.

[284] A "pluripotency factor," or "reprogramming factor," refers to an agent capable of increasing the developmental potency of a cell, either alone or in combination with other agents. Pluripotency factors include, without limitation, polynucleotides, polypeptides, and small molecules capable of increasing the developmental potency of a cell. Exemplary pluripotency factors include, for example, transcription factors and small molecule reprogramming agents.

[285] A number of various cell types from all three germ layers have been shown to be suitable for somatic cell reprogramming, including, but not limited to liver and stomach (Aoi et al., 2008); pancreatic β cells (Stadtfeld et al., 2008); mature B lymphocytes (Hanna et al., 2008); human dermal fibroblasts (Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008;

Aasen et al., 2008); meningiocytes (Qin et al., 2008); neural stem cells (DiSteffano et al., 2008); and neural progenitor cells (Eminli et al., 2008). Thus, the present disclosure contemplates, in part, methods to reprogram and/or program cells from any cell lineage.

[286] The present disclosure contemplates, in part, to alter the potency of a cell by contacting the cell with one or more repressors and/or activators to modulate the epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity of a component of a cellular pathway associated with determining or influencing cell potency.

[287] Thus, in various embodiments, the present disclosure uses predictable and highly controlled methods for gene expression, as discussed elsewhere herein, that enable the reprogramming or de-differentiation and programming or differentiation of somatic cells ex vivo or in vivo. As, noted above, the intentional genetic engineering of cells, however, is not preferred, since it alters the cellular genome and would likely result in genetic or epigenetic abnormalities. In contrast, the compositions and methods of the present disclosure provide repressors and/or activators that non-genetically alter the potency of a cell by mimicking the cell's endogenous developmental potency pathways to achieve reprogramming and/or programming of the cell.

Small Molecules in Reprogramming

[288] Reprogramming of somatic cells into induced pluripotent stem cells has also been achieved by retroviral infection of defined genes (*e.g.*, Oct-3/4, Sox-2, Klf-4, c-Myc, and Lin28, and the like) in combination with small molecules.

[289] In some embodiments, the present disclosure provides a method of altering the potency of a cell that comprises contacting the cell with one or more repressors and/or activators or a composition comprising the same, wherein said one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the potency of the cell, thereby altering the potency of the cell. In particular embodiments, the one or more repressors and/or activators modulate one or more components of a cellular pathway associated with the potency of the cell and thereby alter the potency of the cell. In certain embodiments, the one or more repressors and/or activators modulate one or more components of one or more cellular pathways associated with the potency of the cell and thereby alter the potency of the cell. In certain related embodiments, the modulation of the component(s) is synergistic and increases the overall efficacy of altering the potency of a cell. The potency of the cell can be altered, compared to the ground potency state, to a more potent

state (*e.g.*, from a differentiated cell to a multipotent, pluripotent, or totipotent cell) or a less potent state (*e.g.*, from a totipotent, pluripotent, or multipotent cell to a differentiated somatic cell). In still yet other embodiments, the potency of a cell may be altered more than once. For example, a cell may first be reprogrammed to a more potent state, then programmed to a particular somatic cell.

[290] In another embodiment, the methods of the present disclosure provide for increasing the potency a cell, wherein the cell is reprogrammed or dedifferentiated to a totipotent state, comprising contacting the cell with a composition comprising one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the totipotency of the cell, thereby increasing the potency of the cell to a totipotent state.

[291] In a particular embodiment, a method of increasing the potency a cell to a pluripotent state comprises contacting the cell with one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the potency of the cell, thereby increasing the potency of the cell to a pluripotent state.

[292] In another particular embodiment, a method of increasing the potency a cell to a multipotent state comprises contacting the cell with one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the potency of the cell, thereby increasing the potency of the cell to a multipotent state.

[293] In certain embodiments, a method of increasing the potency of a cell further comprises a step of contacting the totipotent cell, the pluripotent cell or the multipotent cell with a second composition, wherein the second composition modulates the at least one component of a cellular potency pathway to decrease the totipotency, pluripotency or multipotency of the cell and differentiate the cell to a mature somatic cell.

[294] In another related embodiment, the present disclosure provides a method of reprogramming a cell that comprises contacting the cell with a composition comprising one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway or pathways associated with the reprogramming of a cell, thereby reprogramming the cell.

[295] In other embodiments, the present disclosure provides a method of dedifferentiating a cell to a more potent state, comprising contacting the cell with the composition comprising one/or more activators, wherein the one or more repressors and/or activators modulates at

least one component of a cellular pathway or pathways associated with the dedifferentiation of the cell to the more potent state, thereby dedifferentiating the cell to an impotent state.

[296] According to various embodiments of the present disclosure a repressor can be an antibody or an antibody fragment, an intrabody, a transbody, a DNAzyme, an ssRNA, a dsRNA, an mRNA, an antisense RNA, a ribozyme, an antisense oligonucleotide, a pri-miRNA, an shRNA, an antagomir, an aptamer, an siRNA, a dsDNA, a ssDNA; a polypeptide or an active fragment thereof, a peptidomimetic, a peptoid, or a small organic molecule. Polypeptide-based repressors include, but are not limited to fusion polypeptides. Polypeptide-based repressors also include transcriptional repressors, which can further be fusion polypeptides and/or artificially designed transcriptional repressors as described elsewhere herein.

[297] According to other various embodiments, an activator can be an antibody or an antibody fragment, an mRNA, a bifunctional antisense oligonucleotide, a dsDNA, a polypeptide or an active fragment thereof, a peptidomimetic, a peptoid, or a small organic molecule.

[298] In some embodiments, repressors modulate at least one component of a cellular potency pathway by a) repressing the at least one component; b) de-repressing a repressor of the at least one component; or c) repressing an activator of the at least one component. In related embodiments, one or more repressors can modulate at least one component of a pathway associated with the potency of a cell by a) de-repressing the at least one component; b) repressing a repressor of the at least one component; or c) de-repressing an activator of the at least one component.

[299] In certain embodiments, one or more repressors modulates at least one component of a cellular pathway associated with the potency of a cell by a) repressing a histone methyltransferase or repressing the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity; or b) de-repressing a demethylase or activating the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity.

[300] In related embodiments, activators modulate at least one component of a cellular pathway associated with the potency of a cell by a) activating the at least one component; b)

activating a repressor of a repressor of the at least one component; or c) activating an activator of the at least one component.

[301] In certain embodiments, one or more activators modulates at least one component by a) activating a histone demethylase or activating the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity; or b) activating a repressor of a histone methyltransferase or activating a repressor of the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity.

[302] In various other embodiments, the present disclosure contemplates, in part, a method of reprogramming a cell, comprising contacting the cell with one or more repressors, wherein the one or more repressors modulates at least one component of a cellular pathway associated with the reprogramming of a cell, thereby reprogramming the cell.

[303] In various other embodiments, the present disclosure contemplates, in part, a method of reprogramming a cell, comprising contacting the cell with a composition comprising one or more activators, wherein the one or more activators modulates at least one component of a cellular pathway associated with the reprogramming of a cell, thereby re-programming the cell.

[304] While some exemplary methods for reprogramming/NK cell differentiation are provided herein, these are exemplary and not meant to limit the scope of the present disclosure. Additional suitable methods for reprogramming/NK cell differentiation will be apparent to those of skill in the art based on the present disclosure in view of the knowledge in the art.

Methods for culturing NK cells on feeder layers or with feeder cells are described in detail in, for *e.g.*, EP3184109 by Valamehr *et al.* ("Valamehr") incorporated in its entirety herein by reference.

[305] In general, any type of NK cell population can be cultured using a variety of methods and devices. Selection of culture apparatus is usually based on the scale and purpose of the culture. Scaling up of cell culture preferably involves the use of dedicated devices. Apparatus for large scale, clinical grade NK cell production is detailed, for example, in Spanholtz *et al.* (PLoS ONE 2010;5:e9221) and Sutlu *et al.* (Cytotherapy 2010, Early Online 1-12).

[306] The methods described hereinabove for *ex vivo* culturing NK cells populations can result, inter alia, in a cultured population of NK cells.

Types of Edits

[307] Some aspects of the present disclosure provide complex editing strategies, and resulting NK cells having complex genomic alterations, that allow for the generation of advanced NK cell products for clinical applications, *e.g.*, for immunooncology therapeutic approaches. In some embodiments, the modified NK cells provided herein can serve as an off-the-shelf clinical solution for patients having, or having been diagnosed with, a hyperproliferative disease, such as, for example, a cancer. In some embodiments, the modified NK cells exhibit an enhanced survival, proliferation, NK cell response level, NK cell response duration, resistance against NK cell exhaustion, and/or target recognition as compared to non-modified NK cells. For example, the modified NK cells provided herein may comprise genomic edits that result in: expression of a chimeric antigen receptor (CAR) of interest, *e.g.,* a CAR targeting mesothelin, EGFR, HER2 and/or MICA/B; may express a CD16 variant, *e.g.*, hnCD16; expression of an IL15/IL15RA fusion; a loss-of-function in TGF beta receptor 2 (TGFbetaR2); and/or expression of a dominant-negative variant of TGFbetaR2; a loss-of-function of ADORA2A; a loss-of-function of B2M; expression of HLA-G: a loss-of-function of a CIITA ; a loss-of-function of a PD1 ; a loss-of-function of TIGIT ; and/or a loss-of-function of CISH; or any combination of two or more thereof in the modified NK cell.

[308] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[309] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, expression of a soluble MICA and/or MICB, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[310] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of

an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, expression of a soluble MICA and/or MICB, expression of an exogenous IL-12, expression of an exogenous IL-18, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[311] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFbetaR2, expression of an exogenous IL-12, expression of an exogenous IL-18, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[312] The modified NK cells may exhibit one or more edits in their genome that results in a loss-of-function in a target gene, and/or one or more modifications that results in a gain-of-function, or an overexpression, of a gene product, *e.g.*, of a protein, from an exogenous nucleic acid construct, *e.g.*, from an expression construct comprising a cDNA encoding for the gene product that is integrated into the genome of the modified NK cell or provided in an extrachromosomal manner, *e.g.*, in the form of an episomal expression construct.

[313] A loss-of-function of a target gene is characterized by a decrease in the expression of a target gene based on a genomic modification, *e.g.*, an RNA-guided nuclease-mediated cut in the target gene that results in an inactivation, or in diminished expression or function, of the encoded gene product.

[314] A gain-of-function of a gene product is characterized by an increased expression (also referred to herein as overexpression) of a gene product, *e.g.*, of a protein, in a cell, which can include, for example, an increased expression level of the gene product, or expression of the gene product in a cell that does not express the gene product endogenously, *e.g.*, from an endogenous gene.

[315] In some embodiments, increased expression of a gene product is effected by introducing an exogenous nucleic acid construct that encodes the gene product into a cell, *e.g.*, an exogenous nucleic acid construct that comprises a cDNA encoding the gene product under the control of a heterologous promoter. In some embodiments, the exogenous nucleic acid construct is integrated into a specific locus, *e.g.*, via HDR-mediated gene editing, as described in more detail elsewhere herein. Methods for effecting loss-of-function edits as well as methods for effecting increased expression of gene products, *e.g.*, via RNA-guided nuclease technology are well known to those of ordinary skill in the art.

[316] Some exemplary gene products, one or more of which may be overexpressed in a modified NK cells provided in some embodiments of this disclosure are provided in Table 10 below:

TABLE 10:

CAR Chimeric antigen receptor (*e.g.*, binding to Her2, EGFR, alpha folate receptor, CEA, cMET, MUC1, Mesothelin, ROR1, or other targets.)
CD16 or CD16 variant (*e.g.*, hnCD16)
IL-15/IL-15R/IL-15RA
IL-12/IL-12R/IL-12RA
IL-2/IL-2R/IL-2RA
HLA-G
HLA-E
CD47
CXCR1
CX3CR1
mTRAIL
TOSO

[317] Some exemplary target genes, one or more of which are modified to exhibit a loss-of-function in modified NK cells provided in some embodiments of this disclosure are provided in Table 11 below.

TABLE 11:

TGFβR2
ADORA2A
TIGIT
B2M
PD-1
CISH
CIITA
HLA class II histocompatibility antigen alpha chain genes, e.g., HLA-DQA1, HLA-DRA, HLA-DPA1, HLA-DMA, HLA-DQA2, and/or HLA-DOA
HLA class II histocompatibility antigen beta chain genes, e.g., HLA-DMB, HLA-

DOB, HLA-DPB1, HLA-DQB1, HLA-DQB2, HLA-DQB3, HLA-DRB1, HLA-DRB3, HLA-DRB4, and/or HLA-DRB5
CD32B (FCGR2B)
CTLA4
NKG2A
BIM
CBLB
CCR5
CCR7
CD96
CDK8
CXCR3
EP4 (PGE2 receptor)
Fas
GITR
IL1R8
KIRDL1
KIR2DL1-3
LAG3
SOCS genes
Sortilin
TIM3
TRAC
NLRC5

[318] The present disclosure embraces modified NK cells exhibiting any of the edits and/or increased expression of gene products listed in TABLES 7 and TABLES 8 combined, as well as any combination of such edits and/or increased expression of gene products listed in these tables. For example, it is to be understood that the present disclosure embraces embodiments in which modified NK cells are provided that comprise a single edit listed in TABLE 10 or TABLE 11, *e.g.*, loss of function of ADORA2A, or loss of function of B2M, or increased expression of HLA-G, etc. It is to be understood that the present disclosure embraces embodiments in which modified NK cells are provided that comprise a single edit listed in

TABLE 11 and increased expression of a gene product listed in TABLE 10, *e.g.*, loss of function of ADORA2A or loss of function of B2M; and increased expression of HLA-G. It is further to be understood that the present disclosure embraces embodiments in which modified NK cells are provided that comprise two or more edits listed in TABLE 11, and increased expression of a single gene product listed in TABLE 10. It is further to be understood that the present disclosure embraces embodiments in which modified NK cells are provided that comprise a single edit listed in TABLE 11, and increased expression of two or more gene products listed in TABLE 10. It is further to be understood that the present disclosure embraces embodiments in which modified NK cells are provided that comprise two or more edits listed in TABLE 11, and increased expression of two or more gene products listed in TABLE 10.

[319] In order to illustrate some of the configurations of modified NK cells embraced by the present disclosure, some exemplary, non-limiting embodiments are provided below and elsewhere herein. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of B2M. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of TGFbRII. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M. In some embodiments, modified NK cells are provided that exhibit a gain-of-function of hnCD16. In some embodiments, modified NK cells are provided that exhibit a gain-of-function of a CAR, *e.g.*, a CAR binding Her2, EGFR, alpha folate receptor, CEA, cMET, MUC1, Mesothelin, ROR1, or a different target, *e.g.*, as disclosed herein or otherwise known in the art. In some embodiments, modified NK cells are provided that exhibit a gain-of-function of HLA-G. In some embodiments, modified NK cells are provided that exhibit a gain-of-function of a single-chain IL-15/IL-15R fusion protein. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of hnCD16. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of a CAR, *e.g.*, a CAR binding Her2, EGFR, alpha folate receptor, CEA, cMET, MUC1, Mesothelin, ROR1, or a different target, *e.g.*, as disclosed herein or otherwise known in the art. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of HLA-G. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of a single-chain IL-15/IL-15R fusion protein. In some embodiments, modified NK cells are provided that

exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of hnCD16 and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of a CAR, *e.g.*, a CAR binding Her2, EGFR, alpha folate receptor, CEA, cMET, MUC1, Mesothelin, ROR1, or a different target, *e.g.*, as disclosed herein or otherwise known in the art, and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of HLA-G and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of a single-chain IL-15/IL-15R fusion protein, and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A, CISH, and B2M, and a gain-of-function of hnCD16 and HLA-G. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A and B2M, and a gain-of-function of a single-chain IL-15/IL-15R fusion protein, HLA-G, and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of TIGIT and B2M, and a gain-of-function of hnCD16 and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of TIGIT and B2M, and a gain-of-function of a CAR, *e.g.*, a CAR binding Her2, EGFR, alpha folate receptor, CEA, cMET, MUC1, Mesothelin, ROR1, or a different target, *e.g.*, as disclosed herein or otherwise known in the art, and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of TIGIT and B2M, and a gain-of-function of HLA-G and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of TIGIT and B2M, and a gain-of-function of a single-chain IL-15/IL-15R fusion protein, and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of TIGIT, CISH, and B2M, and a gain-of-function of hnCD16 and HLA-G. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of TIGIT and B2M, and a gain-of-function of a single-chain IL-15/IL-15R fusion protein, HLA-G, and a dominant-negative TGFbRII variant. In some embodiments, modified NK cells are provided that exhibit a loss-of-function of ADORA2A, TIGIT, PD-1, and B2M, and a gain-of-function of a single-chain IL-15/IL-15R fusion protein, HLA-G, and a dominant-negative TGFbRII variant.

[320] It is to be understood that the exemplary embodiments provided herein are meant to illustrate some examples of NK cells embraced by the present disclosure. Additional

configurations are embraced that are not described here in detail for the sake of brevity, but such embodiments will be immediately apparent to those of skill in the art based on the present disclosure.

Chimeric Antigen Receptors (CARs)

[321] 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 NK 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.

[322] 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).

[323] 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 (ie. 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.

[324] Modified NK cells provided herein may, in some embodiments, comprise a CAR and a CD16 variant, *e.g.*, hnCD16, or a CAR and no CD16 variant. Any cell expressing CD16, or a variant thereof, would be suitable for combination therapy with a monoclonal antibody, *e.g.*, a monoclonal antibody used in cancer therapy, or with an Fc fusion protein targeting pathological cells.

Knock-Ins and Knock-Outs

[325] In some embodiments, a modified cell may express one or more of an exogenous hnCD16 , an exogenous IL-15, an exogenous IL-15RA, a loss of function in TGFbetaR2, an exogenous DN-TGFbetaR2, and/or a loss of function in ADORA2A. In yet another embodiment, the modified cell may comprise a loss of function in B2M, an exogenous HLA-G, a loss of function in CIITA, a loss of function in PD1, a loss of function in TIGIT, or a loss of function in CISH.

[326] In some embodiments, a modified cell may express one or more of an exogenous hnCD16, an exogenous IL-15, an exogenous IL-15RA, an exogenous HLA-G, an exogenous DN-TGFbetaR2, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function in PD1, a loss of function in TIGIT, and/or a loss of function in ADORA2A.

[327] In some embodiments, a modified cell may express one or more of an exogenous hnCD16, an exogenous IL-15, an exogenous IL-15RA, an exogenous HLA-G, an exogenous DN-TGFbetaR2, a soluble MICA and/or MICB, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function in PD1, a loss of function in TIGIT, and/or a loss of function in ADORA2A.

[328] In some embodiments, a modified cell may express one or more of an exogenous hnCD16, an exogenous IL-15, an exogenous IL-15RA, an exogenous HLA-G, an exogenous DN-TGFbetaR2, an exogenous IL-12, an exogenous IL-18, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function in PD1, a loss of function in TIGIT, and/or a loss of function in ADORA2A.

[329] In some embodiments, a modified cell may express one or more of an exogenous hnCD16, an exogenous IL-15, an exogenous IL-15RA, an exogenous HLA-G, an exogenous DN-TGFbetaR2, an exogenous IL-12, an exogenous IL-18, a soluble MICA and/or MICB, a loss of function in TGFbetaR2, a loss of function in B2M, a loss of function in PD1, a loss of function in TIGIT, and/or a loss of function in ADORA2A.

[330] As used herein, the term "express" or "expression" refers to the process to produce a polypeptide, including transcription and translation. Expression may be, *e.g.*, increased by a number of approaches, including: increasing the number of genes encoding the polypeptide,

increasing the transcription of the gene (such as by placing the gene under the control of a constitutive promoter), increasing the translation of the gene, knocking out of a competitive gene, or a combination of these and/or other approaches.

[331] As used herein, the term "knock-in" refers to the addition of a target gene into a genetic locus of a cell.

[332] As used herein, the term "knock-out" refers to an inactivating mutation in a target gene, wherein the product of the target gene comprises a loss of function.

[333] As used herein, the term "loss of function" refers to an inactivating mutation in a target gene, wherein the gene product has less, or no, function (being partially or wholly inactivated). As used herein the term "complete loss of function" refers to an inactivating mutation in a target gene, wherein the gene product has no function (wholly inactivated).

[334] As used herein, the term "hnCD16a" refers to a high affinity, non-cleavable variant of CD16 (a low-affinity Fcγ receptor involved in antibody-dependent cellular cytotoxicity (ADCC). Typically, CD16 is cleaved during ADCC – the hnCD16 CAR does not undergo this cleavage and thus sustains an ADCC signal longer. In some embodiments, the hnCD16a is disclosed in *Blood* 2016 128:3363, the entire contents of which are expressly incorporated herein by reference.

[335] As used herein, the term "MICA/B" refers to MHC class I chain-related protein A (MICA) and B (MICB) are polymorphic proteins induced upon stress, damage or (malignant) transformation of cells, and act as a 'kill me' signal through the natural-killer group 2, member D receptor expressed on cytotoxic lymphocytes. MICA/B are not thought to be constitutively expressed by healthy normal cells, but expression has been reported for most tumor types. Exemplary sequences for MICA are provided in NG_034139.1, and exemplary sequences for MICB are provided in NG_021405.1.

[336] As used herein, the term "AAVSI" refers to Adeno associated integration site 1.

[337] As used herein, the term "2A" refers to self-cleaving 2A peptide.

[338] As used herein, the term "TGFβRII" or "TGFbetaR2" refers to a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with TGF-beta receptor type-1, and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of genes related to cell proliferation, cell cycle arrest, wound healing, immunosuppression, and tumorigenesis. Exemplary sequences of TGFβRII are set forth in KR710923.1, NM_001024847.2, and NM_003242.5.

[339] As used herein, the term "DN-TGFβRII" refers to dominant negative TGF beta receptor II (could be expressed from an NK-specific promoter) TGFβRII plays an important role in T-cell differentiation, and KO in iPSCs would prevent CD34+ differentiation; KO would have to be performed later, but DN could be expressed from NK specific promoter (would turn on after CD34+ diff). In some embodiments, DN-TGFβRII is disclosed in *Immunity. 2000 Feb;12(2):171-81,* the entire contents of which are expressly incorporated herein by reference.

[340] The strategy used by tumor cells to protect themselves against the effects of TGF-β can be manipulated to shield tumor-specific cytotoxic T lymphocytes (CTLs) from the inhibitory effects of tumor-secreted TGF-β. Tumor-specific CTLs expressing a dominant negative TGF beta receptor II (for *e.g.*, a TGFβRIIDNR sequence) have a selective functional and survival advantage over unmodified CTLs in the presence of TGF-β–secreting tumors (Bollard *et al.*, 2002 Blood. 2002 May 1;99(9):3179-87; incorporated in its entirety herein by reference). Accordingly, in some embodiments, the modified cell of the disclosure expresses a DN-TGFβRII construct. In some embodiments, the DN-TGFβRII construct is driven by an EF1a long promoter. In some embodiments, the DN-TGFβRII construct is knocked into an ADORA2A locus by using an *S. pyogenes* gRNA. In some embodiments, the DN-TGFβRII construct comprises a TGFβRIIDNR sequence, immediately followed by a 2A sequence, and further followed by a truncated EGFR sequence (EGFRt), to enable tracking of cells that efficiently express the construct. In some embodiments, the DN-TGFβRII construct is produced as a long single stranded DNA molecule. In some embodiments, the DN-TGFβRII construct is delivered to cells in an RNP. In some embodiments, the DN-TGFβRII construct is delivered to cells by AAV delivery (for *e.g.*, via AAV6).

[341] As used herein, the term "Neural cell adhesion molecule" (NCAM), also called CD56, refers to a homophilic binding glycoprotein expressed on the surface of neurons, glia and skeletal muscle and certain cells of the hematopoietic system. Expression of CD56 is associated with, but not limited to, natural killer cells. Exemplary sequences for NCAM are provided in NM_000615.6, NM_181351.4, NM_001076682.3, NM_001242608.1, and NM_001242607.1.

[342] As used herein, the term "CISH" refers to the Cytokine Inducible SH2 Containing Protein, for *e.g.*, see Delconte *et al.*, Nat Immunol. 2016 Jul;17(7):816-24; incorporated in its entirety herein by reference. Exemplary sequences for CISH are set forth as NG_023194.1.

[343] 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 IL15 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.

[344] IL-15 is a key cytokine in promoting NK cell growth and homeostatic maintenance of memory T cells. IL-15 and its receptor chain, IL-15Ra, are essential for NK survival and do not stimulate regulatory T cells. IL-15/IL-15Ra binds to the beta and gamma subunits of IL-2 receptor and thereby activates JAK1/3 and STAT5. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) expresses an exogenous IL-15/IL-15Ra. In some embodiments, the exogenous IL-15/IL-15Ra is expressed as a membrane-bound IL15.IL15Ra complex, as described in Imamura et al., Blood. 2014 Aug 14;124(7):1081-8 and Hurton LV et al., PNAS, 2016; incorporated in their entirety herein by reference. In some embodiments, the exogenous IL-15/IL-15Ra is expressed as a soluble IL15Ra.IL15 complex, as described in Mortier E et al, JBC 2006; Bessard A, Mol Cancer Ther 2009; and Desbois M, JI 2016; incorporated in their entirety herein by reference. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) expresses a membrane-bound IL15.IL15Ra complex and a soluble IL15Ra.IL15 complex. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) express a membrane-bound form of IL15.IL15Ra complex with a cleavable linker. A knockout of CISH is associated with further promoting the IL-15 signaling, as described in Delconte P, Nat Immunol 2016; incorporated in its entirety herein by reference. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) expresses a loss of function in CISH. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) express exogenous IL-15/IL-15Ra and a loss of function in CISH.

[345] As used herein, the term "ADORA2A" refers to the adenosine A2A receptor encodes a member of the guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) superfamily, which is subdivided into classes and subtypes. The receptors are seven-pass transmembrane proteins that respond to extracellular cues and activate intracellular signal transduction pathways. 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. It plays an important role in many biological functions, such as cardiac rhythm and circulation, cerebral and renal blood flow, immune function, pain regulation, and sleep. It has been implicated in pathophysiological conditions such as inflammatory diseases and neurodegenerative disorders. Exemplary sequences of ADORA2a are provided in NG_052804.1.

[346] 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. The protein has a predominantly beta-pleated sheet structure that can form amyloid fibrils in some pathological conditions. The encoded antimicrobial protein displays antibacterial activity in amniotic fluid. Exemplary sequences for B2M are set forth as NG_012920.2.

[347] As used herein, the term "CD32B" 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., CD32B, the human inhibitory Fc-gamma receptor IIB, as a target for monoclonal antibody therapy of B-cell lymphoma. Blood 2006 108(7):2384-91, the entire contents of which are incorporated herein by reference.

[348] 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.

[349] 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.

[350] In some embodiments, two or more HLA class II histocompatibility antigen alpha chain genes and/or two or more HLA class II histocompatibility antigen alpha chain genes are knocked out, e.g., by genomic editing, in the modified lymphocytes provided herein. 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 knocked out. For another example, in some embodiments, the 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 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.

[351]

[352] 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.

[353] 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. Once in the nucleus it does not bind DNA but rather uses an intrinsic acetyltransferase (AT) activity to act in a coactivator-like fashion. 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.

[354] 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. This prevents autoimmune diseases, but it can also prevent the immune system from killing cancer cells. PD-1 is an immune checkpoint and guards against autoimmunity through two mechanisms. First, it promotes apoptosis (programmed cell death) of antigen-specific T-cells in lymph nodes. Second, it reduces apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells). Exemplary sequences for PD1 are set forth as NM_005018.3.

[355] As used herein, the term "TIGIT" refers to a member of the PVR (poliovirus receptor) family of immunoglobin proteins. The product of this gene is expressed on several classes of T cells including follicular B helper T cells (TFH). The protein has been shown to bind PVR with high affinity; this binding is thought to assist interactions between TFH and dendritic cells to regulate T cell dependent B cell responses. Exemplary sequences for TIGIT are set forth in NM_173799.4.

[356] As used herein, the term "NLRC5" refers to a NOD-like receptor family CARD domain containing 5 intracellular protein that plays a role in the immune system. NLRC5 is a pattern recognition receptor implicated in innate immunity to viruses potentially by regulating interferon activity. Exemplary sequences forNLRC5 are set forth as NM_032206.4.

[357] As used herein, the term "CTLA4" refers to a member of the immunoglobulin superfamily which transmits an inhibitory signal to T cells. The protein contains a V domain, a transmembrane domain, and a cytoplasmic tail. Exemplary sequences forCTLA4 are set forth as AF414120.1.

[358] As used herein, the term "LAG3" refers to the lymphocyte-activation protein 3, which belongs to the Ig superfamily and contains 4 extracellular Ig-like domains. Exemplary sequences forLAG3 are set forth as NM_002286.6.

[359] As used herein, the term "CBLB" refers to a E3 ubiquitin-protein ligase which promotes proteosome-mediated protein degradation by transferring ubiquitin from an E2 ubiquitin-conjugating enzyme to a substrate. The encoded protein is involved in the regulation of immune response by limiting T-cell receptor, B-cell receptor, and high affinity immunoglobulin epsilon receptor activation. Exemplary sequences for CBLB are set forth as KR709533.1.

[360] As used herein, the term "NKG2A" 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. This protein forms a complex with another family member, KLRD1/CD94, and has been implicated in the recognition of the MHC class I HLA-E molecules in NK cells. See, e.g., Kamiya-T et al., J Clin Invest 2019 https://doi.org/10.1172/JCI123955, the entire contents of which are incorporated herein by reference. Exemplary sequences forNKG2A are set forth as AF461812.1.

[361] As used herein, the term "CCR5" refers to a member of the beta chemokine receptor family, which is predicted to be a seven transmembrane protein similar to G protein-coupled receptors. This protein is expressed by T cells and macrophages, and is known to be an important co-receptor for macrophage-tropic virus, including HIV, to enter host cells. Exemplary sequences forCCR5 are set forth as U54994.1 .

[362] As used herein, the term "SOCS" refers to a family of genes involved in inhibiting the JAK-STAT signaling pathway.

[363] As used herein, the term "BIM" refers to a pro-apoptotic member of the BCL-2 protein family, which interacts with other members of the BCL-2 protein family, including BCL2, BCL2L1/BCL-X(L), and MCL1, and act as an apoptotic activator.

[364] As used herein, the term "FAS" refers to a member of the TNF-receptor superfamily. This receptor contains a death domain. It has been shown to play a central role in the physiological regulation of programmed cell death.

[365] As used herein, the term "GITR" refers to a Tumor necrosis factor receptor superfamily member 18 (TNFRSF18) also known as activation-inducible TNFR family receptor (AITR) or glucocorticoid-induced TNFR-related protein. It involved in interactions between activated T-lymphocytes and endothelial cells and in the regulation of T-cell receptor-mediated cell death.

[366] As used herein, the term "sortilin" refers to the VPS10-related sortilin family of proteins.

[367] As used herein, the term "TIM3" refers to a T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) protein that in humans is encoded by the HAVCR2 gene.

[368] As used herein, the term "CD96" or "TACTILE" refers to a type I membrane protein that plays a role in the adhesive interactions of activated T and NK cells during the late phase of the immune response.

[369] As used herein, the term "IL1R8" refers to a member of the interleukin 1 receptor family and is similar to the interleukin 1 accessory proteins.

[370] As used herein, the term , "KIR2DL1", "KIR2DL2" and "KIR2DL3" refer to killer cell immunoglobulin-like receptors (KIRs), which are transmembrane glycoproteins expressed by natural killer cells and subsets of T cells.

[371] As used herein, the term "CDK8" refers to a member of the cyclin-dependent protein kinase (CDK) family, that functions as a regulator of cell cycle progression.

[372] As used herein, the term "CXCR3" refers to a G protein-coupled receptor with selectivity for three chemokines, termed CXCL9/Mig (monokine induced by interferon-g), CXCL10/IP10 (interferon-g-inducible 10 kDa protein) and CXCL11/I-TAC (interferon-inducible T cell a-chemoattractant).

[373] As used herein, the term "CCR7" refers to a member of the G protein-coupled receptor family. This receptor is expressed in various lymphoid tissues and activates B and T lymphocytes.

[374] As used herein, the term "EP4" refers to a member of the G-protein coupled receptor family. This protein is one of four receptors identified for prostaglandin E2 (PGE2). This receptor can activate T-cell factor signaling.

[375] As used herein, the term "IL-2" refers to interleukin-2, a secreted cytokine that is important for the proliferation of T and B lymphocytes.

[376] As used herein, the term "IL-12" refers to interleukin-12, a cytokine that acts on T and natural killer cells.

[377] As used herein, the term "IL-18" refers to interleukin-18, a proinflammatory cytokine primarily involved in polarized T-helper 1 (Th1) cell and natural killer (NK) cell immune responses.

[378]

[379] As used herein, the term "CXCR1" refers to a member of the G-protein-coupled receptor family. This protein is a receptor for interleukin 8 (IL8).

[380] As used herein, the term "CX3CR1" refers to a transmembrane protein and chemokine involved in the adhesion and migration of leukocytes.

[381] As used herein, the term "mTRAIL" refers to a cytokine that belongs to the tumor necrosis factor (TNF) ligand family. This protein preferentially induces apoptosis in transformed and tumor cells.

[382] As used herein, the term "TOSO" refers to an Fc Fragment of the IgM Receptor

[383] As used herein, the term "CD16" refers to a receptor 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.

[384] In some embodiments, modified cells are provided herein that exhibit a loss of function of TRAC. The term "TRAC" refers to the T-cell receptor alpha subunit (constant), encoded by the TRAC locus. Cells exhibiting a loss-of-function of TRAC do not express a T-cell receptor (TCR). In some embodiments, modified cells, e.g., pluripotent or multipotent stem cells or differentiated daughter cells thereof (e.g., iNK cells), are provided herein that are derived from a cell expressing a TCR or from a cell having a rearranged endogenous TCR locus, e.g., from a T-cell. In some embodiments, such cells comprise a modification that effects a loss-of-function of TRAC and thus do not express a functional TCR. Suitable methods and compositions for effecting a loss-of-function of TRAC will be apparent to those of ordinary skill in the art based on the present disclosure. Such methods and compositions include, without limitation, those disclosed in PCT Application PCT/US2015/026504, entitled "CRISPR-CAS-related methods, compositions and components for cancer immunotherapy"; PCT Application PCT/US2016/024353, entitled "CRISPR-CAS-related methods, compositions and components"; and PCT Application PCT/US2017/020598, entitled "CRISPR-CPF1-related methods, compositions and components for cancer immunotherapy"; the entire contents of each of which are incorporated herein by reference.

[385] The disclosure specifically encompasses variants of the above genes and CARs, including variants having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% percent identity to the above-identified gene sequences. As used herein, the term "percent (%) sequence identity" or "percent (%) identity," also including "homology," is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference sequences after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Optimal alignment of the sequences for comparison may be

produced, besides manually, by means of the local homology algorithm of Smith and Waterman, 1981, Ads App. Math. 2, 482, by means of the local homology algorithm of Neddleman and Wunsch, 1970, J. Mol. Biol. 48, 443, by means of the similarity search method of Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85, 2444, or by means of computer programs which use these algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

[386] Knock-ins and knock-outs can be effected by genome editing technologies known to those of skill in the art and include CRISPR/Cas technologies. Single-cut as well as multiplex editing strategies are suitable to achieve the desired product configurations provided herein, and such strategies are described herein or otherwise known to those of ordinary skill in the art.

[387] In some embodiments, exemplary modified cells, e.g., modified pluripotent cells or differentiated progeny thereof, e.g., iNK cells or other modified lymphocyte types, are evaluated for their ability to escape the immune system of a non-autologous host, e.g., a patient in need of immunotherapy. In some embodiments, such an evaluation includes an in vitro assay. Suitable in vitro assays for such evaluations are known to those of ordinary skill in the relevant art, and include, without limitation, mixed lymphocyte reactivity (MLR) assays. This assay and other suitable assays are described, e.g., in Abbas et al., Cellular and Molecular Immunology, 7th edition, ISBN 9781437735734, the entire contents of which are incorporated herein by reference. Other suitable assays will be apparent to the skilled artisan in view of the present disclosure.

Methods of Use

[388] A variety of diseases may be ameliorated by introducing the modified cells of the invention to a subject. Examples of diseases are, including but not limited to, cancer, including but not limited to solid tumors, including but not limited to, tumor 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, including but not limited to, acute and chronic leukemias, lymphomas, multiple myeloma and myelodysplastic syndromes.

[389] Particular embodiments of the present invention are directed to methods of treating a subject in need thereof by administering to the subject a composition comprising any of the cells described herein. In particular embodiments, the terms “treating,” “treatment,” and the

like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, and includes: preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; inhibiting the disease, *i.e.*, arresting its development; or relieving the disease, *i.e.*, causing regression of the disease. The therapeutic agent or composition may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest.

[390] In particular embodiments, the subject has a disease, condition, and/or an injury that can be treated, ameliorated, and/or improved by a cell therapy. Some embodiments contemplate that a subject in need of cell therapy is a subject with an injury, disease, or condition, whereby a cell therapy, *e.g.*, a therapy in which a cellular material is administered to the subject, can treat, ameliorate, improve, and/or reduce the severity of at least one symptom associated with the injury, disease, or condition. Certain embodiments contemplate that a subject in need of cell therapy, includes, but is not limited to, a candidate for bone marrow or stem cell transplantation, 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, a subject who has or is at risk of having a viral infection or a disease associated with a viral infection.

[391] According, the present invention further provides pharmaceutical compositions comprising the pluripotent cell derived hematopoietic lineage cells made by the methods and composition disclosed herein, wherein the pharmaceutical compositions further comprise a pharmaceutically acceptable medium. In some embodiments, the pharmaceutical composition comprises the pluripotent cell derived T cells made by the methods and composition disclosed herein. In some embodiments, the pharmaceutical composition comprises the pluripotent cell derived NK cells made by the methods and composition disclosed herein. In some embodiments, the pharmaceutical composition comprises the pluripotent cell derived CD34 HE cells made by the methods and composition disclosed herein. In some embodiments, the pharmaceutical composition comprises the pluripotent cell derived HSCs made by the methods and composition disclosed herein.

[392] Additionally, the present invention provides therapeutic use of the above pharmaceutical compositions by introducing the composition to a subject suitable for adoptive cell therapy, wherein the subject has an autoimmune disorder; a hematological malignancy; a solid tumor; or an infection associated with HIV, RSV, EBV, CMV, adenovirus, or BK polyomavirus.

[393] The isolated pluripotent stem cell derived hematopoietic lineage cells can have at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs. In some embodiments, the isolated pluripotent stem cell derived hematopoietic lineage cells has about 95% to about 100% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs. In some embodiments, the present invention provides pharmaceutical compositions having purified T cells, NK cells, NKT cells, CD34+ HE cells or HSCs, such as a composition having an isolated population of about 95% T cells, NK cells, NKT cells, CD34+ HE cells or HSCs to treat a subject in need of the cell therapy.

[394] In some embodiments, the pharmaceutical composition includes an isolated population of pluripotent stem cell derived hematopoietic lineage cells, wherein population has less than about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 15%, 20%, 25%, or 30% iPSC derived T cells, NK cells, NKT cells, CD34+ HE cells or HSCs. The isolated population of derived hematopoietic lineage cells in some embodiments can have 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. In other embodiments, the isolated population of derived hematopoietic lineage cells can have 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.

[395] In particular embodiments, the derived hematopoietic lineage cells can have 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.

[396] As a person of ordinary skill in the art would understand, both autologous and allogeneic immune cells can be used in cell therapies. Autologous cell therapies can have reduced infection, low probability for GvHD, and rapid immune reconstitution. Allogeneic cell therapies can have an immune mediated graft-versus-malignancy (GVM) effect, and low

rate of relapse. Based on the specific conditions of the patients or subject in need of the cell therapy, a person of ordinary skill in the art would be able to determine which specific type of therapy to administer.

[397] In particular embodiments, the derived hematopoietic lineage cells of the pharmaceutical composition of the invention are allogeneic to a subject. In particular embodiments, the derived hematopoietic lineage cells of the pharmaceutical formulation of the invention are autologous to a subject. For autologous transplantation, the isolated population of derived hematopoietic lineage cells are either complete or partial HLA-match with the patient. In another embodiment, the derived hematopoietic lineage cells are not HLA-matched to the subject.

[398] The derived hematopoietic lineage cells provided by the invention can be administration 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. The modulated population of derived hematopoietic lineage cells can be washed to remove the treatment agent(s), and the improved population is administered to a patient without further expansion of the population in vitro.

[399] In other embodiments, the invention provides an isolated population of derived hematopoietic lineage cells that are expanded prior to modulating the isolated population or subpopulation of T lymphocytes with one or more agents. The isolated population of derived hematopoietic lineage cells can be recombinantly produced to express TCR, CAR or other proteins.

[400] 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

[401] Cancers that are suitable 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, the cancer may specifically be of the following histological type, though it is not limited to these: 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's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's 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; 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.

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

[403] In some embodiments, solid cancer indications that can be treated with the modified NK 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;

[404] In some embodiments, hematological cancer indications that can be treated with the modified NK cells provided herein, either alone or in combination with one or more additional cancer treatment modality, include: ALL, CLL, NHL, DLBCL, AML, CML, multiple myeloma (MM).

[405] As used herein, the term "cancer" (also used interchangeably with the terms, "hyperproliferative" and "neoplastic") 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. The term "cancer" includes malignancies of the various organ systems, such as those affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as 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 cancer of the esophagus. The term "carcinoma" is art recognized and 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. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term "carcinoma" also includes carcinosarcomas, *e.g.*, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures. The term "sarcoma" is art recognized and refers to malignant tumors of mesenchymal derivation.

[406] 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.

[407] 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.

[408] 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.

[409] 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.

[410] Contemplated useful secondary or adjunctive therapeutic agents in this context 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.

[411] 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 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

[412] All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

[413] 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.

[414] 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.

[415] 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.

EXAMPLES

[416] The following Examples are merely illustrative and are not intended to limit the scope or content of the disclosure in any way.

Example 1: Generation of modified iNK cells from iPS cells

[417] The use of iPS cell technology for implementing a complex editing strategy and subsequent derivation of iNK cells or other lymphocytes, for example, enable the generation of an iNK cell that express a CAR of interest such as mesothelin, EGFR, HER2, and MICA/B, and/or having one or more edits from List A and/or Table 10, and one or more edits from List B and/or Table 11.

[418] List A:

- Exogenous expression of an enhanced variant of CD16, *e.g.*, of hnCD16a (high affinity, non-cleavable variant of CD 16 - a low-affinity Fcy receptor involved in antibody-dependent cellular cytotoxicity (ADCC)). Typically, CD16 is cleaved during ADCC by proteases, the hnCD16 CAR does not undergo this cleavage and thus sustains an ADCC signal longer.
- Exogenous expression of IL-15/IL 15RA
- Loss-of-function of TGFbR2, or exogenous expression of a dominant-negative variant of TGFbR2 (dominant negative TGF beta receptor II is expressed from an NK-specific promoter, in order to not interfere with TGFbRII's role in the differentiation of CD34 cells, which can be derived from iPS cells, and typically serve as a cell type from which heme lineages (like NK cells) are differentiated)
- Loss-of-function of ADORA2A

[419] List B:

- Loss-of-function of B2M (*e.g.*, eliminate MHC class I expression by targeting B2M expression) Exogenous expression of HLA-G
- Loss-of-function of CIITA (*e.g.*, eliminate MHC class II expression by targeting CIITA)
- Loss-of-function of PD1
- Loss-of-function of TIGIT
- Loss-of-function CISH (cytokine inducible SH2 containing protein)

[420] Loss-of-function preferably includes complete elimination of surface expression of the respective protein.

[421] For example, iNK cells with exogenous expression of a CAR and a CD16 variant (*e.g.*, hnCD16), or a CAR and no CD16 variant can be generated. Cells expressing no CAR but a CD16 variant can also be generated. Any cell expressing CD16, or an enhanced variant thereof (*e.g.*, hnCD16), would be suitable for combination therapy with a monoclonal antibody (*e.g.*, used in cancer therapy), or with an Fc fusion protein targeting pathological cells.

[422] If more than two transgenes are knocked-in, a multicistronic expression construct, or a 2A construct may be advantageous in order to avoid having to insert an individual construct for each transgene.

[423] Such iNK cells are useful for a wide range of immunotherapy applications, including, but not limited to the treatment of proliferative diseases, *e.g.*, certain forms of cancers. When using the CARs outlined above, applications in breast cancer, colon cancer, gastric cancer, renal cell carcinoma, and NSCLC are envisioned. The altered surface molecule repertoire of such cells would also enable the successful treatment of solid tumors, which has been proven difficult with current NK-cell based strategies.

[424] Exemplary iNK cells obtained from reprogrammed somatic cells (or their daughter cells) comprise one or more (*e.g.,* one or more, two or more, three or more, four or more, five or more, or six or more) of the following characteristics:

- They comprise a rearranged endogenous TCR locus (*e.g.*, TCRa VJ and/or TCRf3 V(D)J section rearrangement & complete V-domain exons);
- They do not express an endogenous T cell co-receptor, *e.g.*, CD3, CD4, and/or CD8;
- They express an NK-cell biomarker, for example:

- CD56 (NCAM), CD49, and/or CD45;
- NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII,cluster of differentiation 16 (CD16));
- natural killer group-2 member 0 (NKG2D, MICAIB stress ligand receptor);
- CD69;
- a natural cytotoxicity receptor (*e.g.*, NKp30; NKp44; NKp46; and/or CD158b); or any combination of two or more of these;

- They may express:
  - a chimeric antigen receptor (CAR),
  - a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcyRIII, CD16)
  - an interleukin 15 (IL-15) pathway agonist, e.g., interleukin-15 (IL-15), interleukin 15 receptor (IL-15R) or a variant thereof (e.g., a constitutively active variant of IL-15R, e.g., an IL-15R fused to an IL-15R agonist (IL-15RA); other interleukin pathway agonists are also contemplated, either in the alternative or in combination with the IL-15 pathway agonist, *e.g.*, an interleukin 2 (IL-2) pathway agonist, e.g., IL-2, interleukin 2 receptor (IL-2R) or a variant thereof (e.g., a constitutively active variant of IL-2R, e.g., an IL-2R fused to an IL-2R agonist (IL-2RA); and/or 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); a combination of two or more interleukins is also contemplated, e.g., a combination of an IL-15 pathway agonist or an IL-2 agonist and an IL-12 agonist, e.g., an IL-15R fused to an IL-15R agonist (IL-15RA) in combination with an IL-12R fused to an IL-12R agonist (IL-15RA).
  - human leukocyte antigen G (HLA-G); or any combination of two or more thereof;
  - human leukocyte antigen E (HLA-E)
  - leukocyte surface antigen cluster of differentiation CD47 (CD47)
  - and

- They may exhibit a loss of function of:
    - transforming growth factor beta receptor 2 (TGFbetaR2, *e.g.*, either by modification of the encoding sequence or by expression of a dominant negative variant);
    - adenosine A2a receptor (ADORA2A);
    - T cell immunoreceptor with Ig and ITIM domains (TIGIT);
    - β-2 microgobulin (B2M);
    - class II, major histocompatibility complex, transactivator (CIITA);
    - programmed cell death protein 1 (PD-1, CD279), or express a PD-1 antagonist;
    - cytokine inducible SH2 containing protein (CISH);
    - natural killer cell receptor NKG2A (natural killer group 2A);
    - two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;
    - cluster of differentiation 32B (CD32B, FCGR2B);
    - or any combination of two or more thereof.

[425] It is desirable to achieve specific combinations of these characteristics, *e.g.*, an iNK cell expressing a CAR, IL-15, and HLA-G, and exhibiting a loss-of-function in B2M and PD-1 by minimizing the number of edits. For example, an expression construct encoding the CAR could be inserted into the B2M locus and an expression construct encoding IL-15 and HLA-G could be inserted into the B2M locus. Similar strategies would apply to other combinations.

[426] The iNK cells could be used as a monotherapy, and those expressing a CAR (*e.g.*, a CAR binding mesothelin, EGFR, or HER2) would be particularly suitable for therapeutic approaches specifically targeting cells expressing a surface antigen the CAR binds. Some iNK cells envisioned may also be suitable for combination therapy approaches, *e.g.*, in combination with a monoclonal antibody targeting cancer cells.

[427] In some embodiments, the generation of iPS cells would include obtaining a donor cell, e.g., a somatic cell from a healthy donor individual. In some embodiments, a donor cell or cell population is confirmed to be karyotypically normal, and not to exhibit expression of a gene or a combination of genes known to be associated with a pathological state, e.g., a malignant state. In some embodiments, the somatic cell is edited and then reprogrammed to a pluripotent state. In some embodiments, the somatic cell is reprogrammed and at the same time edited. In some embodiments, the somatic cell is reprogrammed and a resulting

pluripotent cell is edited. In some embodiments, the generation of iPS cells comprises clonal expansion of reprogrammed cell lines, characterization of a number of such clonal iPS cell lines, and selection of a line that includes all the desired edits while being karyotypically normal.

[428] The end product for clinical use is a population of iNK cells carrying the respective edits. The number of cells would be sufficient to elicit a desired immune response after administration to a subject. The precise number would depend on the specific desired clinical outcome, the patient, and the disease to be treated, amongst other factors, and may vary greatly. It is anticipated that a suitable cell population for administration may range from about 1,000 cells to about 100,000,000 cells. The iNK cell population for clinical use should be free of remaining stem cells, *e.g.*, of iPS cells expressing Oct-4 and/or Sox2, should ideally be free of or contain only a minimal amount of cells harboring episomal expression constructs, *e.g.*, episomal expression constructs used during reprogramming of T cells; should be free of, or not contain more than 1 %, 5%, or 10%, of cells not expressing the desired combination of cell markers and overexpressed surface molecules.

Example 2: Use of T cells as cells of origin for a complex editing strategy and subsequent derivation of iNK cells

[429] The use of T cells as cells of origin for a complex editing strategy and subsequent derivation of iNK cells or other lymphocytes, for example, enable the generation of an iNK cell that express a CAR of interest such as mesothelin, EGFR, HER2, and MICA/B, and/or having one or more edits from List A and/or Table 10, and one or more edits from List B and/or Table 11.

[430] List A:

- Exogenous expression of an enhanced variant of CD16, *e.g.*, of hnCD16a (high affinity, non-cleavable variant of CD 16 - a low-affinity Fcy receptor involved in antibody-dependent cellular cytotoxicity (ADCC)). Typically, CD16 is cleaved during ADCC by proteases, the hnCD16 CAR does not undergo this cleavage and thus sustains an ADCC signal longer.
- Exogenous expression of IL-15/IL 15RA
- Loss-of-function of TGFbR2, or exogenous expression of a dominant-negative variant of TGFbR2 (dominant negative TGF beta receptor II is expressed from an NK-specific promoter, in order to not interfere with TGFbRII's role in the differentiation

of CD34 cells, which can be derived from iPS cells, and typically serve as a cell type from which heme lineages (like NK cells) are differentiated)

- Loss-of-function of ADORA2A

[431] List B:

- Loss-of-function of B2M (*e.g.*, eliminate MHC class I expression by targeting B2M expression) Exogenous expression of HLA-G
- Loss-of-function of CIITA (*e.g.*, eliminate MHC class II expression by targeting CIITA)
- Loss-of-function of PD1
- Loss-of-function of TIGIT
- Loss-of-function CISH (cytokine inducible SH2 containing protein)

[432] Loss-of-function preferably includes complete elimination of surface expression of the respective protein.

[433] For example, iNK cells with exogenous expression of a CAR and a CD16 variant (*e.g.*, hnCD16), or a CAR and no CD16 variant can be generated. Cells expressing no CAR but a CD16 variant can also be generated. Any cell expressing CD16, or an enhanced variant thereof (*e.g.*, hnCD16), would be suitable for combination therapy with a monoclonal antibody (*e.g.*, used in cancer therapy), or with an Fc fusion protein targeting pathological cells.

[434] If more than two transgenes are knocked-in, a multicistronic expression construct, or a 2A construct may be advantageous in order to avoid having to insert an individual construct for each transgene.

[435] Such iNK cells are useful for a wide range of immunotherapy applications, including, but not limited to the treatment of proliferative diseases, *e.g.*, certain forms of cancers. When using the CARs outlined above, applications in breast cancer, colon cancer, gastric cancer, renal cell carcinoma, and NSCLC are envisioned. The altered surface molecule repertoire of such cells would also enable the successful treatment of solid tumors, which has been proven difficult with current NK-cell based strategies.

[436] Exemplary iNK cells obtained from reprogrammed/edited T cells (or their daughter cells) comprise one or more (*e.g.,* one or more, two or more, three or more, four or more, five or more, or six or more) of the following characteristics:

- They comprise a rearranged endogenous TCR locus (*e.g.*, TCRa VJ and/or TCRf3 V(D)J section rearrangement & complete V-domain exons);
- They do not express an endogenous T cell co-receptor, *e.g.*, CD3, CD4, and/or CD8;
- They express an NK-cell biomarker, for example:
  - CD56 (NCAM), CD49, and/or CD45;
  - NK cell receptor immunoglobulin gamma Fc region receptor III (FcγRIII,cluster of differentiation 16 (CD16));
  - natural killer group-2 member 0 (NKG2D, MICAIB stress ligand receptor);
  - CD69;
  - a natural cytotoxicity receptor (*e.g.*, NKp30; NKp44; NKp46; and/or CD158b); or any combination of two or more of these;
- They may express:
  - a chimeric antigen receptor (CAR),
  - a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcyRIII, CD16)
  - an interleukin 15 (IL-15) pathway agonist, e.g., interleukin-15 (IL-15), interleukin 15 receptor (IL-15R) or a variant thereof (e.g., a constitutively active variant of IL-15R, e.g., an IL-15R fused to an IL-15R agonist (IL-15RA); other interleukin pathway agonists are also contemplated, either in the alternative or in combination with the IL-15 pathway agonist, *e.g.*, an interleukin 2 (IL-2) pathway agonist, e.g., IL-2, interleukin 2 receptor (IL-2R) or a variant thereof (e.g., a constitutively active variant of IL-2R, e.g., an IL-2R fused to an IL-2R agonist (IL-2RA); and/or 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); a combination of two or more interleukins is also contemplated, e.g., a combination of an IL-15 pathway agonist or an IL-2 agonist and an IL-12 agonist, e.g., an IL-15R fused to an IL-15R agonist (IL-15RA) in combination with an IL-12R fused to an IL-12R agonist (IL-15RA).
  - human leukocyte antigen G (HLA-G); or any combination of two or more thereof;
  - human leukocyte antigen E (HLA-E)
  - leukocyte surface antigen cluster of differentiation CD47 (CD47)

- and

- They may exhibit a loss of function of:
  - transforming growth factor beta receptor 2 (TGFf3R2, *e.g.*, either by modification of the encoding sequence or by expression of a dominant negative variant);
  - adenosine A2a receptor (ADORA2A);
  - T cell immunoreceptor with Ig and ITIM domains (TIGIT);
  - β-2 microgobulin (B2M);
  - class II, major histocompatibility complex, transactivator (CIITA);
  - programmed cell death protein 1 (PD-1, CD279), or express a PD-1 antagonist;
  - cytokine inducible SH2 containing protein (CISH);
  - natural killer cell receptor NKG2A (natural killer group 2A);
  - two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes;
  - cluster of differentiation 32B (CD32B, FCGR2B);
  - T cell receptor alpha constant (TRAC);
  - or any combination of two or more thereof.

[437] It is desirable to achieve specific combinations of these characteristics, *e.g.*, an iNK cell expressing a CAR, IL-15, and HLA-G, and exhibiting a loss-of-function in B2M and PD-1 by minimizing the number of edits. For example, an expression construct encoding the CAR could be inserted into the B2M locus and an expression construct encoding IL-15 and HLA-G could be inserted into the B2M locus. Similar strategies would apply to other combinations.

[438] The iNK cells could be used as a monotherapy, and those expressing a CAR (*e.g.*, a CAR binding mesothelin, EGFR, or HER2) would be particularly suitable for therapeutic approaches specifically targeting cells expressing a surface antigen the CAR binds. Some the iNK cells envisioned may also be suitable for combination therapy approaches, *e.g.*, in combination with a monoclonal antibody targeting cancer cells.

[439] The generation of iPS cells would include the clonal expansion of reprogrammed cell lines, the characterization of a number of such clonal iPS cell lines, and the selection of a line that includes all the desired edits while being karyotypically normal.

[440] The end product for clinical use is a population of iNK cells carrying the respective edits. The number of cells would be sufficient to elicit a desired immune response after

administration to a subject. The precise number would depend on the specific desired clinical outcome, the patient, and the disease to be treated, amongst other factors, and may vary greatly. It is anticipated that a suitable cell population for administration may range from about 1,000 cells to about 100,000,000 cells. The iNK cell population for clinical use should be free of remaining stem cells, *e.g.*, of iPS cells expressing Oct-4 and/or Sox2, should ideally be free of or contain only a minimal amount of cells harboring episomal expression constructs, *e.g.*, episomal expression constructs used during reprogramming of T cells; should be free of, or not contain more than 1 %, 5%, or 10%, of cells not expressing the desired combination of cell markers and overexpressed surface molecules.

Example 3: iPS/iNK cells for clinical applications

[441] For clinical use as an immunotherapeutic, e.g., in the context of immunooncology applications, modified lymphocytes, here iNK cells, are generated that comprise a loss-of-function of B2M; a loss-of-function of CIITA; and an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding HLA-G. These edits allow the edited cells, and/or differentiated iNK cells derived therefrom, to escape the immune system of a non-autologous host. Additional edits may be made to enhance the clinical properties of the iNK cells. These iNK cells are obtained by reprogramming a somatic donor cell from a healthy donor, reprogramming the donor cell into a pluripotent state and effecting the desired edits. Once edited, the pluripotent cells are differentiated into NK cells, resulting in a population of modified iNK cells for clinical application.

Example 4: iPS/iNK cells for clinical applications

[442] For clinical use as an immunotherapeutic, e.g., in the context of immunooncology applications, modified lymphocytes, here iNK cells, are generated that comprise a loss-of-function of B2M; a loss-of-function of CIITA; and an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding HLA-E. In some embodiments, the cells further comprise a loss-of function of NKG2A. These edits allow the edited cells, and/or differentiated iNK cells derived therefrom, to escape the immune system of a non-autologous host. Additional edits may be made to enhance the clinical properties of the iNK cells. These iNK cells are obtained by reprogramming a somatic donor cell from a healthy donor, reprogramming the donor cell into a pluripotent state and effecting the desired edits. Once edited, the pluripotent cells are differentiated into NK cells, resulting in a population of modified iNK cells for clinical application.

Example 5: iPS/iNK cells for clinical applications

[443] For clinical use as an immunotherapeutic, e.g., in the context of immunooncology applications, modified lymphocytes, here iNK cells, are generated that comprise a loss-of-function of B2M; a loss-of-function of CIITA; and an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding CD47. These edits allow the edited cells, and/or differentiated iNK cells derived therefrom, to escape the immune system of a non-autologous host. Additional edits may be made to enhance the clinical properties of the iNK cells. These iNK cells are obtained by reprogramming a somatic donor cell from a healthy donor, reprogramming the donor cell into a pluripotent state and effecting the desired edits. Once edited, the pluripotent cells are differentiated into NK cells, resulting in a population of modified iNK cells for clinical application.

Example 6: iPS/iNK cells for clinical applications

[444] For clinical use as an immunotherapeutic, e.g., in the context of immunooncology applications, modified lymphocytes, here iNK cells, are generated that comprise a loss-of-function of B2M; a loss-of-function of HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5, HLA-DQB1, and HLA-DPB1; and an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding HLA-G. These edits allow the edited cells, and/or differentiated iNK cells derived therefrom, to escape the immune system of a non-autologous host. Additional edits may be made to enhance the clinical properties of the iNK cells. These iNK cells are obtained by reprogramming a somatic donor cell from a healthy donor, reprogramming the donor cell into a pluripotent state and effecting the desired edits. Once edited, the pluripotent cells are differentiated into NK cells, resulting in a population of modified iNK cells for clinical application.

Example 7: iPS/iNK cells for clinical applications

[445] For clinical use as an immunotherapeutic, e.g., in the context of immunooncology applications, modified lymphocytes, here iNK cells, are generated that comprise a loss-of-function of B2M; a loss-of-function of HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5, HLA-DQB1, and HLA-DPB1; and an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding HLA-E. In some embodiments, the cells further comprise a loss-of function of NKG2A. These edits allow the edited cells, and/or differentiated iNK cells derived therefrom, to escape the immune system of a non-autologous

host. Additional edits may be made to enhance the clinical properties of the iNK cells. These iNK cells are obtained by reprogramming a somatic donor cell from a healthy donor, reprogramming the donor cell into a pluripotent state and effecting the desired edits. Once edited, the pluripotent cells are differentiated into NK cells, resulting in a population of modified iNK cells for clinical application.

Example 8: iPS/iNK cells for clinical applications

[446] For clinical use as an immunotherapeutic, e.g., in the context of immunooncology applications, modified lymphocytes, here iNK cells, are generated that comprise a loss-of-function of B2M; a loss-of-function of HLA-DRB1, HLA-DRB3, HLA-DRB4, HLA-DRB5, HLA-DQB1, and HLA-DPB1; and an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding CD47. These edits allow the edited cells, and/or differentiated iNK cells derived therefrom, to escape the immune system of a non-autologous host. Additional edits may be made to enhance the clinical properties of the iNK cells. These iNK cells are obtained by reprogramming a somatic donor cell from a healthy donor, reprogramming the donor cell into a pluripotent state and effecting the desired edits. Once edited, the pluripotent cells are differentiated into NK cells, resulting in a population of modified iNK cells for clinical application.

Example 9: iPS/iNK cells for clinical applications

[447] Additional edits are made to the cells provided in examples 3-8 that enhance the effectiveness of the iNK cell as a therapeutic.

[448] These edits include, in some embodiments, knock-in of an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding a variant of IL-15R, here a fusion of IL-15R with its ligand (IL-15, or an IL-15-binding fragment thereof), resulting in a constitutively active IL-15 pathway in the iNK cells.

[449] These edits further include, in some embodiments, knock-in of an exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding transforming growth factor beta receptor 2 (TGFβR2) under the control of an NK-cell specific promoter, e.g., a CD45 promoter.

[450] These edits further include, in some embodiments, a loss-of-function of CD32B(FCGR2).

Example 10: Gene edited NK cells, exhibiting loss-of-function of CISH and/or TGFBR2, demonstrate improved effector function in response to tumor cells

[451] A next-generation allogeneic NK cell therapy was developed using CRISPR-Cpf1 gene editing to enhance NK cell function through knockout of the CISH and TGFBR2 genes.

[452] NK cells were expanded from $CD3^-$ PBMC cultures in 20ng/mL IL-15. Gene editing was performed at different NK cell expansion stages (between day 8-21). For editing of CISH and TGFBR2, guides for either targets were complexed with Cpf1 nuclease at a 2:1 ratio to form ribonucleoproteins (RNPs). Where cells were edited with both targets, RNP complexation for each target was done separately and then mixed at a 1:1 ratio prior to electroporation.

[453] For electroporation, NK cells were suspended in HyClone buffer at a density of $80x10^6$ cells/mL. Ninety microliters of NK cells were mixed with 10 microliters of the appropriate RNPs. The cell and RNP mixtures were then transferred to a MaxCyte OC-100 or OC-400 cassette for electroporation. Immediately post-electroporation, NK cells were recovered in 100microliters of culture media for 10 minutes at 37°C, before transferring to a 24-well Grex plate for post editing recovery and functional analyses.

[454] The following guide RNA sequences were used for editing of CISH and TGFBR2: Both guides were generated with a targeting domain consisting of RNA, an AsCpf1 scaffold of the sequence UAAUUUCUACUCUUGUAGAU 5' of the targeting domain, and a 25-mer DNA extension of the sequence ATGTGTTTTTGTCAAAAGACCTTTT at the 5' terminus of the scaffold sequence.

Table 12:

Target	**gRNA targeting domain Sequence (DNA)**	**gRNA sequence**
CISH 7050	GGTGTACAGCAGTGGCTGGT	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrGrGrUrGrUrArCrArGrCrArGrUrGrGrCrUrGrGrU
TGFBR2 24026	TGATGTGAGATTTTCCACCT	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrUrGrArUrGrUrGrArGrArUrUrUrUrCrCrArCrCrU

[455] As demonstrated in **Figs. 1A-1B**, robust single and double-gene editing of TGFBR2 and CISH was achieved in NK cells. Both single and simultaneous targeting of TGFBR2 and CISH in NK cells using CRISPR-Cpf1 produced indels at both targets in greater than 80% of NK cells, with greater than 90% of edited NK cells viable at 72 hours post-editing.

[456] The efficacy of the effector cells was assessed *in vitro* by 3D tumor spheroid assays

[457] To form spheroids, 5,000 NucLight Red labeled PC-3 or SK-OV-3 tumor cells were plated in a single well of ultra-low attachment 96 well plates, centrifuged at 1,000rpm for 10 minutes, and incubated for 96 hours at 37° C. At 96 hours, effector cells (primary human NK cells treated with different RNPs) were added to spheroids at multiple effector to target cell ratios with or without 10ng/mL of TGF-beta. Red object intensity was measured every two hours for 6 days on an Incucyte imaging system. 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 (**Figs. 2A-2B**).

[458] Moreover, CISH KO NK cells reduced the growth of SK-OV-3 ovarian (**Figs. 3A-3B** and **Fig. 5A**) and PC-3 prostate tumor spheroids (**Figs. 4A-4B** and **Fig. 5B**) by an average of 23% and 12% ($p<0.0001$ in both cases), respectively, in comparison to unedited controls. However, the activity of CISH KO NK cells were dampened by addition of exogenous TGF-β.

[459] Given this observation, a knockout of the TGF-β receptor gene, TGFBR2, with the CISH KO was generated. Single knockout of TGFBR2 rendered NK cells resistant to TGFβ inhibition ($p<0.0001$). Importantly, across 4 unique donors and 7 independent experiments, TGFBR2/CISH double knockout (DKO) NK cells demonstrated superior effector function and attenuated SK-OV-3 and PC-3 tumor spheroid growth by greater than 60% for both tumor types, with supplement of exogenous TGF-β (**Figs. 3A-3B** and **Figs. 4A-4B**) and without supplement of exogenous TGF-β (**Fig. 5A-5B**). These effector functions were statistically greater than control NK cells or TGFBR2 and CISH single knockout NK cells ($p<0.0001$ in all cases). In addition, TGFBR2/CISH DKO NK cells produced higher concentrations of TNF-α (**Fig. 6A**) and IFN-γ (**Fig. 6B**) $p<0.01$ in both cases as assessed by ELISA.

[460] Double KO NK cells expressed significantly higher levels of activation markers CD25 and CD69 as compared to control NK cells (**Fig. 6C**).

[461] Anti-tumor activity of edited NK cells was measured in an in vivo model. NSG mice received an intraperitoneal injection of 500,000 SKOV3 tumor cells labeled with luciferase. Seven days post-tumor implantation, 10 million edited (CISH/TGFBR2 double-knockout) or unedited (control) NK cells were injected into the peritoneal cavity of the tumor-bearing mice. Tumor burden was monitored weekly by IP administration of luciferin and IVIS imaging.

Two-way ANOVA analysis was performed at day 34 to determine statistical significance between control and DKO NK cell groups (****, P<0.0001) (**Fig 6.D**).

[462] These results demonstrate efficient editing of primary human NK cells at two unique targets simultaneously with CRISPR-Cpf1. Together, the increased effector function of CISH/TGFBR2 DKO primary human NK cells *in vitro* and *in vivo* relative to either single knockout or unedited NK cells indicates an enhanced and synergistic effect of the CISH/TGFBR2 DKO.

Example 11: Gene edited NK cells, exhibiting loss-of-function of TIGIT, NKG2A or ADORA2A, demonstrate improved effector function in response to tumor cells

[463] A next-generation allogeneic NK cell therapy was developed using CRISPR-Cpf1 gene editing to enhance NK cell function through knockout of the TIGIT, NKG2A, or ADORA2A genes.

[464] NK cells were expanded as previously described in Example 10. Briefly, NK cells were expanded *ex-vivo* for 14 days in IL15, and then edited with the respective targeting RNP complex. Gene editing was performed at different NK cell expansion stages (between day 8-21). For editing of TIGIT, NKG2A, or ADORA2A, guides for the corresponding targets were complexed with Cpf1 nuclease at a 2:1 ratio to form ribonucleoproteins (RNPs). Where cells were edited with both targets, RNP complexation for each target was done separately and then mixed at a 1:1 ratio prior to electroporation. NK cells were electroporated as previously described in Example 10.

[465] The following guide RNA sequences were used for editing of TIGIT, NKG2A, or ADORA2A:

Table 13:

Target	**gRNA targeting domain Sequence (DNA)**	**gRNA sequence**
TIGIT	TGCAGAGAAAGGTGGCTCTA	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArArUrUrGrCrArGrArGrArArArGrGrUrGrGrCrUrCrUrA
NKG2A	GCAACTGAACAGGAAATAACC	UAAUUUCUACUCUUGUAGAUGCAACUGAACAGGAAAUAACC
ADORA2A	CCTGTGTGCTGGTGCCCCTG	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArArUrCrCrUrGrUrGrUrGrUrGrCrUrGrGrUrGrCrCrCrCrUrG

[466] As demonstrated in **Figs. 7A-7C**, robust single gene editing of TIGIT, NKG2A, and ADORA2A was achieved in NK cells.

[467] The efficacy of the effector cells (primary human NK cells treated with different RNPs), was assessed *in vitro* to determine the function of TIGIT single KO **(Figs. 8A-8B)**, NKG2A single KO **(Figs. 9A-9B)** and ADORA2A single KO **(Figs. 10A-10B)** by 3D tumor spheroid assays

[468] To form spheroids, 5,000 NucLight Red labeled PC-3 or SK-OV-3 tumor cells were plated in a single well of ultra-low attachment 96 well plates, centrifuged at 1,000rpm for 10 minutes, and incubated for 96 hours at 37° C. At 96 hours, effector cells (primary human NK cells treated with different RNPs) were added to spheroids at multiple effector to target cell ratios with or without 10ng/mL of TGF-beta. Red object intensity was measured every two hours for 6 days on an Incucyte imaging system. Data shown are normalized to the red object intensity at time of effector addition.

[469] Across 2 unique donors and 2 independent experiments, TIGIT single KO **(Figs. 8A-8B)**, NKG2A single KO **(Figs. 9A-9B)** and ADORA2A single KO **(Figs. 10A-10B)** NK cells demonstrated superior effector function and attenuated SK-OV-3 and PC-3 tumor spheroid growth . These data demonstrate efficient editing of primary human NK cells at three independent unique targets with CRISPR-Cpf1, resulting in increased effector function of TIGIT single KO, NKG2A single KO and ADORA2A single KO primary human NK cells *in vitro* relative to unedited NK cells.

Example 12: Gene edited NK cells, exhibiting loss-of-function of CISH, TGFBR2 and TIGIT, demonstrate improved effector function in response to tumor cells

[470] A next-generation allogeneic NK cell therapy was developed using CRISPR-Cpf1 gene editing to enhance NK cell function through knockout of the CISH, TGFBR2 and TIGIT genes.

[471] NK cells were expanded as previously described in Example 10. Briefly, NK cells were expanded *ex-vivo* for 14 days in IL15, and then edited with the respective targeting RNP complex. Gene editing was performed at different NK cell expansion stages (between day 8-21). For editing of CISH, TGFBR2 and TIGIT, guides for the targets were complexed with Cpf1 nuclease to form ribonucleoproteins (RNPs), RNP complexation for each target was

done separately and then mixed at a 1:1 ratio prior to electroporation. NK cells were electroporated as previously described in Example 10.

[472] The guide RNA sequences that were used for editing of CISH, TGFBR2 and TIGIT are indicated in Table 14 below:

Table 14

Target	**gRNA targeting domain Sequence (DNA)**	**gRNA sequence**
CISH	ACTGACAGCGTGAACAGGTAG	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrArCrUrGrArCrArGrCrGrUrGrArArCrArGrGrUrArG
TGFBR2	TGATGTGAGATTTTCCACCT	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrUrGrArUrGrUrGrArGrArUrUrUrUrCrCrArCrCrU
TIGIT	TGCAGAGAAAGGTGGCTCTA	ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrUrCrUrArCrUrCrUrUrGrUrArGrArUrUrGrCrArGrArGrArArArGrGrUrGrGrCrUrCrUrA

[473] As demonstrated in **Fig. 11**, robust triple-gene editing of TGFBR2, CISH and TIGIT was achieved in NK cells.

[474] The efficacy of the effector cells was assessed *in vitro* by 3D tumor spheroid assays.

[475] To form spheroids, 5,000 NucLight Red labeled PC-3 or SK-OV-3 tumor cells were plated in a single well of ultra-low attachment 96 well plates, centrifuged at 1,000rpm for 10 minutes, and incubated for 96 hours at 37° C. At 96 hours, effector cells (primary human NK cells treated with different RNPs) were added to spheroids at multiple effector to target cell ratios with or without 10ng/mL of TGF-beta. Red object intensity was measured every two hours for 6 days on an Incucyte imaging system. 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.

[476] TGFBR2/CISH/TIGIT triple knockout (TKO) NK cells demonstrated superior effector function and attenuated SK-OV-3 and PC-3 tumor spheroid growth (**Figs. 12A-12B**). These effector functions were statistically greater than control NK cells. These results demonstrate efficient editing of primary human NK cells at three unique targets simultaneously with CRISPR-Cpf1. Together, the increased effector function of CISH/TGFBR2/TIGIT TKO primary human NK cells *in vitro* and relative to unedited NK cells indicates an enhanced effect of the CISH/TGFBR2/TIGIT TKO.

CLAIMS

1. A modified lymphocyte, wherein the modified lymphocyte:

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

(b) expresses at least one endogenous gene encoding:

(i) CD56 (NCAM), CD49, and/or CD45;

(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;

wherein the modified lymphocyte further:

(1) comprises at least one exogenous nucleic acid expression construct comprising 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) IL-15 receptor (IL-15R), or a variant thereof;

(v) interleukin 12 (IL-12);

(vi) IL-12 receptor (IL-12R), or a variant thereof;

(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) exhibits a loss of function of at least one of:

(i) transforming growth factor beta receptor 2 (TGFβR2);

(ii) adenosine A2a receptor (ADORA2A);

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

(iv) β-2 microgobulin (B2M);

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

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

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

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

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

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

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

or any combination of two or more thereof.

2. The modified lymphocyte of claim 1, wherein the lymphocyte exhibits a loss of function of :

(i) TGFβR2, CISH, TIGIT, ADORA2A, or NKG2A;

(ii) TGFβR2 and CISH, TGFβR2 and TIGIT, TGFβR2 and ADORA2A, TGFβR2 and NKG2A, CISH and TIGIT, CISH and ADORA2A, CISH and NKG2A, TIGIT and ADORA2A, TIGIT and NKG2A, or ADORA2A and NKG2A; or

(iii) TGFβR2, CISH and TIGIT; TGFβR2, CISH and ADORA2A; TGFβR2, CISH and NKG2A; TGFβR2, TIGIT and ADORA2A; TGFβR2, TIGIT and NKG2A; TGFβR2, ADORA2A and NKG2A; CISH, TIGIT and ADORA2A; CISH, TIGIT and NKG2A; CISH, ADORA2A and NKG2A; or TIGIT, ADORA2A and NKG2A.

3. The modified lymphocyte of claim 1 or 2, wherein the lymphocyte comprises a rearranged endogenous T-cell receptor (TCR) locus.

4. The modified lymphocyte of claim 3, wherein the rearranged TCR comprises TCRα VJ and/or TCRβ V(D)J section rearrangements and complete V-domain exons.

5. The modified lymphocyte of claim 1, 2, 3 or 4 wherein the natural cytotoxicity receptor is NKp30, NKp44, NKp46, and/or CD158b.

6. The modified lymphocyte of any of claims 1-5, wherein the IL-15R variant is a constitutively active IL-15R variant and/or wherein the IL12-R variant is a constitutively active IL12-R variant.

7. The modified lymphocyte of claim 6, wherein the constitutively active IL-15R variant is a fusion between IL-15R and an IL-15R agonist (IL-15RA), and/or wherein the constitutively active IL-12R variant is a fusion between IL-12R and an IL-12R agonist (IL-12RA).

8. The modified lymphocyte of claim 7, wherein the IL-15R agonist is IL-15, or an IL-15R-binding variant thereof; and/or wherein the IL-12R agonist is IL-12, or an IL-12R-binding variant thereof.

9. The modified lymphocyte of any one of claims 1-8, wherein the loss of TGFβR2 is associated with exogenous expression of a dominant-negative variant of TGFβ receptor II (DN-TGFβR2).

10. The modified lymphocyte of claim 1, wherein the CAR is capable of binding mesothelin, EGFR, HER2, MICA/B, BCMA, CD19, CD22, CD20, CD33, CD123, androgen receptor, PSMA, PSCA, Muc1, HPV viral peptides (ie. E7), EBV viral peptides, CD70, WT1, CEA, EGFRvIII, IL13Rα2, and GD2, CA125, CD7, EpCAM, Muc16, or CD30,

11. The modified lymphocyte of any one of claims 1-10, wherein the lymphocyte is derived from a pluripotent or multipotent stem cell.

12. The modified lymphocyte of claim 11, wherein the multipotent stem cell is a hematopoietic stem cell (HSC).

13. The modified lymphocyte of claim 11, wherein the pluripotent stem cell is an induced pluripotent stem cell (iPSC).

14. The modified lymphocyte of claim 11, wherein the pluripotent stem cell is an embryonic stem cell (ESC).

15. The modified lymphocyte of any one of claims 1-11, wherein the lymphocyte is derived from a pluripotent or multipotent stem cell that comprises at least one or more

exogenous nucleic acid constructs encoding any of (1)(i)-(1)(xi), or any combination thereof; and/or at least one genomic alteration that effects the loss-of-function of any of (2)(i)-(2)(xi), or any combination thereof, in the lymphocyte.

16. The modified lymphocyte of claim 15, wherein the lymphocyte is derived from a pluripotent or multipotent stem cell that comprises at least one genomic alteration that effects the loss-of-function of any of (2)(i)-(2)(xi), or any combination thereof, in the lymphocyte.

17. The modified lymphocyte of claim 15 or claim 16, wherein the at least one genomic alteration that effects the loss-of-function of one or more (2)(i)-(2)(xi) in the lymphocyte comprises an insertion of an exogenous nucleic acid construct.

18. The modified lymphocyte of claim 17, wherein the exogenous nucleic acid construct encodes any of (1)(i)-(1)(ix), or any combination thereof.

19. The modified lymphocyte of any one of claims 1-18, wherein the lymphocyte exhibits a loss-of-function in two or more of the genes/proteins listed under (2).

20. The modified lymphocyte of any one of claims 1-19, wherein the lymphocyte comprises an indel or an insertion of an exogenous nucleotide construct into a genomic locus harboring a gene or encoding a protein under (2).

21. The modified lymphocyte of any one of claims 1-20, wherein the lymphocyte comprises an indel or an insertion of an exogenous nucleotide construct into two or more genomic loci harboring a gene or encoding a protein under (2).

22. The modified lymphocyte of any one of claims 1-21, wherein the lymphocyte is obtained by editing a genomic locus with an RNA-guided nuclease.

23. The modified lymphocyte of claim 22, wherein the RNA-guided nuclease is a CRISPR/Cas nuclease.

24. The modified lymphocyte of claim 22, wherein the RNA-guided nuclease is selected from the group consisting of SpCas9, SaCas9, (KKH) SaCas9, AsCpf1 (AsCas12a), LbCpf1,

(LbCas12a), CasX, CasY, Cas12h1, Cas12i1, Cas12c1, Cas12c2, eSpCas9, Cas9-HF1, HypaCas9, dCas9-Fokl, Sniper-Cas9, xCas9, AaCas12b, evoCas9, SpCas9-NG, VRQR, VRER, NmeCas9, CjCas9, BhCas12b, and BhCas12b V4.

25. The modified lymphocyte of any one of claims 1-24, wherein the lymphocyte is obtained by editing two or more genomic loci harboring genes encoding any of the proteins under (2).

26. The modified lymphocyte of claim 25, wherein at least two of the two or more genomic loci harboring genes encoding any of the proteins under (2) have been edited by a different RNA-guided nuclease.

27. The modified lymphocyte of claim 25, wherein at least one of the two or more genomic loci harboring genes encoding any of the proteins under (2) has been edited by Cas9, and wherein at least one of the loci has been edited by Cpf1.

28. The modified lymphocyte of any one of claims 1-27, wherein the modified lymphocyte expresses endogenous CD56, CD49, and CD45.

29. The modified lymphocyte of any one of claims 1-28, wherein the lymphocyte is a natural killer (NK) cell.

30. A modified cell, wherein the modified cell

(1) comprises at least one exogenous nucleic acid expression construct comprising 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) IL-15 receptor (IL-15R), or a variant thereof;

(v) interleukin 12 (IL-12);

(vi) IL-12 receptor (IL-12R), or a variant thereof;

(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) exhibits a loss of function of at least one of:

(i) transforming growth factor beta receptor 2 (TGFβR2);

(ii) adenosine A2a receptor (ADORA2A);

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

(iv) β-2 microgobulin (B2M);

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

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

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

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

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

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

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

or any combination of two or more thereof.

31. The modified cell of claim 30, wherein the modified cell exhibits a loss of function of :

(i) TGFβR2, CISH, TIGIT, ADORA2A, or NKG2A;

(ii) TGFβR2 and CISH, TGFβR2 and TIGIT, TGFβR2 and ADORA2A, TGFβR2 and NKG2A, CISH and TIGIT, CISH and ADORA2A, CISH and NKG2A, TIGIT and ADORA2A, TIGIT and NKG2A, or ADORA2A and NKG2A; or

(iii) TGFβR2, CISH and TIGIT; TGFβR2, CISH and ADORA2A; TGFβR2, CISH and NKG2A; TGFβR2, TIGIT and ADORA2A; TGFβR2, TIGIT and NKG2A; TGFβR2, ADORA2A and NKG2A; CISH, TIGIT and ADORA2A; CISH, TIGIT and NKG2A; CISH, ADORA2A and NKG2A; or TIGIT, ADORA2A and NKG2A.

32. The modified cell of claim 30 or 31, wherein the modified cell is an immune cell.

33. The modified cell of claim 32, wherein the immune cell is a lymphocyte.

34. The modified cell of claim 33, wherein the lymphocyte is an NK cell.

35. The modified cell of claim 30, wherein the cell is a pluripotent stem cell, or a differentiated daughter cell derived therefrom.

36. The modified cell of claim 34 or claim 35, wherein the modified cell does not express an endogenous T-cell co-receptor.

37. The modified cell of claim 33, wherein the lymphocyte is a T cell.

38. The modified cell of claim 30, wherein the cell comprises a rearranged endogenous TCR locus, wherein the rearranged TCR comprises TCRα VJ and/or TCRβ V(D)J section rearrangements and complete V-domain exons.

39. The modified cell of any one of claims 30-38, wherein the modified cell expresses at least one endogenous gene encoding:

(i) CD56 (NCAM), CD49, and/or CD45;

(ii) FcγRIII (CD16);

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

(iv) CD69;

(v) a natural cytotoxicity receptor;

or any combination of two or more thereof.

40. The modified cell of claim 39, wherein the natural cytotoxicity receptor is NKp30, NKp44, NKp46, and/or CD158b.

41. The modified cell of any one of claims 30-40, wherein the cell expresses at least one NK cell biomarker.

42. The modified cell of claim 41, wherein the NK cell biomarker is CD56, CD49, and/or CD45.

43. A population of cells comprising the modified lymphocyte of any one of claims 1-28, or the modified cell of any one of claims 30-42.

44. A pharmaceutical composition comprising the population of cells of claim 43.

45. An isolated population of lymphocytes, wherein the population comprises at least $1x10^3$, at least $1x10^4$, at least $1x10^5$, at least $2x10^5$, at least $3x10^5$, at least $4x10^5$, at least $5x10^5$,at least $1x10^6$, at least $2x10^6$, at least $3x10^6$, at least $4x10^6$, at least $5x10^6$,at least $1x10^7$, at least $1x10^7$, at least $2x10^7$, at least $3x10^7$, at least $4x10^7$, at least $5x10^7$,at least $1x10^8$, at least $2x10^8$, at least $3x10^8$, at least $4x10^8$, at least $5x10^8$,at least $1x10^9$, at least $1x10^9$, at least $2x10^9$, at least $3x10^9$, at least $4x10^9$, at least $5x10^9$,at least $1x10^{10}$, at least $2x10^{10}$, at least $3x10^{10}$, at least $4x10^{10}$, at least $5x10^{10}$, at least $1x10^{11}$, or at least $1x10^{12}$ cells, and wherein at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, at least 99.9%, at least 99.99%, at least 99.999%, or virtually 100% of the lymphocytes in the population:

(a) comprise a rearranged T-cell receptor (TCR) locus;

(b) do not express endogenous CD3;

(c) express endogenous CD56 (NCAM), CD49, and/or CD45; and

(d) expresses at least one endogenous gene encoding:

(i) FcγRIII (CD16);

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

(iii) CD69;

(iv) a natural cytotoxicity receptor;

or any combination of two or more thereof; and

wherein the modified lymphocyte further:

(1) comprises at least one exogenous nucleic acid expression construct comprising a nucleic acid sequence encoding:

(i) chimeric antigen receptor (CAR);

(ii) non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16);

(iii) interleukin 15 (IL-15);

(iv) IL-15 receptor (IL-15R), or a variant thereof;

(v) interleukin 12 (IL-12);

(vi) IL-12 receptor (IL-12R), or a variant thereof;

(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) exhibits a loss of function of at least one of:

(i) transforming growth factor beta receptor 2 (TGFβR2);

(ii) adenosine A2a receptor (ADORA2A);

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

(iv) β-2 microgobulin (B2M);

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

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

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

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

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

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

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

or any combination of two or more thereof.

46. The isolated population of lymphocytes of claim 45, wherein the modified lymphocyte exhibits a loss of function of :

(i) TGFβR2, CISH, TIGIT, ADORA2A, or NKG2A;

(ii) TGFβR2 and CISH, TGFβR2 and TIGIT, TGFβR2 and ADORA2A, TGFβR2 and NKG2A, CISH and TIGIT, CISH and ADORA2A, CISH and NKG2A, TIGIT and ADORA2A, TIGIT and NKG2A, or ADORA2A and NKG2A; or

(iii) TGFβR2, CISH and TIGIT; TGFβR2, CISH and ADORA2A; TGFβR2, CISH and NKG2A; TGFβR2, TIGIT and ADORA2A; TGFβR2, TIGIT and NKG2A; TGFβR2, ADORA2A and NKG2A; CISH, TIGIT and ADORA2A; CISH, TIGIT and NKG2A; CISH, ADORA2A and NKG2A; or TIGIT, ADORA2A and NKG2A.

47. The isolated population of lymphocytes of claim 45 or 46, wherein the rearranged TCR locus comprises of TCRα VJ and/or TCRβ V(D)J section rearrangements and complete V-domain exons.

48. The isolated *in vitro* population of lymphocytes of claim 47, wherein the rearranged endogenous TCR locus consists of no more than two rearranged alleles.

49. The isolated *in vitro* population of lymphocytes of claim 45, wherein the natural cytotoxicity receptor is NKp30, NKp44, NKp46, and/or CD158b.

50. The isolated *in vitro* population of lymphocytes of any one of claims 45-49, wherein the population does not comprise more than 1%, more than 0.1%, more than 0.001%, more than 0.0001%, more than 0.00001%, more than 0.000001%, more than 0.0000001%, more than 0.00000001%, more than 0.000000001%, more than 0.0000000001%, or more than more than 0.00000000001% of cells expressing a reprogramming factor from an exogenous nucleic acid construct.

51. The isolated *in vitro* population of lymphocytes of claim 50, wherein the population does not comprise a cell expressing a reprogramming factor from an exogenous nucleic acid construct.

52. The isolated *in vitro* population of lymphocytes of claim 50 or claim 51, wherein the reprogramming factor is Oct-4 and/or Sox-2.

53. The isolated *in vitro* population of lymphocytes of claim 45, wherein the population does not comprise cells harboring episomal expression constructs encoding a reprogramming factor.

54. The isolated *in vitro* population of lymphocytes of any one of claims 45-53, wherein each cell in the population of cells comprises the same combination of (1) and (2).

55. The isolated *in vitro* population of lymphocytes of any one of claims 45-54, wherein the cell population comprises less than 0.001%, less than 0.002%, less than 0.003%, less than

0.004%, less than 0.005%, less than 0.006%, less than 0.007%, less than 0.008%, less than 0.009%, less than 0.01%, less than 0.02%, less than 0.03%, less than 0.04%, less than 0.05%, less than 0.06%, less than 0.07%, less than 0.08%, less than 0.09%, less than 0.1%, less than 0.2%, less than 0.3%, less than 0.4%, less than 0.5%, less than 0.6%, less than 0.7%, less than 0.8%, less than 0.9%, less than 1%, less than 2%, less than 3%, less than 4%, less than 5%, less than 6%, less than 7%, less than 8%, less than 9%, or less than 10% cell that harbor a chromosomal translocation.

56. A method of treating a subject, the method comprising administering the lymphocyte of any one of claims 1-29, the modified cell of any one of claims 30-42, the population of cells of claim 41, the pharmaceutical composition of claim 44, or the isolated *in vitro* population of lymphocytes of any one of claims 45-55, to a subject in need thereof.

57. The method of claim 56, wherein the subject has, or is diagnosed with, a proliferative disease.

58. The method of claim 57, wherein the proliferative disease is cancer.

59. The method of claim 58, wherein the cancer is breast cancer, colorectal cancer, gastric cancer, renal cell carcinoma (RCC), or non-small cell lung cancer (NSCLC), solid tumors, 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, soft tissue sarcomas, and hematological cancers like ALL, CLL, NHL, DLBCL, AML, CML, multiple myeloma (MM).

60. A method of generating the lymphocyte of any one of claims 1-29, the modified cell of any one of claims 30-42, the population of cells of claim 43, or the isolated *in vitro* population of lymphocytes of any one of claims 44-54, the method comprising:

(a) obtaining an induced pluripotent stem cell (iPSC);

(b) modifying the iPSC, or an undifferentiated or differentiated daughter cell thereof, to comprise express at least one exogenous nucleic acid expression construct of (1) and/or to comprise a loss of function in at least one gene of (2);

(c) directing differentiation of the iPSC to hematopoietic lineage cells,

wherein the hematopoietic lineage cells retain the edited genetic loci comprised in the iPSCs.

61. The method of claim 60, wherein directing differentiation comprises:

(i) contacting iPSCs with a composition comprising a BMP pathway activator, and optionally bFGF, to obtain mesodermal cells; and

(ii) contacting the mesodermal cells with a composition comprising a BMP pathway activator, bFGF, and a WNT pathway activator, to obtain mesodermal cells having definitive hemogenic endothelium (HE) potential, wherein the mesodermal cells having definitive hemogenic endothelium (HE) potential are capable of providing hematopoietic lineage cells;

wherein mesodermal cells and mesodermal cells having definitive HE potential are obtained in steps (i) and (ii) without the step of forming embryoid bodies;

wherein the hematopoietic lineage cells comprise definitive hemogenic endothelium cells, hematopoietic stem and progenitor cells (HSC), hematopoietic multipotent progenitor cell (MPP), pre-T cell progenitor cells, pre-NK cell progenitor cells, T cell progenitor cells, NK cell progenitor cells, T cells, NK cells, NKT cells, or B cells.

62. The method of claim 61, wherein directing differentiation of iPSCs to hematopoietic lineage cells further comprises: contacting the mesodermal cells having definitive HE potential with a composition comprising bFGF and a ROCK inhibitor to obtain definitive HE cells.

63. The method of claim 60, claim 61, or claim 62, further comprising: contacting the definitive HE cells with a composition comprising a BMP activator, and optionally a ROCK inhibitor, and one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GMCSF, EPO, bFGF, VEGF, SCF, IL6, Flt3L and IL11 to obtain hematopoietic multipotent progenitor cells (MPP).

64. The method of any one of claims 60-63, further comprising: contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7; and optionally one or more of a BMP activator, a ROCK inhibitor, TPO, VEGF and bFGF to obtain pre-T cell progenitors, T cell progenitors, and/or T cells.

65. The method of any one of claims 60-63, further comprising: contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, TPO, IL7 and IL15, and optionally one or more of a BMP activator, a ROCK inhibitor, VEGF and bFGF to obtain pre-NK cell progenitors, NK cell progenitors, and/or NK cells.

66. The method of any one of claims 60-65, further comprising: prior to step c), contacting the pluripotent stem cells with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, to seed and expand the cells.

67. The method of any one of claims 60-65, further comprising detecting a rearranged T-cell receptor (TCR) locus in the hematopoietic lineage cells.

68. The method of claim 67, further comprising selecting the hematopoietic lineage cells comprising the rearranged TCR locus based on the TCR encoded by the rearranged TCR locus binding an antigen of interest.

69. The method of claim 68, wherein the antigen of interest is a tumor antigen.

70. A method, the method comprising:
reprogramming a donor cell to a pluripotent state;
editing a target locus in the donor cell genome; and
differentiating the reprogrammed donor cell into a lymphocyte.

71. The method of claim 70, wherein the editing is performed before or during the step of reprogramming of the donor cell to a pluripotent state.

72. The method of claim 70 or 71, wherein the donor cell is a fibroblast, a peripheral blood cell, a lymphocyte, or a T cell.

73. A method, the method comprising:
differentiating a genetically modified pluripotent stem cell into a lymphocyte, wherein the genetically modified pluripotent stem cell comprises:
(1) an exogenous nucleic acid expression construct comprising:

(i) a nucleic acid sequence encoding a chimeric antigen receptor (CAR);

(ii) a nucleic acid sequence encoding a non-naturally occurring variant of FcγRIII (CD16);

(iii) a nucleic acid sequence encoding interleukin 15 (IL-15);

(iv) a nucleic acid sequence encoding interleukin 15 receptor (IL-15R) or a variant thereof;

(v) a nucleic acid sequence encoding interleukin 12 (IL12);

(vi) a nucleic acid sequence encoding interleukin-12 receptor (IL-12R) or a variant thereof;

(vii) a nucleic acid sequence encoding human leukocyte antigen G (HLA-G);

(viii) a nucleic acid sequence encoding human leukocyte antigen E (HLA-E);

(ix) a nucleic acid sequence encoding leukocyte surface antigen cluster of differentiation CD47 (CD47);

or any combination of two or more thereof; and

(2) an indel, or an insertion of an exogenous nucleic acid in one or more of the following genetic loci:

(i) transforming growth factor beta receptor 2 (TGFβR2);

(ii) adenosine A2a receptor (ADORA2A);

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

(iv) β-2 microgobulin (B2M);

(v) programmed cell death protein 1 (PD-1, CD279);

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

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

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

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

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

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

or any combination of two or more thereof,

wherein the indel or insertion results in a loss-of-function of a gene product encoded by the respective genetic locus or loci.

74. The method of claim 73, wherein the indel, or the insertion of an exogenous nucleic acid is in the following genetic loci :

(i) TGFβR2, CISH, TIGIT, ADORA2A, or NKG2A;

(ii) TGFβR2 and CISH, TGFβR2 and TIGIT, TGFβR2 and ADORA2A, TGFβR2 and NKG2A, CISH and TIGIT, CISH and ADORA2A, CISH and NKG2A, TIGIT and ADORA2A, TIGIT and NKG2A, or ADORA2A and NKG2A; or

(iii) TGFβR2, CISH and TIGIT; TGFβR2, CISH and ADORA2A; TGFβR2, CISH and NKG2A; TGFβR2, TIGIT and ADORA2A; TGFβR2, TIGIT and NKG2A; TGFβR2, ADORA2A and NKG2A; CISH, TIGIT and ADORA2A; CISH, TIGIT and NKG2A; CISH, ADORA2A and NKG2A; or TIGIT, ADORA2A and NKG2A, wherein the indel or insertion results in a loss-of-function of a gene product encoded by the respective genetic locus or loci.

75. The method of claim 73 or claim 74, wherein the exogenous nucleic acid of (2) is the exogenous nucleic acid of (1).

76. The method of claim 73 or claim 75, wherein the pluripotent stem cell is an iPS cell.

77. The method of claim 73 or claim 75, wherein the differentiating comprises contacting the pluripotent stem cell with a differentiation medium or a sequence of differentiation media.

78. The modified lymphocyte of any preceding claim, wherein the two or more HLA class II histocompatibility antigen alpha chain genes are selected from HLA-DQA1, HLA-DRA, HLA-DPA1, HLA-DMA, HLA-DQA2, and HLA-DOA.

79. The modified lymphocyte of any preceding claim, wherein the two or more HLA class II histocompatibility antigen beta chain genes are selected from HLA-DMB, HLA-DOB, HLA-DPB1, HLA-DQB1, HLA-DQB3, HLA-DQB2, HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5.

80. The modified lymphocyte of any one of claims 1-29 and 78-79, the modified cell of any one of claims 30-42, the population of cells of claim 43, the pharmaceutical composition of claim 44, the isolated *in vitro* population of lymphocytes of any one of claims 45-55, or the method of any one of claims 56-77, wherein the exogenous nucleic acid expression construct

comprises the encoding nucleic acid sequence listed under (1) under the control of a heterologous promoter.

81. The modified lymphocyte, modified cell, population of cells, pharmaceutical composition, isolated *in vitro* population of lymphocytes, or method of claim 80, wherein the heterologous promoter is an NK-cell specific promoter.

82. The modified lymphocyte, modified cell, population of cells, pharmaceutical composition, isolated *in vitro* population of lymphocytes, or method of claim 80, wherein the NK-cell specific promoter is a promoter of a gene known to be expressed specifically in NK cells, or a variant thereof.

83. The modified lymphocyte, modified cell, population of cells, pharmaceutical composition, isolated *in vitro* population of lymphocytes, or method of claim 80, wherein the NK-cell specific promoter is a CD56 (NCAM), CD49, or CD45 promoter, or a variant thereof.

84. The modified lymphocyte, modified cell, population of cells, pharmaceutical composition, isolated in vitro population of lymphocytes, or method of claim 80, wherein the NK-cell specific promoter is an FcγRIII promoter, an NKG2D promoter, a CD69 promoter, or a natural cytotoxicity receptor promoter, or a variant thereof.

85. A method, the method comprising:

administering the modified lymphocyte, the modified cell, or the population of cells of any of the preceding claims to a subject in need thereof.

86. The method of claim 85, wherein the subject has, or is diagnosed with, a proliferative disease.

87. The method of claim 86, wherein the proliferative disease is cancer.

88. The method of claim 87, wherein the cancer is breast cancer, colorectal cancer, gastric cancer, renal cell carcinoma (RCC), or non-small cell lung cancer (NSCLC), solid tumors, 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, soft tissue sarcomas, and hematological cancers like ALL, CLL, NHL, DLBCL, AML, CML, multiple myeloma (MM).