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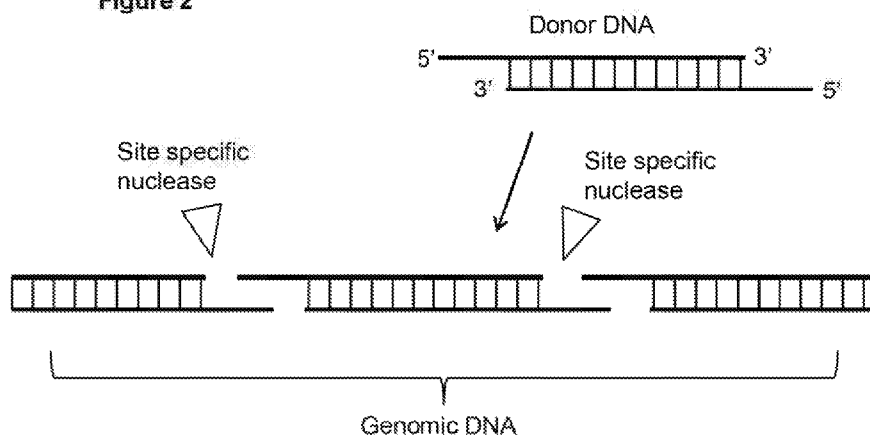
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(54) Title: METHODS AND COMPOSITIONS FOR GENOME EDITING

Figure 2



(57) Abstract: Provided are methods and compositions for genome editing using sticky ends. Subject methods include (a) generating a staggered cut at each of two locations in genomic DNA of a target cell, thus generating two genomic staggered ends; and (b) providing/introducing a linear double stranded donor DNA that has staggered ends (i.e., sticky ends) that match/respond to the sticky ends of the genomic DNA such that the sticky ends of the donor DNA hybridize with the sticky ends of the genomic DNA and the donor DNA is inserted into the genome. In some cases, the staggered cuts are generated by introducing into a target cell one or more sequence specific nucleases (or one or more nucleic acids encoding the one or more sequence specific nucleases).



METHODS AND COMPOSITIONS FOR GENOME EDITING

CROSS-REFERENCE

This application claims the benefit of U.S. Provisional Patent Application No.

- 5 62/659,627, filed April 18, 2018, of U.S. Provisional Patent Application No. 62/685,243, filed June 14, 2018, and of U.S. Provisional Patent Application No. 62/736,400, filed September 25, 2018, all of which applications are incorporated herein by reference in their entirety.

INTRODUCTION

- 10 Genome editing remains an inefficient process in most circumstances. Compositions and methods for efficient genome editing remain an important unmet need.

SUMMARY

- 15 Provided are compositions and methods for genome editing using sticky ends. In some embodiments, subject methods include (a) generating a staggered cut at each of two locations in genomic DNA of a target cell, thus generating two sticky ends (genomic staggered ends); and (b) providing/introducing a linear double stranded donor DNA that has staggered ends (i.e., sticky ends) that correspond to the sticky ends of the genomic DNA such that the sticky ends of the donor DNA hybridize with the sticky ends of the genomic DNA and the donor DNA is
20 inserted into the genome. This method is also referred to herein generally as “tetris” or “tetris-mediated”. In some cases, the staggered cuts are generated by introducing into a target cell one or more sequence specific nucleases (or one or more nucleic acids encoding the one or more sequence specific nucleases), e.g., a meganuclease, a homing endonuclease, a zinc finger nuclease (ZFN), a TALEN, a class 2 CRISPR/Cas effector protein (an RNA-guided
25 CRISPR/Cas polypeptide) such as Cas9, CasX, CasY, Cpf1 (Cas12a), Cas13, MAD7, and the like.

- In some cases, the donor DNA and one or more sequence specific nucleases (or one or more nucleic acids encoding the one or more sequence specific nucleases) are payloads of the same delivery vehicle (which can be introduced into a cell/delivered to a cell, e.g., *in vitro*, *ex vivo*, or *in vivo*). One advantage of delivering multiple payloads as part of the same delivery
30 vehicle (e.g., nanoparticle) is that the efficiency of each payload is not diluted. As an illustrative example, if payload A and payload B are delivered in two separate packages/vehicles (package A and package B, respectively), then the efficiencies are multiplicative, e.g., if package A and package B each have a 1% transfection efficiency, the chance of delivering payload A and
35 payload B to the same cell is 0.01% (1% X 1%). However, if payload A and payload B are both delivered as part of the same delivery vehicle, then the chance of delivering payload A and

payload B to the same cell is 1%, a 100-fold improvement over 0.01%.

In some embodiments, the donor DNA (e.g., the ends of the donor DNA) is bound to one or more sequence specific nucleases (e.g., nuclease pair(s)) when delivered (e.g., as part of the same delivery vehicle), e.g., the donor DNA can be 'pre-assembled' with one or more nucleases. Co-delivery of the donor DNA with a nuclease can lead to thermodynamic "switching" during binding to the genomic cut site, whereby the nuclease (e.g., nuclease pair(s)) is displaced from the donor DNA onto the genome, and the donor DNA slots into the genome. The subject compositions and methods provide a way to insert donor DNA into a DNA target without using homology directed repair (HDR) - insertion is instead mediated by matching the 'sticky ends.'

Delivery vehicles can include, but are not limited to, non-viral vehicles, viral vehicles, nanoparticles (e.g., a nanoparticle that includes a targeting ligand and/or a core comprising an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition), liposomes, micelles, water-oil-water emulsion particles, oil-water emulsion micellar particles, multilamellar water-oil-water emulsion particles, a targeting ligand (e.g., peptide targeting ligand) conjugated to a charged polymer polypeptide domain (wherein the targeting ligand provides for targeted binding to a cell surface protein, and the charged polymer polypeptide domain is condensed with a nucleic acid payload and/or is interacting electrostatically with a protein payload), a targeting ligand (e.g., peptide targeting ligand) conjugated to payload (where the targeting ligand provides for targeted binding to a cell surface protein), etc. In some cases payloads are introduced into the cell as a deoxyribonucleoprotein complex or a ribo-deoxyribonucleoprotein complex.

The provided compositions and methods can be used for genome editing at any locus in any cell type (e.g., to engineer T-cells, e.g., in vivo). For example, a CD8+ T-cell population or mixture of CD8+ and CD4+ T-cells can be programmed to transiently or permanently express an appropriate TCR α /TCR β pair of CDR1, CDR2, and/or CDR3 domains for antigen recognition.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

Figure 1 depicts a schematic representation of example embodiments of a subject linear double stranded donor DNA with sticky ends. In one depicted case, both ends have 5' overhangs and in the other depicted case, both ends have 3' overhangs.

Figure 2 depicts a schematic representation of one example of a subject method.

5 **Figure 3** depicts a schematic representation of an example embodiment of a delivery package (in the depicted case, one type of nanoparticle).

Figure 4 depicts a schematic representation of an example embodiment of a delivery package (in the depicted case, one type of nanoparticle). In this case, the depicted nanoparticle is multi-layered, having a core (which includes a first payload) surrounded by a first sheddable layer, which is surrounded by an intermediate layer (which includes an additional payload),
10 which is surrounded by a second sheddable layer, which is surface coated (i.e., includes an outer shell).

Figure 5 (panels A-B) depicts schematic representations of example configurations of a targeting ligand of a surface coat of a subject nanoparticle. The delivery molecules depicted
15 include a targeting ligand conjugated to an anchoring domain that is interacting electrostatically with a sheddable layer of a nanoparticle. Note that the targeting ligand can be conjugated at the N- or C-terminus (left of each panel), but can also be conjugated at an internal position (right of each panel). The molecules in panel A include a linker while those in panel B do not.

Figure 6 (panels A-D) provides schematic drawings of an example embodiment of a
20 delivery package (in the depicted case, example configurations of a subject delivery molecule). Note that the targeting ligand can be conjugated at the N- or C-terminus (left of each panel), but can also be conjugated at an internal position (right of each panel). The molecules in panels A and C include a linker while those of panels B and D do not. **(panels A-B)** delivery molecules that include a targeting ligand conjugated to a payload. **(panels C-D)** delivery molecules that
25 include a targeting ligand conjugated to a charged polymer polypeptide domain that is condensed with a nucleic acid payload (and/or interacting, e.g., electrostatically, with a protein payload).

Figure 7 provides non-limiting examples of nuclear localization signals (NLSs) that can be used (e.g., as part of a nanoparticle, e.g., as an NLS-containing peptide; as part
30 of/conjugated to an NLS-containing peptide, an anionic polymer, a cationic polymer, and/or a cationic polypeptide; and the like). The figure is adapted from Kosugi et al., J Biol Chem. 2009 Jan 2;284(1):478-85. (Class 1, top to bottom (SEQ ID NOs: 201-221); Class 2, top to bottom (SEQ ID NOs: 222-224); Class 4, top to bottom (SEQ ID NOs: 225-230); Class 3, top to bottom (SEQ ID NOs: 231-245); Class 5, top to bottom (SEQ ID NOs: 246-264)).

35 **Figure 8** (panels A-B) depicts schematic representations of the mouse (panel A) and human (panel B) hematopoietic cell lineage, and markers that have been identified for various

cells within the lineage.

Figure 9 (panels A-B) depicts schematic representations of miRNA (panel A) and protein (panel B) factors that can be used to influence cell differentiation and/or proliferation.

Figures 10-57 depict experimental results – see “Experimental” section.

5 **Figure 58** depicts example target loci for T Cell receptor editing.

Figure 59 depicts examples of CRISPR/CAS guide sequences and TALEN sequences designed to generate double strand breaks at exon 1 and the promoter region of TCR alpha and TCR beta.

10 **Figure 60** depicts how sgRNAs were designed for Cpf1 (Cas12a), which creates staggered cuts at +24 and +19 from TTTV PAM sequence on opposite strands of the genome. dsDNA inserts with compatible overhangs were created by annealing two oligos (ssDNA1 and ssDNA2). GFP gene insertions were not detected with the single-cut Cpf1 approach, whereas successful tetris-mediated (i.e., two staggered end cuts + a double stranded insert with staggered ends) GFP insertion was seen when performing double cuts at the TRBC1 & TRBC2
15 loci. The insert encodes Flag or GFP; compatible overhangs are shown underlined in this figure. 60pmol Cpf1 RNPs and 4ug dsDNA were introduced to stimulated T-Cells via nucleofection. On Day 4-10 post nucleofection, cells were assayed for TCR knock-down by flow cytometry and PCR amplification of either TRBC1-TRBC2, GFP-GFP, or TRBC2-GFP to confirm genomic deletions, presence of GFP donor, and GFP insertion into the TRBC1-TRBC2
20 loci, respectively.

Figure 61 depicts flow cytometry results (Attune NxT) of cryopreserved human primary T Cells that were thawed and stimulated for 2 days the day after culturing with CD3/CD28 beads. 1.27% of cells were GFP+ following double-cut Cpf1-mediated editing of the TRBC1/C2 loci, and subsequent insertion via a tetrisDNA template (i.e., a double stranded insert with
25 staggered ends) encoding GFP. The day after bead removal, cells were electroporated with the Lonza Amaxa 4D system, P3 Primary Cell kit. RNPs were formed by incubating 64pmol A.s. Cpf1 (IDT, catalog 1081068) and 128 pmol sgRNA (IDT) at room temperature for 10-20 minutes, then added to 4μg of dsDNA insert or IDT's Cpf1 electroporation enhancer (Catalog #1076301) and incubated for 10 minutes. 1x10e6 Stimulated T Cells in 20μL were added and
30 then transferred to the cuvette, then electroporated with pulse EH-115 (B, RNP alone) or EO-115 (C, RNP+DNA). On Day 7 post nucleofection, TCRa/b and GFP expression were assayed by flow cytometry. Figure shows cells in live population (Annexin and Sytox negative). DNA was collected from cells using QuickExtract (Lucigen).

Figure 62 depicts GFP knock-in (lanes 4 + 5, bands inside square) and successful
35 TRBC1-TRBC2 knockout (lanes 1 + 2) with Cpf1 gRNAs targeting TRBC1 & TRBC2 loci in human Pan-T cells. GFP donor amplification (lanes 7 + 8) is presumably due to non-integrated

donor DNA in the cell, but is controlled for with GFP-TRBC2 primers (lanes 4 + 5). TRBC1-TRBC2 deletion bands (731bp) and GFP-GFP bands (774bp) are clearly seen for wells 1-2 and 7-8, respectively. A 525bp knock-in band is visible in lanes 4 and 5, corresponding to ~1.27% efficient gene insertion via flow cytometry and GFP+ cells.

5 **Figure 63** depicts positive and negative bands seen in Figure 62.

Figure 64 depicts Sanger sequencing trace plots of LL003 sgRNA - Cpf1 complexes targeting the TRB exon 1 via a Cpf1 guide which has specificity for both C1 and C2 loci and performs two cuts in the genome. Its corresponding sequence is TAATTTCTACTCTTGTAGATGGTGTGGGAGATCTCTGCTTCTGA. Either a FLAG sequence or a T2A-GFP sequence was inserted into the TRAC locus of stimulated human primary T cells. In this figure, cells were untransfected.

Figure 65 depicts Sanger sequencing trace plots of LL003 sgRNA - Cpf1 complexes targeting the TRB exon 1 via a Cpf1 guide which has specificity for both C1 and C2 loci and performs two cuts in the genome. Its corresponding sequence is TAATTTCTACTCTTGTAGATGGTGTGGGAGATCTCTGCTTCTGA. Either a FLAG sequence or a T2A-GFP sequence was inserted into the TRAC locus of stimulated human primary T cells. In this figure, no donor DNA was used.

Figure 66 depicts Sanger sequencing trace plots of LL003 sgRNA - Cpf1 complexes targeting the TRB exon 1 via a Cpf1 guide which has specificity for both C1 and C2 loci and performs two cuts in the genome. Its corresponding sequence is TAATTTCTACTCTTGTAGATGGTGTGGGAGATCTCTGCTTCTGA. Either a FLAG sequence or a T2A-GFP sequence was inserted into the TRAC locus of stimulated human primary T cells. In this figure, a FLAG donor DNA (with staggered ends) was utilized.

25

DETAILED DESCRIPTION

As summarized above, provided are compositions and methods for genome editing using sticky ends. Subject methods can include (a) generating a staggered cut at each of two locations in genomic DNA of a target cell, thus generating two sticky ends (genomic staggered ends); and (b) providing/introducing a linear double stranded donor DNA that has staggered ends (i.e., sticky ends) that correspond to the sticky ends of the genomic DNA such that the sticky ends of the donor DNA hybridize with the sticky ends of the genomic DNA and the donor DNA is inserted into the genome. In some cases, the staggered cuts are generated by introducing into a target cell one or more sequence specific nucleases (or one or more nucleic acids encoding the one or more sequence specific nucleases), e.g., a meganuclease, a homing

endonuclease, a zinc finger nuclease (ZFN), a TALEN, a class 2 CRISPR/Cas effector protein such as Cas9, Cpf1, and the like. In some cases, the donor DNA and one or more sequence specific nucleases (or one or more nucleic acids encoding the one or more sequence specific nucleases) are payloads of the same delivery vehicle. In some cases, the delivery vehicle is a
5 nanoparticles (e.g., a nanoparticle that includes a targeting ligand and/or a core comprising an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition) – and in some cases the payloads are part of the core of the nanoparticle. In some cases, the delivery vehicle is a subject delivery molecule having a targeting ligand (e.g., peptide targeting ligand) conjugated to a charged polymer polypeptide domain (where the
10 targeting ligand provides for targeted binding to a cell surface protein, and the charged polymer polypeptide domain interacts with the payload, e.g., is condensed with a nucleic acid payload and/or is interacting electrostatically with a protein payload).

Before the present methods and compositions are described, it is to be understood that
15 this invention is not limited to the particular methods or compositions described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the
20 tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range
25 where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, all technical and scientific terms used herein have the same
30 meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with
35 which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the endonuclease" includes reference to one or more endonucleases and equivalents thereof, known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any element, e.g., any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

Methods and Compositions

Provided are methods and compositions for efficient genome editing. In some embodiments, a subject method includes (a) generating double stranded cuts with staggered ends at two locations within a target cell's genome, thereby producing a first genomic staggered end and a second genomic staggered end; and (b) introducing a linear double stranded donor DNA having a 5' or 3' overhang at each end, where one end of the donor DNA hybridizes with the first genomic staggered end and the other end of the donor DNA hybridizes with the second genomic staggered end, thereby resulting in insertion of the linear double stranded donor DNA into the target cell's genome.

A nucleic acid encoding a site-specific nuclease can be any nucleic acid of interest, e.g., as a nucleic acid payload of a delivery vehicle it can be linear or circular, and can be a plasmid, a viral genome, an RNA, etc. The term "nucleic acid" encompasses modified nucleic acids. For example, the nucleic acid molecule can be a mimetic, can include a modified sugar backbone, one or more modified internucleoside linkages (e.g., one or more phosphorothioate and/or heteroatom internucleoside linkages), one or more modified bases, and the like. In some embodiments, a subject payload includes triplex-forming peptide nucleic acids (PNAs) (see,

e.g., McNeer et al., Gene Ther. 2013 Jun;20(6):658-69). A subject donor DNA is double stranded, linear, and has staggered ends (i.e., each end of the linear donor DNA has an overhang).

5 **Generating genomic staggered ends at two locations**

In some cases, in order to generate the staggered cuts, a site-specific nuclease (one or more site-specific nucleases) (or a nucleic acid encoding same, e.g., one or more nucleic acids) is introduced into a target cell. If the target cell is *in vivo*, this can be accomplished by administering the appropriate components (e.g., as part of one or more delivery vehicles) to an individual. In some cases, the target cell includes DNA encoding a site-specific nuclease (which can be, e.g., operably linked – under the control of – an inducible promoter) and the ‘generating’ step of a subject method includes inducing expression of the site-specific nuclease.

Each overhang of the two genomic staggered ends (after cutting the genome in two locations) can be, independently, 5’ or 3’ single stranded overhangs. For example, in some cases both genomic staggered ends (after cutting the genome in two locations) can have a 5’ overhang. In some cases, both staggered ends of the genome have a 3’ overhang. In some cases, one genomic staggered end (at one of the two cut locations) has a 5’ overhang while the other genomic staggered end (at the other cut location) has a 3’ overhang.

Each overhang of the two genomic staggered ends (after cutting the genome in two locations) can be any convenient length. In some embodiments each overhang of the two genomic staggered ends (after cutting the genome in two locations), independently, can be 2-20 nucleotides (nt) long (e.g., 2-18, 2-15, 2-12, 2-10, 2-8, 2-7, 2-6, 2-5, 3-20, 3-18, 3-15, 3-12, 3-10, 3-8, 3-7, 3-6, 3-5, 4-20, 4-18, 4-15, 4-12, 4-10, 4-8, 4-7, or 4-6 nt). In some cases, each overhang of the two genomic staggered ends (after cutting the genome in two locations), independently, can be 2-20 nucleotides long. In some cases, each overhang of the two genomic staggered ends (after cutting the genome in two locations), independently, can be 2-15 nucleotides long. In some cases, each overhang of the two genomic staggered ends (after cutting the genome in two locations), independently, can be 2-10 nucleotides long.

In some embodiments, prior to generating the two staggered end cuts (two locations in the genome), the two locations are separated by 1,000,000 base pairs (bp) or less (e.g., 500,000 bp or less, 100,000 bp or less, 50,000 bp or less, 10,000 bp or less, 1,000 bp or less, 750 bp or less, or 500 bp or less). In some cases, the two locations are separated by 100,000 bp or less. In some cases, the two locations are separated by 50,000 bp or less. In some embodiments, prior to generating the two staggered end cuts (two locations in the genome), the two locations are separated by a range of from 5 to 1,000,000 base pairs (bp) (e.g., from 5 to 500,000, 5 to 100,000, 5 to 50,000, 5 to 10,000, 5 to 5,000, 5 to 1,000, 5 to 500, 10 to

1,000,000, 10 to 500,000, 10 to 100,000, 10 to 50,000, 10 to 10,000, 10 to 5,000, 10 to 1,000, 10 to 500, 50 to 1,000,000, 50 to 500,000, 50 to 100,000, 50 to 50,000, 50 to 10,000, 50 to 5,000, 50 to 1,000, 50 to 500, 100 to 1,000,000, 100 to 500,000, 100 to 100,000, 100 to 50,000, 100 to 10,000, 100 to 5,000, 100 to 1,000, 100 to 500, 300 to 1,000,000, 300 to 500,000, 300 to 100,000, 300 to 50,000, 300 to 10,000, 300 to 5,000, 300 to 1,000, 300 to 500, 500 to 1,000,000, 500 to 500,000, 500 to 100,000, 500 to 50,000, 500 to 10,000, 500 to 5,000, 500 to 1,000, 1,000 to 1,000,000, 1,000 to 500,000, 1,000 to 100,000, 1,000 to 50,000, 1,000 to 10,000, or 1,000 to 5,000 bp).

In some cases, the two locations are separated by a range of from 20 to 1,000,000 bp.

In some cases, the two locations are separated by a range of from 20 to 500,000 bp. In some cases, the two locations are separated by a range of from 20 to 150,000 bp. In some cases, the two locations are separated by a range of from 20 to 50,000 bp. In some cases, the two locations are separated by a range of from 20 to 20,000 bp. In some cases, the two locations are separated by a range of from 20 to 15,000 bp. In some cases, the two locations are separated by a range of from 20 to 10,000 bp.

In some cases, the two locations are separated by a range of from 500 to 1,000,000 bp. In some cases, the two locations are separated by a range of from 500 to 500,000 bp. In some cases, the two locations are separated by a range of from 500 to 150,000 bp. In some cases, the two locations are separated by a range of from 500 to 50,000 bp. In some cases, the two locations are separated by a range of from 500 to 20,000 bp. In some cases, the two locations are separated by a range of from 500 to 15,000 bp. In some cases, the two locations are separated by a range of from 500 to 10,000 bp.

In some cases, the two locations are separated by a range of from 1,000 to 1,000,000 bp. In some cases, the two locations are separated by a range of from 1,000 to 500,000 bp. In some cases, the two locations are separated by a range of from 1,000 to 150,000 bp. In some cases, the two locations are separated by a range of from 1,000 to 50,000 bp. In some cases, the two locations are separated by a range of from 1,000 to 20,000 bp. In some cases, the two locations are separated by a range of from 1,000 to 15,000 bp. In some cases, the two locations are separated by a range of from 1,000 to 10,000 bp.

In some cases, the two locations are separated by a range of from 5,000 to 1,000,000 bp. In some cases, the two locations are separated by a range of from 5,000 to 500,000 bp. In some cases, the two locations are separated by a range of from 5,000 to 150,000 bp. In some cases, the two locations are separated by a range of from 5,000 to 50,000 bp. In some cases, the two locations are separated by a range of from 5,000 to 20,000 bp. In some cases, the two locations are separated by a range of from 5,000 to 15,000 bp. In some cases, the two locations are separated by a range of from 5,000 to 10,000 bp.

A subject site-specific nuclease is one that can introduce a double stranded cut in genomic DNA to generate a staggered end (e.g., via two offset single stranded cuts in opposite stands of the DNA). In some cases, a site-specific nuclease such as meganuclease (or a class 2 CRISPR/Cas effector protein such as Cpf1) naturally generates a staggered end. Some site-specific nucleases are engineered proteins (e.g., zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)) and in some cases such proteins are used as protein pairs to generate a staggered ends. In some cases, a site-specific nuclease is one that naturally generates a blunt single strand cut (e.g., a class 2 CRISPR/Cas effector protein such as Cas9), but has been mutated such that the protein is a nickase (cuts only one strand of DNA). Nickase proteins such as a mutated nickase Cas9 can be used to generate a staggered end by using two guide RNAs that target opposite strands of the target DNA. Thus, in some cases a subject method includes using a sequence specific nickase (e.g., a nickase class 2 CRISPR/Cas effector protein such as a nickase Cas9) with two guide RNAs to generate a staggered cut at (at least) one of two genomic locations. In some cases, a subject method includes using a sequence specific nickase (e.g., a nickase class 2 CRISPR/Cas effector protein such as a nickase Cas9) with four guide RNAs to generate two staggered cuts at two genomic locations.

Any convenient site-specific nuclease (e.g., gene editing protein such as any convenient programmable gene editing protein) can be used. Examples of suitable programmable gene editing proteins include but are not limited to transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and CRISPR/Cas RNA-guided polypeptides such as Cas9, CasX, CasY, Cpf1, Cas13, MAD7, and the like). Examples of site-specific nuclease that can be used include but are not limited to transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and CRISPR/Cas RNA-guided polypeptides such as Cas9, CasX, CasY, Cpf1, Cas13, MAD7, and the like); meganucleases (e.g., I-SceI, I-CeuI, I-CreI, I-DmI, I-ChuI, I-DiI, I-FlmI, I-FlmI, I-AniI, I-SceIV, I-CsmI, I-PanI, I-PanII, I-PanMI, I-Scell, I-PpoI, I-ScellI, I-LtrI, I-GpiI, I-GZeI, I-OnuI, I-HjeMI, I-MsoI, I-TevI, I-TevII, I-TevIII, PI-MleI, PI-MtuI, PI-Pspl, PI-Tli I, PI-Tli II, PI-SceV, and the like); and homing endonucleases.

In some cases, a delivery vehicle is used to deliver a nucleic acid encoding a gene editing tool (i.e., a component of a gene editing system, e.g., a site-specific cleaving system such as a programmable gene editing system). For example, a nucleic acid payload can include one or more of: (i) a CRISPR/Cas guide RNA, (ii) a DNA encoding a CRISPR/Cas guide RNA, (iii) a DNA and/or RNA encoding a programmable gene editing protein such as a zinc finger protein (ZFP) (e.g., a zinc finger nuclease – ZFN), a transcription activator-like effector (TALE) protein (e.g., fused to a nuclease – TALEN), and/or a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, Cpf1, Cas13, MAD7, and the like); (iv) a DNA and/or

RNA encoding a meganuclease; (v) a DNA and/or RNA encoding a homing endonuclease; and (iv) a Donor DNA molecule.

In some cases, a subject delivery vehicle is used to deliver a protein payload, e.g., a protein such as a ZFN, a TALEN, a CRISPR/Cas RNA-guided polypeptide (Class 2
5 CRISPR/Cas effector protein) (e.g., Cas9, CasX, CasY, Cpf1, Cas13, MAD7, and the like), a meganuclease, and a homing endonuclease. Cas13, MAD7,

Depending on the nature of the system and the desired outcome, a gene editing system (e.g. a site-specific gene editing system such as a programmable gene editing system) can include a single component (e.g., a ZFP, a ZFN, a TALE, a TALEN, a meganuclease, and the
10 like) or can include multiple components. In some cases, a gene editing system includes at least two components. For example, in some cases a gene editing system (e.g. a programmable gene editing system) includes (i) a donor DNA molecule nucleic acid; and (ii) a gene editing protein (e.g., a programmable gene editing protein such as a ZFP, a ZFN, a TALE, a TALEN, a DNA-guided polypeptide such as *Natronobacterium gregoryi* Argonaute (NgAgo), a
15 CRISPR/Cas RNA-guided polypeptide such as Cas9, CasX, CasY, or Cpf1, Cas13, MAD7, and the like), or a nucleic acid molecule encoding the gene editing protein (e.g., DNA or RNA such as a plasmid or mRNA). As another example, in some cases a gene editing system (e.g. a programmable gene editing system) includes (i) a CRISPR/Cas guide RNA, or a DNA encoding the CRISPR/Cas guide RNA; and (ii) a CRISPR/CAS RNA-guided polypeptide (e.g., Cas9,
20 CasX, CasY, Cpf1, Cas13, MAD7, and the like), or a nucleic acid molecule encoding the RNA-guided polypeptide (e.g., DNA or RNA such as a plasmid or mRNA). As another example, in some cases a gene editing system (e.g. a programmable gene editing system) includes (i) an NgAgo-like guide DNA; and (ii) a DNA-guided polypeptide (e.g., NgAgo), or a nucleic acid molecule encoding the DNA-guided polypeptide (e.g., DNA or RNA such as a plasmid or
25 mRNA). In some cases, a gene editing system (e.g. a programmable gene editing system) includes at least three components: (i) a donor DNA molecule; (ii) a CRISPR/Cas guide RNA, or a DNA encoding the CRISPR/Cas guide RNA; and (iii) a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, or Cpf1), or a nucleic acid molecule encoding the RNA-guided polypeptide (e.g., DNA or RNA such as a plasmid or mRNA). In some cases, a gene
30 editing system (e.g. a programmable gene editing system) includes at least three components: (i) a donor DNA molecule; (ii) an NgAgo-like guide DNA, or a DNA encoding the NgAgo-like guide DNA; and (iii) a DNA-guided polypeptide (e.g., NgAgo), or a nucleic acid molecule encoding the DNA-guided polypeptide (e.g., DNA or RNA such as a plasmid or mRNA).

In some embodiments, a payload of a delivery vehicle includes one or more gene
35 editing tools. The term "gene editing tool" is used herein to refer to one or more components of a gene editing system. Thus, in some cases the payload includes a gene editing system and in

some cases the payload includes one or more components of a gene editing system (i.e., one or more gene editing tools). For example, a target cell might already include one of the components of a gene editing system and the user need only add the remaining components. In such a case the payload of a subject nanoparticle does not necessarily include all of the components of a given gene editing system. As such, in some cases a payload includes one or more gene editing tools.

As an illustrative example, a target cell might already include a gene editing protein (e.g., a ZFP, a TALE, a DNA-guided polypeptide (e.g., NgAgo), a CRISPR/Cas RNA-guided polypeptide such as Cas9, CasX, CasY, Cpf1, Cas13, MAD7, and the like, and/or a DNA or RNA encoding the protein, and therefore the payload can include one or more of: (i) a donor DNA molecule; and (ii) a CRISPR/Cas guide RNA, or a DNA encoding the CRISPR/Cas guide RNA; or an NgAgo-like guide DNA. Likewise, the target cell may already include a CRISPR/Cas guide RNA and/or a DNA encoding the guide RNA or an NgAgo-like guide DNA, and the payload can include one or more of: (i) a donor DNA molecule; and (ii) a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, Cpf1, Cas13, MAD7, and the like), or a nucleic acid molecule encoding the RNA-guided polypeptide (e.g., DNA or RNA such as a plasmid or mRNA); or a DNA-guided polypeptide (e.g., NgAgo), or a nucleic acid molecule encoding the DNA-guided polypeptide.

For additional information related to programmable gene editing tools (e.g., CRISPR/Cas RNA-guided proteins such as Cas9, CasX, CasY, and Cpf1, Zinc finger proteins such as Zinc finger nucleases, TALE proteins such as TALENs, CRISPR/Cas guide RNAs, and the like) refer to, for example, Dreier, et al., (2001) J Biol Chem 276:29466-78; Dreier, et al., (2000) J Mol Biol 303:489-502; Liu, et al., (2002) J Biol Chem 277:3850-6; Dreier, et al., (2005) J Biol Chem 280:35588-97; Jamieson, et al., (2003) Nature Rev Drug Discov 2:361-8; Durai, et al., (2005) Nucleic Acids Res 33:5978-90; Segal, (2002) Methods 26:76-83; Porteus and Carroll, (2005) Nat Biotechnol 23:967-73; Pabo, et al., (2001) Ann Rev Biochem 70:313-40; Wolfe, et al., (2000) Ann Rev Biophys Biomol Struct 29:183-212; Segal and Barbas, (2001) Curr Opin Biotechnol 12:632-7; Segal, et al., (2003) Biochemistry 42:2137-48; Beerli and Barbas, (2002) Nat Biotechnol 20:135-41; Carroll, et al., (2006) Nature Protocols 1:1329; Ordiz, et al., (2002) Proc Natl Acad Sci USA 99:13290-5; Guan, et al., (2002) Proc Natl Acad Sci USA 99:13296-301; Sanjana et al., Nature Protocols, 7:171-192 (2012); Zetsche et al, Cell. 2015 Oct 22;163(3):759-71; Makarova et al, Nat Rev Microbiol. 2015 Nov;13(11):722-36; Shmakov et al., Mol Cell. 2015 Nov 5;60(3):385-97; Jinek et al., Science. 2012 Aug 17;337(6096):816-21; Chylinski et al., RNA Biol. 2013 May;10(5):726-37; Ma et al., Biomed Res Int. 2013;2013:270805; Hou et al., Proc Natl Acad Sci U S A. 2013 Sep 24;110(39):15644-9; Jinek et al., Elife. 2013;2:e00471; Pattanayak et al., Nat Biotechnol. 2013 Sep;31(9):839-43; Qi et al,

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 8,771,945; and 8,697,359; all of which are hereby incorporated by reference in their entirety.

30 ***Donor DNA and staggered ends of the genome***

A subject donor DNA is a linear double stranded DNA with sticky ends (i.e., staggered
 ends) (see, e.g., Figure 1). A subject donor DNA is linear and has (i) two strands of DNA that
 are hybridized to one another forming base pairs, and (ii) single stranded overhangs on each
 end. In some cases, two donor DNAs are used (e.g., to edit two sections of genomic DNA), in
 35 which case 4 staggered cuts are introduced into the genome – two per donor DNA.

In some cases, the two strands of the donor DNA are hybridized to one another forming

a total of 10 or more base pairs (bp) (e.g., 20 or more, 30 or more, 50 or more, 100 or more, or 200 or more bp). In other words, in some cases a subject donor DNA has 10 or more bp (e.g., 20 or more, 30 or more, 50 or more, 100 or more, or 200 or more bp).

In some cases a subject donor DNA has a total of from 10 base pairs (bp) to 100

5 kilobase pairs (kbp) (e.g., from 10 bp to 70 kbp, 10 bp to 50 kbp, 10 bp to 40 kbp, 10 bp to 25 kbp, 10 bp to 15 kbp, 10 bp to 10 kbp, 10 bp to 1 kbp, 10 bp to 750 bp, 10 bp to 500 bp, 10 bp to 250 bp, 10 bp to 150 bp, 10 bp to 100 bp, 10 bp to 50 bp, 18 bp to 100 kbp, 18 bp to 70 kbp, 18 bp to 50 kbp, 18 bp to 40 kbp, 18 bp to 25 kbp, 18 bp to 15 kbp, 18 bp to 10 kbp, 18 bp to 1 kbp, 18 bp to 750 bp, 18 bp to 500 bp, 18 bp to 250 bp, 18 bp to 150 bp, 25 bp to 100 kbp, 25
10 bp to 70 kbp, 25 bp to 50 kbp, 25 bp to 40 kbp, 25 bp to 25 kbp, 25 bp to 15 kbp, 25 bp to 10 kbp, 25 bp to 1 kbp, 25 bp to 750 bp, 25 bp to 500 bp, 25 bp to 250 bp, 25 bp to 150 bp, 50 bp to 100 kbp, 50 bp to 70 kbp, 50 bp to 50 kbp, 50 bp to 40 kbp, 50 bp to 25 kbp, 50 bp to 15 kbp, 50 bp to 10 kbp, 50 bp to 1 kbp, 50 bp to 750 bp, 50 bp to 500 bp, 50 bp to 250 bp, 50 bp to 150 bp, 100 bp to 100 kbp, 100 bp to 70 kbp, 100 bp to 50 kbp, 100 bp to 40 kbp, 100 bp to 25
15 kbp, 100 bp to 15 kbp, 100 bp to 10 kbp, 100 bp to 1 kbp, 100 bp to 750 bp, 100 bp to 500 bp, 100 bp to 250 bp, 200 bp to 100 kbp, 200 bp to 70 kbp, 200 bp to 50 kbp, 200 bp to 40 kbp, 200 bp to 25 kbp, 200 bp to 15 kbp, 200 bp to 10 kbp, 200 bp to 1 kbp, 200 bp to 750 bp, or 200 bp to 500 bp). In other words, in some cases, the two strands of the donor DNA are hybridized to one another forming a total of from 10 bp to 100 kbp. In some cases, a subject donor DNA has
20 a total of from 10 bp to 50 kbp. In some cases, a subject donor DNA has a total of from 10 bp to 10 kbp. In some cases, a subject donor DNA has a total of from 10 bp to 1 kbp. In some cases, a subject donor DNA has a total of from 20 bp to 50 kbp. In some cases, a subject donor DNA has a total of from 20 bp to 10 kbp. In some cases, a subject donor DNA has a total of from 20 bp to 1 kbp.

25 In some embodiments the lengths of the donor DNA overhangs are known and well defined. For example, if a donor DNA is cut from a larger template using a nuclease such as a TALEN – this can lead to a population of donor DNAs with a variety of undefined and unknown overhang lengths. On the other hand, donor DNAs can be synthesized (e.g., in vitro synthesis) such that the population of donor DNAs are copies of the same donor DNA, with the same,
30 known, defined overhangs. In some cases, donor DNAs are produced as PCR products that are subsequently digested with an enzyme (e.g., restriction enzyme or a class 2 CRISPR/Cas effector protein such as Cas9) to generate the sticky ends.

Each end of a subject donor DNA, independently, can have a 5' or 3' single stranded overhang. For example, in some cases both ends of the donor DNA have a 5' overhang. In
35 some cases, both ends of the donor DNA have a 3' overhang. In some cases, one end of the donor DNA has a 5' overhang while the other end has a 3' overhang. Each overhang can be

any convenient length. In some cases, the length of each overhang can be, independently, 2-200 nucleotides (nt) long (see, e.g., 2-150, 2-100, 2-50, 2-25, 2-20, 2-15, 2-12, 2-10, 2-8, 2-7, 2-6, 2-5, 3-150, 3-100, 3-50, 3-25, 3-20, 3-15, 3-12, 3-10, 3-8, 3-7, 3-6, 3-5, 4-150, 4-100, 4-50, 4-25, 4-20, 4-15, 4-12, 4-10, 4-8, 4-7, 4-6, 5-150, 5-100, 5-50, 5-25, 5-20, 5-15, 5-12, 5-10, 5-8, or 5-7 nt). In some cases, the length of each overhang can be, independently, 2-20 nt long. In some cases, the length of each overhang can be, independently, 2-15 nt long. In some cases, the length of each overhang can be, independently, 2-10 nt long. In some cases, the length of each overhang can be, independently, 2-7 nt long.

When the donor DNA inserts into the two staggered ends of genome (also referred to herein as genomic staggered ends) (after the genome has been cut in two locations), each end of the donor DNA, independently, can hybridize with the overhang of the genome over a total of 2-20 base pairs (bp) (e.g., 2-18, 2-16, 2-15, 2-12, 2-10, 2-8, 2-6, 2-5, 3-20, 3-18, 3-16, 3-15, 3-12, 3-10, 3-8, 3-6, 3-5, 4-20, 4-18, 4-16, 4-15, 4-12, 4-10, 4-8, 4-6, 5-20, 5-18, 5-16, 5-15, 5-12, 5-10, 8-20, 8-18, 8-16, 8-15, 8-12, 8-10, 5-8, 10-20, 10-18, 10-16, 10-15, or 10-12 bp). In some cases, the length of the overhangs of the donor DNA are equal to or less than the length of the overhangs of the genome. In some cases, the length of the overhangs of the genome are equal to or less than the length of the overhangs of the donor DNA.

In some embodiments the donor DNA has at least one adenylated 3' end.

In some cases, the donor DNA include a mimetic, can include a modified sugar backbone, one or more modified internucleoside linkages (e.g., one or more phosphorothioate and/or heteroatom internucleoside linkages), one or more modified bases, and the like.

Delivery vehicles / Payloads

In some embodiments, subject compositions (e.g., one or more sequence specific nucleases, one or more nucleic acids encoding one or more sequence specific nucleases, a linear double stranded donor DNA, and the like) are delivered to a cell as a payload of a delivery vehicle (e.g., in some cases as payloads of the same delivery vehicle). For example, in some cases, a subject linear double stranded donor DNA (with overhangs on each end) and one or more sequence specific nucleases (such as a meganuclease, a Homing Endonuclease, a Zinc Finger Nuclease, a TALEN, a CRISPR/Cas effector protein) (or more nucleic acids encoding one or more sequence specific nucleases), are payloads of the same delivery vehicle. In some such cases the payloads bind together and form a deoxyribonucleoprotein complex (e.g., a complex that includes the donor DNA and a nuclease) or a ribo-deoxyribonucleoprotein complex (e.g., a complex that further includes a CRISPR/Cas guide RNA).

Delivery vehicles can include, but are not limited to, non-viral vehicles, viral vehicles, nanoparticles (e.g., a nanoparticle that includes a targeting ligand and/or a core comprising an

anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition), liposomes, micelles, water-oil-water emulsion particles, oil-water emulsion micellar particles, multilamellar water-oil-water emulsion particles, a targeting ligand (e.g., peptide targeting ligand) conjugated to a charged polymer polypeptide domain (wherein the targeting ligand provides for targeted binding to a cell surface protein, and the charged polymer polypeptide domain is condensed with a nucleic acid payload and/or is interacting electrostatically with a protein payload), a targeting ligand (e.g., peptide targeting ligand) conjugated to payload (where the targeting ligand provides for targeted binding to a cell surface protein).

In some cases, a delivery vehicle is a water-oil-water emulsion particle. In some cases, a delivery vehicle is an oil-water emulsion micellar particle. In some cases, a delivery vehicle is a multilamellar water-oil-water emulsion particle. In some cases, a delivery vehicle is a multilayered particle. In some cases, a delivery vehicle is a DNA origami nanobot. For any of the above a payload (nucleic acid and/or protein) can be inside of the particle, either covalently, bound as nucleic acid complementary pairs, or within a water phase of a particle. In some cases, a delivery vehicle includes a targeting ligand, e.g., in some cases a targeting ligand (described in more detail elsewhere herein) coated upon a water-oil-water emulsion particle, upon an oil-water emulsion micellar particle, upon a multilamellar water-oil-water emulsion particle, upon a multilayered particle, or upon a DNA origami nanobot. In some cases, a delivery vehicle has a metal particle core, and the payload (e.g., donor DNA and/or site-specific nuclease – or nucleic acid encoding same) can be conjugated to (covalently bound to) the metal core.

Nanoparticles

Nanoparticles of the disclosure include a payload, which can be made of nucleic acid and/or protein. For example, in some cases a subject nanoparticle is used to deliver a nucleic acid payload (e.g., a DNA and/or RNA). In some cases, the core of the nanoparticle includes the payload(s). In some such cases a nanoparticle core can also include an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition. In some cases, the nanoparticle has a metallic core and the payload associates with (in some cases is conjugated to, e.g., the outside of) the core. In some embodiments, the payload is part of the nanoparticle core. Thus the core of a subject nanoparticle can include nucleic acid, DNA, RNA, and/or protein. Thus, in some cases a subject nanoparticle includes nucleic acid (DNA and/or RNA) and protein. In some cases, a subject nanoparticle core includes a ribonucleoprotein (RNA and protein) complex. In some cases, a subject nanoparticle core includes a deoxyribonucleoprotein (DNA and protein, e.g., donor DNA and ZFN, TALEN, or CRISPR/Cas

effector protein) complex. In some cases, a subject nanoparticle core includes a ribo-deoxyribonucleoprotein (RNA and DNA and protein, e.g., a guide RNA, a donor DNA and a CRISPR/Cas effector protein) complex. In some cases, a subject nanoparticle core includes PNAs. In some cases, a subject core includes PNAs and DNAs.

5 A subject nucleic acid payload (e.g., a donor DNA and/or a nucleic acid encoding a sequence specific nuclease) can include a morpholino backbone structure. In some case, a subject nucleic acid payload (e.g., a donor DNA and/or a nucleic acid encoding a sequence specific nuclease) can have one or more locked nucleic acids (LNAs). Suitable sugar
10 substituent groups include methoxy (-O-CH₃), aminopropoxy (-O-CH₂-CH₂-CH₂-NH₂), allyl (-CH₂-CH=CH₂), -O-allyl (-O-CH₂-CH=CH₂) and fluoro (F). 2'-sugar substituent groups may be in the arabino (up) position or ribo (down) position. Suitable base modifications include synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil,
15 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH₃) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-
20 amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido(5,4-b)(1,4)benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido(5,4-b)(1,4)benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido(5,4-(b) (1,4)benzoxazin-2(3H)-one),
25 carbazole cytidine (2H-pyrimido(4,5-b)indol-2-one), pyridoindole cytidine (H-pyrido(3',2':4,5)pyrrolo(2,3-d)pyrimidin-2-one).

In some cases, a nucleic acid payload can include a conjugate moiety (e.g., one that enhances the activity, stability, cellular distribution or cellular uptake of the nucleic acid payload). These moieties or conjugates can include conjugate groups covalently bound to
30 functional groups such as primary or secondary hydroxyl groups. Conjugate groups include, but are not limited to, intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Suitable conjugate groups include, but are not limited to, cholesterol, lipids, phospholipids, biotin, phenazine, folate,
35 phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties include groups that improve uptake,

enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties include groups that improve uptake, distribution, metabolism or excretion of a subject nucleic acid.

Any convenient polynucleotide can be used as a subject nucleic acid payload that is not the donor DNA (e.g., for delivering a site-specific nuclease). Examples include but are not limited to: species of RNA and DNA including mRNA, m1A modified mRNA (monomethylation at position 1 of Adenosine), morpholino RNA, peptoid and peptide nucleic acids, cDNA, DNA origami, DNA and RNA with synthetic nucleotides, DNA and RNA with predefined secondary structures, and multimers and oligomers of the aforementioned.

In some embodiments, more than one payload is delivered as part of the same package (e.g., nanoparticle), e.g., in some cases different payloads are part of different cores. One advantage of delivering multiple payloads as part of the same delivery vehicle (e.g., nanoparticle) is that the efficiency of each payload is not diluted. As an illustrative example, if payload A and payload B are delivered in two separate packages/vehicles (package A and package B, respectively), then the efficiencies are multiplicative, e.g., if package A and package B each have a 1% transfection efficiency, the chance of delivering payload A and payload B to the same cell is 0.01% (1% X 1%). However, if payload A and payload B are both delivered as part of the same delivery vehicle, then the chance of delivering payload A and payload B to the same cell is 1%, a 100-fold improvement over 0.01%.

Likewise, in a scenario where package A and package B each have a 0.1% transfection efficiency, the chance of delivering payload A and payload B to the same cell is 0.0001% (0.1% X 0.1%). However, if payload A and payload B are both delivered as part of the same package (e.g., part of the same nanoparticle - package A) in this scenario, then the chance of delivering payload A and payload B to the same cell is 0.1%, a 1000-fold improvement over 0.0001%.

As such, in some embodiments, one or more gene editing tools (e.g., as described above) and a donor DNA are delivered in combination with (e.g., as part of the same nanoparticle) a protein (and/or a DNA or mRNA encoding same) and/or a non-coding RNA that increases genomic editing efficiency. In some cases, one or more gene editing tools (e.g., as described above) and a donor DNA are delivered in combination with (e.g., as part of the same nanoparticle) a protein (and/or a DNA or mRNA encoding same) and/or a non-coding RNA that controls cell division and/or differentiation.

As non-limiting examples of the above, in some embodiments one or more gene editing tools and a donor DNA can be delivered in combination with one or more of: SCF (and/or a DNA or mRNA encoding SCF), HoxB4 (and/or a DNA or mRNA encoding HoxB4), BCL-XL (and/or a DNA or mRNA encoding BCL-XL), SIRT6 (and/or a DNA or mRNA encoding SIRT6),

a nucleic acid molecule (e.g., an siRNA and/or an LNA) that suppresses miR-155, a nucleic acid molecule (e.g., an siRNA, an shRNA, a microRNA) that reduces ku70 expression, and a nucleic acid molecule (e.g., an siRNA, an shRNA, a microRNA) that reduces ku80 expression.

For examples of microRNAs that can be delivered in combination with a gene editing tool (e.g., a site-specific nuclease) and a donor DNA, see Figure 9A. For example, the following microRNAs can be used for the following purposes: for blocking differentiation of a pluripotent stem cell toward ectoderm lineage: miR-430/427/302 (see, e.g., MiR Base accession: MI0000738, MI0000772, MI0000773, MI0000774, MI0006417, MI0006418, MI0000402, MI0003716, MI0003717, and MI0003718); for blocking differentiation of a pluripotent stem cell toward endoderm lineage: miR-109 and/or miR-24 (see, e.g., MiR Base accession: MI0000080, MI0000081, MI0000231, and MI0000572); for driving differentiation of a pluripotent stem cell toward endoderm lineage: miR-122 (see, e.g., MiR Base accession: MI0000442 and MI0000256) and/or miR-192 (see, e.g., MiR Base accession: MI0000234 and MI0000551); for driving differentiation of an ectoderm progenitor cell toward a keratinocyte fate: miR-203 (see, e.g., MiR Base accession: MI0000283, MI0017343, and MI0000246); for driving differentiation of a neural crest stem cell toward a smooth muscle fate: miR-145 (see, e.g., MiR Base accession: MI0000461, MI0000169, and MI0021890); for driving differentiation of a neural stem cell toward a glial cell fate and/or toward a neuron fate: miR-9 (see, e.g., MiR Base accession: MI0000466, MI0000467, MI0000468, MI0000157, MI0000720, and MI0000721) and/or miR-124a (see, e.g., MiR Base accession: MI0000443, MI0000444, MI0000445, MI0000150, MI0000716, and MI0000717); for blocking differentiation of a mesoderm progenitor cell toward a chondrocyte fate: miR-199a (see, e.g., MiR Base accession: MI0000242, MI0000281, MI0000241, and MI0000713); for driving differentiation of a mesoderm progenitor cell toward an osteoblast fate: miR-296 (see, e.g., MiR Base accession: MI0000747 and MI0000394) and/or miR-2861 (see, e.g., MiR Base accession: MI0013006 and MI0013007); for driving differentiation of a mesoderm progenitor cell toward a cardiac muscle fate: miR-1 (see, e.g., MiR Base accession: MI0000437, MI0000651, MI0000139, MI0000652, MI0006283); for blocking differentiation of a mesoderm progenitor cell toward a cardiac muscle fate: miR-133 (see, e.g., MiR Base accession: MI0000450, MI0000451, MI0000822, MI0000159, MI0000820, MI0000821, and MI0021863); for driving differentiation of a mesoderm progenitor cell toward a skeletal muscle fate: miR-214 (see, e.g., MiR Base accession: MI0000290 and MI0000698), miR-206 (see, e.g., MiR Base accession: MI0000490 and MI0000249), miR-1 and/or miR-26a (see, e.g., MiR Base accession: MI0000083, MI0000750, MI0000573, and MI0000706); for blocking differentiation of a mesoderm progenitor cell toward a skeletal muscle fate: miR-133 (see, e.g., MiR Base accession: MI0000450, MI0000451, MI0000822, MI0000159, MI0000820, MI0000821, and MI0021863), miR-221 (see, e.g., MiR Base accession: MI0000298 and

MI0000709), and/or miR-222 (see, e.g., MiR Base accession: MI0000299 and MI0000710); for driving differentiation of a hematopoietic progenitor cell toward differentiation: miR-223 (see, e.g., MiR Base accession: MI0000300 and MI0000703); for blocking differentiation of a hematopoietic progenitor cell toward differentiation: miR-128a (see, e.g., MiR Base accession: MI0000447 and MI0000155) and/or miR-181a (see, e.g., MiR Base accession: MI0000269, MI0000289, MI0000223, and MI0000697); for driving differentiation of a hematopoietic progenitor cell toward a lymphoid progenitor cell: miR-181 (see, e.g., MiR Base accession: MI0000269, MI0000270, MI0000271, MI0000289, MI0000683, MI00003139, MI0000223, MI0000723, MI0000697, MI0000724, MI0000823, and MI00005450); for blocking differentiation of a hematopoietic progenitor cell toward a lymphoid progenitor cell: miR-146 (see, e.g., MiR Base accession: MI0000477, MI00003129, MI00003782, MI0000170, and MI00004665); for blocking differentiation of a hematopoietic progenitor cell toward a myeloid progenitor cell: miR-155, miR-24a, and/or miR-17 (see, e.g., MiR Base accession: MI0000071 and MI0000687); for driving differentiation of a lymphoid progenitor cell toward a T cell fate: miR-150 (see, e.g., MiR Base accession: MI0000479 and MI0000172); for blocking differentiation of a myeloid progenitor cell toward a granulocyte fate: miR-223 (see, e.g., MiR Base accession: MI0000300 and MI0000703); for blocking differentiation of a myeloid progenitor cell toward a monocyte fate: miR-17-5p (see, e.g., MiR Base accession: MIMAT0000070 and MIMAT0000649), miR-20a (see, e.g., MiR Base accession: MI0000076 and MI0000568), and/or miR-106a (see, e.g., MiR Base accession: MI0000113 and MI0000406); for blocking differentiation of a myeloid progenitor cell toward a red blood cell fate: miR-150 (see, e.g., MiR Base accession: MI0000479 and MI0000172), miR-155, miR-221 (see, e.g., MiR Base accession: MI0000298 and MI0000709), and/or miR-222 (see, e.g., MiR Base accession: MI0000299 and MI0000710); and for driving differentiation of a myeloid progenitor cell toward a red blood cell fate: miR-451 (see, e.g., MiR Base accession: MI0001729, MI0017360, MI0001730, and MI0021960) and/or miR-16 (see, e.g., MiR Base accession: MI0000070, MI0000115, MI0000565, and MI0000566).

For examples of signaling proteins (e.g., extracellular signaling proteins) that can be delivered (e.g., as protein or as DNA or RNA encoding the protein) in combination with a gene editing tool and a donor DNA, see Figure 9B. The same proteins can be used as part of the outer shell of a subject nanoparticle in a similar manner as a targeting ligand, e.g., for the purpose of biasing differentiation in target cells that receive the nanoparticle. For example, the following signaling proteins (e.g., extracellular signaling proteins) can be used for the following purposes: for driving differentiation of a hematopoietic stem cell toward a common lymphoid progenitor cell lineage: IL-7 (see, e.g., NCBI Gene ID 3574); for driving differentiation of a hematopoietic stem cell toward a common myeloid progenitor cell lineage: IL-3 (see, e.g., NCBI Gene ID 3562), GM-CSF (see, e.g., NCBI Gene ID 1437), and/or M-CSF (see, e.g., NCBI Gene

ID 1435); for driving differentiation of a common lymphoid progenitor cell toward a B-cell fate: IL-3, IL-4 (see, e.g., NCBI Gene ID: 3565), and/or IL-7; for driving differentiation of a common lymphoid progenitor cell toward a Natural Killer Cell fate: IL-15 (see, e.g., NCBI Gene ID 3600); for driving differentiation of a common lymphoid progenitor cell toward a T-cell fate: IL-2 (see, e.g., NCBI Gene ID 3558), IL-7, and/or Notch (see, e.g., NCBI Gene IDs 4851, 4853, 4854, 4855); for driving differentiation of a common lymphoid progenitor cell toward a dendritic cell fate: Flt-3 ligand (see, e.g., NCBI Gene ID 2323); for driving differentiation of a common myeloid progenitor cell toward a dendritic cell fate: Flt-3 ligand, GM-CSF, and/or TNF-alpha (see, e.g., NCBI Gene ID 7124); for driving differentiation of a common myeloid progenitor cell toward a granulocyte-macrophage progenitor cell lineage: GM-CSF; for driving differentiation of a common myeloid progenitor cell toward a megakaryocyte-erythroid progenitor cell lineage: IL-3, SCF (see, e.g., NCBI Gene ID 4254), and/or Tpo (see, e.g., NCBI Gene ID 7173); for driving differentiation of a megakaryocyte-erythroid progenitor cell toward a megakaryocyte fate: IL-3, IL-6 (see, e.g., NCBI Gene ID 3569), SCF, and/or Tpo; for driving differentiation of a megakaryocyte-erythroid progenitor cell toward a erythrocyte fate: erythropoietin (see, e.g., NCBI Gene ID 2056); for driving differentiation of a megakaryocyte toward a platelet fate: IL-11 (see, e.g., NCBI Gene ID 3589) and/or Tpo; for driving differentiation of a granulocyte-macrophage progenitor cell toward a monocyte lineage: GM-CSF and/or M-CSF; for driving differentiation of a granulocyte-macrophage progenitor cell toward a myeloblast lineage: GM-CSF; for driving differentiation of a monocyte toward a monocyte-derived dendritic cell fate: Flt-3 ligand, GM-CSF, IFN-alpha (see, e.g., NCBI Gene ID 3439), and/or IL-4; for driving differentiation of a monocyte toward a macrophage fate: IFN-gamma, IL-6, IL-10 (see, e.g., NCBI Gene ID 3586), and/or M-CSF; for driving differentiation of a myeloblast toward a neutrophil fate: G-CSF (see, e.g., NCBI Gene ID 1440), GM-CSF, IL-6, and/or SCF; for driving differentiation of a myeloblast toward a eosinophil fate: GM-CSF, IL-3, and/or IL-5 (see, e.g., NCBI Gene ID 3567); and for driving differentiation of a myeloblast toward a basophil fate: G-CSF, GM-CSF, and/or IL-3.

Examples of proteins that can be delivered (e.g., as protein and/or a nucleic acid such as DNA or RNA encoding the protein) in combination with a gene editing tool and a donor DNA include but are not limited to: SOX17, HEX, OSKM (Oct4/Sox2/Klf4/c-myc), and/or bFGF (e.g., to drive differentiation toward hepatic stem cell lineage); HNF4a (e.g., to drive differentiation toward hepatocyte fate); Poly (I:C), BMP-4, bFGF, and/or 8-Br-cAMP (e.g., to drive differentiation toward endothelial stem cell/progenitor lineage); VEGF (e.g., to drive differentiation toward arterial endothelium fate); Sox-2, Brn4, Myt1l, Neurod2, Ascl1 (e.g., to drive differentiation toward neural stem cell/progenitor lineage); and BDNF, FCS, Forskolin, and/or SHH (e.g., to drive differentiation neuron, astrocyte, and/or oligodendrocyte fate).

Examples of signaling proteins (e.g., extracellular signaling proteins) that can be delivered (e.g., as protein and/or a nucleic acid such as DNA or RNA encoding the protein) in combination with a gene editing tool and a donor DNA include but are not limited to: cytokines (e.g., IL-2 and/or IL-15, e.g., for activating CD8⁺ T-cells); ligands and or signaling proteins that modulate one or more of the Notch, Wnt, and/or Smad signaling pathways; SCF; stem cell programming factors (e.g. Sox2, Oct3/4, Nanog, Klf4, c-Myc, and the like); and temporary surface marker “tags” and/or fluorescent reporters for subsequent isolation/purification/concentration. For example, a fibroblast may be converted into a neural stem cell via delivery of Sox2, while it will turn into a cardiomyocyte in the presence of Oct3/4 and small molecule “epigenetic resetting factors.” In a patient with Huntington’s disease or a CXCR4 mutation, these fibroblasts may respectively encode diseased phenotypic traits associated with neurons and cardiac cells. By delivering gene editing corrections and these factors in a single package, the risk of deleterious effects due to one or more, but not all of the factors/payloads being introduced can be significantly reduced.

Because the timing and/or location of payload release can be controlled (described in more detail elsewhere in this disclosure), the packaging of multiple payloads in the same package (e.g., same nanoparticle) does not preclude one from achieving different release times/rates and/or locations for different payloads. For example the release of the above proteins (and/or a DNAs or mRNAs encoding same) and/or non-coding RNAs can be controlled separately from the release of the one or more gene editing tools that are part of the same package. For example, proteins and/or nucleic acids (e.g., DNAs, mRNAs, non-coding RNAs, miRNAs) that control cell proliferation and/or differentiation can be released earlier than the one or more gene editing tools or can be released later than the one or more gene editing tools. This can be achieved, e.g., by using more than one sheddable layer and/or by using more than one core (e.g., where one core has a different release profile than the other, e.g., uses a different D- to L- isomer ratio, uses a different ESP:ENP:EPP profile, and the like). In this way, a donor and nuclease may be released in a stepwise manner that allows for optimal editing and insertion efficiencies.

Nanoparticle core

The core of a subject nanoparticle can include an anionic polymer composition (e.g., poly(glutamic acid)), a cationic polymer composition (e.g., poly(arginine), a cationic polypeptide composition (e.g., a histone tail peptide), and a payload (e.g., nucleic acid and/or protein payload, e.g., a donor RNA and/or a site-specific nuclease or a nucleic acid encoding the site-specific nuclease). In some cases, the core is generated by condensation of a cationic amino acid polymer and payload in the presence of an anionic amino acid polymer (and in some

cases in the presence of a cationic polypeptide of a cationic polypeptide composition). In some embodiments, condensation of the components that make up the core can mediate increased transfection efficiency compared to conjugates of cationic polymers with a payload. Inclusion of an anionic polymer in a nanoparticle core may prolong the duration of intracellular residence of the nanoparticle and release of payload.

For the cationic and anionic polymer compositions of the core, ratios of D-isomer polymers to L-isomer polymers can be controlled in order to control the timed release of payload, where increased ratio of D-isomer polymers to L-isomer polymers leads to increased stability (reduced payload release rate), which for example can enable longer lasting gene expression from a payload delivered by a subject nanoparticle. In some cases, modifying the ratio of D-to-L isomer polypeptides within the nanoparticle core can cause gene expression profiles (e.g., expression of a protein encoded by a payload molecule) to be on the order of from 1-90 days (e.g. from 1-80, 1-70, 1-60, 1-50, 1-40, 1-30, 1-25, 1-20, 1-15, 1-10, 3-90, 3-80, 3-70, 3-60, 3-50, 3-40, 3-30, 3-25, 3-20, 3-15, 3-10, 5-90, 5-80, 5-70, 5-60, 5-50, 5-40, 5-30, 5-25, 5-20, 5-15, or 5-10 days). The control of payload release (e.g., when delivering a gene editing tool), can be particularly effective for performing genomic edits e.g., in some cases where homology-directed repair is desired.

In some embodiments, a nanoparticle includes a core and a sheddable layer encapsulating the core, where the core includes: (a) an anionic polymer composition; (b) a cationic polymer composition; (c) a cationic polypeptide composition; and (d) a nucleic acid and/or protein payload, where one of (a) and (b) includes a D-isomer polymer of an amino acid, and the other of (a) and (b) includes an L-isomer polymer of an amino acid, and where the ratio of the D-isomer polymer to the L-isomer polymer is in a range of from 10:1 to 1.5:1 (e.g., from 8:1 to 1.5:1, 6:1 to 1.5:1, 5:1 to 1.5:1, 4:1 to 1.5:1, 3:1 to 1.5:1, 2:1 to 1.5:1, 10:1 to 2:1; 8:1 to 2:1, 6:1 to 2:1, 5:1 to 2:1, 10:1 to 3:1; 8:1 to 3:1, 6:1 to 3:1, 5:1 to 3:1, 10:1 to 4:1; 4:1 to 2:1, 6:1 to 4:1, or 10:1 to 5:1), or from 1:1.5 to 1:10 (e.g., from 1:1.5 to 1:8, 1:1.5 to 1:6, 1:1.5 to 1:5, 1:1.5 to 1:4, 1:1.5 to 1:3, 1:1.5 to 1:2, 1:2 to 1:10, 1:2 to 1:8, 1:2 to 1:6, 1:2 to 1:5, 1:2 to 1:4, 1:2 to 1:3, 1:3 to 1:10, 1:3 to 1:8, 1:3 to 1:6, 1:3 to 1:5, 1:4 to 1:10, 1:4 to 1:8, 1:4 to 1:6, or 1:5 to 1:10). In some such cases, the ratio of the D-isomer polymer to the L-isomer polymer is not 1:1. In some such cases, the anionic polymer composition includes an anionic polymer selected from poly(D-glutamic acid) (PDEA) and poly(D-aspartic acid) (PDDA), where (optionally) the cationic polymer composition can include a cationic polymer selected from poly(L-arginine), poly(L-lysine), poly(L-histidine), poly(L-ornithine), and poly(L-citrulline). In some cases, the cationic polymer composition comprises a cationic polymer selected from poly(D-arginine), poly(D-lysine), poly(D-histidine), poly(D-ornithine), and poly(D-citrulline), where (optionally) the anionic polymer composition can include an anionic polymer selected

from poly(L-glutamic acid) (PLEA) and poly(L-aspartic acid) (PLDA).

In some embodiments, a nanoparticle includes a core and a sheddable layer encapsulating the core, where the core includes: (i) an anionic polymer composition; (ii) a cationic polymer composition; (iii) a cationic polypeptide composition; and (iv) a nucleic acid and/or protein payload, wherein (a) said anionic polymer composition includes polymers of D-isomers of an anionic amino acid and polymers of L-isomers of an anionic amino acid; and/or (b) said cationic polymer composition includes polymers of D-isomers of a cationic amino acid and polymers of L-isomers of a cationic amino acid. In some such cases, the anionic polymer composition comprises a first anionic polymer selected from poly(D-glutamic acid) (PDEA) and poly(D-aspartic acid) (PDDA); and comprises a second anionic polymer selected from poly(L-glutamic acid) (PLEA) and poly(L-aspartic acid) (PLDA). In some cases, the cationic polymer composition comprises a first cationic polymer selected from poly(D-arginine), poly(D-lysine), poly(D-histidine), poly(D-ornithine), and poly(D-citrulline); and comprises a second cationic polymer selected from poly(L-arginine), poly(L-lysine), poly(L-histidine), poly(L-ornithine), and poly(L-citrulline). In some cases, the polymers of D-isomers of an anionic amino acid are present at a ratio, relative to said polymers of L-isomers of an anionic amino acid, in a range of from 10:1 to 1:10. In some cases, the polymers of D-isomers of a cationic amino acid are present at a ratio, relative to said polymers of L-isomers of a cationic amino acid, in a range of from 10:1 to 1:10.

nanoparticle components (timing)

In some embodiments, timing of payload release can be controlled by selecting particular types of proteins, e.g., as part of the core (e.g., part of a cationic polypeptide composition, part of a cationic polymer composition, and/or part of an anionic polymer composition). For example, it may be desirable to delay payload release for a particular range of time, or until the payload is present at a particular cellular location (e.g., cytosol, nucleus, lysosome, endosome) or under a particular condition (e.g., low pH, high pH, etc.). As such, in some cases a protein is used (e.g., as part of the core) that is susceptible to a specific protein activity (e.g., enzymatic activity), e.g., is a substrate for a specific protein activity (e.g., enzymatic activity), and this is in contrast to being susceptible to general ubiquitous cellular machinery, e.g., general degradation machinery. A protein that is susceptible to a specific protein activity is referred to herein as an 'enzymatically susceptible protein' (ESP). Illustrative examples of ESPs include but are not limited to: (i) proteins that are substrates for matrix metalloproteinase (MMP) activity (an example of an extracellular activity), e.g., a protein that includes a motif recognized by an MMP; (ii) proteins that are substrates for cathepsin activity (an example of an intracellular endosomal activity), e.g., a protein that includes a motif

recognized by a cathepsin; and (iii) proteins such as histone tails peptides (HTPs) that are substrates for methyltransferase and/or acetyltransferase activity (an example of an intracellular nuclear activity), e.g., a protein that includes a motif that can be enzymatically methylated/de-methylated and/or a motif that can be enzymatically acetylated/de-acetylated. For example, in some cases a nucleic acid payload is condensed with a protein (such as a histone tails peptide) that is a substrate for acetyltransferase activity, and acetylation of the protein causes the protein to release the payload – as such, one can exercise control over payload release by choosing to use a protein that is more or less susceptible to acetylation.

In some cases, a core of a subject nanoparticle includes an enzymatically neutral polypeptide (ENP), which is a polypeptide homopolymer (i.e., a protein having a repeat sequence) where the polypeptide does not have a particular activity and is neutral. For example, unlike NLS sequences and HTPs, both of which have a particular activity, ENPs do not.

In some cases, a core of a subject nanoparticle includes an enzymatically protected polypeptide (EPP), which is a protein that is resistant to enzymatic activity. Examples of PPs include but are not limited to: (i) polypeptides that include D-isomer amino acids (e.g., D-isomer polymers), which can resist proteolytic degradation; and (ii) self-sheltering domains such as a polyglutamine repeat domains (e.g., QQQQQQQQQQ) (SEQ ID NO: 170).

By controlling the relative amounts of susceptible proteins (ESPs), neutral proteins (ENPs), and protected proteins (EPPs), that are part of a subject nanoparticle (e.g., part of the nanoparticle core), one can control the release of payload. For example, use of more ESPs can in general lead to quicker release of payload than use of more EPPs. In addition, use of more ESPs can in general lead to release of payload that depends upon a particular set of conditions/circumstances, e.g., conditions/circumstances that lead to activity of proteins (e.g., enzymes) to which the ESP is susceptible.

Anionic polymer composition of a nanoparticle

An anionic polymer composition can include one or more anionic amino acid polymers. For example, in some cases a subject anionic polymer composition includes a polymer selected from: poly(glutamic acid)(PEA), poly(aspartic acid)(PDA), and a combination thereof. In some cases a given anionic amino acid polymer can include a mix of aspartic and glutamic acid residues. Each polymer can be present in the composition as a polymer of L-isomers or D-isomers, where D-isomers are more stable in a target cell because they take longer to degrade. Thus, inclusion of D-isomer poly(amino acids) in the nanoparticle core delays degradation of the core and subsequent payload release. The payload release rate can therefore be controlled and is proportional to the ratio of polymers of D-isomers to polymers of L-isomers, where a

higher ratio of D-isomer to L-isomer increases duration of payload release (i.e., decreases release rate). In other words, the relative amounts of D- and L- isomers can modulate the nanoparticle core's timed release kinetics and enzymatic susceptibility to degradation and payload release.

5 In some cases an anionic polymer composition of a subject nanoparticle includes polymers of D-isomers and polymers of L-isomers of an anionic amino acid polymer (e.g., poly(glutamic acid)(PEA) and poly(aspartic acid)(PDA)). In some cases the D- to L- isomer ratio is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1).

Thus, in some cases an anionic polymer composition includes a first anionic polymer (e.g., amino acid polymer) that is a polymer of D-isomers (e.g., selected from poly(D-glutamic acid) (PDEA) and poly(D-aspartic acid) (PDDA)); and includes a second anionic polymer (e.g., amino acid polymer) that is a polymer of L-isomers (e.g., selected from poly(L-glutamic acid) (PLEA) and poly(L-aspartic acid) (PLDA)). In some cases the ratio of the first anionic polymer (D-isomers) to the second anionic polymer (L-isomers) is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1)

15 In some embodiments, an anionic polymer composition of a core of a subject nanoparticle includes (e.g., in addition to or in place of any of the foregoing examples of anionic polymers) a glycosaminoglycan, a glycoprotein, a polysaccharide, poly(mannuronic acid), poly(guluronic acid), heparin, heparin sulfate, chondroitin, chondroitin sulfate, keratan, keratan sulfate, aggrecan, poly(glucosamine), or an anionic polymer that comprises any combination thereof.

30 In some embodiments, an anionic polymer within the core can have a molecular weight in a range of from 1-200 kDa (e.g., from 1-150, 1-100, 1-50, 5-200, 5-150, 5-100, 5-50, 10-200, 10-150, 10-100, 10-50, 15-200, 15-150, 15-100, or 15-50 kDa). As an example, in some cases an anionic polymer includes poly(glutamic acid) with a molecular weight of approximately 15 kDa.

35 In some cases, an anionic amino acid polymer includes a cysteine residue, which can facilitate conjugation, e.g., to a linker, an NLS, and/or a cationic polypeptide (e.g., a histone or

HTP). For example, a cysteine residue can be used for crosslinking (conjugation) via sulfhydryl chemistry (e.g., a disulfide bond) and/or amine-reactive chemistry. Thus, in some embodiments an anionic amino acid polymer (e.g., poly(glutamic acid) (PEA), poly(aspartic acid) (PDA), poly(D-glutamic acid) (PDEA), poly(D-aspartic acid) (PDDA), poly(L-glutamic acid) (PLEA),
 5 poly(L-aspartic acid) (PLDA)) of an anionic polymer composition includes a cysteine residue. In some cases the anionic amino acid polymer includes cysteine residue on the N- and/or C-terminus. In some cases the anionic amino acid polymer includes an internal cysteine residue.

In some cases, an anionic amino acid polymer includes (and/or is conjugated to) a nuclear localization signal (NLS) (described in more detail below). Thus, in some embodiments
 10 an anionic amino acid polymer (e.g., poly(glutamic acid) (PEA), poly(aspartic acid) (PDA), poly(D-glutamic acid) (PDEA), poly(D-aspartic acid) (PDDA), poly(L-glutamic acid) (PLEA), poly(L-aspartic acid) (PLDA)) of an anionic polymer composition includes (and/or is conjugated to) one or more (e.g., two or more, three or more, or four or more) NLSs. In some cases the anionic amino acid polymer includes an NLS on the N- and/or C-terminus. In some cases the
 15 anionic amino acid polymer includes an internal NLS.

In some cases, an anionic polymer is added prior to a cationic polymer when generating a subject nanoparticle core.

Cationic polymer composition of a nanoparticle

20 A cationic polymer composition can include one or more cationic amino acid polymers. For example, in some cases a subject cationic polymer composition includes a polymer selected from: poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), poly(citrulline), and a combination thereof. In some cases a given cationic amino acid polymer can include a mix of arginine, lysine, histidine, ornithine, and citrulline residues (in any
 25 convenient combination). Each polymer can be present in the composition as a polymer of L-isomers or D-isomers, where D-isomers are more stable in a target cell because they take longer to degrade. Thus, inclusion of D-isomer poly(amino acids) in the nanoparticle core delays degradation of the core and subsequent payload release. The payload release rate can therefore be controlled and is proportional to the ratio of polymers of D-isomers to polymers of
 30 L-isomers, where a higher ratio of D-isomer to L-isomer increases duration of payload release (i.e., decreases release rate). In other words, the relative amounts of D- and L- isomers can modulate the nanoparticle core's timed release kinetics and enzymatic susceptibility to degradation and payload release.

In some cases a cationic polymer composition of a subject nanoparticle includes
 35 polymers of D-isomers and polymers of L-isomers of an cationic amino acid polymer (e.g., poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), poly(citrulline)). In

some cases the D- to L- isomer ratio is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1).

Thus, in some cases a cationic polymer composition includes a first cationic polymer (e.g., amino acid polymer) that is a polymer of D-isomers (e.g., selected from poly(D-arginine), poly(D-lysine), poly(D-histidine), poly(D-ornithine), and poly(D-citrulline)); and includes a second cationic polymer (e.g., amino acid polymer) that is a polymer of L-isomers (e.g., selected from poly(L-arginine), poly(L-lysine), poly(L-histidine), poly(L-ornithine), and poly(L-citrulline)). In some cases the ratio of the first cationic polymer (D-isomers) to the second cationic polymer (L-isomers) is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1).

In some embodiments, an cationic polymer composition of a core of a subject nanoparticle includes (e.g., in addition to or in place of any of the foregoing examples of cationic polymers) poly(ethylenimine), poly(amidoamine) (PAMAM), poly(aspartamide), polypeptoids (e.g., for forming "spiderweb"-like branches for core condensation), a charge-functionalized polyester, a cationic polysaccharide, an acetylated amino sugar, chitosan, or a cationic polymer that comprises any combination thereof (e.g., in linear or branched forms).

In some embodiments, an cationic polymer within the core can have a molecular weight in a range of from 1-200 kDa (e.g., from 1-150, 1-100, 1-50, 5-200, 5-150, 5-100, 5-50, 10-200, 10-150, 10-100, 10-50, 15-200, 15-150, 15-100, or 15-50 kDa). As an example, in some cases an cationic polymer includes poly(L-arginine), e.g., with a molecular weight of approximately 29 kDa. As another example, in some cases a cationic polymer includes linear poly(ethylenimine) with a molecular weight of approximately 25 kDa (PEI). As another example, in some cases a cationic polymer includes branched poly(ethylenimine) with a molecular weight of approximately 10 kDa. As another example, in some cases a cationic polymer includes branched poly(ethylenimine) with a molecular weight of approximately 70 kDa. In some cases a cationic polymer includes PAMAM.

In some cases, a cationic amino acid polymer includes a cysteine residue, which can facilitate conjugation, e.g., to a linker, an NLS, and/or a cationic polypeptide (e.g., a histone or

HTP). For example, a cysteine residue can be used for crosslinking (conjugation) via sulfhydryl chemistry (e.g., a disulfide bond) and/or amine-reactive chemistry. Thus, in some embodiments a cationic amino acid polymer (e.g., poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), and poly(citrulline), poly(D-arginine)(PDR), poly(D-lysine)(PDK), poly(D-histidine)(PDH), poly(D-ornithine), and poly(D-citrulline), poly(L-arginine)(PLR), poly(L-lysine)(PLK), poly(L-histidine)(PLH), poly(L-ornithine), and poly(L-citrulline)) of a cationic polymer composition includes a cysteine residue. In some cases the cationic amino acid polymer includes cysteine residue on the N- and/or C- terminus. In some cases the cationic amino acid polymer includes an internal cysteine residue.

In some cases, a cationic amino acid polymer includes (and/or is conjugated to) a nuclear localization signal (NLS) (described in more detail below). Thus, in some embodiments a cationic amino acid polymer (e.g., poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), and poly(citrulline), poly(D-arginine)(PDR), poly(D-lysine)(PDK), poly(D-histidine)(PDH), poly(D-ornithine), and poly(D-citrulline), poly(L-arginine)(PLR), poly(L-lysine)(PLK), poly(L-histidine)(PLH), poly(L-ornithine), and poly(L-citrulline)) of a cationic polymer composition includes (and/or is conjugated to) one or more (e.g., two or more, three or more, or four or more) NLSs. In some cases the cationic amino acid polymer includes an NLS on the N- and/or C- terminus. In some cases the cationic amino acid polymer includes an internal NLS.

Cationic polypeptide composition of a nanoparticle

In some embodiments the cationic polypeptide composition of a nanoparticle can mediate stability, subcellular compartmentalization, and/or payload release. As one example, fragments of the N-terminus of histone proteins, referred to generally as histone tail peptides, within a subject nanoparticle core are in some case not only capable of being deprotonated by various histone modifications, such as in the case of histone acetyltransferase-mediated acetylation, but may also mediate effective nuclear-specific unpackaging of components (e.g., a payload) of a nanoparticle core. In some cases a cationic polypeptide composition includes a histone and/or histone tail peptide (e.g., a cationic polypeptide can be a histone and/or histone tail peptide). In some cases a cationic polypeptide composition includes an NLS- containing peptide (e.g., a cationic polypeptide can be an NLS- containing peptide). In some cases, a cationic polypeptide composition includes one or more NLS-containing peptides separated by cysteine residues to facilitate crosslinking. In some cases a cationic polypeptide composition includes a peptide that includes a mitochondrial localization signal (e.g., a cationic polypeptide can be a peptide that includes a mitochondrial localization signal).

Sheddable layer (shedtable coat) of a nanoparticle

In some embodiments, a subject nanoparticle includes a sheddable layer (also referred to herein as a “transient stabilizing layer”) that surrounds (encapsulates) the core. In some cases a subject sheddable layer can protect the payload before and during initial cellular uptake. For example, without a sheddable layer, much of the payload can be lost during cellular internalization. Once in the cellular environment, a sheddable layer ‘sheds’ (e.g., the layer can be pH- and/or or glutathione-sensitive), exposing the components of the core.

In some cases a subject sheddable layer includes silica. In some cases, when a subject nanoparticle includes a sheddable layer (e.g., of silica), greater intracellular delivery efficiency can be observed despite decreased probability of cellular uptake. Without wishing to be bound by any particular theory, coating a nanoparticle core with a sheddable layer (e.g., silica coating) can seal the core, stabilizing it until shedding of the layer, which leads to release of the payload (e.g., upon processing in the intended subcellular compartment). Following cellular entry through receptor-mediated endocytosis, the nanoparticle sheds its outermost layer, the sheddable layer degrades in the acidifying environment of the endosome or reductive environment of the cytosol, and exposes the core, which in some cases exposes localization signals such as nuclear localization signals (NLSs) and/or mitochondrial localization signals. Moreover, nanoparticle cores encapsulated by a sheddable layer can be stable in serum and can be suitable for administration *in vivo*.

Any desired sheddable layer can be used, and one of ordinary skill in the art can take into account where in the target cell (e.g., under what conditions, such as low pH) they desire the payload to be released (e.g., endosome, cytosol, nucleus, lysosome, and the like). Different sheddable layers may be more desirable depending on when, where, and/or under what conditions it would be desirable for the sheddable coat to shed (and therefore release the payload). For example, a sheddable layer can be acid labile. In some cases the sheddable layer is an anionic sheddable layer (an anionic coat). In some cases the sheddable layer comprises silica, a peptoid, a polycysteine, and/or a ceramic (e.g., a bioceramic). In some cases the sheddable includes one or more of: calcium, manganese, magnesium, iron (e.g., the sheddable layer can be magnetic, e.g., Fe_3MnO_2), and lithium. Each of these can include phosphate or sulfate. As such, in some cases the sheddable includes one or more of: calcium phosphate, calcium sulfate, manganese phosphate, manganese sulfate, magnesium phosphate, magnesium sulfate, iron phosphate, iron sulfate, lithium phosphate, and lithium sulfate; each of which can have a particular effect on how and/or under which conditions the sheddable layer will ‘shed.’ Thus, in some cases the sheddable layer includes one or more of: silica, a peptoid, a polycysteine, a ceramic (e.g., a bioceramic), calcium, calcium phosphate, calcium sulfate, calcium oxide, hydroxyapatite, manganese, manganese phosphate,

manganese sulfate, manganese oxide, magnesium, magnesium phosphate, magnesium sulfate, magnesium oxide, iron, iron phosphate, iron sulfate, iron oxide, lithium, lithium phosphate, and lithium sulfate (in any combination thereof) (e.g., the sheddable layer can be a coating of silica, peptoid, polycysteine, a ceramic (e.g., a bioceramic), calcium phosphate, calcium sulfate, manganese phosphate, manganese sulfate, magnesium phosphate, magnesium sulfate, iron phosphate, iron sulfate, lithium phosphate, lithium sulfate, or a combination thereof). In some cases the sheddable layer includes silica (e.g., the sheddable layer can be a silica coat). In some cases the sheddable layer includes an alginate gel.

In some cases different release times for different payloads are desirable. For example, in some cases it is desirable to release a payload early (e.g., within 0.5 – 7 days of contacting a target cell) and in some cases it is desirable to release a payload late (e.g., within 6 days-30 days of contacting a target cell). For example, in some cases it may be desirable to release a payload (e.g., a gene editing tool such as a CRISPR/Cas guide RNA, a DNA molecule encoding said CRISPR/Cas guide RNA, a CRISPR/Cas RNA-guided polypeptide, and/or a nucleic acid molecule encoding said CRISPR/Cas RNA-guided polypeptide) within 0.5-7 days of contacting a target cell (e.g., within 0.5-5 days, 0.5-3 days, 1-7 days, 1-5 days, or 1-3 days of contacting a target cell). In some cases it may be desirable to release a payload (e.g., a Donor DNA molecule) within 6-40 days of contacting a target cell (e.g., within 6-30, 6-20, 6-15, 7-40, 7-30, 7-20, 7-15, 9-40, 9-30, 9-20, or 9-15 days of contacting a target cell). In some cases release times can be controlled by delivering nanoparticles having different payloads at different times. In some cases release times can be controlled by delivering nanoparticles at the same time (as part of different formulations or as part of the same formulation), where the components of the nanoparticle are designed to achieve the desired release times. For example, one may use a sheddable layer that degrades faster or slower, core components that are more or less resistant to degradation, core components that are more or less susceptible to de-condensation, etc. – and any or all of the components can be selected in any convenient combination to achieve the desired timing.

In some cases it is desirable to delay the release of a payload (e.g., a Donor DNA molecule) relative to another payload (e.g., one or more gene editing tools). As an example, in some cases a first nanoparticle includes a donor DNA molecule as a payload is designed such that the payload is released within 6-40 days of contacting a target cell (e.g., within 6-30, 6-20, 6-15, 7-40, 7-30, 7-20, 7-15, 9-40, 9-30, 9-20, or 9-15 days of contacting a target cell), while a second nanoparticle that includes one or more gene editing tools (e.g., a ZFP or nucleic acid encoding the ZFP, a TALE or a nucleic acid encoding the TALE, a ZFN or nucleic acid encoding the ZFN, a TALEN or a nucleic acid encoding the TALEN, a CRISPR/Cas guide RNA or DNA molecule encoding the CRISPR/Cas guide RNA, a CRISPR/Cas RNA-guided

polypeptide or a nucleic acid molecule encoding the CRISPR/Cas RNA-guided polypeptide, and the like) as a payload is designed such that the payload is released within 0.5-7 days of contacting a target cell (e.g., within 0.5-5 days, 0.5-3 days, 1-7 days, 1-5 days, or 1-3 days of contacting a target cell). The second nanoparticle can be part of the same or part of a different
 5 formulation as the first nanoparticle.

In some cases, a nanoparticle includes more than one payload, where it is desirable for the payloads to be released at different times. This can be achieved in a number of different ways. For example, a nanoparticle can have more than one core, where one core is made with components that can release the payload early (e.g., within 0.5-7 days of contacting a target
 10 cell, e.g., within 0.5-5 days, 0.5-3 days, 1-7 days, 1-5 days, or 1-3 days of contacting a target cell) (e.g., an siRNA, an mRNA, and/or a genome editing tool such as a ZFP or nucleic acid encoding the ZFP, a TALE or a nucleic acid encoding the TALE, a ZFN or nucleic acid encoding the ZFN, a TALEN or a nucleic acid encoding the TALEN, a CRISPR/Cas guide RNA or DNA molecule encoding the CRISPR/Cas guide RNA, a CRISPR/Cas RNA-guided
 15 polypeptide or a nucleic acid molecule encoding the CRISPR/Cas RNA-guided polypeptide, and the like) and the other is made with components that can release the payload (e.g., a Donor DNA molecule) later (e.g., within 6-40 days of contacting a target cell, e.g., within 6-30, 6-20, 6-15, 7-40, 7-30, 7-20, 7-15, 9-40, 9-30, 9-20, or 9-15 days of contacting a target cell).

As another example, a nanoparticle can include more than one sheddable layer, where
 20 the outer sheddable layer is shed (releasing a payload) prior to an inner sheddable layer being shed (releasing another payload). In some cases, the inner payload is a Donor DNA molecule and the outer payload is one or more gene editing tools (e.g., a ZFN or nucleic acid encoding the ZFN, a TALEN or a nucleic acid encoding the TALEN, a CRISPR/Cas guide RNA or DNA molecule encoding the CRISPR/Cas guide RNA, a CRISPR/Cas RNA-guided polypeptide or a
 25 nucleic acid molecule encoding the CRISPR/Cas RNA-guided polypeptide, and the like). The inner and outer payloads can be any desired payload and either or both can include, for example, one or more siRNAs and/or one or more mRNAs. As such, in some cases a nanoparticle can have more than one sheddable layer and can be designed to release one payload early (e.g., within 0.5-7 days of contacting a target cell, e.g., within 0.5-5 days, 0.5-3
 30 days, 1-7 days, 1-5 days, or 1-3 days of contacting a target cell) (e.g., an siRNA, an mRNA, a genome editing tool such as a ZFP or nucleic acid encoding the ZFP, a TALE or a nucleic acid encoding the TALE, a ZFN or nucleic acid encoding the ZFN, a TALEN or a nucleic acid encoding the TALEN, a CRISPR/Cas guide RNA or DNA molecule encoding the CRISPR/Cas guide RNA, a CRISPR/Cas RNA-guided polypeptide or a nucleic acid molecule encoding the
 35 CRISPR/Cas RNA-guided polypeptide, and the like), and another payload (e.g., an siRNA, an mRNA, a Donor DNA molecule) later (e.g., within 6-40 days of contacting a target cell, e.g.,

within 6-30, 6-20, 6-15, 7-40, 7-30, 7-20, 7-15, 9-40, 9-30, 9-20, or 9-15 days of contacting a target cell).

In some embodiments (e.g., in embodiments described above), time of altered gene expression can be used as a proxy for the time of payload release. As an illustrative example, if one desires to determine if a payload has been released by day 12, one can assay for the desired result of nanoparticle delivery on day 12. For example, if the desired result was to reduce the expression of a target gene of the target cell, e.g., by delivering an siRNA, then the expression of the target gene can be assayed/monitored to determine if the siRNA has been released. As another example, if the desired result was to express a protein of interest, e.g., by delivering a DNA or mRNA encoding the protein of interest, then the expression of the protein of interest can be assayed/monitored to determine if the payload has been released. As yet another example, if the desired result was to alter the genome of the target cell, e.g., via cleaving genomic DNA and/or inserting a sequence of a donor DNA molecule, the expression from the targeted locus and/or the presence of genomic alterations can be assayed/monitored to determine if the payload has been released.

As such, in some cases a sheddable layer provides for a staged release of nanoparticle components. For example, in some cases, a nanoparticle has more than one (e.g., two, three, or four) sheddable layers. For example, for a nanoparticle with two sheddable layers, such a nanoparticle can have, from inner-most to outer-most: a core, e.g., with a first payload; a first sheddable layer, an intermediate layer e.g., with a second payload; and a second sheddable layer surrounding the intermediate layer (see, e.g., **Figure 4**). Such a configuration (multiple sheddable layers) facilitates staged release of various desired payloads. As a further illustrative example, a nanoparticle with two sheddable layers (as described above) can include one or more desired gene editing tools in the core (e.g., one or more of: a Donor DNA molecule, a CRISPR/Cas guide RNA, a DNA encoding a CRISPR/Cas guide RNA, and the like), and another desired gene editing tool in the intermediate layer (e.g., one or more of: a programmable gene editing protein such as a CRISPR/Cas protein, a ZFP, a ZFN, a TALE, a TALEN, etc.; a DNA or RNA encoding a programmable gene editing protein; a CRISPR/Cas guide RNA; a DNA encoding a CRISPR/Cas guide RNA; and the like) – in any desired combination.

Alternative packaging (e.g., lipid formulations)

In some embodiments, a subject core (e.g., including any combination of components and/or configurations described above) is part of a lipid-based delivery system, e.g., a cationic lipid delivery system (see, e.g., Chesnoy and Huang, *Annu Rev Biophys Biomol Struct.* 2000, 29:27-47; Hirko et al., *Curr Med Chem.* 2003 Jul 10(14):1185-93; and Liu et al., *Curr Med*

Chem. 2003 Jul 10(14):1307-15). In some cases a subject core (e.g., including any combination of components and/or configurations described above) is not surrounded by a sheddable layer. As noted above a core can include an anionic polymer composition (e.g., poly(glutamic acid)), a cationic polymer composition (e.g., poly(arginine), a cationic polypeptide composition (e.g., a histone tail peptide), and a payload (e.g., nucleic acid and/or protein payload).

In some cases in which the core is part of a lipid-based delivery system, the core was designed with timed and/or positional (e.g., environment-specific) release in mind. For example, in some cases the core includes ESPs, ENPs, and/or EPPs, and in some such cases these components are present at ratios such that payload release is delayed until a desired condition (e.g., cellular location, cellular condition such as pH, presence of a particular enzyme, and the like) is encountered by the core (e.g., described above). In some such embodiments the core includes polymers of D-isomers of an anionic amino acid and polymers of L-isomers of an anionic amino acid, and in some cases the polymers of D- and L- isomers are present, relative to one another, within a particular range of ratios (e.g., described above). In some cases the core includes polymers of D-isomers of a cationic amino acid and polymers of L-isomers of a cationic amino acid, and in some cases the polymers of D- and L- isomers are present, relative to one another, within a particular range of ratios (e.g., described above). In some cases the core includes polymers of D-isomers of an anionic amino acid and polymers of L-isomers of a cationic amino acid, and in some cases the polymers of D- and L- isomers are present, relative to one another, within a particular range of ratios (e.g., described elsewhere herein). In some cases the core includes a protein that includes an NLS (e.g., described elsewhere herein). In some cases the core includes an HTP (e.g., described elsewhere herein).

Cationic lipids are nonviral vectors that can be used for gene delivery and have the ability to condense plasmid DNA. After synthesis of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride for lipofection, improving molecular structures of cationic lipids has been an active area, including head group, linker, and hydrophobic domain modifications. Modifications have included the use of multivalent polyamines, which can improve DNA binding and delivery via enhanced surface charge density, and the use of sterol-based hydrophobic groups such as 3B-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol, which can limit toxicity. Helper lipids such as dioleoyl phosphatidylethanolamine (DOPE) can be used to improve transgene expression via enhanced liposomal hydrophobicity and hexagonal inverted-phase transition to facilitate endosomal escape. In some cases a lipid formulation includes one

or more of: DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, DLin-MC3-DMA, 98N12-5, C12-200, a cholesterol a PEG-lipid, a lipidopolyamine, dexamethasone-spermine (DS), and disubstituted spermine (D₂S) (e.g., resulting from the conjugation of dexamethasone to polyamine spermine). DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, 98N12-5, C12-200 and DLin-MC3-DMA can be synthesized by methods outlined in the art (see, e.g., Heyes et. al, J. Control Release, 2005, 107, 276-287; Semple et. al, Nature Biotechnology, 2010, 28, 172-176; Akinc et. al, Nature Biotechnology, 2008, 26, 561-569; Love et. al, PNAS, 2010, 107, 1864-1869; international patent application publication WO2010054401; all of which are hereby incorporated by reference in their entirety.

Examples of various lipid-based delivery systems include, but are not limited to those described in the following publications: international patent publication No. WO2016081029; U.S. patent application publication Nos. US20160263047 and US20160237455; and U.S. patent Nos. 9,533,047; 9,504,747; 9,504,651; 9,486,538; 9,393,200; 9,326,940; 9,315,828; and 9,308,267; all of which are hereby incorporated by reference in their entirety.

As such, in some cases a subject core is surrounded by a lipid (e.g., a cationic lipid such as a LIPOFECTAMINE transfection reagent). In some cases a subject core is present in a lipid formulation (e.g., a lipid nanoparticle formulation). A lipid formulation can include a liposome and/or a lipoplex. A lipid formulation can include a Spontaneous Vesicle Formation by Ethanol Dilution (SNALP) liposome (e.g., one that includes cationic lipids together with neutral helper lipids which can be coated with polyethylene glycol (PEG) and/or protamine).

A lipid formulation can be a lipidoid-based formulation. The synthesis of lipidoids has been extensively described and formulations containing these compounds can be included in a subject lipid formulation (see, e.g., Mahon et al., Bioconjug Chem. 2010 21:1448-1454; Schroeder et al., J Intern Med. 2010 267:9-21; Akinc et al., Nat Biotechnol. 2008 26:561-569; Love et al., Proc Natl Acad Sci USA. 2010 107:1864-1869; and Siegwart et al., Proc Natl Acad Sci USA. 2011 108:12996-3001; all of which are incorporated herein by reference in their entirety). In some cases a subject lipid formulation can include one or more of (in any desired combination): 1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC); 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE); N-[1-(2,3-Dioleoyloxy)propyl]N,N,N-trimethylammonium chloride (DOTMA); 1,2-Dioleoyloxy-3-trimethylammonium-propane (DOTAP); Dioctadecylamidoglycylspermine (DOGS); N-(3-Aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1 (GAP-DLRIE); propanaminium bromide; cetyltrimethylammonium bromide (CTAB); 6-Lauroxyhexyl ornithinate (LHON); 1-(2,3-Dioleoyloxypropyl)-2,4,6-trimethylpyridinium (2Oc); 2,3-Dioleoyloxy-N-[2(sperminecarboxamido-ethyl)]-N,N-dimethyl-1 (DOSPA); propanaminium trifluoroacetate; 1,2-Dioleoyl-3-trimethylammonium-propane (DOPA); N-(2-Hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1 (MDRIE); propanaminium bromide;

dimyristooxypropyl dimethyl hydroxyethyl ammonium bromide (DMRI); 3.β-[N-(N',N'-Dimethylaminoethane)-carbamoyl]cholesterol DC-Chol; bis-guanidium-tren-cholesterol (BGTC); 1,3-Diideoxy-2-(6-carboxy-spermyl)-propylamide (DOSPER); Dimethyloctadecylammonium bromide (DDAB); Dioctadecylamidoglycylspermidin (DSL); rac-[(2,3-Dioctadecyloxypropyl)(2-hydroxyethyl)]-dimethylammonium (CLIP-1); chloride rac-[2(2,3-Dihexadecyloxypropyl (CLIP-6); oxymethyloxy)ethyl]trimethylammonium bromide; ethyldimyristoylphosphatidylcholine (EDMPC); 1,2-Distearyloxy-N,N-dimethyl-3-aminopropane (DSDMA); 1,2-Dimyristoyl-trimethylammonium propane (DMTAP); O,O'-Dimyristyl-N-lysyl aspartate (DMKE); 1,2-Distearoyl-sn-glycero-3-ethylphosphocholine (DSEPC); N-Palmitoyl D-erythro-sphingosyl carbamoyl-spermine (CCS); N-t-Butyl-N0-tetradecyl-3-tetradecylaminopropionamidine; diC14-amidine; octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium (DOTIM); chloride N1-Cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN); 2-[3-[bis(3-aminopropyl)amino]propylamino]-N-[2-[di(tetradecyl)amino]-2-oxoethyl]acetamide (RPR209120); ditetradecylcarbamoylme-ethyl-acetamide; 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA); 2,2-dilinoley-4-dimethylaminoethyl-[1,3]-dioxolane; DLin-KC2-DMA; dilinoley-methyl-4-dimethylaminobutyrate; DLin-MC3-DMA; DLin-K-DMA; 98N12-5; C12-200; a cholesterol; a PEG-lipid; a lipopolyamine; dexamethasone-spermine (DS); and disubstituted spermine (D₂S).

20 Surface Coat (Outer shell) of a nanoparticle

In some cases, the sheddable layer (the coat), is itself coated by an additional layer, referred to herein as an “outer shell,” “outer coat,” or “surface coat.” A surface coat can serve multiple different functions. For example, a surface coat can increase delivery efficiency and/or can target a subject nanoparticle to a particular cell type. The surface coat can include a peptide, a polymer, or a ligand-polymer conjugate. The surface coat can include a targeting ligand. For example, an aqueous solution of one or more targeting ligands (with or without linker domains) can be added to a coated nanoparticle suspension (suspension of nanoparticles coated with a sheddable layer). For example, in some cases the final concentration of protonated anchoring residues (of an anchoring domain) is between 25 and 300 μM. In some cases, the process of adding the surface coat yields a monodispersed suspension of particles with a mean particle size between 50 and 150 nm and a zeta potential between 0 and -10 mV.

In some cases, the surface coat interacts electrostatically with the outermost sheddable layer. For example, in some cases, a nanoparticle has two sheddable layers (e.g., from inner-most to outer-most: a core, e.g., with a first payload; a first sheddable layer, an intermediate layer e.g., with a second payload; and a second sheddable layer surrounding the intermediate

layer), and the outer shell (surface coat) can interact with (e.g., electrostatically) the second sheddable layer. In some cases, a nanoparticle has only one sheddable layer (e.g., an anionic silica layer), and the outer shell can in some cases electrostatically interact with the sheddable layer.

Thus, in cases where the sheddable layer (e.g., outermost sheddable layer) is anionic (e.g., in some cases where the sheddable layer is a silica coat), the surface coat can interact electrostatically with the sheddable layer if the surface coat includes a cationic component. For example, in some cases the surface coat includes a delivery molecule in which a targeting ligand is conjugated to a cationic anchoring domain. The cationic anchoring domain interacts electrostatically with the sheddable layer and anchors the delivery molecule to the nanoparticle. Likewise, in cases where the sheddable layer (e.g., outermost sheddable layer) is cationic, the surface coat can interact electrostatically with the sheddable layer if the surface coat includes an anionic component.

In some embodiments, the surface coat includes a cell penetrating peptide (CPP). In some cases, a polymer of a cationic amino acid can function as a CPP (also referred to as a 'protein transduction domain' - PTD), which is a term used to refer to a polypeptide, polynucleotide, carbohydrate, or organic or inorganic compound that facilitates traversing a lipid bilayer, micelle, cell membrane, organelle membrane, or vesicle membrane. A PTD attached to another molecule (e.g., embedded in and/or interacting with a sheddable layer of a subject nanoparticle), which can range from a small polar molecule to a large macromolecule and/or a nanoparticle, facilitates the molecule traversing a membrane, for example going from extracellular space to intracellular space, or cytosol to within an organelle (e.g., the nucleus).

Examples of CPPs include but are not limited to a minimal undecapeptide protein transduction domain (corresponding to residues 47-57 of HIV-1 TAT comprising YGRKKRRQRRR (SEQ ID NO: 160); a polyarginine sequence comprising a number of arginines sufficient to direct entry into a cell (e.g., 3, 4, 5, 6, 7, 8, 9, 10, or 10-50 arginines); a VP22 domain (Zender et al. (2002) *Cancer Gene Ther.* 9(6):489-96); an *Drosophila* Antennapedia protein transduction domain (Noguchi et al. (2003) *Diabetes* 52(7):1732-1737); a truncated human calcitonin peptide (Trehin et al. (2004) *Pharm. Research* 21:1248-1256); polylysine (Wender et al. (2000) *Proc. Natl. Acad. Sci. USA* 97:13003-13008); RRQRRTSKLMKR (SEQ ID NO: 161); Transportan GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO: 162); KALAEAKLAKALAKALAKHLAKALAKALKCEA (SEQ ID NO: 163); and RQIKIWFQNRRMKWKK (SEQ ID NO: 164). Example CPPs include but are not limited to: YGRKKRRQRRR (SEQ ID NO: 160), RKKRRQRRR (SEQ ID NO: 165), an arginine homopolymer of from 3 arginine residues to 50 arginine residues, RKKRRQRR (SEQ ID NO: 166), YARAAARQARA (SEQ ID NO: 167), THRLPRRRRRR (SEQ ID NO: 168), and

GGRRARRRRRR (SEQ ID NO: 169). In some embodiments, the CPP is an activatable CPP (ACPP) (Aguilera et al. (2009) *Integr Biol (Camb)* June; 1(5-6): 371-381). ACPPs comprise a polycationic CPP (e.g., Arg9 or "R9") connected via a cleavable linker to a matching polyanion (e.g., Glu9 or "E9"), which reduces the net charge to nearly zero and thereby inhibits adhesion and uptake into cells. Upon cleavage of the linker, the polyanion is released, locally unmasking the polyarginine and its inherent adhesiveness, thus "activating" the ACPP to traverse the membrane

In some cases a CPP can be added to the nanoparticle by contacting a coated core (a core that is surrounded by a sheddable layer) with a composition (e.g., solution) that includes the CPP. The CPP can then interact with the sheddable layer (e.g., electrostatically).

In some cases, the surface coat includes a polymer of a cationic amino acid (e.g., a poly(arginine) such as poly(L-arginine) and/or poly(D-arginine), a poly(lysine) such as poly(L-lysine) and/or poly(D-lysine), a poly(histidine) such as poly(L-histidine) and/or poly(D-histidine), a poly(ornithine) such as poly(L-ornithine) and/or poly(D-ornithine), poly(citrulline) such as poly(L-citrulline) and/or poly(D-citrulline), and the like). As such, in some cases the surface coat includes poly(arginine), e.g., poly(L-arginine).

In some embodiments, the surface coat includes a heptapeptide such as selank (TKPRPGP - SEQ ID NO: 147) (e.g., N-acetyl selank) and/or semax (MEHFPGP - SEQ ID NO: 148) (e.g., N-acetyl semax). As such, in some cases the surface coat includes selank (e.g., N-acetyl selank). In some cases the surface coat includes semax (e.g., N-acetyl semax).

In some embodiments the surface coat includes a delivery molecule. A delivery molecule includes a targeting ligand and in some cases the targeting ligand is conjugated to an anchoring domain (e.g. a cationic anchoring domain or anionic anchoring domain). In some cases a targeting ligand is conjugated to an anchoring domain (e.g. a cationic anchoring domain or anionic anchoring domain) via an intervening linker.

Multivalent surface coat

In some cases the surface coat includes any one or more of (in any desired combination): (i) one or more of the above described polymers, (ii) one or more targeting ligands, one or more CPPs, and one or more heptapeptides. For example, in some cases a surface coat can include one or more (e.g., two or more, three or more) targeting ligands, but can also include one or more of the above described cationic polymers. In some cases a surface coat can include one or more (e.g., two or more, three or more) targeting ligands, but can also include one or more CPPs. Further, a surface coat may include any combination of glycopeptides to promote stealth functionality, that is, to prevent serum protein adsorption and complement activity. This may be accomplished through Azide-alkyne click chemistry, coupling

a peptide containing propargyl modified residues to azide containing derivatives of sialic acid, neuraminic acid, and the like.

In some cases, a surface coat includes a combination of targeting ligands that provides for targeted binding to CD34 and heparin sulfate proteoglycans. For example, poly(L-arginine) can be used as part of a surface coat to provide for targeted binding to heparin sulfate proteoglycans. As such, in some cases, after surface coating a nanoparticle with a cationic polymer (e.g., poly(L-arginine)), the coated nanoparticle is incubated with hyaluronic acid, thereby forming a zwitterionic and multivalent surface.

In some embodiments, the surface coat is multivalent. A multivalent surface coat is one that includes two or more targeting ligands (e.g., two or more delivery molecules that include different ligands). An example of a multimeric (in this case trimeric) surface coat (outer shell) is one that includes the targeting ligands stem cell factor (SCF) (which targets c-Kit receptor, also known as CD117), CD70 (which targets CD27), and SH2 domain-containing protein 1A (SH2D1A) (which targets CD150). For example, in some cases, to target hematopoietic stem cells (HSCs) [KLS (c-Kit⁺ Lin⁻ Sca-1⁺) and CD27⁺/IL-7Ra⁺/CD150⁺/CD34⁻], a subject nanoparticle includes a surface coat that includes a combination of the targeting ligands SCF, CD70, and SH2 domain-containing protein 1A (SH2D1A), which target c-Kit, CD27, and CD150, respectively (see, e.g., Table 1). In some cases, such a surface coat can selectively target HSPCs and long-term HSCs (c-Kit⁺/Lin⁻/Sca-1⁺/CD27⁺/IL-7Ra⁺/CD150⁺/CD34⁻) over other lymphoid and myeloid progenitors.

In some example embodiments, all three targeting ligands (SCF, CD70, and SH2D1A) are anchored to the nanoparticle via fusion to a cationic anchoring domain (e.g., a poly-histidine such as 6H, a poly-arginine such as 9R, and the like). For example, (1) the targeting polypeptide SCF (which targets c-Kit receptor) can include

XMEGICRNRVTNNVKDVTCLVANLPKDYMITLKYPGMVDLP SHCWISEM VVQLSDSLTDLLD KFSNISEGLSNYSIIDKLVNIVDDLVECVKENSSKDLKKSFKSPEPRLFTPEEFFRIFNRSIDAFKD FWASETSDCWSSSTLSPEKDSRVSVTKPFMLPPV**X** (SEQ ID NO: 194), where the X is a cationic anchoring domain (e.g., a poly-histidine such as 6H, a poly-arginine such as 9R, and the like), e.g., which can in some cases be present at the N- and/or C-terminal end, or can be embedded within the polypeptide sequence; (2) the targeting polypeptide CD70 (which targets CD27) can include

XPEEGSGCSVRRRPYGCVLRAALVPLVAGLVICLVCIQRFAQAQQQLPLESLGWDVAELQLN HTGPQQDPRLYWQGGPALGRSFLHGPELDKGQLRIHRDGIYMMHIQVTLAICSSTTASRHHPT TLAVGICSPASRSISLLRLSFHQGCTIASQRLTPLARGDTLCTNLTGTLLPSRNTDETFFGVQWV RP**X** (SEQ ID NO: 195), where the X is a cationic anchoring domain (e.g., a poly-histidine such as 6H, a poly-arginine such as 9R, and the like), e.g., which can in some cases be present at

the N- and/or C-terminal end, or can be embedded within the polypeptide sequence; and (3) the targeting polypeptide SH2D1A (which targets CD150) can include

XSSGLVPRGSHMDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSSESVPGVYCLCVLYHGYIY
TYRVSQTETGSWSAETAPGVHKRYFRKIKNLISAFQKPDQGVIPLQYPVEKKSSARSTQGTTG

5 **IREDPDVCLKAP** (SEQ ID NO: 196), where the X is a cationic anchoring domain (e.g., a poly-histidine such as 6H, a poly-arginine such as 9R, and the like), e.g., which can in some cases be present at the N- and/or C-terminal end, or can be embedded within the polypeptide sequence (e.g., such as

MGSSXSGLVPRGSHMDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSSESVPGVYCLCVLYH
 10 **GYIYTYRVSQTETGSWSAETAPGVHKRYFRKIKNLISAFQKPDQGVIPLQYPVEKKSSARSTQ**
GTTGIREDPDVCLKAP (SEQ ID NO: 197)).

As noted above, nanoparticles of the disclosure can include multiple targeting ligands (as part of a surface coat) in order to target a desired cell type, or in order to target a desired combination of cell types. Examples of cells of interest within the mouse and human hematopoietic cell lineages are depicted in **Figure 8** (panels A-B), along with markers that have been identified for those cells. For example, various combinations of cell surface markers of interest include, but are not limited to: [Mouse] (i) CD150; (ii) Sca1, cKit, CD150; (iii) CD150 and CD49b; (iv) Sca1, cKit, CD150, and CD49b; (v) CD150 and Flt3; (vi) Sca1, cKit, CD150, and Flt3; (vii) Flt3 and CD34; (viii) Flt3, CD34, Sca1, and cKit; (ix) Flt3 and CD127; (x) Sca1, cKit, Flt3, and CD127; (xi) CD34; (xii) cKit and CD34; (xiii) CD16/32 and CD34; (xiv) cKit, CD16/32, and CD34; and (xv) cKit; and [Human] (i) CD90 and CD49f; (ii) CD34, CD90, and CD49f; (iii) CD34; (iv) CD45RA and CD10; (v) CD34, CD45RA, and CD10; (vi) CD45RA and CD135; (vii) CD34, CD38, CD45RA, and CD135; (viii) CD135; (ix) CD34, CD38, and CD135; and (x) CD34 and CD38. Thus, in some cases a surface coat includes one or more targeting ligands that provide targeted binding to a surface protein or combination of surface proteins selected from: [Mouse] (i) CD150; (ii) Sca1, cKit, CD150; (iii) CD150 and CD49b; (iv) Sca1, cKit, CD150, and CD49b; (v) CD150 and Flt3; (vi) Sca1, cKit, CD150, and Flt3; (vii) Flt3 and CD34; (viii) Flt3, CD34, Sca1, and cKit; (ix) Flt3 and CD127; (x) Sca1, cKit, Flt3, and CD127; (xi) CD34; (xii) cKit and CD34; (xiii) CD16/32 and CD34; (xiv) cKit, CD16/32, and CD34; and (xv) cKit; and [Human] (i) CD90 and CD49f; (ii) CD34, CD90, and CD49f; (iii) CD34; (iv) CD45RA and CD10; (v) CD34, CD45RA, and CD10; (vi) CD45RA and CD135; (vii) CD34, CD38, CD45RA, and CD135; (viii) CD135; (ix) CD34, CD38, and CD135; and (x) CD34 and CD38. Because a subject nanoparticle can include more than one targeting ligand, and because some cells include overlapping markers, multiple different cell types can be targeted using combinations of surface coats, e.g., in some cases a surface coat may target one specific

cell type while in other cases a surface coat may target more than one specific cell type (e.g., 2 or more, 3 or more, 4 or more cell types). For example, any combination of cells within the hematopoietic lineage can be targeted. As an illustrative example, targeting CD34 (using a targeting ligand that provides for targeted binding to CD34) can lead to nanoparticle delivery of a payload to several different cells within the hematopoietic lineage (see, e.g., **Figure 8**, panels A and B).

Delivery molecules

Provided are delivery molecules that include a targeting ligand (a peptide) conjugated to (i) a protein or nucleic acid payload, or (ii) a charged polymer polypeptide domain. The targeting ligand provides for (i) targeted binding to a cell surface protein, and in some cases (ii) engagement of a long endosomal recycling pathway. In some cases when the targeting ligand is conjugated to a charged polymer polypeptide domain, the charged polymer polypeptide domain interacts with (e.g., is condensed with) a nucleic acid payload and/or a protein payload. In some cases the targeting ligand is conjugated via an intervening linker. Refer to **Figure 6** for examples of different possible conjugation strategies (i.e., different possible arrangements of the components of a subject delivery molecule). In some cases, the targeting ligand provides for targeted binding to a cell surface protein, but does not necessarily provide for engagement of a long endosomal recycling pathway. Thus, also provided are delivery molecules that include a targeting ligand (e.g., peptide targeting ligand) conjugated to a protein or nucleic acid payload, or conjugated to a charged polymer polypeptide domain, where the targeting ligand provides for targeted binding to a cell surface protein (but does not necessarily provide for engagement of a long endosomal recycling pathway).

In some cases, the delivery molecules disclosed herein are designed such that a nucleic acid or protein payload reaches its extracellular target (e.g., by providing targeted binding to a cell surface protein) and is preferentially not destroyed within lysosomes or sequestered into 'short' endosomal recycling endosomes. Instead, delivery molecules of the disclosure can provide for engagement of the 'long' (indirect/slow) endosomal recycling pathway, which can allow for endosomal escape and/or endosomal fusion with an organelle.

For example, in some cases, β -arrestin is engaged to mediate cleavage of seven-transmembrane GPCRs (McGovern et al., *Handb Exp Pharmacol.* 2014;219:341-59; Goodman et al., *Nature.* 1996 Oct 3;383(6599):447-50; Zhang et al., *J Biol Chem.* 1997 Oct 24;272(43):27005-14) and/or single-transmembrane receptor tyrosine kinases (RTKs) from the actin cytoskeleton (e.g., during endocytosis), triggering the desired endosomal sorting pathway. Thus, in some embodiments the targeting ligand of a delivery molecule of the disclosure

provides for engagement of β -arrestin upon binding to the cell surface protein (e.g., to provide for signaling bias and to promote internalization via endocytosis following orthosteric binding).

Charged polymer polypeptide domain

5 In some case a targeting ligand (e.g., of a subject delivery molecule) is conjugated to a charged polymer polypeptide domain (an anchoring domain such as a cationic anchoring domain or an anionic anchoring domain) (see e.g., **Figure 5** and **Figure 6**). Charged polymer polypeptide domains can include repeating residues (e.g., cationic residues such as arginine, lysine, histidine). In some cases, a charged polymer polypeptide domain (an anchoring domain)
 10 has a length in a range of from 3 to 30 amino acids (e.g., from 3-28, 3-25, 3-24, 3-20, 4-30, 4-28, 4-25, 4-24, or 4-20 amino acids; or e.g., from 4-15, 4-12, 5-30, 5-28, 5-25, 5-20, 5-15, 5-12 amino acids). In some cases, a charged polymer polypeptide domain (an anchoring domain) has a length in a range of from 4 to 24 amino acids. In some cases, a charged polymer polypeptide domain (an anchoring domain) has a length in a range of from 5 to 10 amino acids.
 15 Suitable examples of a charged polymer polypeptide domain include, but are not limited to: RRRRRRRRRR (9R)(SEQ ID NO: 15) and HHHHHH (6H)(SEQ ID NO: 16).

A charged polymer polypeptide domain (a cationic anchoring domain, an anionic anchoring domain) can be any convenient charged domain (e.g., cationic charged domain). For example, such a domain can be a histone tail peptide (HTP) (described elsewhere herein in
 20 more detail). In some cases a charged polymer polypeptide domain includes a histone and/or histone tail peptide (e.g., a cationic polypeptide can be a histone and/or histone tail peptide). In some cases a charged polymer polypeptide domain includes an NLS- containing peptide (e.g., a cationic polypeptide can be an NLS- containing peptide). In some cases a charged polymer polypeptide domain includes a peptide that includes a mitochondrial localization signal (e.g., a
 25 cationic polypeptide can be a peptide that includes a mitochondrial localization signal).

In some cases, a charged polymer polypeptide domain of a subject delivery molecule is used as a way for the delivery molecular to interact with (e.g., interact electrostatically, e.g., for condensation) the payload (e.g., nucleic acid payload and/or protein payload).

In some cases, a charged polymer polypeptide domain of a subject delivery molecule is
 30 used as an anchor to coat the surface of a nanoparticle with the delivery molecule, e.g., so that the targeting ligand is used to target the nanoparticle to a desired cell/cell surface protein (see e.g., **Figure 5**). Thus, in some cases, the charged polymer polypeptide domain interacts electrostatically with a charged stabilization layer of a nanoparticle. For example, in some cases a nanoparticle includes a core (e.g., including a nucleic acid, protein, and/or
 35 ribonucleoprotein complex payload) that is surrounded by a stabilization layer (e.g., a silica, peptoid, polycysteine, or calcium phosphate coating). In some cases, the stabilization layer has

a negative charge and a positively charged polymer polypeptide domain can therefore interact with the stabilization layer, effectively anchoring the delivery molecule to the nanoparticle and coating the nanoparticle surface with a subject targeting ligand (see, e.g., **Figure 5**). In some cases, the stabilization layer has a positive charge and a negatively charged polymer

polypeptide domain can therefore interact with the stabilization layer, effectively anchoring the delivery molecule to the nanoparticle and coating the nanoparticle surface with a subject targeting ligand. Conjugation can be accomplished by any convenient technique and many different conjugation chemistries will be known to one of ordinary skill in the art. In some cases the conjugation is via sulfhydryl chemistry (e.g., a disulfide bond). In some cases the conjugation is accomplished using amine-reactive chemistry. In some cases, the targeting ligand and the charged polymer polypeptide domain are conjugated by virtue of being part of the same polypeptide.

In some cases a charged polymer polypeptide domain (cationic) can include a polymer selected from: poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), poly(citrulline), and a combination thereof. In some cases a given cationic amino acid polymer can include a mix of arginine, lysine, histidine, ornithine, and citrulline residues (in any convenient combination). Polymers can be present as a polymer of L-isomers or D-isomers, where D-isomers are more stable in a target cell because they take longer to degrade. Thus, inclusion of D-isomer poly(amino acids) delays degradation (and subsequent payload release). The payload release rate can therefore be controlled and is proportional to the ratio of polymers of D-isomers to polymers of L-isomers, where a higher ratio of D-isomer to L-isomer increases duration of payload release (i.e., decreases release rate). In other words, the relative amounts of D- and L- isomers can modulate the release kinetics and enzymatic susceptibility to degradation and payload release.

In some cases a cationic polymer includes D-isomers and L-isomers of an cationic amino acid polymer (e.g., poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), poly(citrulline)). In some cases the D- to L- isomer ratio is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1).

Thus, in some cases a cationic polymer includes a first cationic polymer (e.g., amino acid polymer) that is a polymer of D-isomers (e.g., selected from poly(D-arginine), poly(D-lysine), poly(D-histidine), poly(D-ornithine), and poly(D-citrulline)); and includes a second cationic polymer (e.g., amino acid polymer) that is a polymer of L-isomers (e.g., selected from

poly(L-arginine), poly(L-lysine), poly(L-histidine), poly(L-ornithine), and poly(L-citrulline)). In some cases the ratio of the first cationic polymer (D-isomers) to the second cationic polymer (L-isomers) is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1)

In some embodiments, a cationic polymer includes (e.g., in addition to or in place of any of the foregoing examples of cationic polymers) poly(ethylenimine), poly(amidoamine) (PAMAM), poly(aspartamide), polypeptoids (e.g., for forming "spiderweb"-like branches for core condensation), a charge-functionalized polyester, a cationic polysaccharide, an acetylated amino sugar, chitosan, or a cationic polymer that includes any combination thereof (e.g., in linear or branched forms).

In some embodiments, an cationic polymer can have a molecular weight in a range of from 1-200 kDa (e.g., from 1-150, 1-100, 1-50, 5-200, 5-150, 5-100, 5-50, 10-200, 10-150, 10-100, 10-50, 15-200, 15-150, 15-100, or 15-50 kDa). As an example, in some cases a cationic polymer includes poly(L-arginine), e.g., with a molecular weight of approximately 29 kDa. As another example, in some cases a cationic polymer includes linear poly(ethylenimine) with a molecular weight of approximately 25 kDa (PEI). As another example, in some cases a cationic polymer includes branched poly(ethylenimine) with a molecular weight of approximately 10 kDa. As another example, in some cases a cationic polymer includes branched poly(ethylenimine) with a molecular weight of approximately 70 kDa. In some cases a cationic polymer includes PAMAM.

In some cases, a cationic amino acid polymer includes a cysteine residue, which can facilitate conjugation, e.g., to a linker, an NLS, and/or a cationic polypeptide (e.g., a histone or HTP). For example, a cysteine residue can be used for crosslinking (conjugation) via sulfhydryl chemistry (e.g., a disulfide bond) and/or amine-reactive chemistry. Thus, in some embodiments a cationic amino acid polymer (e.g., poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), and poly(citrulline), poly(D-arginine)(PDR), poly(D-lysine)(PDK), poly(D-histidine)(PDH), poly(D-ornithine), and poly(D-citrulline), poly(L-arginine)(PLR), poly(L-lysine)(PLK), poly(L-histidine)(PLH), poly(L-ornithine), and poly(L-citrulline)) of a cationic polymer composition includes a cysteine residue. In some cases the cationic amino acid polymer includes cysteine residue on the N- and/or C- terminus. In some cases the cationic amino acid polymer includes an internal cysteine residue.

In some cases, a cationic amino acid polymer includes (and/or is conjugated to) a nuclear localization signal (NLS) (described in more detail below). Thus, in some embodiments

a cationic amino acid polymer (e.g., poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), and poly(citrulline), poly(D-arginine)(PDR), poly(D-lysine)(PDK), poly(D-histidine)(PDH), poly(D-ornithine), and poly(D-citrulline), poly(L-arginine)(PLR), poly(L-lysine)(PLK), poly(L-histidine)(PLH), poly(L-ornithine), and poly(L-citrulline)) includes one or
 5 more (e.g., two or more, three or more, or four or more) NLSs. In some cases the cationic amino acid polymer includes an NLS on the N- and/or C- terminus. In some cases the cationic amino acid polymer includes an internal NLS.

In some cases, the charged polymer polypeptide domain is condensed with a nucleic acid payload and/or a protein payload (see e.g., **Figure 6**). In some cases, the charged
 10 polymer polypeptide domain interacts electrostatically with a protein payload. In some cases, the charged polymer polypeptide domain is co-condensed with silica, salts, and/or anionic polymers to provide added endosomal buffering capacity, stability, and, e.g., optional timed release. In some cases, a charged polymer polypeptide domain of a subject delivery molecule is a stretch of repeating cationic residues (such as arginine, lysine, and/or histidine), e.g., in
 15 some 4-25 amino acids in length or 4-15 amino acids in length. Such a domain can allow the delivery molecule to interact electrostatically with an anionic sheddable matrix (e.g., a co-condensed anionic polymer). Thus, in some cases, a subject charged polymer polypeptide domain of a subject delivery molecule is a stretch of repeating cationic residues that interacts (e.g., electrostatically) with an anionic sheddable matrix and with a nucleic acid and/or protein
 20 payload. Thus, in some cases a subject delivery molecule interacts with a payload (e.g., nucleic acid and/or protein) and is present as part of a composition with an anionic polymer (e.g., co-condenses with the payload and with an anionic polymer).

The anionic polymer of an anionic sheddable matrix (i.e., the anionic polymer that interacts with the charged polymer polypeptide domain of a subject delivery molecule) can be
 25 any convenient anionic polymer/polymer composition. Examples include, but are not limited to: poly(glutamic acid) (e.g., poly(D-glutamic acid) (PDE), poly(L-glutamic acid) (PLE), both PDE and PLE in various desired ratios, etc.) In some cases, PDE is used as an anionic sheddable matrix. In some cases, PLE is used as an anionic sheddable matrix (anionic polymer). In some cases, PDE is used as an anionic sheddable matrix (anionic polymer). In some cases, PLE and
 30 PDE are both used as an anionic sheddable matrix (anionic polymer), e.g., in a 1:1 ratio (50% PDE, 50% PLE).

Anionic polymer

An anionic polymer can include one or more anionic amino acid polymers. For example,
 35 in some cases a subject anionic polymer composition includes a polymer selected from: poly(glutamic acid)(PEA), poly(aspartic acid)(PDA), and a combination thereof. In some cases

a given anionic amino acid polymer can include a mix of aspartic and glutamic acid residues. Each polymer can be present in the composition as a polymer of L-isomers or D-isomers, where D-isomers are more stable in a target cell because they take longer to degrade. Thus, inclusion of D-isomer poly(amino acids) can delay degradation and subsequent payload

5 release. The payload release rate can therefore be controlled and is proportional to the ratio of polymers of D-isomers to polymers of L-isomers, where a higher ratio of D-isomer to L-isomer increases duration of payload release (i.e., decreases release rate). In other words, the relative amounts of D- and L- isomers can modulate the nanoparticle core's timed release kinetics and enzymatic susceptibility to degradation and payload release.

10 In some cases an anionic polymer composition includes polymers of D-isomers and polymers of L-isomers of an anionic amino acid polymer (e.g., poly(glutamic acid)(PEA) and poly(aspartic acid)(PDA)). In some cases the D- to L- isomer ratio is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1).

Thus, in some cases an anionic polymer composition includes a first anionic polymer (e.g., amino acid polymer) that is a polymer of D-isomers (e.g., selected from poly(D-glutamic acid) (PDEA) and poly(D-aspartic acid) (PDDA)); and includes a second anionic polymer (e.g., amino acid polymer) that is a polymer of L-isomers (e.g., selected from poly(L-glutamic acid) (PLEA) and poly(L-aspartic acid) (PLDA)). In some cases the ratio of the first anionic polymer (D-isomers) to the second anionic polymer (L-isomers) is in a range of from 10:1-1:10 (e.g., from 8:1-1:10, 6:1-1:10, 4:1-1:10, 3:1-1:10, 2:1-1:10, 1:1-1:10, 10:1-1:8, 8:1-1:8, 6:1-1:8, 4:1-1:8, 3:1-1:8, 2:1-1:8, 1:1-1:8, 10:1-1:6, 8:1-1:6, 6:1-1:6, 4:1-1:6, 3:1-1:6, 2:1-1:6, 1:1-1:6, 10:1-1:4, 8:1-1:4, 6:1-1:4, 4:1-1:4, 3:1-1:4, 2:1-1:4, 1:1-1:4, 10:1-1:3, 8:1-1:3, 6:1-1:3, 4:1-1:3, 3:1-1:3, 2:1-1:3, 1:1-1:3, 10:1-1:2, 8:1-1:2, 6:1-1:2, 4:1-1:2, 3:1-1:2, 2:1-1:2, 1:1-1:2, 10:1-1:1, 8:1-1:1, 6:1-1:1, 4:1-1:1, 3:1-1:1, or 2:1-1:1)

15 In some embodiments, an anionic polymer composition includes (e.g., in addition to or in place of any of the foregoing examples of anionic polymers) a glycosaminoglycan, a glycoprotein, a polysaccharide, poly(mannuronic acid), poly(guluronic acid), heparin, heparin sulfate, chondroitin, chondroitin sulfate, keratan, keratan sulfate, aggrecan, poly(glucosamine), or an anionic polymer that comprises any combination thereof.

20 In some embodiments, an anionic polymer can have a molecular weight in a range of from 1-200 kDa (e.g., from 1-150, 1-100, 1-50, 5-200, 5-150, 5-100, 5-50, 10-200, 10-150, 10-100, 10-50, 15-200, 15-150, 15-100, or 15-50 kDa). As an example, in some cases an anionic

polymer includes poly(glutamic acid) with a molecular weight of approximately 15 kDa.

In some cases, an anionic amino acid polymer includes a cysteine residue, which can facilitate conjugation, e.g., to a linker, an NLS, and/or a cationic polypeptide (e.g., a histone or HTP). For example, a cysteine residue can be used for crosslinking (conjugation) via sulfhydryl chemistry (e.g., a disulfide bond) and/or amine-reactive chemistry. Thus, in some embodiments an anionic amino acid polymer (e.g., poly(glutamic acid) (PEA), poly(aspartic acid) (PDA), poly(D-glutamic acid) (PDEA), poly(D-aspartic acid) (PDDA), poly(L-glutamic acid) (PLEA), poly(L-aspartic acid) (PLDA)) of an anionic polymer composition includes a cysteine residue. In some cases the anionic amino acid polymer includes cysteine residue on the N- and/or C-terminus. In some cases the anionic amino acid polymer includes an internal cysteine residue.

In some cases, an anionic amino acid polymer includes (and/or is conjugated to) a nuclear localization signal (NLS) (described in more detail below). Thus, in some embodiments an anionic amino acid polymer (e.g., poly(glutamic acid) (PEA), poly(aspartic acid) (PDA), poly(D-glutamic acid) (PDEA), poly(D-aspartic acid) (PDDA), poly(L-glutamic acid) (PLEA), poly(L-aspartic acid) (PLDA)) of an anionic polymer composition includes (and/or is conjugated to) one or more (e.g., two or more, three or more, or four or more) NLSs. In some cases the anionic amino acid polymer includes an NLS on the N- and/or C-terminus. In some cases the anionic amino acid polymer includes an internal NLS.

In some cases, an anionic polymer is conjugated to a targeting ligand.

Linker

In some embodiments a targeting ligand is conjugated to an anchoring domain (e.g., a cationic anchoring domain, an anionic anchoring domain) or to a payload via an intervening linker. The linker can be a protein linker or non-protein linker. A linker can in some cases aid in stability, prevent complement activation, and/or provide flexibility to the ligand relative to the anchoring domain.

Conjugation of a targeting ligand to a linker or a linker to an anchoring domain can be accomplished in a number of different ways. In some cases the conjugation is via sulfhydryl chemistry (e.g., a disulfide bond, e.g., between two cysteine residues). In some cases the conjugation is accomplished using amine-reactive chemistry. In some cases, a targeting ligand includes a cysteine residue and is conjugated to the linker via the cysteine residue; and/or an anchoring domain includes a cysteine residue and is conjugated to the linker via the cysteine residue. In some cases, the linker is a peptide linker and includes a cysteine residue. In some cases, the targeting ligand and a peptide linker are conjugated by virtue of being part of the same polypeptide; and/or the anchoring domain and a peptide linker are conjugated by virtue of being part of the same polypeptide.

In some cases, a subject linker is a polypeptide and can be referred to as a polypeptide linker. It is to be understood that while polypeptide linkers are contemplated, non-polypeptide linkers (chemical linkers) are used in some cases. For example, in some embodiments the linker is a polyethylene glycol (PEG) linker. Suitable protein linkers include polypeptides of
 5 between 4 amino acids and 60 amino acids in length (e.g., 4-50, 4-40, 4-30, 4-25, 4-20, 4-15, 4-10, 6-60, 6-50, 6-40, 6-30, 6-25, 6-20, 6-15, 6-10, 8-60, 8-50, 8-40, 8-30, 8-25, 8-20, or 8-15 amino acids in length).

In some embodiments, a subject linker is rigid (e.g., a linker that include one or more proline residues). One non-limiting example of a rigid linker is GAPGAPGAP (SEQ ID NO: 17).

10 In some cases, a polypeptide linker includes a C residue at the N- or C-terminal end. Thus, in some case a rigid linker is selected from: GAPGAPGAPC (SEQ ID NO: 18) and CGAPGAPGAP (SEQ ID NO: 19).

Peptide linkers with a degree of flexibility can be used. Thus, in some cases, a subject linker is flexible. The linking peptides may have virtually any amino acid sequence, bearing in
 15 mind that flexible linkers will have a sequence that results in a generally flexible peptide. The use of small amino acids, such as glycine and alanine, are of use in creating a flexible peptide. The creation of such sequences is routine to those of skill in the art. A variety of different linkers are commercially available and are considered suitable for use. Example linker polypeptides include glycine polymers (G)_n, glycine-serine polymers (including, for example, (GS)_n, GSGGS_n
 20 (SEQ ID NO: 20), GSGGS_n (SEQ ID NO: 21), and GGGS_n (SEQ ID NO: 22), where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers. Example linkers can comprise amino acid sequences including, but not limited to, GGSG (SEQ ID NO: 23), GGSGG (SEQ ID NO: 24), GSGSG (SEQ ID NO: 25), GSGGG (SEQ ID NO: 26), GGGSG (SEQ ID NO: 27), GSSSG (SEQ ID NO: 28), and the like. The ordinarily skilled artisan will recognize that
 25 design of a peptide conjugated to any elements described above can include linkers that are all or partially flexible, such that the linker can include a flexible linker as well as one or more portions that confer less flexible structure. Additional examples of flexible linkers include, but are not limited to: GGGGSGGGGG (SEQ ID NO: 29) and GGGGSGGGGS (SEQ ID NO: 30). As noted above, in some cases, a polypeptide linker includes a C residue at the N- or C-terminal end. Thus, in some cases a flexible linker includes an amino acid sequence selected
 30 from: GGGGSGGGGGC (SEQ ID NO: 31), CGGGGSGGGGG (SEQ ID NO: 32), GGGGSGGGGSC (SEQ ID NO: 33), and CGGGGSGGGGS (SEQ ID NO: 34).

In some cases, a subject polypeptide linker is endosomolytic. Endosomolytic polypeptide linkers include but are not limited to: KALA (SEQ ID NO: 35) and GALA (SEQ ID
 35 NO: 36). As noted above, in some cases, a polypeptide linker includes a C residue at the N- or C-terminal end. Thus, in some cases a subject linker includes an amino acid sequence

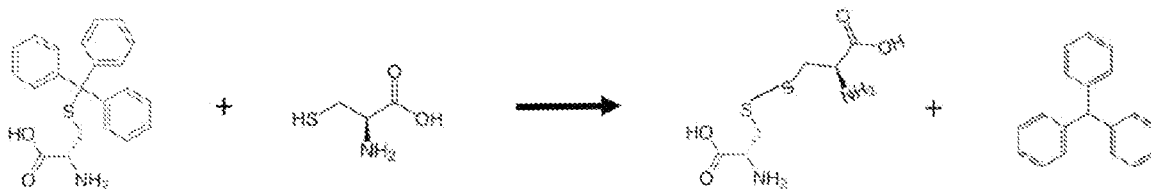
selected from: CKALA (SEQ ID NO: 37), KALAC (SEQ ID NO: 38), CGALA (SEQ ID NO: 39), and GALAC (SEQ ID NO: 40).

Illustrative examples of sulfhydryl coupling reactions

- 5 (e.g., for conjugation via sulfhydryl chemistry, e.g., using a cysteine residue)
 (e.g., for conjugating a targeting ligand or glycopeptide to a linker, conjugating a targeting ligand or glycopeptide to an anchoring domain (e.g., cationic anchoring domain), conjugating a linker to an anchoring domain (e.g., cationic anchoring domain), and the like)

10 *Disulfide bond*

Cysteine residues in the reduced state, containing free sulfhydryl groups, readily form disulfide bonds with protected thiols in a typical disulfide exchange reaction.



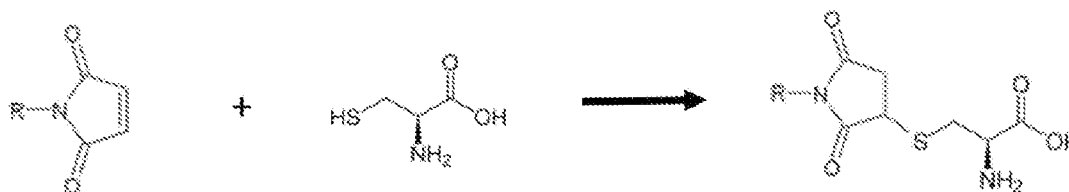
15

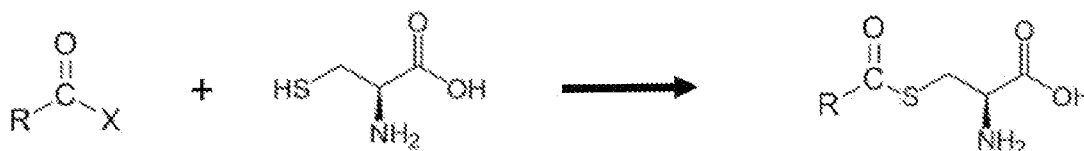
Thioether/Thioester bond

Sulfhydryl groups of cysteine react with maleimide and acyl halide groups, forming stable thioether and thioester bonds respectively.

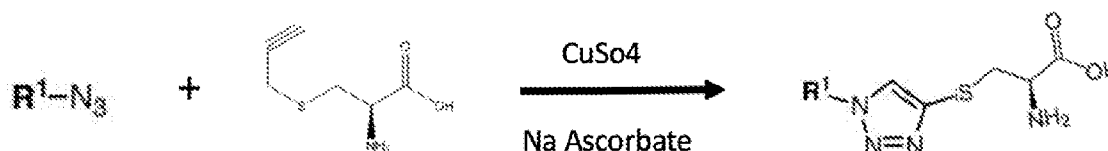
20

Maleimide



Acyl Halide*Azide - alkyne Cycloaddition*

- 5 This conjugation is facilitated by chemical modification of the cysteine residue to contain an alkyne bond, or by the use of an L-propargyl amino acid derivative (e.g., L-propargyl cysteine - pictured below) in synthetic peptide preparation (e.g., solid phase synthesis). Coupling is then achieved by means of Cu promoted click chemistry.

10 *Examples of targeting ligands*

Examples of targeting ligands include, but are not limited to, those that include the following amino acid sequences:

SCF (targets/binds to c-Kit receptor)

- 15 EGICRNRVTNNVKDVTCLVANLPKDYMITLKYVPGMDVLPSHCWISEMVVQLSDSLTDLLDKFS
NISEGLSNYSIIDKLVNIVDDLVECVKENSSKDLKKSFKSPEPRLFTPEEFFRIFNRSIDAFKDFW
ASETSDCWSSTLSPEKDSRVSVTKPFMLPPVA (SEQ ID NO: 184);

CD70 (targets/binds to CD27)

- 20 PEEGSGCSVRRRPYGCVLRAALVPLVAGLVICLVVCIQRFAQAQQQLPLESLGWDVAELQLNH
TGPQQDPRLYWQGGPALGRSFLHGPELDKGQLRIHRDGYMMHIQVTLAICSSTTASRHHPTT
LAVGICSPASRSISLLRLSFHQGCTIASQRLTPLARGDTLCTNLTGTLLPSRNTDETFFGVQWV
RP (SEQ ID NO: 185); and

- 25 SH2 domain-containing protein 1A (SH2D1A) (targets/binds to CD150)

SSGLVPRGSHMDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVLYHGYIYT

YRVSQTETGWSAETAPGVHKRYFRKIKNLISAFQKPDQGVIPLQYPVEKKSSARSTQGTGTGI
REDPDVCLKAP (SEQ ID NO: 186)

Thus, non-limiting examples of targeting ligands (which can be used alone or in combination
5 with other targeting ligands) include:

9R-SCF

RRRRRRRRRRMEGICRNRVTNNVKDVTCLVANLPKDYMITLKYPGMDVLPSCWCISEMVVQL
SDSLTDLLDKFSNISEGLSNYSIIDKLVNIVDDLVECVKENSSKDLKKSFKSPEPRLFTPEEFFRIF
10 NRSIDAFKDFVASETSDCVSSTLSPEKDSRVSVTKPFMLPPVA (SEQ ID NO: 189)

9R-CD70

RRRRRRRRRRPEEGSGCSVRRRPYGCVLRAALVPLVAGLVICLVVCIQRFQAQQAQQLPLESLG
WDVAELQLNHTGPQQDPRLYWQGGPALGRSFLHGPELDKGQLRIHRDGIYMMHIQVTLAICSS
15 TTASRHHPTTLAVGICSPASRSISLLRLSFHQGCTIASQRLTPLARGDTLCTNLTGTLLPSRNTD
ETFFGVQWVRP (SEQ ID NO: 190)

CD70-9R

PEEGSGCSVRRRPYGCVLRAALVPLVAGLVICLVVCIQRFQAQQAQQLPLESLGWDVAELQLNH
20 TGPQQDPRLYWQGGPALGRSFLHGPELDKGQLRIHRDGIYMMHIQVTLAICSSTTASRHHPTT
LAVGICSPASRSISLLRLSFHQGCTIASQRLTPLARGDTLCTNLTGTLLPSRNTDETFFGVQWV
R**PPRRRRRRRRRR** (SEQ ID NO: 191)

6H-SH2D1A

25 **MGSSHHHHHH**SSGLVPRGSHMDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYC
LCVLYHGYITYRVSTETGWSAETAPGVHKRYFRKIKNLISAFQKPDQGVIPLQYPVEKKSS
ARSTQGTGTGIREDPDVCLKAP (SEQ ID NO: 192)

6H-SH2D1A

30 **RRRRRRRRRR**SSGLVPRGSHMDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCL
CVLYHGYITYRVSTETGWSAETAPGVHKRYFRKIKNLISAFQKPDQGVIPLQYPVEKKSSA
RSTQGTGTGIREDPDVCLKAP (SEQ ID NO: 193)

Illustrative examples of delivery molecules and components

35 (0a) Cysteine conjugation anchor 1 (CCA1)

[anchoring domain (e.g., cationic anchoring domain) - linker (GAPGAPGAP) - cysteine]

RRRRRRRRR GAPGAPGAP C (SEQ ID NO: 41)

(0b) Cysteine conjugation anchor 2 (CCA2)

[cysteine - linker (GAPGAPGAP) - anchoring domain (e.g., cationic anchoring domain)]

5 C GAPGAPGAP RRRRRRRRR (SEQ ID NO: 42)

(1a) $\alpha 5\beta 1$ ligand

[anchoring domain (e.g., cationic anchoring domain) - linker (GAPGAPGAP) - Targeting ligand]

RRRRRRRRR GAPGAPGAP RRETAWA (SEQ ID NO: 45)

10

(1b) $\alpha 5\beta 1$ ligand

[Targeting ligand - linker (GAPGAPGAP) - anchoring domain (e.g., cationic anchoring domain)]

RRETAWA GAPGAPGAP RRRRRRRRR (SEQ ID NO: 46)

15 (1c) $\alpha 5\beta 1$ ligand - Cys left

CGAPGAPGAP (SEQ ID NO: 19)

Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

20 (1d) $\alpha 5\beta 1$ ligand - Cys right

GAPGAPGAPC (SEQ ID NO: 18)

Note: This can be conjugated to CCA2 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

25 (2a) RGD $\alpha 5\beta 1$ ligand

[anchoring domain (e.g., cationic anchoring domain) - linker (GAPGAPGAP) - Targeting ligand]

RRRRRRRRR GAPGAPGAP RGD (SEQ ID NO: 47)

(2b) RGD $\alpha 5\beta 1$ ligand

30 [Targeting ligand - linker (GAPGAPGAP) - anchoring domain (e.g., cationic anchoring domain)]

RGD GAPGAPGAP RRRRRRRRR (SEQ ID NO: 48)

(2c) RGD ligand - Cys left

CRGD (SEQ ID NO: 49)

35 Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

(2d) RGD ligand - Cys right

RGDC (SEQ ID NO: 50)

Note: This can be conjugated to CCA2 (see above) either via sulfhydryl chemistry (e.g., a
5 disulfide bond) or amine-reactive chemistry.

(3a) Transferrin ligand

[anchoring domain (e.g., cationic anchoring domain) - linker (GAPGAPGAP) - Targeting ligand]

RRRRRRRRR GAPGAPGAP THRPPMWSPVWP (SEQ ID NO: 51)

10

(3b) Transferrin ligand

[Targeting ligand - linker (GAPGAPGAP) - anchoring domain (e.g., cationic anchoring domain)]

THRPPMWSPVWP GAPGAPGAP RRRRRRRRR (SEQ ID NO: 52)

15 *(3c) Transferrin ligand - Cys left*

CTHRPPMWSPVWP (SEQ ID NO: 53)

CPTHRPPMWSPVWP (SEQ ID NO: 54)

Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a
disulfide bond) or amine-reactive chemistry.

20

(3d) Transferrin ligand - Cys right

THRPPMWSPWPC (SEQ ID NO: 55)

Note: This can be conjugated to CCA2 (see above) either via sulfhydryl chemistry (e.g., a
disulfide bond) or amine-reactive chemistry.

25

(4a) E-selectin ligand [1-21]

[anchoring domain (e.g., cationic anchoring domain) - linker (GAPGAPGAP) - Targeting ligand]

RRRRRRRRR GAPGAPGAP MIASQFLSALTLLIKESGA (SEQ ID NO: 56)

30 *(4b) E-selectin ligand [1-21]*

[Targeting ligand - linker (GAPGAPGAP) - anchoring domain (e.g., cationic anchoring domain)]

MIASQFLSALTLLIKESGA GAPGAPGAP RRRRRRRRR (SEQ ID NO: 57)

(4c) E-selectin ligand [1-21] - Cys left

35 CMIASQFLSALTLLIKESGA (SEQ ID NO: 58)

Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a

disulfide bond) or amine-reactive chemistry.

(4d) E-selectin ligand [1-21] - Cys right

MIASQFLSALTLVLLIKESGAC (SEQ ID NO: 59)

- 5 Note: This can be conjugated to CCA2 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

(5a) FGF fragment [26-47]

[anchoring domain (e.g., cationic anchoring domain) - linker (GAPGAPGAP) - Targeting ligand]

- 10 RRRRRRRRRR GAPGAPGAP KNNGFFLRIHPDGRVDGVREKS (SEQ ID NO: 60)

Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

(5b) FGF fragment [26-47]

- 15 [Targeting ligand - linker (GAPGAPGAP) - anchoring domain (e.g., cationic anchoring domain)]

KNNGFFLRIHPDGRVDGVREKS GAPGAPGAP RRRRRRRRRR (SEQ ID NO: 61)

Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

- 20 *(5c) FGF fragment [25-47] - Cys on left is native*

CKNNGFFLRIHPDGRVDGVREKS (SEQ ID NO: 43)

Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

- 25 *(5d) FGF fragment [26-47] - Cys right*

KNNGFFLRIHPDGRVDGVREKSC (SEQ ID NO: 44)

Note: This can be conjugated to CCA2 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

- 30 *(6a) Exendin (S11C) [1-39]*

HGEGTFTSDLCKQMEEEAVRLFIEWLKNNGPSSGAPPPS (SEQ ID NO: 2)

Note: This can be conjugated to CCA1 (see above) either via sulfhydryl chemistry (e.g., a disulfide bond) or amine-reactive chemistry.

Targeting ligand

A variety of targeting ligands (e.g., as part of a subject delivery molecule, e.g., as part of a nanoparticle) can be used and numerous different targeting ligands are envisioned. In some embodiments the targeting ligand is a fragment (e.g., a binding domain) of a wild type protein.

5 For example, in some cases a peptide targeting ligand of a subject delivery molecule can have a length of from 4-50 amino acids (e.g., from 4-40, 4-35, 4-30, 4-25, 4-20, 4-15, 5-50, 5-40, 5-35, 5-30, 5-25, 5-20, 5-15, 7-50, 7-40, 7-35, 7-30, 7-25, 7-20, 7-15, 8-50, 8-40, 8-35, 8-30, 8-25, 8-20, or 8-15 amino acids). The targeting ligand can be a fragment of a wild type protein, but in some cases has a mutation (e.g., insertion, deletion, substitution) relative to the wild type amino
10 acid sequence (i.e., a mutation relative to a corresponding wild type protein sequence). For example, a targeting ligand can include a mutation that increases or decreases binding affinity with a target cell surface protein.

In some cases the targeting ligand is an antigen-binding region of an antibody (F(ab)). In some cases the targeting ligand is an ScFv. "Fv" is the minimum antibody fragment which
15 contains a complete antigen-recognition and binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species (scFv), one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. For a review
20 of scFv see Pluckthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

In some cases a targeting ligand includes a viral glycoprotein, which in some cases binds to ubiquitous surface markers such as heparin sulfate proteoglycans, and may induce micropinocytosis (and/or macropinocytosis) in some cell populations through membrane ruffling
25 associated processes. Poly(L-arginine) is another example targeting ligand that can also be used for binding to surface markers such as heparin sulfate proteoglycans.

In some cases a targeting ligand is coated upon a particle surface (e.g., nanoparticle surface) either electrostatically or utilizing covalent modifications to the particle surface or one or more polymers on the particle surface. In some cases, a targeting ligand can include a
30 mutation that adds a cysteine residue, which can facilitate conjugation to a linker and/or an anchoring domain (e.g., cationic anchoring domain). For example, cysteine can be used for crosslinking (conjugation) via sulfhydryl chemistry (e.g., a disulfide bond) and/or amine-reactive chemistry.

In some cases, a targeting ligand includes an internal cysteine residue. In some cases,
35 a targeting ligand includes a cysteine residue at the N- and/or C- terminus. In some cases, in order to include a cysteine residue, a targeting ligand is mutated (e.g., insertion or substitution),

e.g., relative to a corresponding wild type sequence. As such, any of the targeting ligands described herein can be modified by inserting and/or substituting in a cysteine residue (e.g., internal, N-terminal, C-terminal insertion of or substitution with a cysteine residue).

By “corresponding” wild type sequence is meant a wild type sequence from which the subject sequence was or could have been derived (e.g., a wild type protein sequence having high sequence identity to the sequence of interest). In some cases, a “corresponding” wild type sequence is one that has 85% or more sequence identity (e.g., 90% or more, 92% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) over the amino acid stretch of interest. For example, for a targeting ligand that has one or more mutations (e.g., substitution, insertion) but is otherwise highly similar to a wild type sequence, the amino acid sequence to which it is most similar may be considered to be a corresponding wild type amino acid sequence.

A corresponding wild type protein/sequence does not have to be 100% identical (e.g., can be 85% or more identical, 90% or more identical, 95% or more identical, 98% or more identical, 99% or more identical, etc.) (outside of the position(s) that is modified), but the targeting ligand and corresponding wild type protein (e.g., fragment of a wild protein) can bind to the intended cell surface protein, and retain enough sequence identity (outside of the region that is modified) that they can be considered homologous. The amino acid sequence of a “corresponding” wild type protein sequence can be identified/evaluated using any convenient method (e.g., using any convenient sequence comparison/alignment software such as BLAST, MUSCLE, T-COFFEE, etc.).

Examples of targeting ligands that can be used as part of a surface coat (e.g., as part of a delivery molecule of a surface coat) include, but are not limited to, those listed in Table 1. Examples of targeting ligands that can be used as part of a subject delivery molecule include, but are not limited to, those listed in Table 3 (many of the sequences listed in Table 3 include the targeting ligand (e.g., SNRWLDVK for row 2) conjugated to a cationic polypeptide domain, e.g., 9R, 6R, etc., via a linker (e.g., GGGSGGGGS). Examples of amino acid sequences that can be included in a targeting ligand include, but are not limited to: NPKLTRMLTFKFY (SEQ ID NO: xx) (IL2), TSVGKYPNTGYYGD (SEQ ID NO: xx) (CD3), SNRWLDVK (Siglec), EKFILKVRPAFKAV (SEQ ID NO: xx) (SCF); EKFILKVRPAFKAV (SEQ ID NO: xx) (SCF), EKFILKVRPAFKAV (SEQ ID NO: xx) (SCF), SNYSIIDKLVNIVDDLVECVKENS (SEQ ID NO: xx) (cKit), and Ac-SNYSaibADKAibANAibADDAibAEAibAKENS (SEQ ID NO: xx) (cKit). Thus in some cases a targeting ligand includes an amino acid sequence that has 85% or more (e.g., 90% or more, 95% or more, 98% or more, 99% or more, or 100%) sequence identity with NPKLTRMLTFKFY (SEQ ID NO: xx) (IL2), TSVGKYPNTGYYGD (SEQ ID NO: xx) (CD3), SNRWLDVK (Siglec), EKFILKVRPAFKAV (SEQ ID NO: xx) (SCF); EKFILKVRPAFKAV (SEQ

ID NO: xx) (SCF), EKFILKVRPAFKAV (SEQ ID NO: xx) (SCF), or
SNYSIIDKLVNIVDDLVECVKENS (SEQ ID NO: xx) (cKit).

Table 1. Examples of Targeting ligands

Cell Surface Protein	Targeting Ligand	Sequence	SEQ ID NO:
Family B GPCR	Exendin	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSG APPPS	1
	Exendin (S11C)	HGEGTFTSDLCKQMEEEAVRLFIEWLKNGGPSSG APPPS	2
FGF receptor	FGF fragment	KRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQL QAEERGVSIGKVCANRYLAMKEDGRLLASKCVT DECFFFERLESNNYNTY	3
	FGF fragment	KNGGFFLRIHPDGRVDGVREKS	4
	FGF fragment	HFKDPK	5
	FGF fragment	LESNNYNT	6
	E-selectin	MIASQFLSALTLLVLLIKESGA	7
	L-selectin	MVFPWRCEGTYWGSRNILKLWVWTLCCDFLIHH GTHC	8
		MIFPWKCQSTQRDLWNIFKLWGW/TMLCCDFLAH HGTDC	9
		MIFPWKCQSTQRDLWNIFKLWGW/TMLCC	10
P-selectin	PSGL-1 (SELPLG)	MAVGASGLEGDKMAGAMPLQLLLLLILLGPGNSL QLWDTWADEAEKALGPLLARDRRQATEYDYLDY DFLPETEPPEMLRNSTD TTPL TGP GTPESTTVEPA ARRSTGLDAGGAVTELTTELANMGNLSTDSAAME IQTTQPAATEAQTTPVP TEAQTTPLAATEAQTTR LTATEAQTTPLAATEAQTTPPAATEAQTTPGLE AQTTPAAMEAQTTPAAMEAQTTPAAMEAQTTP QTAMEAQTTPAEATEAQTTPATAEAQTTPLA MEALSTEPSATEALSMEPTTKRGLIPFSVSSVTH KGIPMAASNLNVNYPVGAPDHISVKQCLLAILLAL VATIFFVCTVVLAVRLSRKGHMYPVRYNYSPTMV CISSLLPDGGEPSATANGGLSKAKSPGLTPEPR EDREGDDLTLHSFLP	271
E-selectin	ESL-1 (GLG1)	MAACGRVRRMFRLSAAHLLLLFAAGAEKLPGQG VHSQQGGPGANFVSFVGQAGGGGPAGQQLPQL PQSSQLQQQQQQQQQQQP QPPQPPFPAGGPP ARRGGAGAGGGWKLAEESCREDDVTRVCPKHT WSNNLAVLECLQDVREPENEISSDCNHLLWNYKL NLTTDPKFESVAREVCKSTITEIKECADEPVGKGY MVSCLVDHRGNITEYQCHQYITKMTAIFSDYRLIC GFMDCKNDINILKCGSIRLGEKDAHSQGEVVSCL EKGLVKEAEEREPIQVSELCKKAILRVAELSSDD FHLDRLHYFACRDDRRFCENTQAGEGRVYKCLF NHKFEESMSEKCREALTTRQKLIAQDYKVSYSYSLAK SCKSDLKKYRCNVENLPRSREARLSYLLMCLESA VHRGRQVSSECQGEMLDYRRMLMEDFSLSP EIL SCRGEIEHHCSGLHRKGR TLHCLMKVV RGEKGNL GMNCQQALQTLIQE TDPGADYRIDRALNEACESVI QTACKHIRSGDPMILSCLMEHLYTEKMVEDCEHR LLELQYFISRDWKLDPVLYRKCCQGDASRLCHTHG WNETSEFMPQGA VFSCLYRHAYRTEEQGRRLSR ECRAEVQRILHQRAMDVKLDPALQDKCLIDLKWK CSEKTETGQELECLQDHLDDLVECDIVGNLTEL	272

Cell Surface Protein	Targeting Ligand	Sequence	SEQ ID NO:
		ESEDIQIEALLMRACEPIIQNFCHDVADNQIDSGDL MECLIQNKHQKDMNEKCAIGVTHFQLVQMKDFRF SYKFKMACKEDVLKLCPNIKKKVDVVICLSTTVRN DTLQEAKEHRVSLKCRRLRVEELEMTEDIRLEP DLYEACKSDIKNFCSAVQYGNAQIECLKENKKQL STRCHQKVFKLQETEMMDPELDYTLMRVCKQMIK RFCPEADSKTMLQCLKQNKNSLMDPKCKQMITK RQITQNTDYRLNPMRLKACKADIPKFCBGILTKAK DDSELEGQVISCLKLRYADQRLSSDCEDQIRIIQE SALDYRLDPQLQLHCSDEISSLCAEEAAAEQQTG QVEECLKVNLLKIKTELCKKEVLNMLKESKADIFVD PVLHTACALDIKHHCAAITPGRGRQMSCLMEALE DKRVRLQPECKRLNDRIEMWSYAAKVAPADGFS DLAMQVMTSPSKNYILSVISGSICILFLIGLMCGRIT KRVRELKDRQLQYRSETMAYKGLVWSQDVTGSP A	
	PSGL-1 (SELPLG)	See above	271
	CD44	MDKFWWHAAWGLCLVPLSLAQIDLNITCRFAGVF HVEKNGRYSISRTEADLCKAFNSTLPTMAQMEK ALSIGFETCRYGFIEGHVVIPRIHPNSICAAANTGV YILTSNTSQYDTYCFNASAPPEEDCTSVTDLPNAF DGPITITIVNRDGRYVQKGEYRTNPEDIYPSNPTD DDVSSGSSSERSSTSGGYIFYTFSVHPIPDEDSP WITDSTDRIPATLMS TSA TA TE TATKRQE TWDW FSWFLPSESKNHLHTTTQMA GTSSNTISAGWEP NEENEDERDRHLSFSGSGIDDDDFISS TISTTPR AFDHTKQNDWTQWNPSHSNPEVLLQTTTRMTD VDRNGTTAYEGNWNPEAHPPLIHHEHHEEEEE TPH STSTIQATPSSTTEE TA TQKEQWFGNRWHEGYR QTPKEDSHSTTG TAAASAHTSHPMQGRTPPSPE DSSWTDFFNPISHPMGRGHQA GRMDMDSSHSI TLQPTANPNTGLVEDLDRTGPLSM TTQQS NSQSF STSHEGLEEDKDHPPTS TL TSSNRNDVTGGRRDP NHSEGSTLLEGY TSHYPHTKESRTFIPVTS AKTG SFGVTAVTVGDSNSNVNRSLSGDQD TFHPSGGS HTHGSESDGHS HGSQE GGANTTSGPI RTPQIPE WLILASLLALALILAVCIAVNSRRRCGQKKKLVINS GNGAVEDRKPSGLNGEASKSQEMVHLVNKESSE TPDQFMTADETRNLQNVDMKIGV	273
	DR3 (TNFRSF25)	MEQRPRGCAAVAAALLVLLGARAQGGTRSPRC DCAGDFHKKIGLFCCRCGPAGHYLKAPCTEPCGN STCLVCPQDTFLAWENHHNSECARCQACDEQAS QVALENC SAVADTRCGCKP GWFVECQVSQCVSS SPFYCQPCLD CGALHRHTRLLCSRRD TDCGTCLP GFYE HGDGCVSCPTPPPSLAGAPWGAVQSAVPL SVAGGRVGVFWVQVLLAGLVVPLLLGATLTYTYR HCWPHKPLVTADEAGMEALTPPPATHLSPLDSA H TLLAPPDSSEKICTVQLVGN SWTPGYPE TQEALC PQVTWSWDQLPSRALGPAAAPTLSPE SPAGSPA MMLQPGPQLYDVMDAVPARRWKEFVRTLGLREA EIEAVEVEIGRFRDQQYEMLKRWRRQQPAGLGA VYAALERMGLDGCVEDLRSRLQGRP	274
	LAMP1	MAAPGSARRPLLLLLLLLLGLMHCSAAMFMVK NGNGTACIMANFSAAFSVNYDTKSGPKNM TFDLP SDATVVLNRSSCGKENTSDPSLVIAFGRGHTLTLN FTRNATRYSVQLMSFVYNLS DTHLFPNASSKEIKT	275

Cell Surface Protein	Targeting Ligand	Sequence	SEQ ID NO:
		VESITDIRADIDKKYRCVSGTQVHMNNVTVTLHDA TIQAYLSNSSFSRGETRCEQDRPSP TTAPPAPPS PSPSPVPKSPSVDKYNVSGTNGTCLLASMGLQLN LTYERKDNTTV TRLLNINPNKTSASGSCGAHLVTL ELHSEGTTVLLFQFGMNASSSRFFLQGIQLNTILP DARDPAFKAANGSLRALQATVGNSYKCAEEHV RVTKAFSVNIFKVVWQAFKVEGGQFGSVEECLLD ENSMLIPIAVGGALAGLVLVLIAYLVGRKRSHAGY QTI	
	LAMP2	MVCFRLFPVPGSGLVLVCLVLGAVRSYALELNLTD SENATCLYAKWQMNFVRYE TTNKTYKTVTISDH GTVTYNGSICGDDQNGPKIAVQFGPGFSWIANFT KAASTYSIDSVSFSYNTGDNTTFPDAEDK GIL TVD ELLAIRIPLNDLFRCNLSLTLEKNDVVQHYWDVLV QAFVQNGTVSTNEFLCDKDKTS TVAP TIHTTVPS TTTTPTPEKPEAGTYSVNNGND TCLA TMGLQLNI TQDKVASVININPNTTHS TGS CRSHTALLRLNSS TI KYLDFVFAVKNENRFYLKEVNISMVYLVNGSVFSIA NNNLSYWDAPLGSSYMCNKEQTVSVSGAFQINTF DLRVQPFNVTQGKYSTA QDCSADDDNFLVPIAVG AALAGVLILVLLAYFIGLKH HHAGYE QF	276
	Mac2-BP (galectin 3 binding protein) (LGALS3BP)	MTPPRLFWVWLLVAGTQGVNDGDMRLADGGAT NQGRVEIFYRGQWGTVCNLDLTDASVVCRAL GFENATQALGRAAFGQSGP IMLDEVQCTGTEAS LADCKSLGWLKSNCRHERDAGVVC TNE TRS THTL DLSRELSEALGQIFDSQRGCDLSISVNVQGEDALG FCGHTVILTANLEAQA LWKEPGSNV TMSVDAE CV PMVRDLLRYFYSSRIDITLSSVKCFHKLASAYGAR QLQGYCASLFAILLPQDPSFQMP LDLYAYAVATGD ALLEKLCLQFLAWNFEALTQAEAWPSVPTDLLQLL LPRSDLAVPSELALLKA VDTWSWGERASHEEVEG LVEKIRFPMMLELPELFELQFNLSLYWSHEALFQKK TLQALEFHTVPFQLLARYKGLNLTEDTYKPRIYTSP TWSAFVTDSSWSARKSQLVYQSRGPLVKYSSD YFQAPSDYRYYPYQSFQTPQHPSFLFQDKRVSW SLVYLPTIQSCWNYGFSCSSDELPVLGLTKSGGS DRTIAYENKALMLCEGLFVADVDFEGWKAAIPSA LDTNSSKSTSSFPCPAGHFNGFRTVIRPFYLTNSS GVD	277
Transferrin receptor	Transferrin ligand	THRPPMWSPVWP	11
$\alpha 5\beta 1$ integrin	$\alpha 5\beta 1$ ligand	RRETAWA	12
		RGD	
		RGDGW	181
integrin	Integrin binding peptide	(Ac)-GCGYGRGDSPG-(NH ₂)	188
		GCGYGRGDSPG	182
$\alpha 5\beta 3$ integrin	$\alpha 5\beta 3$ ligand	DGARYCRGDGCFDG	187
	rabies virus glycoprotein (RVG)	YTIWMPENPRPGTPCDIF TNSRGKRASNGGGG	183
c-Kit receptor (CD117)	stem cell factor (SCF)	EGICRNRVTNNVKDVTCLVANLPKDYMITLKYVPG MDVLP SHCWISEMVVQLSDSLDLDKFSNISEGL SNYSIDKLVNIVDDLVECVKENSSKDLKKSFKSPE PRLFTPEEFFRIFNRSIDAFKDFVASE TSDCVVSS TLSPEKDSRVSVTKPFMLPPVA	184

Cell Surface Protein	Targeting Ligand	Sequence	SEQ ID NO:
CD27	CD70	PEEGSGCSVRRRPYGCVLRAALVPLVAGLVICLV VCIQRFQAQQLPLESLGWDVAELQLNHTGPQ QDPRLYWQGGPALGRSFLHGPELDKGQLRIHRD GIYMVHIQVTLAICSS TTASRHHP TTLAVGICSPAS RSISLLRLSFHQGCTIASQRLTPLARGDTLCTNLTG TLLPSRNTDE TFFGVQWVRP	185
CD150	SH2 domain-containing protein 1A (SH2D1A)	SSGLVPRGSHMDAVAVYHGKISRETGEKLLLATG LDGSYLLRDSSESVPGVYCLCVLYHGYIYTYRVSQT ETGWSAETAPGVHCRYFRKIKNLISAFQKPDQGI VIPLQYPVEKKSSARSTQGTGIREDPDVCLKAP	186
IL2R	IL2	NPKLTRMLTFKIFY	
CD3	Cde3-epsilon	NFYLYRA-NH2	
CD8	peptide-HLA-A*2402	RYPLTFGWCF-NH2	
CD28	CD80	VVLKYEKDAFKR	
CD28	CD86	ENLVLE	

A targeting ligand (e.g., of a delivery molecule) can include the amino acid sequence RGD and/or an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100%

5 sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 1-12. In some cases, a targeting ligand includes the amino acid sequence RGD and/or the amino acid sequence set forth in any one of SEQ ID NOs: 1-12. In some embodiments, a targeting ligand can include a cysteine (internal, C-terminal, or N-terminal), and can also include the amino acid sequence RGD and/or an amino acid sequence having 85% or more sequence identity (e.g.,
10 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 1-12.

A targeting ligand (e.g., of a delivery molecule) can include the amino acid sequence RGD and/or an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100%
15 sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 1-12 and 181-187. In some cases, a targeting ligand includes the amino acid sequence RGD and/or the amino acid sequence set forth in any one of SEQ ID NOs: 1-12 and 181-187. In some embodiments, a targeting ligand can include a cysteine (internal, C-terminal, or N-terminal), and can also include the amino acid sequence RGD and/or an amino acid sequence having 85% or
20 more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in

any one of SEQ ID NOs: 1-12 and 181-187.

A targeting ligand (e.g., of a delivery molecule) can include the amino acid sequence RGD and/or an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 1-12, 181-187, and 271-277. In some cases, a targeting ligand includes the amino acid sequence RGD and/or the amino acid sequence set forth in any one of SEQ ID NOs: 1-12, 181-187, and 271-277. In some embodiments, a targeting ligand can include a cysteine (internal, C-terminal, or N-terminal), and can also include the amino acid sequence RGD and/or an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 1-12, 181-187, and 271-277.

In some cases, a targeting ligand (e.g., of a delivery molecule) can include an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 181-187, and 271-277. In some cases, a targeting ligand includes the amino acid sequence set forth in any one of SEQ ID NOs: 181-187, and 271-277. In some embodiments, a targeting ligand can include a cysteine (internal, C-terminal, or N-terminal), and can also include an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 181-187, and 271-277.

In some cases, a targeting ligand (e.g., of a delivery molecule) can include an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 181-187. In some cases, a targeting ligand includes the amino acid sequence set forth in any one of SEQ ID NOs: 181-187. In some embodiments, a targeting ligand can include a cysteine (internal, C-terminal, or N-terminal), and can also include an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 181-187.

In some cases, a targeting ligand (e.g., of a delivery molecule) can include an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 271-277. In some cases, a targeting ligand includes the amino acid sequence set forth in any one of SEQ ID NOs: 271-277. In some

embodiments, a targeting ligand can include a cysteine (internal, C-terminal, or N-terminal), and can also include an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth in any one of SEQ ID NOs: 271-277.

5 The terms “targets” and “targeted binding” are used herein to refer to specific binding. The terms “specific binding,” “specifically binds,” and the like, refer to non-covalent or covalent preferential binding to a molecule relative to other molecules or moieties in a solution or reaction mixture (e.g., an antibody specifically binds to a particular polypeptide or epitope relative to other available polypeptides, a ligand specifically binds to a particular receptor
10 relative to other available receptors). In some embodiments, the affinity of one molecule for another molecule to which it specifically binds is characterized by a K_d (dissociation constant) of 10^{-5} M or less (e.g., 10^{-6} M or less, 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less, 10^{-10} M or less, 10^{-11} M or less, 10^{-12} M or less, 10^{-13} M or less, 10^{-14} M or less, 10^{-15} M or less, or 10^{-16} M or less). “Affinity” refers to the strength of binding, increased binding affinity correlates with a lower
15 K_d .

 In some cases, the targeting ligand provides for targeted binding to a cell surface protein selected from a family B G-protein coupled receptor (GPCR), a receptor tyrosine kinase (RTK), a cell surface glycoprotein, and a cell-cell adhesion molecule. Consideration of a ligand’s spatial arrangement upon receptor docking can be used to accomplish a desired
20 functional selectivity and endosomal sorting biases, e.g., so that the structure function relationship between the ligand and the target is not disrupted due to the conjugation of the targeting ligand to the payload or anchoring domain (e.g., cationic anchoring domain). For example, conjugation to a nucleic acid, protein, ribonucleoprotein, or anchoring domain (e.g., cationic anchoring domain) could potentially interfere with the binding cleft(s).

25 Thus, in some cases, where a crystal structure of a desired target (cell surface protein) bound to its ligand is available (or where such a structure is available for a related protein), one can use 3D structure modeling and sequence threading to visualize sites of interaction between the ligand and the target. This can facilitate, e.g., selection of internal sites for placement of substitutions and/or insertions (e.g., of a cysteine residue).

30 As an example, in some cases, the targeting ligand provides for binding to a family B G protein coupled receptor (GPCR) (also known as the ‘secretin-family’). In some cases, the targeting ligand provides for binding to both an allosteric-affinity domain and an orthosteric domain of the family B GPCR to provide for the targeted binding and the engagement of long endosomal recycling pathways, respectively.

35 G-protein-coupled receptors (GPCRs) share a common molecular architecture (with seven putative transmembrane segments) and a common signaling mechanism, in that they

interact with G proteins (heterotrimeric GTPases) to regulate the synthesis of intracellular second messengers such as cyclic AMP, inositol phosphates, diacylglycerol and calcium ions. Family B (the secretin-receptor family or 'family 2') of the GPCRs is a small but structurally and functionally diverse group of proteins that includes receptors for polypeptide hormones and molecules thought to mediate intercellular interactions at the plasma membrane (see e.g.,
5 Harmar et al., *Genome Biol.* 2001;2(12):REVIEWS3013). There have been important advances in structural biology as relates to members of the secretin-receptor family, including the publication of several crystal structures of their N-termini, with or without bound ligands, which work has expanded the understanding of ligand binding and provides a useful platform for
10 structure-based ligand design (see e.g., Poyner et al., *Br J Pharmacol.* 2012 May;166(1):1-3).

For example, one may desire to use a subject delivery molecule to target the pancreatic cell surface protein GLP1R (e.g., to target β -islets) using the Exendin-4 ligand, or a derivative thereof (e.g., a cysteine substituted Exendin-4 targeting ligand such as that presented as SEQ ID NO: 2). Because GLP1R is abundant within the brain and pancreas, a targeting ligand that
15 provides for targeting binding to GLP1R can be used to target the brain and pancreas. Thus, targeting GLP1R facilitates methods (e.g., treatment methods) focused on treating diseases (e.g., via delivery of one or more gene editing tools) such as Huntington's disease (CAG repeat expansion mutations), Parkinson's disease (LRRK2 mutations), ALS (SOD1 mutations), and other CNS diseases. Targeting GLP1R also facilitates methods (e.g., treatment methods)
20 focused on delivering a payload to pancreatic β -islets for the treatment of diseases such as diabetes mellitus type I, diabetes mellitus type II, and pancreatic cancer (e.g., via delivery of one or more gene editing tools).

When targeting GLP1R using a modified version of exendin-4, an amino acid for cysteine substitution and/or insertion (e.g., for conjugation to a nucleic acid payload) can be
25 identified by aligning the Exendin-4 amino acid sequence, which is HEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS (SEQ ID NO. 1), to crystal structures of glucagon-GCGR (4ERS) and GLP1-GLP1R-ECD complex (PDB: 3IOL), using PDB 3 dimensional renderings, which may be rotated in 3D space in order to anticipate the direction that a cross-linked complex must face in order not to disrupt the two binding clefts.
30 When a desirable cross-linking site (e.g., site for substitution/insertion of a cysteine residue) of a targeting ligand (that targets a family B GPCR) is sufficiently orthogonal to the two binding clefts of the corresponding receptor, high-affinity binding may occur as well as concomitant long endosomal recycling pathway sequestration (e.g., for optimal payload release). The cysteine substitution at amino acid positions 10, 11, and/or 12 of SEQ ID NO: 1 confers bimodal binding
35 and specific initiation of a Gs-biased signaling cascade, engagement of beta arrestin, and receptor dissociation from the actin cytoskeleton. In some cases, this targeting ligand triggers

internalization of the nanoparticle via receptor-mediated endocytosis, a mechanism that is not engaged via mere binding to the GPCR's N-terminal domain without concomitant orthosteric site engagement (as is the case with mere binding of the affinity strand, Exendin-4 [31-39]).

In some cases, a subject targeting ligand includes an amino acid sequence having 85% or more (e.g., 90% or more, 95% or more, 98% or more, 99% or more, or 100%) identity to the exendin-4 amino acid sequence (SEQ ID NO: 1). In some such cases, the targeting ligand includes a cysteine substitution or insertion at one or more of positions corresponding to L10, S11, and K12 of the amino acid sequence set forth in SEQ ID NO: 1. In some cases, the targeting ligand includes a cysteine substitution or insertion at a position corresponding to S11 of the amino acid sequence set forth in SEQ ID NO: 1. In some cases, a subject targeting ligand includes an amino acid sequence having the exendin-4 amino acid sequence (SEQ ID NO: 1). In some cases, the targeting ligand is conjugated (with or without a linker) to an anchoring domain (e.g., a cationic anchoring domain).

As another example, in some cases a targeting ligand according to the present disclosure provides for binding to a receptor tyrosine kinase (RTK) such as fibroblast growth factor (FGF) receptor (FGFR). Thus in some cases the targeting ligand is a fragment of an FGF (i.e., comprises an amino acid sequence of an FGF). In some cases, the targeting ligand binds to a segment of the RTK that is occupied during orthosteric binding (e.g., see the examples section below). In some cases, the targeting ligand binds to a heparin-affinity domain of the RTK. In some cases, the targeting ligand provides for targeted binding to an FGF receptor and comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence KNGGFFLRIHPDGRVDGVREKS (SEQ ID NO: 4). In some cases, the targeting ligand provides for targeted binding to an FGF receptor and comprises the amino acid sequence set forth as SEQ ID NO: 4.

In some cases, small domains (e.g., 5-40 amino acids in length) that occupy the orthosteric site of the RTK may be used to engage endocytotic pathways relating to nuclear sorting of the RTK (e.g., FGFR) without engagement of cell-proliferative and proto-oncogenic signaling cascades, which can be endemic to the natural growth factor ligands. For example, the truncated bFGF (tbFGF) peptide (a.a.30-115), contains a bFGF receptor binding site and a part of a heparin-binding site, and this peptide can effectively bind to FGFRs on a cell surface, without stimulating cell proliferation. The sequences of tbFGF are KRL YCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVSIGV CANRYLAMKEDGRLL ASKCVTDECF FFERLESNNYNTY (SEQ ID NO: 13) (see, e.g., Cai et al., Int J Pharm. 2011 Apr 15;408(1-2):173-82).

In some cases, the targeting ligand provides for targeted binding to an FGF receptor

and comprises the amino acid sequence HFKDPK (SEQ ID NO: 5) (see, e.g., the examples section below). In some cases, the targeting ligand provides for targeted binding to an FGF receptor, and comprises the amino acid sequence LESNNYNT (SEQ ID NO: 6) (see, e.g., the examples section below).

5 In some cases, a targeting ligand according to the present disclosure provides for targeted binding to a cell surface glycoprotein. In some cases, the targeting ligand provides for targeted binding to a cell-cell adhesion molecule. For example, in some cases, the targeting ligand provides for targeted binding to CD34, which is a cell surface glycoprotein that functions as a cell-cell adhesion factor, and which is protein found on hematopoietic stem cells (e.g., of
10 the bone marrow). In some cases, the targeting ligand is a fragment of a selectin such as E-selectin, L-selectin, or P-selectin (e.g., a signal peptide found in the first 40 amino acids of a selectin). In some cases a subject targeting ligand includes sushi domains of a selectin (e.g., E-selectin, L-selectin, P-selectin).

In some cases, the targeting ligand comprises an amino acid sequence having 85% or
15 more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence MIASQFLSALTLVLLIKESGA (SEQ ID NO: 7). In some cases, the targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 7. In some cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more,
20 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence MVFPWRCEGTYWGSRNILKLWVWTLLCCDFLIHHGTHC (SEQ ID NO: 8). In some cases, the targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 8. In some cases, targeting ligand comprises an amino acid sequence having 85% or more sequence identity
25 (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence MIFPWKCQSTQRDLWNIFKLWGWMTMLCCDFLAHHGTDC (SEQ ID NO: 9). In some cases, targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 9. In some cases, targeting ligand comprises an amino acid sequence having 85% or more sequence identity
30 (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence MIFPWKCQSTQRDLWNIFKLWGWMTMLCC (SEQ ID NO: 10). In some cases, targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 10.

Fragments of selectins that can be used as a subject targeting ligand (e.g., a signal
35 peptide found in the first 40 amino acids of a selectin) can in some cases attain strong binding to specifically-modified sialomucins, e.g., various Sialyl Lewis^x modifications / O-sialylation of

extracellular CD34 can lead to differential affinity for P-selectin, L-selectin and E-selectin to bone marrow, lymph, spleen and tonsillar compartments. Conversely, in some cases a targeting ligand can be an extracellular portion of CD34. In some such cases, modifications of sialylation of the ligand can be utilized to differentially target the targeting ligand to various selectins.

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to E-selectin. E-selectin can mediate the adhesion of tumor cells to endothelial cells and ligands for E-selectin can play a role in cancer metastasis. As an example, P-selectin glycoprotein -1 (PSGL-1) (e.g., derived from human neutrophils) can function as a high-efficiency ligand for E-selectin (e.g., expressed by the endothelium), and a subject targeting ligand can therefore in some cases include the PSGL-1 amino acid sequence (or a fragment thereof the binds to E-selectin). As another example, E-selectin ligand-1 (ESL-1) can bind E-selectin and a subject targeting ligand can therefore in some cases include the ESL-1 amino acid sequence (or a fragment thereof the binds to E-selectin). In some cases, a targeting ligand with the PSGL-1 and/or ESL-1 amino acid sequence (or a fragment thereof the binds to E-selectin) bears one or more sialyl Lewis modifications in order to bind E-selectin. As another example, in some cases CD44, death receptor-3 (DR3), LAMP1, LAMP2, and Mac2-BP can bind E-selectin and a subject targeting ligand can therefore in some cases include the amino acid sequence (or a fragment thereof the binds to E-selectin) of any one of: CD44, death receptor-3 (DR3), LAMP1, LAMP2, and Mac2-BP.

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to P-selectin. In some cases PSGL-1 can provide for such targeted binding. In some cases a subject targeting ligand can therefore in some cases include the PSGL-1 amino acid sequence (or a fragment thereof the binds to P-selectin). In some cases, a targeting ligand with the PSGL-1 amino acid sequence (or a fragment thereof the binds to P-selectin) bears one or more sialyl Lewis modifications in order to bind P-selectin.

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to a target selected from: CD3, CD8, CD4, CD28, CD90, CD45f, CD34, CD80, CD86, CD19, CD20, CD22, CD47, CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, NKp44, NKp46, NKp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL2R, IL7R, IL10R, IL12R, IL15R, IL18R, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to a transferrin receptor. In some such cases, the targeting ligand comprises

an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence THRPPMWSPVWP (SEQ ID NO: 11). In some cases, targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 11.

5 In some cases, a targeting ligand according to the present disclosure provides for targeted binding to an integrin (e.g., $\alpha 5 \beta 1$ integrin). In some such cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence RRETAWA (SEQ ID NO: 12). In some cases, targeting
10 ligand comprises the amino acid sequence set forth as SEQ ID NO: 12. In some cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence RGDGW (SEQ ID NO: 181). In some cases, targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 181. In
15 some cases, the targeting ligand comprises the amino acid sequence RGD.

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to an integrin. In some such cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino
20 acid sequence GCGYGRGDSPG (SEQ ID NO: 182). In some cases, the targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 182. In some cases such a targeting ligand is acetylated on the N-terminus and/or amidated (NH₂) on the C-terminus.

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to an integrin (e.g., $\alpha 5 \beta 3$ integrin). In some such cases, the targeting ligand
25 comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence DGARYCRGDCFDG (SEQ ID NO: 187). In some cases, the targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 187.

In some embodiments, a targeting ligand used to target the brain includes an amino
30 acid sequence from rabies virus glycoprotein (RVG) (e.g., YTIWMPENPRPGTPCDIFTNSRGKRASNGGGG (SEQ ID NO: 183)). In some such cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth as SEQ ID NO: 183. As for
35 any of targeting ligand (as described elsewhere herein), RVG can be conjugated and/or fused to an anchoring domain (e.g., 9R peptide sequence). For example, a subject delivery molecule

used as part of a surface coat of a subject nanoparticle can include the sequence YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRRR (SEQ ID NO: 180).

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to c-Kit receptor. In some such cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth as SEQ ID NO: 184. In some cases, the targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 184.

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to CD27. In some such cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth as SEQ ID NO: 185. In some cases, the targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 185.

In some cases, a targeting ligand according to the present disclosure provides for targeted binding to CD150. In some such cases, the targeting ligand comprises an amino acid sequence having 85% or more sequence identity (e.g., 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, 99.5% or more, or 100% sequence identity) with the amino acid sequence set forth as SEQ ID NO: 186. In some cases, the targeting ligand comprises the amino acid sequence set forth as SEQ ID NO: 186.

In some embodiments, a targeting ligand provides for targeted binding to KLS CD27+/IL-7Ra-/CD150+/CD34- hematopoietic stem and progenitor cells (HSPCs). For example, a gene editing tool(s) (described elsewhere herein) can be introduced in order to disrupt expression of a BCL11a transcription factor and consequently generate fetal hemoglobin. As another example, the beta-globin (HBB) gene may be targeted directly to correct the altered E7V substitution with a corresponding homology-directed repair donor DNA molecule. As one illustrative example, a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, Cpf1) can be delivered with an appropriate guide RNA such that it will bind to loci in the HBB gene and create double-stranded or single-stranded breaks in the genome, initiating genomic repair. In some cases, a Donor DNA molecule (single stranded or double stranded) is introduced (as part of a payload) and is release for 14-30 days while a guide RNA/CRISPR/Cas protein complex (a ribonucleoprotein complex) can be released over the course of from 1-7 days.

In some embodiments, a targeting ligand provides for targeted binding to CD4+ or CD8+ T-cells, hematopoietic stem and progenitor cells (HSPCs), or peripheral blood mononuclear cells (PBMCs), in order to modify the T-cell receptor. For example, a gene editing tool(s)

(described elsewhere herein) can be introduced in order to modify the T-cell receptor. The T-cell receptor may be targeted directly and substituted with a corresponding homology-directed repair donor DNA molecule for a novel T-cell receptor. As one example, a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, Cpf1) can be delivered with an appropriate guide RNA such that it will bind to loci in the TCR gene and create double-stranded or single-stranded breaks in the genome, initiating genomic repair. In some cases, a Donor DNA molecule (single stranded or double stranded) is introduced (as part of a payload). It would be evident to skilled artisans that other CRISPR guide RNA and donor sequences, targeting beta-globin, CCR5, the T-cell receptor, or any other gene of interest, and/or other expression vectors may be employed in accordance with the present disclosure.

In some embodiments, a targeting ligand is a nucleic acid aptamer. In some embodiments, a targeting ligand is a peptoid.

Also provided are delivery molecules with two different peptide sequences that together constitute a targeting ligand. For example, in some cases a targeting ligand is bivalent (e.g., heterobivalent). In some cases, cell-penetrating peptides and/or heparin sulfate proteoglycan binding ligands are used as heterobivalent endocytotic triggers along with any of the targeting ligands of this disclosure. A heterobivalent targeting ligand can include an affinity sequence from one of targeting ligand and an orthosteric binding sequence (e.g., one known to engage a desired endocytic trafficking pathway) from a different targeting ligand.

Anchoring domain

In some embodiments, a delivery molecule includes a targeting ligand conjugated to an anchoring domain (e.g., cationic anchoring domain, an anionic anchoring domain). In some cases a subject delivery vehicle includes a payload that is condensed with and/or interacts electrostatically the anchoring domain (e.g., a delivery molecule can be the delivery vehicle used to deliver the payload). In some cases the surface coat of a nanoparticle includes such a delivery molecule with an anchoring domain, and in some such cases the payload is in the core (interacts with the core) of such a nanoparticle. See the above section describing charged polymer polypeptide domains for additional details related to anchoring domains.

Histone tail peptide (HTPs)

In some embodiments a cationic polypeptide composition of a subject nanoparticle includes a histone peptide or a fragment of a histone peptide, such as an N-terminal histone tail (e.g., a histone tail of an H1, H2 (e.g., H2A, H2AX, H2B), H3, or H4 histone protein). A tail fragment of a histone protein is referred to herein as a histone tail peptide (HTP). Because such a protein (a histone and/or HTP) can condense with a nucleic acid payload as part of the core

of a subject nanoparticle, a core that includes one or more histones or HTPs (e.g., as part of the cationic polypeptide composition) is sometimes referred to herein as a nucleosome-mimetic core. Histones and/or HTPs can be included as monomers, and in some cases form dimers, trimers, tetramers and/or octamers when condensing a nucleic acid payload into a nanoparticle core. In some cases HTPs are not only capable of being deprotonated by various histone modifications, such as in the case of histone acetyltransferase-mediated acetylation, but may also mediate effective nuclear-specific unpackaging of components of the core (e.g., release of a payload). Trafficking of a core that includes a histone and/or HTP may be reliant on alternative endocytotic pathways utilizing retrograde transport through the Golgi and endoplasmic reticulum. Furthermore, some histones include an innate nuclear localization sequence and inclusion of an NLS in the core can direct the core (including the payload) to the nucleus of a target cell.

In some embodiments a subject cationic polypeptide composition includes a protein having an amino acid sequence of an H2A, H2AX, H2B, H3, or H4 protein. In some cases a subject cationic polypeptide composition includes a protein having an amino acid sequence that corresponds to the N-terminal region of a histone protein. For example, the fragment (an HTP) can include the first 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 N-terminal amino acids of a histone protein. In some cases, a subject HTP includes from 5-50 amino acids (e.g., from 5-45, 5-40, 5-35, 5-30, 5-25, 5-20, 8-50, 8-45, 8-40, 8-35, 8-30, 10-50, 10-45, 10-40, 10-35, or 10-30 amino acids) from the N-terminal region of a histone protein. In some cases a subject a cationic polypeptide includes from 5-150 amino acids (e.g., from 5-100, 5-50, 5-35, 5-30, 5-25, 5-20, 8-150, 8-100, 8-50, 8-40, 8-35, 8-30, 10-150, 10-100, 10-50, 10-40, 10-35, or 10-30 amino acids).

In some cases a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide composition includes a post-translational modification (e.g., in some cases on one or more histidine, lysine, arginine, or other complementary residues). For example, in some cases the cationic polypeptide is methylated (and/or susceptible to methylation / demethylation), acetylated (and/or susceptible to acetylation / deacetylation), crotonylated (and/or susceptible to crotonylation / decrotonylation), ubiquitinated (and/or susceptible to ubiquitinylation / deubiquitinylation), phosphorylated (and/or susceptible to phosphorylation / dephosphorylation), SUMOylated (and/or susceptible to SUMOylation / deSUMOylation), farnesylated (and/or susceptible to farnesylation / defarnesylation), sulfated (and/or susceptible to sulfation / desulfation) or otherwise post-translationally modified. In some cases a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide composition is p300/CBP substrate (e.g., see example HTPs below, e.g., SEQ ID NOs: 129-130). In some cases a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic

polypeptide composition includes one or more thiol residues (e.g., can include a cysteine and/or methionine residue) that is sulfated or susceptible to sulfation (e.g., as a thiosulfate sulfurtransferase substrate). In some cases a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide is amidated on the C-terminus.

5 Histones H2A, H2B, H3, and H4 (and/or HTPs) may be monomethylated, dimethylated, or trimethylated at any of their lysines to promote or suppress transcriptional activity and alter nuclear-specific release kinetics.

A cationic polypeptide can be synthesized with a desired modification or can be modified in an *in vitro* reaction. Alternatively, a cationic polypeptide (e.g., a histone or HTP) can be expressed in a cell population and the desired modified protein can be isolated/purified. In
10 some cases the cationic polypeptide composition of a subject nanoparticle includes a methylated HTP, e.g., includes the HTP sequence of H3K4(Me3) - includes the amino acid sequence set forth as SEQ ID NO: 75 or 88). In some cases a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide
15 composition includes a C-terminal amide.

Examples of histones and HTPs

Examples include but are not limited to the following sequences:

20 H2A

SGRGKQGGKARAKAKTRSSR (SEQ ID NO: 62) [1-20]

SGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGGG (SEQ ID NO: 63) [1-39]

MSGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYLA AVL
EYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGKVTIAQGGVLPNIQAVLLP

25 KKTESHKAKGK(SEQ ID NO: 64) [1-130]

H2AX

CKATQASQEY (SEQ ID NO: 65) [134 – 143]

KKTSATVGPKAPSGGKKATQASQEY(SEQ ID NO: 66) [KK 120-129]

30 MSGRGKTGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGYAERVGAGAPVYLA AVL
EYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGGVTIAQGGVLPNIQAVLL
PKKTSATVGPKAPSGGKKATQASQEY(SEQ ID NO: 67) [1-143]

H2B

35 PEPA - K(cr) – SAPAPK (SEQ ID NO: 68) [1-11 H2BK5(cr)]
[cr: crotonylated (crotonylation)]

PEPAKSAPAPK (SEQ ID NO: 69) [1-11]

AQKKDGGKKRKRSRKE (SEQ ID NO: 70) [21-35]

MPEPAKSAPAPKKGSKKAVTKAQKKDGGKKRKRSRKESYSIYVYKVLKQVHPDTGISSK

AMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAVRLLLPGELAKHAVSEGTKA

5 VTKYTSSK (SEQ ID NO: 71) [1-126]

H3

ARTKQTAR (SEQ ID NO: 72) [1-8]

ART - K(Me1) - QTARKS (SEQ ID NO: 73) [1-8 H3K4(Me1)]

10 ART - K(Me2) - QTARKS (SEQ ID NO: 74) [1-8 H3K4(Me2)]

ART - K(Me3) - QTARKS (SEQ ID NO: 75) [1-8 H3K4(Me3)]

ARTKQTARK - pS - TGGKA (SEQ ID NO: 76) [1-15 H3pS10]

ARTKQTARKSTGGKAPRKWC - NH₂ (SEQ ID NO: 77) [1-18 WC, amide]

ARTKQTARKSTGG - K(Ac) - APRKQ (SEQ ID NO: 78) [1-19 H3K14(Ac)]

15 ARTKQTARKSTGGKAPRKQL (SEQ ID NO: 79) [1-20]

ARTKQTAR - K(Ac) - STGGKAPRKQL (SEQ ID NO: 80) [1-20 H3K9(Ac)]

ARTKQTARKSTGGKAPRKQLA (SEQ ID NO: 81) [1-21]

ARTKQTAR - K(Ac) - STGGKAPRKQLA (SEQ ID NO: 82) [1-21 H3K9(Ac)]

ARTKQTAR - K(Me2) - STGGKAPRKQLA (SEQ ID NO: 83) [1-21 H3K9(Me1)]

20 ARTKQTAR - K(Me2) - STGGKAPRKQLA (SEQ ID NO: 84) [1-21 H3K9(Me2)]

ARTKQTAR - K(Me2) - STGGKAPRKQLA (SEQ ID NO: 85) [1-21 H3K9(Me3)]

ART - K(Me1) - QTARKSTGGKAPRKQLA (SEQ ID NO: 86) [1-21 H3K4(Me1)]

ART - K(Me2) - QTARKSTGGKAPRKQLA (SEQ ID NO: 87) [1-21 H3K4(Me2)]

ART - K(Me3) - QTARKSTGGKAPRKQLA (SEQ ID NO: 88) [1-21 H3K4(Me3)]

25 ARTKQTAR - K(Ac) - pS - TGGKAPRKQLA (SEQ ID NO: 89) [1-21 H3K9(Ac), pS10]

ART - K(Me3) - QTAR - K(Ac) - pS - TGGKAPRKQLA (SEQ ID NO: 90) [1-21

H3K4(Me3), K9(Ac), pS10]

ARTKQTARKSTGGKAPRKQLAC (SEQ ID NO: 91) [1-21 Cys]

ARTKQTAR - K(Ac) - STGGKAPRKQLATKA (SEQ ID NO: 92) [1-24 H3K9(Ac)]

30 ARTKQTAR - K(Me3) - STGGKAPRKQLATKA (SEQ ID NO: 93) [1-24 H3K9(Me3)]

ARTKQTARKSTGGKAPRKQLATKAA (SEQ ID NO: 94) [1-25]

ART - K(Me3) - QTARKSTGGKAPRKQLATKAA (SEQ ID NO: 95) [1-25 H3K4(Me3)]

TKQTAR - K(Me1) - STGGKAPR (SEQ ID NO: 96) [3-17 H3K9(Me1)]

TKQTAR - K(Me2) - STGGKAPR (SEQ ID NO: 97) [3-17 H3K9(Me2)]

35 TKQTAR - K(Me3) - STGGKAPR (SEQ ID NO: 98) [3-17 H3K9(Me3)]

KSTGG - K(Ac) - APRKQ (SEQ ID NO: 99) [9-19 H3K14(Ac)]

- QTARKSTGGKAPRKQLASK (SEQ ID NO: 100) [5-23]
 APRKQLATKAARKSAPATGGVKKPH (SEQ ID NO: 101) [15-39]
 ATKAARKSAPATGGVKKPHRYRPG (SEQ ID NO: 102) [21-44]
 KAARKSAPA (SEQ ID NO: 103) [23-31]
 5 KAARKSAPATGG (SEQ ID NO: 104) [23-34]
 KAARKSAPATGGC (SEQ ID NO: 105) [23-34 Cys]
 KAAR - K(Ac) - SAPATGG (SEQ ID NO: 106) [H3K27(Ac)]
 KAAR - K(Me1) - SAPATGG (SEQ ID NO: 107) [H3K27(Me1)]
 KAAR - K(Me2) - SAPATGG (SEQ ID NO: 108) [H3K27(Me2)]
 10 KAAR - K(Me3) - SAPATGG (SEQ ID NO: 109) [H3K27(Me3)]
 AT - K(Ac) - AARKSAPSTGGVKKPHRYRPG (SEQ ID NO: 110) [21-44 H3K23(Ac)]
 ATKAARK - pS - APATGGVKKPHRYRPG (SEQ ID NO: 111) [21-44 pS28]
 ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGV (SEQ ID NO: 112) [1-35]
 STGGV - K(Me1) - KPHRY (SEQ ID NO: 113) [31-41 H3K36(Me1)]
 15 STGGV - K(Me2) - KPHRY (SEQ ID NO: 114) [31-41 H3K36(Me2)]
 STGGV - K(Me3) - KPHRY (SEQ ID NO: 115) [31-41 H3K36(Me3)]
 GTVALREIRRYQ - K(Ac) - STELLIR (SEQ ID NO: 116) [44-63 H3K56(Ac)]
 ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALRE (SEQ ID
 NO: 117) [1-50]
 20 TELLIRKLPFQRLVREIAQDF - K(Me1) - TDLRFQSSAI (SEQ ID NO: 118)
 [H3K79(Me1)]
 EIAQDFKTDLR (SEQ ID NO: 119) [73-83]
 EIAQDF - K(Ac) - TDLR (SEQ ID NO: 120) [73-83 H3K79(Ac)]
 EIAQDF - K(Me3) - TDLR (SEQ ID NO: 121) [73-83 H3K79(Me3)]
 25 RLVREIAQDFKTDLRFQSSAV (SEQ ID NO: 122) [69-89]
 RLVREIAQDFK - (Me1) - TDLRFQSSAV (SEQ ID NO: 123) [69-89 H3K79 (Me1),
 amide]
 RLVREIAQDFK - (Me2) - TDLRFQSSAV (SEQ ID NO: 124) [69-89 H3K79 (Me2),
 amide]
 30 RLVREIAQDFK - (Me3) - TDLRFQSSAV (SEQ ID NO: 125) [69-89 H3K79 (Me3),
 amide]
 KRVTIMPKDIQLARRIRGERA (SEQ ID NO: 126) [116-136]
 MARTKQTARKSTGGKAPRKQLATKVARKSAPATGGVKKPHRYRPGTVALREIRRYQK
 STELLIRKLPFQRLMREIAQDFKTDLRFQSSAVMALQEACESYLVGLFEDTNLCVIHAKR
 35 VTIMPKDIQLARRIRGERA (SEQ ID NO: 127) [1-136]

H4

SGRGKGG (SEQ ID NO: 128) [1-7]

RGKGGKGLGKGA (SEQ ID NO: 129) [4-12]

SGRGKGGKGLGKGGAKRHRKV (SEQ ID NO: 130) [1-21]

5 KGLGKGGAKRHRKVLRDNWC - NH₂ (SEQ ID NO: 131) [8-25 WC, amide]

SGRG - K(Ac) - GG - K(Ac) - GLG - K(Ac) - GGA - K(Ac) - RHRKVLRDNGSGSK (SEQ ID NO: 132) [1-25 H4K5,8,12,16(Ac)]

SGRGKGGKGLGKGGAKRHRK - NH₂ (SEQ ID NO: 133) [1-20 H4 PRMT7 (protein arginine methyltransferase 7) Substrate, M1]

10 SGRG - K(Ac) - GGKGLGKGGAKRHRK (SEQ ID NO: 134) [1-20 H4K5 (Ac)]

SGRGKGG - K(Ac) - GLGKGGAKRHRK (SEQ ID NO: 135) [1-20 H4K8 (Ac)]

SGRGKGGKGLG - K(Ac) - GGAHRK (SEQ ID NO: 136) [1-20 H4K12 (Ac)]

SGRGKGGKGLGKGG - K(Ac) - RHRK (SEQ ID NO: 137) [1-20 H4K16 (Ac)]

KGLGKGGAKRHRKVLRDNWC - NH₂ (SEQ ID NO: 138) [1-25 WC, amide]15 MSGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGV
LKVFLNVIRDAVYTEHAKRKTVMDDVYALKRQGRTLYGFGG (SEQ ID NO: 139)
[1-103]

As such, a cationic polypeptide of a subject cationic polypeptide composition can
20 include an amino acid sequence having the amino acid sequence set forth in any of SEQ ID
NOs: 62-139. In some cases a cationic polypeptide of subject a cationic polypeptide
composition includes an amino acid sequence having 80% or more sequence identity (e.g.,
85% or more, 90% or more, 95% or more, 98% or more, 99% or more, or 100% sequence
identity) with the amino acid sequence set forth in any of SEQ ID NOs: 62-139. In some cases
25 a cationic polypeptide of subject a cationic polypeptide composition includes an amino acid
sequence having 90% or more sequence identity (e.g., 95% or more, 98% or more, 99% or
more, or 100% sequence identity) with the amino acid sequence set forth in any of SEQ ID
NOs: 62-139. The cationic polypeptide can include any convenient modification, and a number
of such contemplated modifications are discussed above, e.g., methylated, acetylated,
30 crotonylated, ubiquitinated, phosphorylated, SUMOylated, farnesylated, sulfated, and the like.

In some cases a cationic polypeptide of a cationic polypeptide composition includes an
amino acid sequence having 80% or more sequence identity (e.g., 85% or more, 90% or more,
95% or more, 98% or more, 99% or more, or 100% sequence identity) with the amino acid
sequence set forth in SEQ ID NO: 94. In some cases a cationic polypeptide of a cationic
35 polypeptide composition includes an amino acid sequence having 95% or more sequence
identity (e.g., 98% or more, 99% or more, or 100% sequence identity) with the amino acid

sequence set forth in SEQ ID NO: 94. In some cases a cationic polypeptide of a cationic polypeptide composition includes the amino acid sequence set forth in SEQ ID NO: 94. In some cases a cationic polypeptide of a cationic polypeptide composition includes the sequence represented by H3K4(Me3) (SEQ ID NO: 95), which comprises the first 25 amino acids of the human histone 3 protein, and tri-methylated on the lysine 4 (e.g., in some cases amidated on the C-terminus).

In some embodiments a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide composition includes a cysteine residue, which can facilitate conjugation to: a cationic (or in some cases anionic) amino acid polymer, a linker, an NLS, and/or other cationic polypeptides (e.g., in some cases to form a branched histone structure). For example, a cysteine residue can be used for crosslinking (conjugation) via sulfhydryl chemistry (e.g., a disulfide bond) and/or amine-reactive chemistry. In some cases the cysteine residue is internal. In some cases the cysteine residue is positioned at the N-terminus and/or C-terminus. In some cases, a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide composition includes a mutation (e.g., insertion or substitution) that adds a cysteine residue. Examples of HTPs that include a cysteine include but are not limited to:

CKATQASQEY (SEQ ID NO: 140) - from H2AX

ARTKQTARKSTGGKAPRKQLAC (SEQ ID NO: 141) - from H3

ARTKQTARKSTGGKAPRKWC (SEQ ID NO: 142)

KAARKSAPATGGC (SEQ ID NO: 143) - from H3

KGLGKGGAHRKVLRLDNWC (SEQ ID NO: 144) – from H4

MARTKQTARKSTGGKAPRKQLATKVARKSAPATGGVKKPHRYRPGTVALREIRRYQK

STELLIRKLPFQRLMREIAQDFKTDLRFQSSAVMALQEACESYLVGLFEDTNLCVIAKR

VTIMPKDIQLARRIRGERA (SEQ ID NO: 145) – from H3

In some embodiments a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide composition is conjugated to a cationic (and/or anionic) amino acid polymer of the core of a subject nanoparticle. As an example, a histone or HTP can be conjugated to a cationic amino acid polymer (e.g., one that includes poly(lysine)), via a cysteine residue, e.g., where the pyridyl disulfide group(s) of lysine(s) of the polymer are substituted with a disulfide bond to the cysteine of a histone or HTP.

Modified / Branching Structure

In some embodiments a cationic polypeptide of a subject a cationic polypeptide composition has a linear structure. In some embodiments a cationic polypeptide of a subject a

cationic polypeptide composition has a branched structure.

For example, in some cases, a cationic polypeptide (e.g., HTPs, e.g., HTPs with a cysteine residue) is conjugated (e.g., at its C-terminus) to the end of a cationic polymer (e.g., poly(L-arginine), poly(D-lysine), poly(L-lysine), poly(D-lysine)), thus forming an extended linear polypeptide. In some cases, one or more (two or more, three or more, etc.) cationic polypeptides (e.g., HTPs, e.g., HTPs with a cysteine residue) are conjugated (e.g., at their C-termini) to the end(s) of a cationic polymer (e.g., poly(L-arginine), poly(D-lysine), poly(L-lysine), poly(D-lysine)), thus forming an extended linear polypeptide. In some cases the cationic polymer has a molecular weight in a range of from 4,500 - 150,000 Da).

As another example, in some cases, one or more (two or more, three or more, etc.) cationic polypeptides (e.g., HTPs, e.g., HTPs with a cysteine residue) are conjugated (e.g., at their C-termini) to the side-chains of a cationic polymer (e.g., poly(L-arginine), poly(D-lysine), poly(L-lysine), poly(D-lysine)), thus forming a branched structure (branched polypeptide). Formation of a branched structure by components of the nanoparticle core (e.g., components of a subject cationic polypeptide composition) can in some cases increase the amount of core condensation (e.g., of a nucleic acid payload) that can be achieved. Thus, in some cases it is desirable to use components that form a branched structure. Various types of branches structures are of interest, and examples of branches structures that can be generated (e.g., using subject cationic polypeptides such as HTPs, e.g., HTPs with a cysteine residue; peptoids, polyamides, and the like) include but are not limited to: brush polymers, webs (e.g., spider webs), graft polymers, star-shaped polymers, comb polymers, polymer networks, dendrimers, and the like.

In some cases, a branched structure includes from 2-30 cationic polypeptides (e.g., HTPs) (e.g., from 2-25, 2-20, 2-15, 2-10, 2-5, 4-30, 4-25, 4-20, 4-15, or 4-10 cationic polypeptides), where each can be the same or different than the other cationic polypeptides of the branched structure. In some cases the cationic polymer has a molecular weight in a range of from 4,500 - 150,000 Da). In some cases, 5% or more (e.g., 10% or more, 20% or more, 25% or more, 30% or more, 40% or more, or 50% or more) of the side-chains of a cationic polymer (e.g., poly(L-arginine), poly(D-lysine), poly(L-lysine), poly(D-lysine)) are conjugated to a subject cationic polypeptide (e.g., HTP, e.g., HTP with a cysteine residue). In some cases, up to 50% (e.g., up to 40%, up to 30%, up to 25%, up to 20%, up to 15%, up to 10%, or up to 5%) of the side-chains of a cationic polymer (e.g., poly(L-arginine), poly(D-lysine), poly(L-lysine), poly(D-lysine)) are conjugated to a subject cationic polypeptide (e.g., HTP, e.g., HTP with a cysteine residue). Thus, an HTP can be branched off of the backbone of a polymer such as a cationic amino acid polymer.

In some cases formation of branched structures can be facilitated using components

such as peptoids (polypeptoids), polyamides, dendrimers, and the like. For example, in some cases peptoids (e.g., polypeptoids) are used as a component of a nanoparticle core, e.g., in order to generate a web (e.g., spider web) structure, which can in some cases facilitate condensation of the nanoparticle core.

One or more of the natural or modified polypeptide sequences herein may be modified with terminal or intermittent arginine, lysine, or histidine sequences. In one embodiment, each polypeptide is included in equal amine molarities within a nanoparticle core. In this embodiment, each polypeptide's C-terminus can be modified with 5R (5 arginines). In some embodiments, each polypeptide's C-terminus can be modified with 9R (9 arginines). In some embodiments, each polypeptide's N-terminus can be modified with 5R (5 arginines). In some embodiments, each polypeptide's N-terminus can be modified with 9R (9 arginines). In some cases, an H2A, H2B, H3 and/or H4 histone fragment (e.g., HTP) are each bridged in series with a FKFL Cathepsin B proteolytic cleavage domain or RGFFP Cathepsin D proteolytic cleavage domain. In some cases, an H2A, H2B, H3 and/or H4 histone fragment (e.g., HTP) can be bridged in series by a 5R (5 arginines), 9R (9 arginines), 5K (5 lysines), 9K (9 lysines), 5H (5 histidines), or 9H (9 histidines) cationic spacer domain. In some cases, one or more H2A, H2B, H3 and/or H4 histone fragments (e.g., HTPs) are disulfide-bonded at their N-terminus to protamine.

To illustrate how to generate a branched histone structure, example methods of preparation are provided. One example of such a method includes the following: covalent modification of equimolar ratios of Histone H2AX [134-143], Histone H3 [1-21 Cys], Histone H3 [23-34 Cys], Histone H4 [8-25 WC] and SV40 T-Ag-derived NLS can be performed in a reaction with 10% pyridyl disulfide modified poly(L-Lysine) [MW = 5400, 18000, or 45000 Da; n = 30, 100, or 250]. In a typical reaction, a 29 μ L aqueous solution of 700 μ M Cys-modified histone/NLS (20 nmol) can be added to 57 μ L of 0.2 M phosphate buffer (pH 8.0). Second, 14 μ L of 100 μ M pyridyl disulfide protected poly(lysine) solution can then be added to the histone solution bringing the final volume to 100 μ L with a 1:2 ratio of pyridyl disulfide groups to Cysteine residues. This reaction can be carried out at room temperature for 3 h. The reaction can be repeated four times and degree of conjugation can be determined via absorbance of pyridine-2-thione at 343nm.

As another example, covalent modification of a 0:1, 1:4, 1:3, 1:2, 1:1, 1:2, 1:3, 1:4, or 1:0 molar ratio of Histone H3 [1-21 Cys] peptide and Histone H3 [23-34 Cys] peptide can be performed in a reaction with 10% pyridyl disulfide modified poly(L-Lysine) or poly(L-Arginine) [MW = 5400, 18000, or 45000 Da; n = 30, 100, or 250]. In a typical reaction, a 29 μ L aqueous solution of 700 μ M Cys-modified histone (20 nmol) can be added to 57 μ L of 0.2 M phosphate buffer (pH 8.0). Second, 14 μ L of 100 μ M pyridyl disulfide protected poly(lysine) solution can

then be added to the histone solution bringing the final volume to 100 μ L with a 1:2 ratio of pyridyl disulfide groups to Cysteine residues. This reaction can be carried out at room temperature for 3 h. The reaction can be repeated four times and degree of conjugation can be determined via absorbance of pyridine-2-thione at 343nm.

5 In some cases, an anionic polymer is conjugated to a targeting ligand.

Nuclear localization sequence (NLS)

In some embodiments a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4) of a cationic polypeptide composition includes (and/or is conjugated to) one or more (e.g., two or more, three or more, or four or more) nuclear localization sequences (NLSs). Thus in some cases the cationic polypeptide composition of a subject nanoparticle includes a peptide that includes an NLS. In some cases a histone protein (or an HTP) of a subject nanoparticle includes one or more (e.g., two or more, three or more) natural nuclear localization signals (NLSs). In some cases a histone protein (or an HTP) of a subject nanoparticle includes one or more (e.g., two or more, three or more) NLSs that are heterologous to the histone protein (i.e., NLSs that do not naturally occur as part of the histone/HTP, e.g., an NLS can be added by humans). In some cases the HTP includes an NLS on the N- and/or C- terminus.

In some embodiments a cationic amino acid polymer (e.g., poly(arginine)(PR), poly(lysine)(PK), poly(histidine)(PH), poly(ornithine), poly(citrulline), poly(D-arginine)(PDR), poly(D-lysine)(PDK), poly(D-histidine)(PDH), poly(D-ornithine), poly(D-citrulline), poly(L-arginine)(PLR), poly(L-lysine)(PLK), poly(L-histidine)(PLH), poly(L-ornithine), or poly(L-citrulline)) of a cationic polymer composition includes (and/or is conjugated to) one or more (e.g., two or more, three or more, or four or more) NLSs. In some cases the cationic amino acid polymer includes an NLS on the N- and/or C- terminus. In some cases the cationic amino acid polymer includes an internal NLS.

In some embodiments an anionic amino acid polymer (e.g., poly(glutamic acid) (PEA), poly(aspartic acid) (PDA), poly(D-glutamic acid) (PDEA), poly(D-aspartic acid) (PDDA), poly(L-glutamic acid) (PLEA), or poly(L-aspartic acid) (PLDA)) of an anionic polymer composition includes (and/or is conjugated to) one or more (e.g., two or more, three or more, or four or more) NLSs. In some cases the anionic amino acid polymer includes an NLS on the N- and/or C- terminus. In some cases the anionic amino acid polymer includes an internal NLS.

Any convenient NLS can be used (e.g., conjugated to a histone, an HTP, a cationic amino acid polymer, an anionic amino acid polymer, and the like). Examples include, but are not limited to Class 1 and Class 2 'monopartite NLSs', as well as NLSs of Classes 3-5 (see, e.g., **Figure 7**, which is adapted from Kosugi et al., J Biol Chem. 2009 Jan 2;284(1):478-85). In

some cases, an NLS has the formula: (K/R) (K/R) $X_{10-12}(K/R)_{3-5}$. In some cases, an NLS has the formula: K(K/R)X(K/R).

In some embodiments a cationic polypeptide of a cationic polypeptide composition includes one more (e.g., two or more, three or more, or four or more) NLSs. In some cases the cationic polypeptide is not a histone protein or histone fragment (e.g., is not an HTP). Thus, in some cases the cationic polypeptide of a cationic polypeptide composition is an NLS-containing peptide.

In some cases, the NLS-containing peptide includes a cysteine residue, which can facilitate conjugation to: a cationic (or in some cases anionic) amino acid polymer, a linker, histone protein for HTP, and/or other cationic polypeptides (e.g., in some cases as part of a branched histone structure). For example, a cysteine residue can be used for crosslinking (conjugation) via sulfhydryl chemistry (e.g., a disulfide bond) and/or amine-reactive chemistry. In some cases the cysteine residue is internal. In some cases the cysteine residue is positioned at the N-terminus and/or C-terminus. In some cases, an NLS-containing peptide of a cationic polypeptide composition includes a mutation (e.g., insertion or substitution) (e.g., relative to a wild type amino acid sequence) that adds a cysteine residue.

Examples of NLSs that can be used as an NLS-containing peptide (or conjugated to any convenient cationic polypeptide such as an HTP or cationic polymer or cationic amino acid polymer or anionic amino acid polymer) include but are not limited to (some of which include a cysteine residue):

PKKKRKV (SEQ ID NO: 151) (T-ag NLS)

PKKKRKVEDPYC (SEQ ID NO: 152) - SV40 T-Ag-derived NLS

PKKKRKVGPKKKRKVGPKKKRKVGPKKKRKVGC (SEQ ID NO: 153) (NLS SV40)

CYGRKKRRQRRR (SEQ ID NO: 154) - N-terminal cysteine of cysteine-TAT

CSIPPEVKFNKPFVYLI (SEQ ID NO: 155)

DRQIKIWFQNRRMKWKK (SEQ ID NO: 156)

PKKKRKVEDPYC (SEQ ID NO: 157) - C-term cysteine of an SV40 T-Ag-derived NLS

PAAKRVKLD (SEQ ID NO: 158) [cMyc NLS]

For non-limiting examples of NLSs that can be used, see, e.g., Kosugi et al., J Biol Chem. 2009 Jan 2;284(1):478-85, e.g., see **Figure 7** of this disclosure.

Mitochondrial localization signal

In some embodiments a cationic polypeptide (e.g., a histone or HTP, e.g., H1, H2, H2A, H2AX, H2B, H3, or H4), an anionic polymer, and/or a cationic polymer of a subject nanoparticle

includes (and/or is conjugated to) one or more (e.g., two or more, three or more, or four or more) mitochondrial localization sequences. Any convenient mitochondrial localization sequence can be used. Examples of mitochondrial localization sequences include but are not limited to: PEDEIWLPEPESVDVPAKPISTSSMMMP (SEQ ID NO: 149), a mitochondrial
 5 localization sequence of SDHB, mono/di/triphenylphosphonium or other phosphoniums, VAMP 1A, VAMP 1B, the 67 N-terminal amino acids of DGAT2, and the 20 N-terminal amino acids of Bax.

Delivery

10 As noted above, in some embodiments a subject method includes generating a staggered cut at each of two locations in genomic DNA. In some cases, in order to generate the staggered cuts, a site-specific nuclease (one or more site-specific nucleases) (or a nucleic acid encoding same, e.g., one or more nucleic acids) is introduced into a target cell. If the target cell is in vivo, this can be accomplished by administering the appropriate components (e.g., as part
 15 of one or more delivery vehicles) to an individual. In some cases, the target cell includes DNA encoding a site-specific nuclease and the 'generating' step of a subject method includes inducing expression of the site-specific nuclease.

Thus, in some cases a subject method includes introducing into a target cell, a site-specific nuclease (e.g., one or more site-specific nucleases) (e.g., via administration to an
 20 individual, via transfection, via a nanoparticle, via a delivery molecule, etc.). In some cases, such a step includes introducing a nucleic acid (e.g., RNA or DNA) that encodes the one or more site-specific nucleases into the cell. Likewise, in some cases a subject method includes introducing a linear double stranded donor DNA into a target cell (e.g., via administration to an individual, via transfection, via a nanoparticle, via a delivery molecule, etc.). In some cases, the
 25 donor DNA and the site-specific nuclease (or nucleic acid encoding same) are introduced into the cell as part of the same delivery vehicle (e.g., nanoparticle, delivery molecule, etc.). The components – a donor DNA and one or more site-specific nucleases (or one or more nucleic acids encoding same) – can be delivered to any desired target cell, e.g., any desired eukaryotic cell.

30 In some cases the target cell is *in vitro* (e.g., the cell is in culture), e.g., the cell can be a cell of an established tissue culture cell line. In some cases the target cell is *ex vivo* (e.g., the cell is a primary cell (or a recent descendant) isolated from an individual, e.g. a patient). In some cases, the target cell is *in vivo* and is therefore inside of (part of) an organism.

A donor DNA and/or one or more site-specific nucleases (or one or more nucleic acids
 35 encoding same), e.g., as payloads of a delivery vehicle, may be introduced to the subject (i.e., administered to an individual) via any of the following routes: systemic, local, parenteral,

subcutaneous (s.c.), intravenous (i.v.), intracranial (i.c.), intraspinal, intraocular, intradermal (i.d.), intramuscular (i.m.), intralymphatic (i.l.), or into spinal fluid. The components may be introduced by injection (e.g., systemic injection, direct local injection, local injection into or near a tumor and/or a site of tumor resection, etc.), catheter, or the like. Examples of methods for local delivery (e.g., delivery to a tumor and/or cancer site) include, e.g., by bolus injection, e.g. by a syringe, e.g. into a joint, tumor, or organ, or near a joint, tumor, or organ; e.g., by continuous infusion, e.g. by cannulation, e.g. with convection (see e.g. US Application No. 20070254842, incorporated here by reference).

The number of administrations of treatment to a subject may vary. Introducing a donor DNA and/or one or more site-specific nucleases (or one or more nucleic acids encoding same), e.g., as payloads of a delivery vehicle, into an individual may be a one-time event; but in certain situations, such treatment may elicit improvement for a limited period of time and require an on-going series of repeated treatments. In other situations, multiple administrations of a donor DNA and/or one or more site-specific nucleases (or one or more nucleic acids encoding same) may be required before an effect is observed. As will be readily understood by one of ordinary skill in the art, the exact protocols depend upon the disease or condition, the stage of the disease and parameters of the individual being treated.

A "therapeutically effective dose" or "therapeutic dose" is an amount sufficient to effect desired clinical results (i.e., achieve therapeutic efficacy). A therapeutically effective dose can be administered in one or more administrations. For purposes of this disclosure, a therapeutically effective dose of a donor DNA and/or one or more site-specific nucleases (or one or more nucleic acids encoding same) is an amount that is sufficient, when administered to the individual, to palliate, ameliorate, stabilize, reverse, prevent, slow or delay the progression of a disease state/ailment.

An example therapeutic intervention is one that creates resistance to HIV infection in addition to ablating any retroviral DNA that has been integrated into the host genome. T-cells are directly affected by HIV and thus a hybrid blood targeting strategy for CD34+ and CD45+ cells may be explored. For example, an effective therapeutic intervention may include simultaneously targeting HSCs and T-cells and delivering an ablation (and replacement sequence) to the CCR5-Δ32 and gag/rev/pol genes through multiple guided nucleases (e.g., within a single particle).

In some cases, the target cell is a mammalian cell (e.g., a rodent cell, a mouse cell, a rat cell, an ungulate cell, a cow cell, a sheep cell, a pig cell, a horse cell, a camel cell, a rabbit cell, a canine (dog) cell, a feline (cat) cell, a primate cell, a non-human primate cell, a human cell). Any cell type can be targeted, and in some cases specific targeting of particular cells depends on the presence of targeting ligands (e.g., as part of a surface coat of a nanoparticle,

as part of a delivery molecule, etc), where the targeting ligands provide for targeting binding to a particular cell type. For example, cells that can be targeted include but are not limited to bone marrow cells, hematopoietic stem cells (HSCs), long-term HSCs, short-term HSCs, hematopoietic stem and progenitor cells (HSPCs), peripheral blood mononuclear cells (PBMCs), myeloid progenitor cells, lymphoid progenitor cells, T-cells, B-cells (e.g., via targeting CD19, CD20, CD22), NKT cells, NK cells, dendritic cells, monocytes, granulocytes, erythrocytes, megakaryocytes, mast cells, basophils, eosinophils, neutrophils, macrophages (e.g., via targeting CD47 via SIRP α -mimetic peptides), erythroid progenitor cells (e.g., HUDEP cells), megakaryocyte-erythroid progenitor cells (MEPs), common myeloid progenitor cells (CMPs), multipotent progenitor cells (MPPs), hematopoietic stem cells (HSCs), short term HSCs (ST-HSCs), IT-HSCs, long term HSCs (LT-HSCs), endothelial cells, neurons, astrocytes, pancreatic cells, pancreatic β -islet cells, muscle cells, skeletal muscle cells, cardiac muscle cells, hepatic cells, fat cells, intestinal cells, cells of the colon, and cells of the stomach.

Examples of various applications (e.g., for targeting neurons, cells of the pancreas, hematopoietic stem cells and multipotent progenitors, etc.) are discussed above, e.g., in the context of targeting ligands. For example, Hematopoietic stem cells and multipotent progenitors can be targeted for gene editing (e.g., insertion) *in vivo*. Even editing 1% of bone marrow cells *in vivo* (approximately 15 billion cells) would target more cells than an *ex vivo* therapy (approximately 10 billion cells). As another example, pancreatic cells (e.g., β islet cells) can be targeted, e.g., to treat pancreatic cancer, to treat diabetes, etc. As another example, somatic cells in the brain such as neurons can be targeted (e.g., to treat indications such as Huntington's disease, Parkinson's (e.g., LRRK2 mutations), and ALS (e.g., SOD1 mutations)). In some cases this can be achieved through direct intracranial injections.

As another example, endothelial cells and cells of the hematopoietic system (e.g., megakaryocytes and/or any progenitor cell upstream of a megakaryocyte such as a megakaryocyte-erythroid progenitor cell (MEP), a common myeloid progenitor cell (CMP), a multipotent progenitor cell (MPP), a hematopoietic stem cells (HSC), a short term HSC (ST-HSC), an IT-HSC, a long term HSC (LT-HSC) – see, e.g., **Figure 8**) can be targeted with a subject nanoparticle (or subject viral or non-viral delivery vehicle) to treat Von Willebrand's disease. For example, a cell (e.g., an endothelial cell, a megakaryocyte and/or any progenitor cell upstream of a megakaryocyte such as an MEP, a CMP, an MPP, an HSC such as an ST-HSC, an IT-HSC, and/or an LT-HSC) harboring a mutation in the gene encoding von Willebrand factor (VWF) can be targeted (*in vitro*, *ex vivo*, *in vivo*) in order to edit (and correct) the mutated gene, e.g., by introducing a replacement sequence (e.g., via delivery of a donor DNA). In some of the above cases (e.g., in cases related to treating Von Willebrand's disease,

in cases related to targeting a cell harboring a mutation in the gene encoding VWF), a subject targeting ligand provides for targeted binding to E-selectin.

Methods and compositions of this disclosure can be used to treat any number of diseases, including any disease that is linked to a known causative mutation, e.g., a mutation in the genome. For example, methods and compositions of this disclosure can be used to treat sickle cell disease, β thalassemia, HIV, myelodysplastic syndromes, JAK2-mediated polycythemia vera, JAK2-mediated primary myelofibrosis, JAK2-mediated leukemia, and various hematological disorders. As additional non-limiting examples, the methods and compositions of this disclosure can also be used for B-cell antibody generation, immunotherapies (e.g., delivery of a checkpoint blocking reagent), and stem cell differentiation applications.

In some embodiments, a targeting ligand provides for targeted binding to KLS CD27+/IL-7Ra-/CD150+/CD34- hematopoietic stem and progenitor cells (HSPCs). For example, the beta-globin (HBB) gene may be targeted directly to correct the altered E7V substitution with an appropriate donor DNA molecule. As one illustrative example, a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, Cpf1) can be delivered with an appropriate guide RNA(s) such that it will bind to loci in the HBB gene and create a staggered end cut at two locations in the genome, initiating insertion of an introduced donor DNA. In some cases, a Donor DNA molecule (single stranded or double stranded) is introduced (as part of a payload) and is release for 14-30 days while a guide RNA/CRISPR/Cas protein complex (a ribonucleoprotein complex) can be released over the course of from 1-7 days.

In some embodiments, a targeting ligand provides for targeted binding to CD4+ or CD8+ T-cells, hematopoietic stem and progenitor cells (HSPCs), or peripheral blood mononuclear cells (PBMCs), in order to modify the T-cell receptor. For example, a gene editing tool(s) (described elsewhere herein) can be introduced in order to modify the T-cell receptor. The T-cell receptor may be targeted directly and substituted with a corresponding homology-directed repair donor DNA molecule for a novel T-cell receptor. As one example, a CRISPR/Cas RNA-guided polypeptide (e.g., Cas9, CasX, CasY, Cpf1) can be delivered with an appropriate guide RNA(s) such that it will bind to loci in the HBB gene and create a staggered end cut at two locations in the genome, initiating insertion of an introduced donor DNA. It would be evident to skilled artisans that other CRISPR guide RNA and donor sequences, targeting beta-globin, CCR5, the T-cell receptor, or any other gene of interest, and/or other expression vectors may be employed in accordance with the present disclosure.

In some cases, a subject method is used to target a locus that encodes a T cell receptor (TCR), which in some cases has nearly 100 domains and as many as 1,000,000 base pairs with the constant region separated from the V(D)J regions by ~100,000 base pairs or more.

In some cases insertion of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) protein. In some such cases the donor DNA encodes amino acids of a CDR1, CDR2, or CDR3 region of the TCR protein. See, e.g., Dash et al., Nature. 2017 Jul 6;547(7661):89-93. Epub 2017 Jun 21; and Glanville et al., Nature. 2017 Jul 6;547(7661):94-98. Epub 2017 Jun 21.

In some cases a subject method is used to insert genes while placing them under the control of (in operable linkage with) specific enhancers as a fail-safe to genome engineering. If the insertion fails, the enhancer is disrupted leading to the subsequent gene and any possible indels being unlikely to express. If the gene insertion succeeds, a new gene can be inserted with a stop codon at its end, which is particularly useful for multi-part genes such as the TCR locus. In some cases, the subject methods can be used to insert a chimeric antigen receptor (CAR) or other construct into a T-cell, or to cause a B-cell to create a specific antibody or alternative to an antibody (such as a nanobody, shark antibody, etc.).

In some cases the donor DNA includes a nucleotide sequence that encodes a chimeric antigen receptor (CAR). In some such cases, insertion of the donor DNA results in operable linkage of the nucleotide sequence encoding the CAR to an endogenous T-cell promoter (i.e., expression of the CAR will be under the control of an endogenous promoter). In some cases the donor DNA includes a nucleotide sequence that is operably linked to a promoter and encodes a chimeric antigen receptor (CAR) – and thus the inserted CAR will be under the control of the promoter that was present on the donor DNA.

In some cases the donor DNA includes a nucleotide sequence encoding a cell-specific targeting ligand that is membrane bound and presented extracellularly. In some cases, insertion of said donor DNA results in operable linkage of the nucleotide sequence encoding the cell-specific targeting ligand to an endogenous promoter. In some cases the donor DNA includes a promoter operably linked to the sequence that encodes a cell-specific targeting ligand that is membrane bound and presented extracellularly – and therefore, after insertion of the donor DNA, expression of the membrane bound targeting ligand will be under the control of the promoter that was present on the donor DNA.

In some embodiments, insertion of a donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit. In some cases, insertion of a donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Gamma subunit. In some cases a subject method and/or composition includes two donor DNAs. In some such cases insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Beta or Gamma subunit.

In some embodiments, insertion of a donor DNA occurs within a nucleotide sequence

that encodes a T cell receptor (TCR) Alpha or Delta subunit constant region. In some cases insertion of a donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Beta or Gamma subunit constant region. In some cases a subject method and/or composition includes two donor DNAs. In some such cases insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit constant region and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Beta or Gamma subunit constant region.

In some embodiments, insertion of a donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Delta subunit promoter. In some cases insertion of a donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Beta or Gamma subunit promoter. In some cases a subject method and/or composition includes two donor DNAs. In some such cases insertion of one donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Delta subunit promoter and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Beta or Gamma subunit promoter.

In some embodiments, insertion of a sequence of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Gamma subunit. In some cases, insertion of a sequence of the donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Delta subunit. In some cases a subject method and/or composition includes two donor DNAs. In some such cases insertion of one sequence of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Gamma subunit and insertion of the sequence of the other donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Beta or Delta subunit.

In some embodiments, insertion of a sequence of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Gamma subunit constant region. In some cases insertion of a sequence of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Beta or Delta subunit constant region. In some cases a subject method and/or composition includes two donor DNAs. In some such cases insertion of one sequence of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Gamma subunit constant region and insertion of the sequence of the other donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Beta or Delta subunit constant region.

In some embodiments, insertion of a sequence of the donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Gamma subunit promoter. In some cases insertion of a sequence of the donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Beta or Delta subunit promoter. In some

cases a subject method and/or composition includes two donor DNAs. In some such cases insertion of one sequence of the donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Gamma subunit promoter and insertion of the sequence of the other donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Beta or Delta subunit promoter.

In some embodiment, insertion of a donor DNA results in operable linkage of the inserted donor DNA with a T cell receptor (TCR) Alpha, Beta, Gamma or Delta endogenous promoter. In some cases, the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a TCR Alpha, Beta, Gamma or Delta promoter such that after insertion, the protein-coding sequence will remain operably linked to (under the control of) the promoter present in the donor DNA. In some cases insertion of said donor DNA results in operable linkage of the inserted donor DNA (e.g., a protein-coding nucleotide sequence such as a CAR, TCR-alphas, TCR-beta, TCR-gamma, or TCR-Delta sequence) with a CD3 or CD28 promoter. In some cases the donor DNA includes a protein-coding nucleotide sequence that is operably linked to a promoter (e.g., a T-cell specific promoter). In some cases insertion of the donor DNA results in operable linkage of the inserted donor DNA with an endogenous promoter (e.g., a stem cell specific or somatic cell specific endogenous promoter). In some cases the donor DNA includes a nucleotide sequence that encodes a reporter protein (e.g., fluorescent protein such as GFP, RFP, YFP, CFP, a near-IR and/or far red reporter protein, etc., e.g., for evaluating gene editing efficiency). In some cases the donor DNA includes a protein-coding nucleotide sequence (e.g., one that encodes all or a portion of a TCR protein) that does not have introns.

In some cases a subject method (and/or subject compositions) can be used for insertion of sequence for applications such as insertion of fluorescent reporters (e.g., a fluorescent protein such green fluorescent protein (GFP)/ red fluorescent protein (RFP)/near-IR/far-red, and the like), e.g., into the C- and/or N-termini of any encoded protein of interest such as

5 transmembrane proteins.

In some embodiments, insertion of the nucleotide sequence of the donor DNA into the cell's genome results in operable linkage of the inserted sequence with an endogenous promoter (e.g., (i) a T-cell specific promoter; (ii) a CD3 promoter; (iii) a CD28 promoter; (iv) a stem cell specific promoter; (v) a somatic cell specific promoter; (vi) a T cell receptor (TCR) Alpha, Beta, Gamma or Delta promoter; (v) a B-cell specific promoter; (vi) a CD19 promoter; 10 (vii) a CD20 promoter; (viii) a CD22 promoter; (ix) a B29 promoter; and (x) a T-cell or B-cell V(D)J-specific promoter). In some cases the nucleotide sequence, of the insert donor composition, that is inserted includes a protein-coding sequence that is operably linked to a promoter (e.g., (i) a T-cell specific promoter; (ii) a CD3 promoter; (iii) a CD28 promoter; (iv) a stem cell specific promoter; (v) a somatic cell specific promoter; (vi) a T cell receptor (TCR) 15

Alpha, Beta, Gamma or Delta promoter; (v) a B-cell specific promoter; (vi) a CD19 promoter; (vii) a CD20 promoter; (viii) a CD22 promoter; (ix) a B29 promoter; and (x) a T-cell or B-cell V(D)J-specific promoter).

In some embodiments the nucleotide sequence that is inserted into the cell's genome encodes a protein. Any convenient protein can be encoded – examples include but are not limited to: a T cell receptor (TCR) protein; a CDR1, CDR2, or CDR3 region of a T cell receptor (TCR) protein; a chimeric antigen receptor (CAR); a cell-specific targeting ligand that is membrane bound and presented extracellularly; a reporter protein (e.g., a fluorescent protein such as GFP, RFP, CFP, YFP, and fluorescent proteins that fluoresce in far red, in near infrared, etc.). In some embodiments the nucleotide sequence that is inserted into the cell's genome encodes a multivalent (e.g., heteromultivalent) surface receptor (e.g., in some cases where a T-cell is the target cell). Any convenient multivalent receptor could be used and non-limiting examples include: bispecific or trispecific CARs and/or TCRs, or other affinity tags on immune cells. Such an insertion would cause the targeted cell to express the receptors. In some cases multivalence is achieved by inserting separate receptors whereby the inserted receptors function as an OR gate (one or the other triggers activation), or as an AND gate (receptor signaling is co-stimulatory and homovalent binding won't activate/stimulate cell, e.g., a targeted T-cell). A protein encoded by the inserted DNA (e.g., a CAR, a TCR, a multivalent surface receptor) can be selected such that it binds to (e.g., functions to target the cell, e.g., T-cell to) one or more targets selected from: CD3, CD8, CD4, CD28, CD90, CD45f, CD34, CD80, CD86, CD19, CD20, CD22, CD47, CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, NKp44, NKp46, NKp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL2R, IL7R, IL10R, IL12R, IL15R, IL18R, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$.

In some cases the inserted nucleotide sequence encodes a receptor whereby the target that is targeted (bound) by the receptor is specific to an individual's disease (e.g., cancer/tumor). In some cases the inserted nucleotide sequence encodes a heteromultivalent receptor, whereby the combination of targets that are targeted by the heteromultivalent receptor are specific to an individual's disease (e.g., cancer/tumor). As one illustrative example, an individual's cancer (e.g., tumor, e.g., via biopsy) can be sequenced (nucleic acid sequence, proteomics, metabolomics etc.) to identify antigens of diseased cells that can be targets (such as antigens that are overexpressed by or are unique to a tumor relative to control cells of the individual), and a nucleotide sequence encoding a receptor (e.g., heteromultivalent receptor) that binds to one or more of those targets (e.g., 2 or more, 3 or more, 5 or more, 10 or more, 15 or more, or about 20 of those targets) can be inserted into an immune cell (e.g., an NK cell, a

B-Cell, a T-Cell, e.g., using a CAR or TCR) so that the immune cell specifically targets the individual's disease cells (e.g., tumor cells). As such, the inserted nucleotide sequence can be designed to be diagnostically responsive – in the sense that the encoded receptor(s) (e.g., heteromultivalent receptor(s)) can be designed after receiving unique insights related to a patient's proteomics, genomics or metabolomics (e.g., through sequencing etc.) – thus generating an avid and specific immune system response. In this way, immune cells (such as NK cells, B cell, T cells, and the like) can be genome edited to express receptors such as CAR and/or TCR proteins (e.g., heteromultivalent versions) that are designed to be effective against an individual's own disease (e.g., cancer). In some cases, regulatory T cells can be given similar avidity for tissues affected by autoimmunity following diagnostically-responsive medicine.

In some cases the nucleotide sequence, of the donor DNA that is inserted into the cell's genome includes a protein-coding nucleotide sequence that does not have introns. In some cases the nucleotide sequence that does not have introns encodes all or a portion of a TCR protein.

In some embodiments more than one delivery vehicle is introduced into a target cell. For example, in some cases a subject method includes introducing a first and a second of said delivery vehicles into the cell, where the nucleotide sequence of the donor DNA of the first delivery vehicle, that is inserted into the cell's genome, encodes a T cell receptor (TCR) Alpha or Delta subunit, and the nucleotide sequence of the donor DNA of the second delivery vehicle, that is inserted into the cell's genome, encodes a TCR Beta or Gamma subunit. In some cases a subject method includes introducing a first and a second of said delivery vehicles into the cell, where the nucleotide sequence of the donor DNA of the first delivery vehicle, that is inserted into the cell's genome, encodes a T cell receptor (TCR) Alpha or Delta subunit constant region, and the nucleotide sequence of the donor DNA of the second delivery vehicle, that is inserted into the cell's genome, encodes a TCR Beta or Gamma subunit constant region.

In some cases a subject method includes introducing a first and a second of said delivery vehicles into the cell, wherein the nucleotide sequence of the donor DNA of the first delivery vehicle is inserted within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Delta subunit promoter, and the nucleotide sequence of the donor DNA of the second delivery vehicle is inserted within a nucleotide sequence that functions as a TCR Beta or Gamma subunit promoter. For more information related to TCR proteins and CDRs, see, e.g., Dash et al., Nature. 2017 Jul 6;547(7661):89-93. Epub 2017 Jun 21; and Glanville et al., Nature. 2017 Jul 6;547(7661):94-98. Epub 2017 Jun 21.

In some cases a subject method includes introducing a first and a second of said delivery vehicles into the cell, where the nucleotide sequence of the donor DNA of the first

delivery vehicle, that is inserted into the cell's genome, encodes a T cell receptor (TCR) Alpha or Gamma subunit, and the nucleotide sequence of the donor DNA of the second delivery vehicle, that is inserted into the cell's genome, encodes a TCR Beta or Delta subunit. In some cases a subject method includes introducing a first and a second of said delivery vehicles into the cell, where the nucleotide sequence of the donor DNA of the first delivery vehicle, that is inserted into the cell's genome, encodes a T cell receptor (TCR) Alpha or Delta subunit constant region, and the nucleotide sequence of the donor DNA of the second delivery vehicle, that is inserted into the cell's genome, encodes a TCR Beta or Gamma subunit constant region. In some cases a subject method includes introducing a first and a second of said delivery vehicles into the cell, wherein the nucleotide sequence of the donor DNA of the first delivery vehicle is inserted within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Gamma subunit promoter, and the nucleotide sequence of the donor DNA of the second delivery vehicle is inserted within a nucleotide sequence that functions as a TCR Beta or Delta subunit promoter. For more information related to TCR proteins and CDRs, see, e.g., Dash et al., Nature. 2017 Jul 6;547(7661):89-93. Epub 2017 Jun 21; and Glanville et al., Nature. 2017 Jul 6;547(7661):94-98. Epub 2017 Jun 21.

Co-delivery (not necessarily a nanoparticle of the disclosure)

As noted above, one advantage of delivering multiple payloads as part of the same package (delivery vehicle) is that the efficiency of each payload is not diluted. In some embodiments a donor DNA and one or more site-specific nucleases (or one or more nucleic acids encoding same) are payloads of the same delivery vehicle. In some embodiments, a donor DNA and/or one or more gene editing tools (e.g., as described elsewhere herein) is delivered in combination with (e.g., as part of the same package/delivery vehicle, where the delivery vehicle does not need to be a nanoparticle of the disclosure) a protein (and/or a DNA or mRNA encoding same) and/or a non-coding RNA that increases genomic editing efficiency. In some embodiments, one or more gene editing tools is delivered in combination with (e.g., as part of the same package/delivery vehicle, where the delivery vehicle does not need to be a nanoparticle of the disclosure) a protein (and/or a DNA or mRNA encoding same) and/or a non-coding RNA that controls cell division and/or differentiation. For example, in some cases one or more gene editing tools is delivered in combination with (e.g., as part of the same package/delivery vehicle, where the delivery vehicle does not need to be a nanoparticle of the disclosure) a protein (and/or a DNA or mRNA encoding same) and/or a non-coding RNA that controls cell division. In some cases one or more gene editing tools is delivered in combination with (e.g., as part of the same package/delivery vehicle, where the delivery vehicle does not need to be a nanoparticle of the disclosure) a protein (and/or a DNA or mRNA encoding same)

and/or a non-coding RNA that controls differentiation. In some cases, one or more gene editing tools is delivered in combination with (e.g., as part of the same package/delivery vehicle, where the delivery vehicle does not need to be a nanoparticle of the disclosure) a protein (and/or a DNA or mRNA encoding same) and/or a non-coding RNA that biases the cell DNA repair machinery.

As noted above, in some cases the delivery vehicle does not need to be a nanoparticle of the disclosure. For example, in some cases the delivery vehicle is viral and in some cases the delivery vehicle is non-viral. Examples of non-viral delivery systems include materials that can be used to co-condense multiple nucleic acid payloads, or combinations of protein and nucleic acid payloads. Examples include, but are not limited to: (1) lipid based particles such as zwitterionic or cationic lipids, and exosome or exosome-derived vesicles; (2) inorganic/hybrid composite particles such as those that include ionic complexes co-condensed with nucleic acids and/or protein payloads, and complexes that can be condensed from cationic ionic states of Ca, Mg, Si, Fe and physiological anions such as O^{2-} , OH, PO_4^{3-} , SO_4^{2-} ; (3) carbohydrate delivery vehicles such as cyclodextrin and/or alginate; (4) polymeric and/or co-polymeric complexes such as poly(amino-acid) based electrostatic complexes, poly(Amido-Amine), and cationic poly(B-Amino Ester); and (5) virus like particles (e.g., protein and nucleic acid based). Examples of viral delivery systems include but are not limited to: AAV, adenoviral, retroviral, and lentiviral.

Examples of payloads for co-delivery

In some embodiments a donor DNA and/or one or more gene editing tools can be delivered in combination with (e.g., as part of the same package/delivery vehicle, where the delivery vehicle does not need to be a nanoparticle of the disclosure) one or more of: SCF (and/or a DNA or mRNA encoding SCF), HoxB4 (and/or a DNA or mRNA encoding HoxB4), BCL-XL (and/or a DNA or mRNA encoding BCL-XL), SIRT6 (and/or a DNA or mRNA encoding SIRT6), a nucleic acid molecule (e.g., an siRNA and/or an LNA) that suppresses miR-155, a nucleic acid molecule (e.g., an siRNA, an shRNA, a microRNA) that reduces ku70 expression, and a nucleic acid molecule (e.g., an siRNA, an shRNA, a microRNA) that reduces ku80 expression.

For examples of microRNAs (delivered as RNAs or as DNA encoding the RNAs) that can be delivered together, see **Figure 9A**. For example, the following microRNAs can be used for the following purposes: for blocking differentiation of a pluripotent stem cell toward ectoderm lineage: miR-430/427/302; for blocking differentiation of a pluripotent stem cell toward endoderm lineage: miR-109 and/or miR-24; for driving differentiation of a pluripotent stem cell toward endoderm lineage: miR-122 and/or miR-192; for driving differentiation of an ectoderm

progenitor cell toward a keratinocyte fate: miR-203; for driving differentiation of a neural crest stem cell toward a smooth muscle fate: miR-145; for driving differentiation of a neural stem cell toward a glial cell fate and/or toward a neuron fate: miR-9 and/or miR-124a; for blocking differentiation of a mesoderm progenitor cell toward a chondrocyte fate: miR-199a; for driving differentiation of a mesoderm progenitor cell toward an osteoblast fate: miR-296 and/or miR-2861; for driving differentiation of a mesoderm progenitor cell toward a cardiac muscle fate: miR-1; for blocking differentiation of a mesoderm progenitor cell toward a cardiac muscle fate: miR-133; for driving differentiation of a mesoderm progenitor cell toward a skeletal muscle fate: miR-214, miR-206, miR-1 and/or miR-26a; for blocking differentiation of a mesoderm progenitor cell toward a skeletal muscle fate: miR-133, miR-221, and/or miR-222; for driving differentiation of a hematopoietic progenitor cell toward differentiation: miR-223; for blocking differentiation of a hematopoietic progenitor cell toward differentiation: miR-128a and/or miR-181a; for driving differentiation of a hematopoietic progenitor cell toward a lymphoid progenitor cell: miR-181; for blocking differentiation of a hematopoietic progenitor cell toward a lymphoid progenitor cell: miR-146; for blocking differentiation of a hematopoietic progenitor cell toward a myeloid progenitor cell: miR-155, miR-24a, and/or miR-17; for driving differentiation of a lymphoid progenitor cell toward a T cell fate: miR-150; for blocking differentiation of a myeloid progenitor cell toward a granulocyte fate: miR-223; for blocking differentiation of a myeloid progenitor cell toward a monocyte fate: miR-17-5p, miR-20a, and/or miR-106a; for blocking differentiation of a myeloid progenitor cell toward a red blood cell fate: miR-150, miR-155, miR-221, and/or miR-222; and for driving differentiation of a myeloid progenitor cell toward a red blood cell fate: miR-451 and/or miR-16.

For examples of signaling proteins (e.g., extracellular signaling proteins) that can be delivered together with a donor DND and/or one or more gene editing tools (e.g., as described elsewhere herein), see Figure 9B. For example, the following signaling proteins (e.g., extracellular signaling proteins) (e.g., delivered as protein or as a nucleic acid such as DNA or RNA encoding the protein) can be used for the following purposes: for driving differentiation of a hematopoietic stem cell toward a common lymphoid progenitor cell lineage: IL-7; for driving differentiation of a hematopoietic stem cell toward a common myeloid progenitor cell lineage: IL-3, GM-CSF, and/or M-CSF; for driving differentiation of a common lymphoid progenitor cell toward a B-cell fate: IL-3, IL-4, and/or IL-7; for driving differentiation of a common lymphoid progenitor cell toward a Natural Killer Cell fate: IL-15; for driving differentiation of a common lymphoid progenitor cell toward a T-cell fate: IL-2, IL-7, and/or Notch; for driving differentiation of a common lymphoid progenitor cell toward a dendritic cell fate: Flt-3 ligand; for driving differentiation of a common myeloid progenitor cell toward a dendritic cell fate: Flt-3 ligand, GM-CSF, and/or TNF-alpha; for driving differentiation of a common myeloid progenitor cell toward a

granulocyte-macrophage progenitor cell lineage: GM-CSF; for driving differentiation of a common myeloid progenitor cell toward a megakaryocyte-erythroid progenitor cell lineage: IL-3, SCF, and/or Tpo; for driving differentiation of a megakaryocyte-erythroid progenitor cell toward a megakaryocyte fate: IL-3, IL-6, SCF, and/or Tpo; for driving differentiation of a
 5 megakaryocyte-erythroid progenitor cell toward a erythrocyte fate: erythropoietin; for driving differentiation of a megakaryocyte toward a platelet fate: IL-11 and/or Tpo; for driving differentiation of a granulocyte-macrophage progenitor cell toward a monocyte lineage: GM-CSF and/or M-CSF; for driving differentiation of a granulocyte-macrophage progenitor cell toward a myeloblast lineage: GM-CSF; for driving differentiation of a monocyte toward a
 10 monocyte-derived dendritic cell fate: Flt-3 ligand, GM-CSF, IFN-alpha, and/or IL-4; for driving differentiation of a monocyte toward a macrophage fate: IFN-gamma, IL-6, IL-10, and/or M-CSF; for driving differentiation of a myeloblast toward a neutrophil fate: G-CSF, GM-CSF, IL-6, and/or SCF; for driving differentiation of a myeloblast toward a eosinophil fate: GM-CSF, IL-3, and/or IL-5; and for driving differentiation of a myeloblast toward a basophil fate: G-CSF, GM-
 15 CSF, and/or IL-3.

Examples of proteins that can be delivered (e.g., as protein and/or a nucleic acid such as DNA or RNA encoding the protein) together with a donor DNA and/or one or more gene editing tools (e.g., as described elsewhere herein) include but are not limited to: SOX17, HEX, OSKM (Oct4/Sox2/Klf4/c-myc), and/or bFGF (e.g., to drive differentiation toward hepatic stem
 20 cell lineage); HNF4a (e.g., to drive differentiation toward hepatocyte fate); Poly (I:C), BMP-4, bFGF, and/or 8-Br-cAMP (e.g., to drive differentiation toward endothelial stem cell/progenitor lineage); VEGF (e.g., to drive differentiation toward arterial endothelium fate); Sox-2, Brn4, Myt1l, Neurod2, Ascl1 (e.g., to drive differentiation toward neural stem cell/progenitor lineage); and BDNF, FCS, Forskolin, and/or SHH (e.g., to drive differentiation neuron, astrocyte, and/or
 25 oligodendrocyte fate).

Examples of signaling proteins (e.g., extracellular signaling proteins) that can be delivered (e.g., as protein and/or a nucleic acid such as DNA or RNA encoding the protein) together with a donor DNA and/or one or more gene editing tools (e.g., as described elsewhere herein) include but are not limited to: cytokines (e.g., IL-2 and/or IL-15, e.g., for activating CD8+
 30 T-cells); ligands and or signaling proteins that modulate one or more of the Notch, Wnt, and/or Smad signaling pathways; SCF; stem cell programming factors (e.g. Sox2, Oct3/4, Nanog, Klf4, c-Myc, and the like); and temporary surface marker "tags" and/or fluorescent reporters for subsequent isolation/purification/concentration. For example, a fibroblast may be converted into a neural stem cell via delivery of Sox2, while it will turn into a cardiomyocyte in the presence of
 35 Oct3/4 and small molecule "epigenetic resetting factors." In a patient with Huntington's disease or a CXCR4 mutation, these fibroblasts may respectively encode diseased phenotypic traits

associated with neurons and cardiac cells. By delivering gene editing corrections and these factors in a single package, the risk of deleterious effects due to one or more, but not all of the factors/payloads being introduced can be significantly reduced.

Applications include *in vivo* approaches wherein a cell death cue may be conditional upon a gene edit not being successful, and cell differentiation/proliferation/activation is tied to a tissue/organ-specific promoter and/or exogenous factor. A diseased cell receiving a gene edit may activate and proliferate, but due to the presence of another promoter-driven expression cassette (e.g. one tied to the absence of tumor suppressor such as p21 or p53), those cells will subsequently be eliminated. The cells expressing desired characteristics, on the other hand, may be triggered to further differentiate into the desired downstream lineages.

Kits

Also within the scope of the disclosure are kits. For example, in some cases a subject kit can include one or more of (in any combination): (i) a donor DNA; (ii) one or more site-specific nucleases (or one or more nucleic acids encoding same) such as a ZFN pair, a TALEN pair, a nickase Cas9, a Cpf1, etc.; (iii) a targeting ligand, (iv) a linker, (v) a targeting ligand conjugated to a linker, (vi) a targeting ligand conjugated to an anchoring domain (e.g., with or without a linker), (vii) an agent for use as a sheddable layer (e.g., silica), (viii) an additional payload, e.g., an siRNA or a transcription template for an siRNA or shRNA; a gene editing tool, and the like, (ix) a polymer that can be used as a cationic polymer, (x) a polymer that can be used as an anionic polymer, (xi) a polypeptide that can be used as a cationic polypeptide, e.g., one or more HTPs, and (xii) a subject viral or non-viral delivery vehicle. In some cases, a subject kit can include instructions for use. Kits typically include a label indicating the intended use of the contents of the kit. The term label includes any writing, or recorded material, e.g., computer-readable media, supplied on or with the kit, or which otherwise accompanies the kit.

First Illustrative Example of nanoparticle synthesis

Procedures were performed within a sterile, dust free environment (BSL-II hood). Gastight syringes were sterilized with 70% ethanol before rinsing 3 times with filtered nuclease free water, and were stored at 4°C before use. Surfaces were treated with RNase inhibitor prior to use.

Nanoparticle Core

A first solution (an anionic solution) was prepared by combining the appropriate amount of payload (in this case plasmid DNA (EGFP-N1 plasmid) with an aqueous mixture (an 'anionic polymer composition') of poly(D-glutamic Acid) and poly(L-glutamic acid). This solution was

diluted to the proper volume with 10mM Tris-HCl at pH 8.5. A second solution (a cationic solution), which was a combination of a 'cationic polymer composition' and a 'cationic polypeptide composition', was prepared by diluting a concentrated solution containing the appropriate amount of condensing agents to the proper volume with 60mM HEPES at pH 5.5.

- 5 In this case, the 'cationic polymer composition' was poly(L-arginine) and the 'cationic polypeptide composition' was 16 µg of H3K4(me3) (tail of histone H3, tri methylated on K4).

Precipitation of nanoparticle cores in batches less than 200 µl can be carried out by dropwise addition of the condensing solution to the payload solution in glass vials or low protein binding centrifuge tubes followed by incubation for 30 minutes at 4°C. For batches greater than
10 200 µl, the two solutions can be combined in a microfluidic format (e.g., using a standard mixing chip (e.g. Dolomite Micromixer) or a hydrodynamic flow focusing chip). Optimal input flowrates can be determined such that the resulting suspension of nanoparticle cores is monodispersed, exhibiting a mean particle size below 100nm.

In this case, the two equal volume solutions from above (one of cationic condensing
15 agents and one of anionic condensing agents) were prepared for mixing. For the solution of cationic condensing agents, polymer/peptide solutions were added to one protein low bind tube (eppendorf) and were then diluted with 60mM HEPES (pH 5.5) to a total volume of 100 µl (as noted above). This solution was kept at room temperature while preparing the anionic solution. For the solution of anionic condensing agents, the anionic solutions were chilled on ice with
20 minimal light exposure. 10µg of nucleic acid in aqueous solution (roughly 1 µg/µl) and 7µg of aqueous poly (D-Glutamic Acid) [.1%] were diluted with 10mM Tris-HCl (pH 8.5) to a total volume of 100 µl (as noted above).

Each of the two solutions was filtered using a 0.2-micron syringe filter and transferred to its own Hamilton 1ml Gastight Syringe (Glass, (insert product number)). Each syringe was
25 placed on a Harvard Pump 11 Elite Dual Syringe Pump. The syringes were connected to appropriate inlets of a Dolomite Micro Mixer chip using tubing, and the syringe pump was run at 120 µl/min for a 100 µl total volume. The resulting solution included the core composition (which now included nucleic acid payload, anionic components, and cationic components).

30 *Core Stabilization (adding a sheddable layer)*

To coat the core with a sheddable layer, the resulting suspension of nanoparticle cores was then combined with a dilute solution of sodium silicate in 10mM Tris HCl (pH8.5, 10 – 500mM) or calcium chloride in 10mM PBS (pH 8.5, 10 – 500mM), and allowed to incubate for 1-2 hours at room temperature. In this case, the core composition was added to a diluted
35 sodium silicate solution to coat the core with an acid labile coating of polymeric silica (an example of a sheddable layer). To do so, 10 µl of stock Sodium Silicate (Sigma) was first

dissolved in 1.99 ml of Tris buffer (10mM Tris pH = 8.5, 1:200 dilution) and was mixed thoroughly. The Silicate solution was filtered using a sterile 0.1-micron syringe filter, and was transferred to a sterile Hamilton Gastight syringe, which was mounted on a syringe pump. The core composition from above was also transferred to a sterile Hamilton Gastight syringe, which
5 was also mounted on the syringe pump. The syringes were connected to the appropriate inlets of a Dolomite Micro Mixer chip using PTFE tubing, and the syringe pump was run at 120 μ l/min.

Stabilized (coated) cores can be purified using standard centrifugal filtration devices (100 kDa Amicon Ultra, Millipore) or dialysis in 30mM HEPES (pH 7.4) using a high molecular weight cutoff membrane. In this case, the stabilized (coated) cores were purified using a
10 centrifugal filtration device. The collected coated nanoparticles (nanoparticle solution) were washed with dilute PBS (1:800) or HEPES and filtered again (the solution can be resuspended in 500 μ l sterile dispersion buffer or nuclease free water for storage). Effective silica coating was demonstrated. The stabilized cores had a size of 110.6 nm and zeta potential of -42.1 mV (95%).

Surface Coat (Outer shell)

Addition of a surface coat (also referred to as an outer shell), sometimes referred to as "surface functionalization," was accomplished by electrostatically grafting ligand species (in this case Rabies Virus Glycoprotein fused to a 9-Arg peptide sequence as a cationic anchoring
20 domain – 'RVG9R') to the negatively charged surface of the stabilized (in this case silica coated) nanoparticles. Beginning with silica coated nanoparticles that were filtered and resuspended in dispersion buffer or water, the final volume of each nanoparticle dispersion was determined, as was the desired amount of polymer or peptide to add such that the final concentration of protonated amine group was at least 75 μ M. The desired surface constituents
25 were added and the solution was sonicated for 20-30 seconds prior to incubate for 1 hour. Centrifugal filtration was performed at 300 kDa (the final product can be purified using standard centrifugal filtration devices, e.g., 300-500kDa from Amicon Ultra Millipore, or dialysis, e.g., in 30mM HEPES (pH 7.4) using a high molecular weight cutoff membrane), and the final resuspension was in either cell culture media or dispersion buffer. In some cases, optimal outer
30 shell addition yields a monodispersed suspension of particles with a mean particle size between 50 and 150 nm and a zeta potential between 0 and -10 mV. In this case, the nanoparticles with an outer shell had a size of 115.8 nm and a Zeta potential of -3.1 mV (100%).

Second Illustrative Example of nanoparticle synthesis

Nanoparticles were synthesized at room temperature, 37C or a differential of 37C and room temperature between cationic and anionic components. Solutions were prepared in aqueous buffers utilizing natural electrostatic interactions during mixing of cationic and anionic components. At the start, anionic components were dissolved in Tris buffer (30mM - 60mM; pH = 7.4 - 9) or HEPES buffer (30mM, pH = 5.5) while cationic components were dissolved in HEPES buffer (30mM - 60mM, pH = 5 - 6.5).

Specifically, payloads (e.g., genetic material (RNA or DNA), genetic material-protein-nuclear localization signal polypeptide complex (ribonucleoprotein), or polypeptide) were reconstituted in a basic, neutral or acidic buffer. For analytical purposes, the payload was manufactured to be covalently tagged with or genetically encode a fluorophore. With pDNA payloads, a Cy5-tagged peptide nucleic acid (PNA) specific to AGAGAG tandem repeats was used to fluorescently tag fluorescent reporter vectors and fluorescent reporter-therapeutic gene vectors. A timed-release component that may also serve as a negatively charged condensing species (e.g. poly(glutamic acid)) was also reconstituted in a basic, neutral or acidic buffer. Targeting ligands with a wild-type derived or wild-type mutated targeting peptide conjugated to a linker-anchor sequence were reconstituted in acidic buffer. In the case where additional condensing species or nuclear localization signal peptides were included in the nanoparticle, these were also reconstituted in buffer as 0.03% w/v working solutions for cationic species, and 0.015% w/v for anionic species. Experiments were also conducted with 0.1% w/v working solutions for cationic species and 0.1% w/v for anionic species. All polypeptides, except those complexing with genetic material, were sonicated for ten minutes to improve solubilization.

Exemplary Non-Limiting Aspects of the Disclosure

Aspects, including embodiments, of the present subject matter described above may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, certain non-limiting aspects of the disclosure are provided below in SET A and SET B. As will be apparent to those of ordinary skill in the art upon reading this disclosure, each of the individually numbered aspects may be used or combined with any of the preceding or following individually numbered aspects. This is intended to provide support for all such combinations of aspects and is not limited to combinations of aspects explicitly provided below. It will be apparent to one of ordinary skill in the art that various changes and modifications can be made without departing from the spirit or scope of the invention.

SET A

1. A method of genome editing in a target cell, comprising:
 - (a) generating double stranded cuts with staggered ends at two locations within the target cell's genome, thereby producing a first genomic staggered end and a second genomic staggered end; and
 - (b) introducing into the target cell a linear double stranded donor DNA having a 5' or 3' overhang at each end,wherein one end of the donor DNA hybridizes with the first genomic staggered end and the other end of the donor DNA hybridizes with the second genomic staggered end, thereby resulting in insertion of the linear double stranded donor DNA into the target cell's genome.
2. The method of 1, wherein at least one end of the donor DNA has a 5' overhang and at least one of the genomic staggered ends has a 5' overhang.
3. The method of 1 or 2, wherein at least one end of the donor DNA has a 3' overhang and at least one of the genomic staggered ends has a 3' overhang.
4. The method of any one of 1-3, wherein said generating comprises introducing one or more sequence specific nucleases, or one or more nucleic acids encoding the one or more sequence specific nucleases, into the target cell to generate said double stranded cuts.
5. The method of 4, wherein the one or more sequence specific nucleases comprises at least one of: a meganuclease, a homing endonuclease, a zinc finger nuclease (ZFN), and a transcription activator-like effector nuclease (TALEN).
6. The method of any one of 4, wherein the one or more sequence specific nucleases comprises a staggered end cutting CRISPR/Cas effector protein.
7. The method of 6, wherein said generating further comprises introducing a CRISPR/Cas guide nucleic acid, or a nucleic acid encoding the CRISPR/Cas guide nucleic acid, into the cell.
8. The method of any one of 4-7, wherein the method comprises introducing into the cell, as payloads of the same delivery vehicle: (i) the one or more sequence specific nucleases, or one or more nucleic acids encoding the one or more sequence specific nucleases, and (ii) the linear double stranded donor DNA.
9. The method of 8, wherein the one or more sequence specific nucleases and the linear double stranded donor DNA are introduced into the cell as a deoxyribonucleoprotein complex or a ribo-deoxyribonucleoprotein complex.
10. The method of 8 or 9, wherein during said introducing, the ends of the donor DNA are bound in a site-specific manner to the one or more sequence specific nucleases.
11. The method of any one of 8-10, wherein the delivery vehicle is non-viral.
12. The method of any one of 8-11, wherein the delivery vehicle is a nanoparticle.
13. The method of 12, wherein, in addition to (i) and (ii), the nanoparticle comprises a core

comprising an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition.

14. The method of 13, wherein said anionic polymer composition comprises an anionic polymer selected from poly(glutamic acid) and poly(aspartic acid).

15. The method of 13 or 14, wherein said cationic polymer composition comprises a cationic polymer selected from poly(arginine), poly(lysine), poly(histidine), poly(ornithine), and poly(citrulline).

16. The method of any one of 13-15, wherein nanoparticle further comprises a sheddable layer encapsulating the core.

17. The method of 16, wherein the sheddable layer is an anionic coat or a cationic coat.

18. The method of 16 or 17, wherein the sheddable layer comprises one or more of: silica, a peptoid, a polycysteine, calcium, calcium oxide, hydroxyapatite, calcium phosphate, calcium sulfate, manganese, manganese oxide, manganese phosphate, manganese sulfate, magnesium, magnesium oxide, magnesium phosphate, magnesium sulfate, iron, iron oxide, iron phosphate, and iron sulfate.

19. The method of any one of 16-18, wherein the nanoparticle further comprises a surface coat surrounding the sheddable layer.

20. The method of 19, wherein the surface coat comprises a cationic or anionic anchoring domain that interacts electrostatically with the sheddable layer.

21. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands.

22. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands selected from the group consisting of: rabies virus glycoprotein (RVG) fragment, ApoE-transferrin, lactoferrin, melanoferritin, ovotransferritin, L-selectin, E-selectin, P-selectin, sialylated peptides, polysialylated O-linked peptides, TPO, EPO, PSGL-1, ESL-1, CD44, death receptor-3 (DR3), LAMP1, LAMP2, Mac2-BP, stem cell factor (SCF), CD70, SH2 domain-containing protein 1A (SH2D1A), a exendin-4, GLP1, RGD, a Transferrin ligand, an FGF fragment, succinic acid, a bisphosphonate, a hematopoietic stem cell chemotactic lipid, sphingosine, ceramide, sphingosine-1-phosphate, ceramide-1-phosphate, and an active targeting fragment of any of the above.

23. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands that provides for targeted binding to a target selected from: CD3, CD28, CD90, CD45f, CD34, CD80, CD86, CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, NKp44, NKp46, NKp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL-2, IL-

7, IL-10, IL-12, IL-15, IL-18, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$.

24. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands that provides for targeted binding to target cells selected from: bone marrow cells, hematopoietic stem cells (HSCs), hematopoietic stem and progenitor cells (HSPCs), peripheral blood mononuclear cells (PBMCs), myeloid progenitor cells, lymphoid progenitor cells, T-cells, B-cells, NKT cells, NK cells, dendritic cells, monocytes, granulocytes, erythrocytes, megakaryocytes, mast cells, basophils, eosinophils, neutrophils, macrophages, erythroid progenitor cells, megakaryocyte-erythroid progenitor cells (MEPs), common myeloid progenitor cells (CMPs), multipotent progenitor cells (MPPs), hematopoietic stem cells (HSCs), short term HSCs (ST-HSCs), IT-HSCs, long term HSCs (LT-HSCs), endothelial cells, neurons, astrocytes, pancreatic cells, pancreatic β -islet cells, liver cells, muscle cells, skeletal muscle cells, cardiac muscle cells, hepatic cells, fat cells, intestinal cells, cells of the colon, and cells of the stomach.

25. The method of any one of 8-10, wherein the delivery vehicle is a targeting ligand conjugated to the payload, wherein the targeting ligand provides for targeted binding to a cell surface protein.

26. The method of any one of 8-10, wherein the delivery vehicle is a targeting ligand conjugated to a charged polymer polypeptide domain, wherein the targeting ligand provides for targeted binding to a cell surface protein, and wherein the charged polymer polypeptide domain is condensed with a nucleic acid payload and/or is interacting electrostatically with a protein payload.

27. The method of 25 or 26, wherein the targeting ligand is a peptide, an ScFv, a F(ab), a nucleic acid aptamer, or a peptoid.

28. The method of 26, wherein the charged polymer polypeptide domain has a length in a range of from 3 to 30 amino acids.

29. The method of any one of 26-28, wherein the delivery vehicle further comprises an anionic polymer interacting with the payload and the charged polymer polypeptide domain.

30. The method of 29, wherein the anionic polymer is selected from poly(glutamic acid) and poly(aspartic acid).

31. The method of any one of 25-30, wherein the targeting ligand has a length of from 5-50 amino acids.

32. The method of any one of 25-31, wherein the targeting ligand provides for targeted binding to a cell surface protein selected from a family B G-protein coupled receptor (GPCR), a receptor tyrosine kinase (RTK), a cell surface glycoprotein, and a cell-cell adhesion molecule.

33. The method of any one of 25-31, wherein the targeting ligand is selected from the group consisting of: rabies virus glycoprotein (RVG) fragment, ApoE-transferrin, lactoferrin, melanoferritin, ovotransferrin, L-selectin, E-selectin, P-selectin, sialylated peptides,

polysialylated O-linked peptides, TPO, EPO, PSGL-1, ESL-1, CD44, death receptor-3 (DR3), LAMP1, LAMP2, Mac2-BP, stem cell factor (SCF), CD70, SH2 domain-containing protein 1A (SH2D1A), a exendin-4, GLP1, RGD, a Transferrin ligand, an FGF fragment, succinic acid, a bisphosphonate, a hematopoietic stem cell chemotactic lipid, sphingosine, ceramide, sphingosine-1-phosphate, ceramide-1-phosphate, and an active targeting fragment of any of the above.

34. The method of any one of 25-31, wherein the targeting ligand provides for targeted binding to a target selected from: CD3, CD28, CD90, CD45f, CD34, CD80, CD86, CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, NKp44, NKp46, NKp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL-2, IL-7, IL-10, IL-12, IL-15, IL-18, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$.

35. The method of any one of 25-31, wherein the targeting ligand provides for binding to a cell type selected from the group consisting of: bone marrow cells, hematopoietic stem cells (HSCs), long-term HSCs, short-term HSCs, hematopoietic stem and progenitor cells (HSPCs), peripheral blood mononuclear cells (PBMCs), myeloid progenitor cells, lymphoid progenitor cells, T-cells, B-cells, NKT cells, NK cells, dendritic cells, monocytes, granulocytes, erythrocytes, megakaryocytes, mast cells, basophils, eosinophils, neutrophils, macrophages, erythroid progenitor cells (e.g., HUDEP cells), megakaryocyte-erythroid progenitor cells (MEPs), common myeloid progenitor cells (CMPs), multipotent progenitor cells (MPPs), hematopoietic stem cells (HSCs), short term HSCs (ST-HSCs), IT-HSCs, long term HSCs (LT-HSCs), endothelial cells, neurons, astrocytes, pancreatic cells, pancreatic β -islet cells, muscle cells, skeletal muscle cells, cardiac muscle cells, hepatic cells, fat cells, intestinal cells, cells of the colon, and cells of the stomach.

36. The method of any one of 1-35, wherein, prior to generating the double stranded cuts at said two locations within the target cell's genome, the two locations are separated by 1,000,000 base pairs or less.

37. The method of any one of 1-35, wherein, prior to generating the double stranded cuts at said two locations within the target cell's genome, the two locations are separated by 100,000 base pairs or less.

38. The method of any one of 1-35, wherein the donor DNA has a total of from 10 base pairs (bp) to 100 kilobase pairs (kbp).

39. The method of any one of 1-38, wherein the insertion of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) protein.

40. The method of 39, wherein the donor DNA encodes amino acids of a CDR1, CDR2, or

CDR3 region of the TCR protein.

41. The method of any one of 1-38, wherein the donor DNA comprises a nucleotide sequence encoding a chimeric antigen receptor (CAR), and wherein insertion of said donor DNA results in operable linkage of the nucleotide sequence encoding the CAR to an endogenous T-cell promoter.

42. The method of any one of 1-38, wherein the donor DNA comprises a nucleotide sequence that is operably linked to a promoter and encodes a chimeric antigen receptor (CAR).

43. The method of any one of 1-38, wherein the donor DNA comprises a nucleotide sequence encoding a cell-specific targeting ligand that is membrane bound and presented extracellularly, and wherein insertion of said donor DNA results in operable linkage of the nucleotide sequence encoding the cell-specific targeting ligand to an endogenous promoter.

44. The method of any one of 1-38, wherein the donor DNA comprises a promoter operably linked to a sequence that encodes a cell-specific targeting ligand that is membrane bound and presented extracellularly.

45. The method of any one of 1-38, wherein the method comprises:
generating double stranded cuts with staggered ends at four locations within the target cell's genome, thereby producing a third genomic staggered end and a fourth genomic staggered end in addition to the first and second genomic staggered ends; and

introducing two linear double stranded donor DNAs, each having a 5' or 3' overhang at each end,

wherein the ends of one donor DNA hybridize with the first and second genomic staggered ends and the ends of the other donor DNA hybridize with the third and fourth genomic staggered ends and the ends,

thereby resulting in insertion of said two donor DNAs into the target cell's genome.

46. The method of 45, wherein insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Gamma subunit.

47. The method of 45, wherein insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit constant region, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Gamma subunit constant region.

48. The method of 45, wherein insertion of one donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Delta subunit promoter, and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a TCR Beta or Gamma subunit promoter.

49. The method of any one of 1-48, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a T cell receptor (TCR) Alpha, Beta, Gamma or Delta endogenous promoter.
50. The method of any one of 1-48, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a TCR Alpha, Beta, Gamma or Delta promoter.
51. The method of any one of 1-48, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a CD3 or CD28 promoter.
52. The method of any one of 1-48, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a T-cell specific promoter.
53. The method of any one of 1-48, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a promoter.
54. The method of any one of 1-48, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a stem cell specific or somatic cell specific endogenous promoter.
55. The method of any one of 1-54, wherein the donor DNA comprises a nucleotide sequence that encodes a reporter protein (e.g., a near-IR and/or far red reporter protein, e.g., for evaluating gene editing efficiency).
56. The method of 55, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with an endogenous promoter.
57. The method of 55, wherein the donor DNA comprises a promoter that is operably linked to the nucleotide sequence that encodes the reporter protein.
58. The method of any one of 1-57, wherein the donor DNA comprises a protein-coding nucleotide sequence that does not have introns.
59. The method of 58, wherein the nucleotide sequence that does not have introns encodes all or a portion of a TCR protein.
60. The method of any one of 1-59, wherein the donor DNA has at least one adenylated 3' end.
61. The method of any one of 1-60, wherein the target cell is a mammalian cell.
62. The method of any one of 1-61, wherein the target cell is a human cell.
63. A kit or composition comprising:
(a) a linear double stranded donor DNA having a 5' or 3' overhang at each end; and
(b) a sequence specific nuclease, or a nucleic acid encoding the sequence specific nucleases,
wherein (a) and (b) are payloads as part of the same delivery vehicle.
64. The kit or composition of 63, wherein the delivery vehicle is a nanoparticle.
65. The kit or composition of 64, wherein the nanoparticle comprises a core comprising (a),

(b), an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition.

66. The kit or composition of 64 or 65, wherein the nanoparticle comprises a targeting ligand that targets the nanoparticle to a cell surface protein.

67. The kit or composition of any one of 63-66, wherein in the linear double stranded donor and the sequence specific nuclease are bound to one another forming a deoxyribonucleoprotein or ribo-deoxyribonucleoprotein complex.

68. The kit or composition of any one of 63-67, wherein the delivery vehicle is a targeting ligand conjugated to a charged polymer polypeptide domain, wherein the targeting ligand provides for targeted binding to a cell surface protein, and wherein the charged polymer polypeptide domain is interacting electrostatically with the payloads.

69. The kit or composition of 68, wherein the delivery vehicle further comprises an anionic polymer interacting with the payload and the charged polymer polypeptide domain.

70. The kit or composition of any one of 63-67, wherein the delivery vehicle is a targeting ligand conjugated to (a) and/or (b), wherein the targeting ligand provides for targeted binding to a cell surface protein.

71. The kit or composition of any one of 63-67, wherein the delivery vehicle includes a targeting ligand coated upon a water-oil-water emulsion particle, upon an oil-water emulsion micellar particle, upon a multilamellar water-oil-water emulsion particle, upon a multilayered particle, or upon a DNA origami nanobot.

72. The method of any one of 66-71, wherein the targeting ligand is a peptide, an ScFv, a F(ab), a nucleic acid aptamer, or a peptoid.

73. The kit or composition of any one of 63-67, wherein the delivery vehicle is non-viral.

SET B

1. A method of genome editing in a target cell, comprising:

(a) generating double stranded cuts with staggered ends at two locations within the target cell's genome, thereby producing a first genomic staggered end and a second genomic staggered end; and

(b) introducing into the target cell a linear double stranded donor DNA having a 5' or 3' overhang at each end,

wherein one end of the donor DNA hybridizes with the first genomic staggered end and the other end of the donor DNA hybridizes with the second genomic staggered end, thereby resulting in insertion of the linear double stranded donor DNA into the target cell's genome.

2. The method of 1, wherein at least one end of the donor DNA has a 5' overhang and at

least one of the genomic staggered ends has a 5' overhang.

3. The method of 1 or 2, wherein at least one end of the donor DNA has a 3' overhang and at least one of the genomic staggered ends has a 3' overhang.

4. The method of any one of 1-3, wherein said generating comprises introducing one or more sequence specific nucleases, or one or more nucleic acids encoding the one or more sequence specific nucleases, into the target cell to generate said double stranded cuts.

5. The method of 4, wherein the one or more sequence specific nucleases comprises at least one of: a meganuclease, a homing endonuclease, a zinc finger nuclease (ZFN), and a transcription activator-like effector nuclease (TALEN).

6. The method of any one of 4, wherein the one or more sequence specific nucleases comprises a staggered end cutting CRISPR/Cas effector protein.

7. The method of 6, wherein said generating further comprises introducing a CRISPR/Cas guide nucleic acid, or a nucleic acid encoding the CRISPR/Cas guide nucleic acid, into the cell.

8. The method of any one of 4-7, wherein the method comprises introducing into the cell, as payloads of the same delivery vehicle: (i) the one or more sequence specific nucleases, or one or more nucleic acids encoding the one or more sequence specific nucleases, and (ii) the linear double stranded donor DNA.

9. The method of 8, wherein the one or more sequence specific nucleases and the linear double stranded donor DNA are introduced into the cell as a deoxyribonucleoprotein complex or a ribo-deoxyribonucleoprotein complex.

10. The method of 8 or 9, wherein during said introducing, the ends of the donor DNA are bound in a site-specific manner to the one or more sequence specific nucleases.

11. The method of any one of 8-10, wherein the delivery vehicle is non-viral.

12. The method of any one of 8-11, wherein the delivery vehicle is a nanoparticle.

13. The method of 12, wherein, in addition to (i) and (ii), the nanoparticle comprises a core comprising an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition.

14. The method of 13, wherein said anionic polymer composition comprises an anionic polymer selected from poly(glutamic acid) and poly(aspartic acid).

15. The method of 13 or 14, wherein said cationic polymer composition comprises a cationic polymer selected from poly(arginine), poly(lysine), poly(histidine), poly(ornithine), and poly(citrulline).

16. The method of any one of 13-15, wherein nanoparticle further comprises a sheddable layer encapsulating the core.

17. The method of 16, wherein the sheddable layer is an anionic coat or a cationic coat.

18. The method of 16 or 17, wherein the sheddable layer comprises one or more of: silica, a peptoid, a polycysteine, calcium, calcium oxide, hydroxyapatite, calcium phosphate, calcium sulfate, manganese, manganese oxide, manganese phosphate, manganese sulfate, magnesium, magnesium oxide, magnesium phosphate, magnesium sulfate, iron, iron oxide, iron phosphate, and iron sulfate.

19. The method of any one of 16-18, wherein the nanoparticle further comprises a surface coat surrounding the sheddable layer.

20. The method of 19, wherein the surface coat comprises a cationic or anionic anchoring domain that interacts electrostatically with the sheddable layer.

21. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands.

22. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands selected from the group consisting of: rabies virus glycoprotein (RVG) fragment, ApoE-transferrin, lactoferrin, melanoferritin, ovotransferrin, L-selectin, E-selectin, P-selectin, sialylated peptides, polysialylated O-linked peptides, TPO, EPO, PSGL-1, ESL-1, CD44, death receptor-3 (DR3), LAMP1, LAMP2, Mac2-BP, stem cell factor (SCF), CD70, SH2 domain-containing protein 1A (SH2D1A), exendin, exendin-S11C, GLP1, RGD, a Transferrin ligand, an FGF fragment, an $\alpha 5\beta 1$ ligand, IL2, Cde3-epsilon, peptide-HLA-A*2402, CD80, CD86, succinic acid, a bisphosphonate, a hematopoietic stem cell chemotactic lipid, sphingosine, ceramide, sphingosine-1-phosphate, ceramide-1-phosphate, and an active targeting fragment of any of the above.

23. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands that provides for targeted binding to a target selected from: CD3, CD8, CD4, CD28, CD90, CD45f, CD34, CD80, CD86, CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, NKp44, NKp46, NKp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL2R, IL7R, IL10R, IL12R, IL15R, IL18R, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$.

24. The method of 19 or 20, wherein the surface coat comprises one or more targeting ligands that provides for targeted binding to target cells selected from: bone marrow cells, hematopoietic stem cells (HSCs), hematopoietic stem and progenitor cells (HSPCs), peripheral blood mononuclear cells (PBMCs), myeloid progenitor cells, lymphoid progenitor cells, T-cells, B-cells, NKT cells, NK cells, dendritic cells, monocytes, granulocytes, erythrocytes, megakaryocytes, mast cells, basophils, eosinophils, neutrophils, macrophages, erythroid progenitor cells, megakaryocyte-erythroid progenitor cells (MEPs), common myeloid progenitor cells (CMPs), multipotent progenitor cells (MPPs), hematopoietic stem cells (HSCs), short term

HSCs (ST-HSCs), IT-HSCs, long term HSCs (LT-HSCs), endothelial cells, neurons, astrocytes, pancreatic cells, pancreatic β -islet cells, liver cells, muscle cells, skeletal muscle cells, cardiac muscle cells, hepatic cells, fat cells, intestinal cells, cells of the colon, and cells of the stomach.

25. The method of any one of 8-10, wherein the delivery vehicle is a targeting ligand conjugated to the payload, wherein the targeting ligand provides for targeted binding to a cell surface protein.

26. The method of any one of 8-10, wherein the delivery vehicle is a targeting ligand conjugated to a charged polymer polypeptide domain, wherein the targeting ligand provides for targeted binding to a cell surface protein, and wherein the charged polymer polypeptide domain is condensed with a nucleic acid payload and/or is interacting electrostatically with a protein payload.

27. The method of 25 or 26, wherein the targeting ligand is a peptide, an ScFv, a F(ab), a nucleic acid aptamer, or a peptoid.

28. The method of 26, wherein the charged polymer polypeptide domain has a length in a range of from 3 to 30 amino acids.

29. The method of any one of 26-28, wherein the delivery vehicle further comprises an anionic polymer interacting with the payload and the charged polymer polypeptide domain.

30. The method of 29, wherein the anionic polymer is selected from poly(glutamic acid) and poly(aspartic acid).

31. The method of any one of 25-30, wherein the targeting ligand has a length of from 5-50 amino acids.

32. The method of any one of 25-31, wherein the targeting ligand provides for targeted binding to a cell surface protein selected from a family B G-protein coupled receptor (GPCR), a receptor tyrosine kinase (RTK), a cell surface glycoprotein, and a cell-cell adhesion molecule.

33. The method of any one of 25-31, wherein the targeting ligand is selected from the group consisting of: rabies virus glycoprotein (RVG) fragment, ApoE-transferrin, lactoferrin, melanoferritin, ovotransferrin, L-selectin, E-selectin, P-selectin, sialylated peptides, polysialylated O-linked peptides, TPO, EPO, PSGL-1, ESL-1, CD44, death receptor-3 (DR3), LAMP1, LAMP2, Mac2-BP, stem cell factor (SCF), CD70, SH2 domain-containing protein 1A (SH2D1A), exendin, exendin-S11C, GLP1, RGD, a Transferrin ligand, an FGF fragment, an $\alpha 5\beta 1$ ligand, IL2, Cde3-epsilon, peptide-HLA-A*2402, CD80, CD86, succinic acid, a bisphosphonate, a hematopoietic stem cell chemotactic lipid, sphingosine, ceramide, sphingosine-1-phosphate, ceramide-1-phosphate, and an active targeting fragment of any of the above.

34. The method of any one of 25-31, wherein the targeting ligand provides for targeted binding to a target selected from: CD3, CD8, CD4, CD28, CD90, CD45f, CD34, CD80, CD86,

CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, Nkp44, Nkp46, Nkp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL2R, IL7R, IL10R, IL12R, IL15R, IL18R, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$.

35. The method of any one of 25-31, wherein the targeting ligand provides for binding to a cell type selected from the group consisting of: bone marrow cells, hematopoietic stem cells (HSCs), long-term HSCs, short-term HSCs, hematopoietic stem and progenitor cells (HSPCs), peripheral blood mononuclear cells (PBMCs), myeloid progenitor cells, lymphoid progenitor cells, T-cells, B-cells, NKT cells, NK cells, dendritic cells, monocytes, granulocytes, erythrocytes, megakaryocytes, mast cells, basophils, eosinophils, neutrophils, macrophages, erythroid progenitor cells (e.g., HUDEP cells), megakaryocyte-erythroid progenitor cells (MEPs), common myeloid progenitor cells (CMPs), multipotent progenitor cells (MPPs), hematopoietic stem cells (HSCs), short term HSCs (ST-HSCs), IT-HSCs, long term HSCs (LT-HSCs), endothelial cells, neurons, astrocytes, pancreatic cells, pancreatic β -islet cells, muscle cells, skeletal muscle cells, cardiac muscle cells, hepatic cells, fat cells, intestinal cells, cells of the colon, and cells of the stomach.

36. The method of any one of 1-35, wherein, prior to generating the double stranded cuts at said two locations within the target cell's genome, the two locations are separated by 1,000,000 base pairs or less.

37. The method of any one of 1-35, wherein, prior to generating the double stranded cuts at said two locations within the target cell's genome, the two locations are separated by 100,000 base pairs or less.

38. The method of any one of 1-35, wherein, the first and second genomic staggered ends are produced at the TCR alpha locus or the TCR beta locus.

39. The method of any one of 1-35, where at least one of the first and second genomic staggered ends are produced (1) using one or more of the CRISPR/Cas guide RNA (gRNA) sequences depicted in Figure 59, and/or (2) by targeting one or more of the TALEN sequences depicted in Figure 59.

40. The method of any one of 1-35, wherein the donor DNA has a total of from 10 base pairs (bp) to 100 kilobase pairs (kbp).

41. The method of any one of 1-40, wherein the insertion of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) protein.

42. The method of 41, wherein the donor DNA encodes amino acids of a CDR1, CDR2, or CDR3 region of the TCR protein.

43. The method of any one of 1-40, wherein the donor DNA comprises a nucleotide

sequence encoding a chimeric antigen receptor (CAR), and wherein insertion of said donor DNA results in operable linkage of the nucleotide sequence encoding the CAR to an endogenous T-cell promoter.

44. The method of any one of 1-40, wherein the donor DNA comprises a nucleotide sequence that is operably linked to a promoter and encodes a chimeric antigen receptor (CAR).

45. The method of any one of 1-40, wherein the donor DNA comprises a nucleotide sequence encoding a cell-specific targeting ligand that is membrane bound and presented extracellularly, and wherein insertion of said donor DNA results in operable linkage of the nucleotide sequence encoding the cell-specific targeting ligand to an endogenous promoter.

46. The method of any one of 1-40, wherein the donor DNA comprises a promoter operably linked to a sequence that encodes a cell-specific targeting ligand that is membrane bound and presented extracellularly.

47. The method of any one of 1-40, wherein the method comprises:

generating double stranded cuts with staggered ends at four locations within the target cell's genome, thereby producing a third genomic staggered end and a fourth genomic staggered end in addition to the first and second genomic staggered ends; and

introducing two linear double stranded donor DNAs, each having a 5' or 3' overhang at each end,

wherein the ends of one donor DNA hybridize with the first and second genomic staggered ends and the ends of the other donor DNA hybridize with the third and fourth genomic staggered ends and the ends,

thereby resulting in insertion of said two donor DNAs into the target cell's genome.

48. The method of 47, wherein:

(1) insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Gamma subunit; or

(2) insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Gamma subunit, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Delta subunit; or

(3) insertion of one donor DNA occurs within a nucleotide sequence that encodes the κ chain of an IgA, IgD, IgE, IgG, or IgM protein, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes the λ chain of an IgA, IgD, IgE, IgG, or IgM protein.

49. The method of 47, wherein insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit constant region, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Gamma subunit constant region.

50. The method of 47, wherein:

(1) insertion of one donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Delta subunit promoter, and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a TCR Beta or Gamma subunit promoter; or

(2) insertion of one donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Gamma subunit promoter, and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a TCR Beta or Delta subunit promoter; or

(3) insertion of one donor DNA occurs within a nucleotide sequence that functions as a promoter for a κ chain of an IgA, IgD, IgE, IgG, or IgM protein, and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a promoter for a λ chain of an IgA, IgD, IgE, IgG, or IgM protein.

51. The method of any one of 1-50, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a T cell receptor (TCR) Alpha, Beta, Gamma or Delta endogenous promoter.

52. The method of any one of 1-50, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a TCR Alpha, Beta, Gamma or Delta promoter.

53. The method of any one of 1-50, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a promoter selected from the group consisting of: (i) a T-cell specific promoter; (ii) a CD3 promoter; (iii) a CD28 promoter; (iv) a stem cell specific promoter; (v) a somatic cell specific promoter; (vi) a T cell receptor (TCR) Alpha, Beta, Gamma or Delta promoter; (v) a B-cell specific promoter; (vi) a CD19 promoter; (vii) a CD20 promoter; (viii) a CD22 promoter; (ix) a B29 promoter; and (x) a T-cell or B-cell V(D)J-specific promoter.

54. The method of any one of 1-50, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a T-cell specific promoter.

55. The method of any one of 1-50, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a promoter.

56. The method of any one of 1-50, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a stem cell specific or somatic cell specific endogenous promoter.

57. The method of any one of 1-56, wherein the donor DNA comprises a nucleotide sequence that encodes a reporter protein (e.g., a near-IR and/or far red reporter protein, e.g., for evaluating gene editing efficiency).

58. The method of 57, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with an endogenous promoter.

59. The method of 57, wherein the donor DNA comprises a promoter that is operably linked

to the nucleotide sequence that encodes the reporter protein.

60. The method of any one of 1-59, wherein the donor DNA comprises a nucleotide sequence that encodes (i) a T cell receptor (TCR) protein; (ii) an IgA, IgD, IgE, IgG, or IgM protein; or (iii) the κ or λ chains of an IgA, IgD, IgE, IgG, or IgM protein.

61. The method of any one of 1-60, wherein the donor DNA comprises a protein-coding nucleotide sequence that does not have introns.

62. The method of 61, wherein the nucleotide sequence that does not have introns encodes all or a portion of a TCR protein or an Immunoglobulin.

63. The method of any one of 1-62, wherein the donor DNA has at least one adenylated 3' end.

64. The method of any one of 1-63, wherein the target cell is a mammalian cell.

65. The method of any one of 1-64, wherein the target cell is a human cell.

66. A kit or composition comprising:

(a) a linear double stranded donor DNA having a 5' or 3' overhang at each end; and

(b) a sequence specific nuclease, or a nucleic acid encoding the sequence specific nucleases,

wherein (a) and (b) are payloads as part of the same delivery vehicle.

67. The kit or composition of 66, wherein the delivery vehicle is a nanoparticle.

68. The kit or composition of 67, wherein the nanoparticle comprises a core comprising (a), (b), an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition.

69. The kit or composition of 67 or 68, wherein the nanoparticle comprises a targeting ligand that targets the nanoparticle to a cell surface protein.

70. The kit or composition of any one of 66-69, wherein in the linear double stranded donor and the sequence specific nuclease are bound to one another forming a deoxyribonucleoprotein or ribo-deoxyribonucleoprotein complex.

71. The kit or composition of any one of 66-70, wherein the delivery vehicle is a targeting ligand conjugated to a charged polymer polypeptide domain, wherein the targeting ligand provides for targeted binding to a cell surface protein, and wherein the charged polymer polypeptide domain is interacting electrostatically with the payloads.

72. The kit or composition of 71, wherein the delivery vehicle further comprises an anionic polymer interacting with the payload and the charged polymer polypeptide domain.

73. The kit or composition of any one of 66-70, wherein the delivery vehicle is a targeting ligand conjugated to (a) and/or (b), wherein the targeting ligand provides for targeted binding to a cell surface protein.

74. The kit or composition of any one of 69-73, wherein the cell surface protein is CD47.

75. The kit or composition of 74, wherein the targeting ligand is a SIRP α protein mimetic.
76. The kit or composition of any one of 69-75, wherein the delivery vehicle further comprises an endocytosis-triggering ligand.
77. The kit or composition of any one of 66-70, wherein the delivery vehicle includes a targeting ligand coated upon a water-oil-water emulsion particle, upon an oil-water emulsion micellar particle, upon a multilamellar water-oil-water emulsion particle, upon a multilayered particle, or upon a DNA origami nanobot.
78. The method of any one of 69-74, wherein the targeting ligand is a peptide, an ScFv, a F(ab), a nucleic acid aptamer, or a peptoid.
79. The kit or composition of any one of 66-70, wherein the delivery vehicle is non-viral.

EXPERIMENTAL

5 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of the invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, *etc.*) but some
10 experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and
15 individually indicated to be incorporated by reference.

The present invention has been described in terms of particular embodiments found or proposed to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the
20 intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence. Moreover, due to biological functional equivalency considerations, changes can be made in protein structure without affecting the biological action in kind or amount. All such modifications are intended to be included within the scope of the appended claims.

25

Example 1 (Figures 10-29)Example 2 (Figures 30-34)5 Example 3 (Figures 35-55)

We characterize cellular uptake and phenotype with flow cytometry and high-content screening, quantifying delivery efficiency and gene editing across various subpopulations of cells.

10 In these experiments, unstimulated human primary Pan-T Cells (a mixture of CD4+ and CD8+ T-cells) and peripheral blood mononuclear cells (PBMCs) were flash transfected (30 minutes incubation with nanoparticles), washed twice with PBS containing 10ug/ml heparan sulfate, and analyzed 24 hours later on an Attune NxT flow cytometer. Cells were stained with antibodies specific for CD4 and CD8, and transduction of EGFP-tagged Cas9 was quantified in each subpopulation.

15

Example 4 (Figures 56-57)

MULTIMODAL DATASETS

Cellular uptake and phenotype were characterized with flow cytometry and high-content screening, quantifying delivery efficiency and gene editing across various subpopulations of
20 cells.

In these experiments, unstimulated human primary Pan-T Cells (a mixture of CD4+ and CD8+ T cells) and peripheral blood mononuclear cells (PBMCs) were flash transfected (30 minutes incubation with nanoparticles), washed twice with PBS containing 10ug/ml heparan sulfate, and analyzed 24 hours later on an Attune NxT flow cytometer. Cells were stained with
25 antibodies specific for CD4 and CD8, and transduction of EGFP-tagged Cas9 was quantified in each subpopulation. The following tables show comparisons of imaging performed 1h post-transfection utilizing a BioTek Cytation 5 Imaging Reader with a 40x objective vs. flow cytometry data gathered at 24h. Without intending to be bound by any particular theory, it is believed that ≥ 24 h time-points determine cellular internalization, whereas early time-points
30 determine cellular affinity. Unsupervised learning was utilized for determining cellular affinity at the 1h time-point from images, and imaging data was compared to cellular uptake at the 24h time-point as assessed via flow cytometry.

Example 5 (Figures 60-66)

35 20M Pan-T cells (IQ Biosciences) were thawed into a flask containing 20mL media. The following day, CD3/CD28 beads (10M beads) were introduced to unstimulated cells. 2d

following thawing and resuspension, cells were pelleted and media was changed. Beads were removed 3d following thawing and resuspension.

For nucleofection, 160pmol sgRNA and 126pmol Cpf1 (A.s. or L.b.) were utilized as in the following examples:

	μM (pmol/ μL)	160pmol sgRNA	126pmol A.s. Cpf1	PBS	126pmol L.b. Cpf1	PBS
LL00 1 0.1%	70.42	2.27208179 49	2	0.72791820 51	1.26	1.46791820 51
LL00 2 0.1%	77.52	2.06398348 81	2	0.93601651 19	1.26	1.67601651 19
LL00 3 0.1%	69.93	2.28800228 8	2	0.71199771 2	1.26	1.45199771 2
LL03 2 0.1%	76.92	2.08008320 33	2	0.91991679 67	1.26	1.65991679 67
Cpf1 pos contr ol 0.1%	75.1	2.13	2	0.87	1.26	1.61
	μM (pmol/ μL)	160pmol sgRNA	*3	126pmol A.s. Cpf1 v3	PBS	
LL00 2 0.1%	77.52	2.06398348 81	6.19195046 44	6	2.80804953 56	
LL00 3 0.1%	69.93	2.28800228 8	6.86400686 4	6	2.13599313 6	
LL03 2	76.92	2.08008320 33	6.24024961	6	2.75975039	

0.1%						
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Cryopreserved human primary T Cells were thawed and stimulated for 2 days the day after culturing with CD3/CD28 beads. 1.27% of cells were GFP+ following double-cut Cpf1-mediated editing of the TRBC1/C2 loci, and subsequent insertion via a donor DNA template with staggered ends encoding GFP. The day after bead removal, cells were electroporated with the Lonza Amaxa 4D system, P3 Primary Cell kit. RNPs were formed by incubating 64pmol A.s. Cpf1 (IDT, catalog 1081068) and 128 pmol sgRNA (IDT) at room temperature for 10-20 minutes, then added to 4µg of dsDNA insert or IDT's Cpf1 electroporation enhancer (Catalog #1076301) and incubated for 10 minutes. 1x10⁶ Stimulated T Cells in 20µL were added and then transferred to the cuvette, then electroporated with pulse EH-115 (B, RNP alone) or EO-115 (C, RNP+DNA) (Figure 62). On Day 7 post nucleofection, TCRa/b and GFP expression were assayed by flow cytometry. Figure shows cells in live population (Annexin and Sytox negative). DNA was collected from cells using QuickExtract (Lucigen).

Comparison of various primers (TRBC1-TRBC2, GFP-GFP, and GFP-TRBC2) resulted in a faint band in double-cut Cpf1 studies, whereby both TRBC1 / TRBC2 loci were cut, forming double stranded breaks with 4bp overhangs. The overhangs were matched to a GFP insertion sequence or FLAG insertion sequence and examined via either flow cytometry and PCR (GFP vs. Cpf1 RNP only), or Sanger sequencing (FLAG vs. Cpf1 RNP only).

Name	Nucleic Acid Type	Description	Sequence
LL001	sgRNA	TRAC exon1 Cpf1 guide	TAATTTCTACTCTTGTAGATCATGTGCAAACGCCTTCAACAA CA
LL002	sgRNA	TRAC exon1 Cpf1 guide	TAATTTCTACTCTTGTAGATCATGTGCAAACGCCTTCAAC
LL003	sgRNA	TRB1 exon1 Cpf1 guide - C1 and C2	TAATTTCTACTCTTGTAGATGGTGTGGGAGATCTCTGCTTCTGA
LL004	sgRNA	TRB promoter Cpf1 guide	TAATTTCTACTCTTGTAGATCAGATGGGCTGAAGTCTCCACTGT
LL032		TRAC exon1 Cpf1 guide	TAATTTCTACTCTTGTAGATTTTGAGAAATCAAAATCGGTG

Table depicts sgRNA sequences used for TRAC, TRB1 C1/C2, and TRB promoter regions

LL300	ssDNA	LL001 Flag tetr ^r donor antisense-phospho anneal with LL301	GTTGcttatcgatcatgctcttgtaac
LL301	ssDNA	LL001 Flag tetr ^r donor sense-phospho anneal with LL300	CAACgattacaaagacgatgacgataag
LL302	ssDNA	LL003 Flag tetr ^r donor antisense-phospho anneal with LL303	TCTGcttatcgatcatgctcttgtaac
LL303	ssDNA	LL003 Flag tetr ^r donor sense-phospho anneal with LL302	CAGAgattacaaagacgatgacgataag

LL304	ssDNA	LL032 Flag tetris donor antisense-phospho anneal with LL305	GAATcttatcgatcgtctttgtaatc
LLtetris GFP	ssDNA	LL003 GFP tetris donor sense-phosphoanneal with antisense	ggctccggcgagggcaggggaagctactaacatgcgggacgtggagga aaatccggc ccaagcaaaggagaagaactttcactggagttgtcccaattcttggaattagatggtgatgta atgggcacaaatttctgtccgtggagaggggaagggtgatgctacaaacggaaaactcaccc ttaaatttattgactactggaaaactacctgttcgtggccaacttgtcactactctgacctat ggtgttcaatgcttttccggtatccggatcacatgaaacggcatgacttttcaagagtccatgc ccgaagggtatgtacaggaacgcactatactttcaagatgacgggacctacaagacgcgtg ctgaagtcaagttgaagggtgatacccttgtaactgtatcgagttaaagggtattgattt aaaga agatggaaacattcttgacacaaactcgagtacaacttaactcacacaatgtatcatcacg gcagacaaacaaaagaatggaatcaaagctaacttcaaaattcgccacaacgttgaaagt gttcggtcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtcctttacc agacaaccattacgtgtgacacaatctgtccttgcgaagatcccaacgaaaagcgtgacca catggtccttctgagtttgtaactgctgctgggattacacatggcatggatgagctctacaaa
LLtetris GFP	ssDNA	LL003 GFP tetris donor antisense – phosphoanneal with sense	CAGAtttgtagagctatccatgccatgtgtaatccagcagcagttacaaactcaagaagg accatgtggcacgcttttctgtgggactttcgaaaggacagattgtgtcgacaggtaatggtgt ctggtaaaaggacagggccatcgccaattggagttttgtgataatggtctgctagtgaacgg aaccatcttcaacgtgtggcgaatttgaagttagctttgattccattctttgtgtctgccgtgatgt atacattgtgtagttaaagttgactcgagttgtgtccaagaatgttccatcttctttaaataat acccttfaactcgatcagattaacaagggtatcaccttcaacttgacttcagcacgcgtctgtga gggtcccgatcatcttgaaagatagtgcttccgtgtacataaccttcgggcatggcactcttgaaa aagtcatgccgtttcatgtgatccggataacgggaaaagcattgaacaccataggtcagagta gtgacaagtgtggccacggaacaggtagtttccagtagtgcaaat aaatttaagggtgagttt ccgtttagcatcaccttcacctctccacggacagaaaattgtgccattaacatccatct aattcaacaagaattgggacaactccagtgaagaagttcttctcttctgtggccgggatttct ccacgtccccgcagttagtagacttcccctgccctcgccggagcc
LL305	ssDNA	LL032 Flag tetris donor sense-phospho anneal with LL304	ATTCgattacaaagacgatgacgataag
LL306	oligos (primer)	LL001 T2A tetris donor BsaI F - PCR with LL307 and digest	atGGTCTCACAACggctccggcgagggcagggg

		with BsaI	
LL307	oligos (primer)	LL001 sfGFP tetris donor BsaI R - PCR with LL306 and digest with BsaI	atGGTCTCACAACCTTAttgttagagctcatcca
LL308	oligos (primer)	LL003 T2A tetris donor BsaI F - PCR with LL309 and digest with BsaI	atGGTCTCACAGAggctccggcgagggcagggg
LL309	oligos (primer)	LL003 sfGFP tetris donor BsaI R - PCR with LL308 and digest with BsaI	atGGTCTCACAGATTAttgttagagctcatcca
LL310	oligos (primer)	LL032 T2A tetris donor BsaI F - PCR with LL311 and digest with BsaI	atGGTCTCAATTGggtccggcgagggcagggg
LL311	oligos (primer)	LL032 sfGFP tetris donor BsaI R - PCR with LL310 and digest with BsaI	atGGTCTCAATTCTTAttgttagagctcatcca

LL238	oligos (primer)	TRBC1&2 primer R	AGCCCGTAGAACTGGACTTGAC
LL239	oligos (primer)	TRBC2 primer F	GGCAAGGAAGGGGTAGAACCAT

LL030	ssDNA	TRAC exon1 double cpf1 Tetris donor sense - anneal with LL031	attcggctccggcgagggcaggggaagtctactaacatgcggggacgtggagga aaatcccgcccaagcaaaggagaagaactttcactggagttgcccaattctgtt gaattagatggtgatgttaatgggcacaaatttctgtccgtggagagggtgaagggtg atgctacaaacggaaaactcaccttaatttattgcaactactggaaaactacctgtt ccgtggccaacacttgtcactactctgacctatggtgttcaatgctttcccggtatccgg atcacatgaaacggcatgacttttcaagagtgccatgccgaagggtatgtacagg aacgcactatatcttcaagatgacgggacctacaagacgcgtgtgaagtcaag tttgaagggtatacccttgtaacgtatcgagttaaagggtattgatttaagaagatg gaaacattcttgacacaaactcgagtacaactttaactcacacaatgtatacatcac ggcagacaaaacaaagaatggaatcaaaacttaactcaaaattcgccacaacggt gaagalgggtccgttcaactagcagaccattatcaacaaaactccaaltggcgtat ggccctgtcctttaccagacaaccattacctgtcgacacaatctgtcctttcgaaaga tcccaacgaaaagcgtgaccacatggtccttcttgagtttgaactgctgctgggatta cacatggcatggatgagctctacaaaTAATAG
LL031	ssDNA	TRAC exon1 double cpf1 Tetris donor antisense - anneal with LL030	gttgCTATTAttttagagctcatccatgccatgtgaatccagcagcagttacaa actcaagaaggaccatgtggcacgcctttcgttgggatcttgcgaaggacagattgt gtcgacaggtaatggtgtctggttaaaggacagggccatcgcaattggagatttt gttgataatggtctgctagttgaacggaaccatctcaacgttggcgaattttgaagt tagctttgattccattctttgttctgtccgtgatgtatatactgtgtgagttaaagttgtac tcgagttgtgtccaagaatgttccatcttctttaaatacaatacccttaactcgatacg attaacaagggtatcaccttcaacttgacttcagcacgcgtctttaggtcccgtcat ctttgaaagatatagtgcgttctgtacataaaccttcgggcattggcactcttgaaaaag tcatgccgtttcatgtgatccggataacgggaaaagcattgaacaccataggtcaga gtagtacaagtgtggccacggaacaggtagtttccagtagtgcaataaatttaa gggtgagttttccgttttagcatcaccttcacctctccacggacagaaaatttgtgcc cattaacatcaccatctaattcaacaagaattgggacaactccagtgaagaagttcttc tcctttgcttgggccgggattttctccacgtcccgcatgttagtagacttcccctgcc tcgccggagcc

CLAIMS

What is claimed is:

1. A method of genome editing in a target cell, comprising:
 - (a) generating double stranded cuts with staggered ends at two locations within the target cell's genome, thereby producing a first genomic staggered end and a second genomic staggered end; and
 - (b) introducing into the target cell a linear double stranded donor DNA having a 5' or 3' overhang at each end,
wherein one end of the donor DNA hybridizes with the first genomic staggered end and the other end of the donor DNA hybridizes with the second genomic staggered end, thereby resulting in insertion of the linear double stranded donor DNA into the target cell's genome.
2. The method of claim 1, wherein at least one end of the donor DNA has a 5' overhang and at least one of the genomic staggered ends has a 5' overhang.
3. The method of claim 1 or claim 2, wherein at least one end of the donor DNA has a 3' overhang and at least one of the genomic staggered ends has a 3' overhang.
4. The method of any one of claims 1-3, wherein said generating comprises introducing one or more sequence specific nucleases, or one or more nucleic acids encoding the one or more sequence specific nucleases, into the target cell to generate said double stranded cuts.
5. The method of claim 4, wherein the one or more sequence specific nucleases comprises at least one of: a meganuclease, a homing endonuclease, a zinc finger nuclease (ZFN), and a transcription activator-like effector nuclease (TALEN).
6. The method of any one of claim 4, wherein the one or more sequence specific nucleases comprises a staggered end cutting CRISPR/Cas effector protein.
7. The method of claim 6, wherein said generating further comprises introducing a CRISPR/Cas guide nucleic acid, or a nucleic acid encoding the CRISPR/Cas guide nucleic acid, into the cell.

8. The method of any one of claims 4-7, wherein the method comprises introducing into the cell, as payloads of the same delivery vehicle: (i) the one or more sequence specific nucleases, or one or more nucleic acids encoding the one or more sequence specific nucleases, and (ii) the linear double stranded donor DNA.
9. The method of claim 8, wherein the one or more sequence specific nucleases and the linear double stranded donor DNA are introduced into the cell as a deoxyribonucleoprotein complex or a ribo-deoxyribonucleoprotein complex.
10. The method of claim 8 or claim 9, wherein during said introducing, the ends of the donor DNA are bound in a site-specific manner to the one or more sequence specific nucleases.
11. The method of any one of claims 8-10, wherein the delivery vehicle is non-viral.
12. The method of any one of claims 8-11, wherein the delivery vehicle is a nanoparticle.
13. The method of claim 12, wherein, in addition to (i) and (ii), the nanoparticle comprises a core comprising an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition.
14. The method of claim 13, wherein said anionic polymer composition comprises an anionic polymer selected from poly(glutamic acid) and poly(aspartic acid).
15. The method of claim 13 or claim 14, wherein said cationic polymer composition comprises a cationic polymer selected from poly(arginine), poly(lysine), poly(histidine), poly(ornithine), and poly(citrulline).
16. The method of any one of claims 13-15, wherein nanoparticle further comprises a sheddable layer encapsulating the core.
17. The method of claim 16, wherein the sheddable layer is an anionic coat or a cationic coat.

18. The method of claim 16 or claim 17, wherein the sheddable layer comprises one or more of: silica, a peptoid, a polycysteine, calcium, calcium oxide, hydroxyapatite, calcium phosphate, calcium sulfate, manganese, manganese oxide, manganese phosphate, manganese sulfate, magnesium, magnesium oxide, magnesium phosphate, magnesium sulfate, iron, iron oxide, iron phosphate, and iron sulfate.

19. The method of any one of claims 16-18, wherein the nanoparticle further comprises a surface coat surrounding the sheddable layer.

20. The method of claim 19, wherein the surface coat comprises a cationic or anionic anchoring domain that interacts electrostatically with the sheddable layer.

21. The method of claim 19 or claim 20, wherein the surface coat comprises one or more targeting ligands.

22. The method of claim 19 or claim 20, wherein the surface coat comprises one or more targeting ligands selected from the group consisting of: rabies virus glycoprotein (RVG) fragment, ApoE-transferrin, lactoferrin, melanoferritin, ovotransferrin, L-selectin, E-selectin, P-selectin, sialylated peptides, polysialylated O-linked peptides, TPO, EPO, PSGL-1, ESL-1, CD44, death receptor-3 (DR3), LAMP1, LAMP2, Mac2-BP, stem cell factor (SCF), CD70, SH2 domain-containing protein 1A (SH2D1A), exendin, exendin-S11C, GLP1, RGD, a Transferrin ligand, an FGF fragment, an $\alpha 5\beta 1$ ligand, IL2, Cde3-epsilon, peptide-HLA-A*2402, CD80, CD86, succinic acid, a bisphosphonate, a hematopoietic stem cell chemotactic lipid, sphingosine, ceramide, sphingosine-1-phosphate, ceramide-1-phosphate, and an active targeting fragment of any of the above.

23. The method of claim 19 or claim 20, wherein the surface coat comprises one or more targeting ligands that provides for targeted binding to a target selected from: CD3, CD8, CD4, CD28, CD90, CD45f, CD34, CD80, CD86, CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, NKp44, NKp46, NKp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL2R, IL7R, IL10R, IL12R, IL15R, IL18R, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$.

24. The method of claim 19 or claim 20, wherein the surface coat comprises one or more targeting ligands that provides for targeted binding to target cells selected from: bone marrow cells, hematopoietic stem cells (HSCs), hematopoietic stem and progenitor cells (HSPCs), peripheral blood mononuclear cells (PBMCs), myeloid progenitor cells, lymphoid progenitor cells, T-cells, B-cells, NKT cells, NK cells, dendritic cells, monocytes, granulocytes, erythrocytes, megakaryocytes, mast cells, basophils, eosinophils, neutrophils, macrophages, erythroid progenitor cells, megakaryocyte-erythroid progenitor cells (MEPs), common myeloid progenitor cells (CMPs), multipotent progenitor cells (MPPs), hematopoietic stem cells (HSCs), short term HSCs (ST-HSCs), IT-HSCs, long term HSCs (LT-HSCs), endothelial cells, neurons, astrocytes, pancreatic cells, pancreatic β -islet cells, liver cells, muscle cells, skeletal muscle cells, cardiac muscle cells, hepatic cells, fat cells, intestinal cells, cells of the colon, and cells of the stomach.

25. The method of any one of claims 8-10, wherein the delivery vehicle is a targeting ligand conjugated to the payload, wherein the targeting ligand provides for targeted binding to a cell surface protein.

26. The method of any one of claims 8-10, wherein the delivery vehicle is a targeting ligand conjugated to a charged polymer polypeptide domain, wherein the targeting ligand provides for targeted binding to a cell surface protein, and wherein the charged polymer polypeptide domain is condensed with a nucleic acid payload and/or is interacting electrostatically with a protein payload.

27. The method of claim 25 or 26, wherein the targeting ligand is a peptide, an ScFv, a F(ab), a nucleic acid aptamer, or a peptoid.

28. The method of claim 26, wherein the charged polymer polypeptide domain has a length in a range of from 3 to 30 amino acids.

29. The method of any one of claims 26-28, wherein the delivery vehicle further comprises an anionic polymer interacting with the payload and the charged polymer polypeptide domain.

30. The method of claim 29, wherein the anionic polymer is selected from poly(glutamic acid) and poly(aspartic acid).

31. The method of any one of claims 25-30, wherein the targeting ligand has a length of from 5-50 amino acids.

32. The method of any one of claims 25-31, wherein the targeting ligand provides for targeted binding to a cell surface protein selected from a family B G-protein coupled receptor (GPCR), a receptor tyrosine kinase (RTK), a cell surface glycoprotein, and a cell-cell adhesion molecule.

33. The method of any one of claims 25-31, wherein the targeting ligand is selected from the group consisting of: rabies virus glycoprotein (RVG) fragment, ApoE-transferrin, lactoferrin, melanoferritin, ovotransferritin, L-selectin, E-selectin, P-selectin, sialylated peptides, polysialylated O-linked peptides, TPO, EPO, PSGL-1, ESL-1, CD44, death receptor-3 (DR3), LAMP1, LAMP2, Mac2-BP, stem cell factor (SCF), CD70, SH2 domain-containing protein 1A (SH2D1A), exendin, exendin-S11C, GLP1, RGD, a Transferrin ligand, an FGF fragment, an $\alpha 5\beta 1$ ligand, IL2, Cde3-epsilon, peptide-HLA-A*2402, CD80, CD86, succinic acid, a bisphosphonate, a hematopoietic stem cell chemotactic lipid, sphingosine, ceramide, sphingosine-1-phosphate, ceramide-1-phosphate, and an active targeting fragment of any of the above.

34. The method of any one of claims 25-31, wherein the targeting ligand provides for targeted binding to a target selected from: CD3, CD8, CD4, CD28, CD90, CD45f, CD34, CD80, CD86, CD3-epsilon, CD3-gamma, CD3-delta; TCR Alpha, TCR Beta, TCR gamma, and/or TCR delta constant regions; 4-1BB, OX40, OX40L, CD62L, ARP5, CCR5, CCR7, CCR10, CXCR3, CXCR4, CD94/NKG2, NKG2A, NKG2B, NKG2C, NKG2E, NKG2H, NKG2D, NKG2F, NKp44, NKp46, NKp30, DNAM, XCR1, XCL1, XCL2, ILT, LIR, Ly49, IL2R, IL7R, IL10R, IL12R, IL15R, IL18R, TNF α , IFN γ , TGF- β , and $\alpha 5\beta 1$.

35. The method of any one of claims 25-31, wherein the targeting ligand provides for binding to a cell type selected from the group consisting of: bone marrow cells, hematopoietic stem cells (HSCs), long-term HSCs, short-term HSCs, hematopoietic stem and progenitor cells (HSPCs), peripheral blood mononuclear cells (PBMCs), myeloid progenitor cells, lymphoid progenitor cells, T-cells, B-cells, NKT cells, NK cells, dendritic cells, monocytes, granulocytes, erythrocytes, megakaryocytes, mast cells, basophils, eosinophils, neutrophils, macrophages, erythroid progenitor cells (e.g., HUDEP cells), megakaryocyte-erythroid progenitor cells (MEPs), common

myeloid progenitor cells (CMPs), multipotent progenitor cells (MPPs), hematopoietic stem cells (HSCs), short term HSCs (ST-HSCs), IT-HSCs, long term HSCs (LT-HSCs), endothelial cells, neurons, astrocytes, pancreatic cells, pancreatic β -islet cells, muscle cells, skeletal muscle cells, cardiac muscle cells, hepatic cells, fat cells, intestinal cells, cells of the colon, and cells of the stomach.

36. The method of any one of claims 1-35, wherein, prior to generating the double stranded cuts at said two locations within the target cell's genome, the two locations are separated by 1,000,000 base pairs or less.

37. The method of any one of claims 1-35, wherein, prior to generating the double stranded cuts at said two locations within the target cell's genome, the two locations are separated by 100,000 base pairs or less.

38. The method of any one of claims 1-35, wherein, the first and second genomic staggered ends are produced at the TCR alpha locus or the TCR beta locus.

39. The method of any one of claims 1-35, where at least one of the first and second genomic staggered ends are produced (1) using one or more of the CRISPR/Cas guide RNA (gRNA) sequences depicted in Figure 59, and/or (2) by targeting one or more of the TALEN sequences depicted in Figure 59.

40. The method of any one of claims 1-35, wherein the donor DNA has a total of from 10 base pairs (bp) to 100 kilobase pairs (kbp).

41. The method of any one of claims 1-40, wherein the insertion of the donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) protein.

42. The method of claim 41, wherein the donor DNA encodes amino acids of a CDR1, CDR2, or CDR3 region of the TCR protein.

43. The method of any one of claims 1-40, wherein the donor DNA comprises a nucleotide sequence encoding a chimeric antigen receptor (CAR), and wherein insertion of said donor DNA

results in operable linkage of the nucleotide sequence encoding the CAR to an endogenous T-cell promoter.

44. The method of any one of claims 1-40, wherein the donor DNA comprises a nucleotide sequence that is operably linked to a promoter and encodes a chimeric antigen receptor (CAR).

45. The method of any one of claims 1-40, wherein the donor DNA comprises a nucleotide sequence encoding a cell-specific targeting ligand that is membrane bound and presented extracellularly, and wherein insertion of said donor DNA results in operable linkage of the nucleotide sequence encoding the cell-specific targeting ligand to an endogenous promoter.

46. The method of any one of claims 1-40, wherein the donor DNA comprises a promoter operably linked to a sequence that encodes a cell-specific targeting ligand that is membrane bound and presented extracellularly.

47. The method of any one of claims 1-40, wherein the method comprises:
generating double stranded cuts with staggered ends at four locations within the target cell's genome, thereby producing a third genomic staggered end and a fourth genomic staggered end in addition to the first and second genomic staggered ends; and

introducing two linear double stranded donor DNAs, each having a 5' or 3' overhang at each end,

wherein the ends of one donor DNA hybridize with the first and second genomic staggered ends and the ends of the other donor DNA hybridize with the third and fourth genomic staggered ends and the ends,

thereby resulting in insertion of said two donor DNAs into the target cell's genome.

48. The method of claim 47, wherein:

(1) insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Gamma subunit; or

(2) insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Gamma subunit, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Delta subunit; or

(3) insertion of one donor DNA occurs within a nucleotide sequence that encodes the κ chain of an IgA, IgD, IgE, IgG, or IgM protein, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes the λ chain of an IgA, IgD, IgE, IgG, or IgM protein.

49. The method of claim 47, wherein insertion of one donor DNA occurs within a nucleotide sequence that encodes a T cell receptor (TCR) Alpha or Delta subunit constant region, and insertion of the other donor DNA occurs within a nucleotide sequence that encodes a TCR Beta or Gamma subunit constant region.

50. The method of claim 47, wherein:

(1) insertion of one donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Delta subunit promoter, and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a TCR Beta or Gamma subunit promoter; or

(2) insertion of one donor DNA occurs within a nucleotide sequence that functions as a T cell receptor (TCR) Alpha or Gamma subunit promoter, and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a TCR Beta or Delta subunit promoter; or

(3) insertion of one donor DNA occurs within a nucleotide sequence that functions as a promoter for a κ chain of an IgA, IgD, IgE, IgG, or IgM protein, and insertion of the other donor DNA occurs within a nucleotide sequence that functions as a promoter for a λ chain of an IgA, IgD, IgE, IgG, or IgM protein.

51. The method of any one of claims 1-50, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a T cell receptor (TCR) Alpha, Beta, Gamma or Delta endogenous promoter.

52. The method of any one of claims 1-50, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a TCR Alpha, Beta, Gamma or Delta promoter.

53. The method of any one of claims 1-50, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a promoter selected from the group consisting of: (i) a T-cell specific promoter; (ii) a CD3 promoter; (iii) a CD28 promoter; (iv) a stem cell specific promoter; (v) a somatic cell specific promoter; (vi) a T cell receptor (TCR) Alpha, Beta, Gamma or Delta promoter; (v) a B-cell specific promoter; (vi) a CD19 promoter; (vii) a CD20 promoter; (viii) a

CD22 promoter; (ix) a B29 promoter; and (x) a T-cell or B-cell V(D)J-specific promoter.

54. The method of any one of claims 1-50, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a T-cell specific promoter.

55. The method of any one of claims 1-50, wherein the donor DNA comprises a protein-coding nucleotide sequence that is operably linked to a promoter.

56. The method of any one of claims 1-50, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with a stem cell specific or somatic cell specific endogenous promoter.

57. The method of any one of claims 1-56, wherein the donor DNA comprises a nucleotide sequence that encodes a reporter protein (e.g., a near-IR and/or far red reporter protein, e.g., for evaluating gene editing efficiency).

58. The method of claim 57, wherein insertion of said donor DNA results in operable linkage of the inserted donor DNA with an endogenous promoter.

59. The method of claim 57, wherein the donor DNA comprises a promoter that is operably linked to the nucleotide sequence that encodes the reporter protein.

60. The method of any one of claims 1-59, wherein the donor DNA comprises a nucleotide sequence that encodes (i) a T cell receptor (TCR) protein; (ii) an IgA, IgD, IgE, IgG, or IgM protein; or (iii) the κ or λ chains of an IgA, IgD, IgE, IgG, or IgM protein.

61. The method of any one of claims 1-60, wherein the donor DNA comprises a protein-coding nucleotide sequence that does not have introns.

62. The method of claim 61, wherein the nucleotide sequence that does not have introns encodes all or a portion of a TCR protein or an Immunoglobulin.

63. The method of any one of claims 1-62, wherein the donor DNA has at least one adenylated

3' end.

64. The method of any one of claims 1-63, wherein the target cell is a mammalian cell.

65. The method of any one of claims 1-64, wherein the target cell is a human cell.

66. A kit or composition comprising:

(a) a linear double stranded donor DNA having a 5' or 3' overhang at each end; and

(b) a sequence specific nuclease, or a nucleic acid encoding the sequence specific nucleases,

wherein (a) and (b) are payloads as part of the same delivery vehicle.

67. The kit or composition of claim 66, wherein the delivery vehicle is a nanoparticle.

68. The kit or composition of claim 67, wherein the nanoparticle comprises a core comprising (a), (b), an anionic polymer composition, a cationic polymer composition, and a cationic polypeptide composition.

69. The kit or composition of claim 67 or claim 68, wherein the nanoparticle comprises a targeting ligand that targets the nanoparticle to a cell surface protein.

70. The kit or composition of any one of claims 66-69, wherein in the linear double stranded donor and the sequence specific nuclease are bound to one another forming a deoxyribonucleoprotein or ribo-deoxyribonucleoprotein complex.

71. The kit or composition of any one of claims 66-70, wherein the delivery vehicle is a targeting ligand conjugated to a charged polymer polypeptide domain, wherein the targeting ligand provides for targeted binding to a cell surface protein, and wherein the charged polymer polypeptide domain is interacting electrostatically with the payloads.

72. The kit or composition of claim 71, wherein the delivery vehicle further comprises an anionic polymer interacting with the payload and the charged polymer polypeptide domain.

73. The kit or composition of any one of claims 66-70, wherein the delivery vehicle is a targeting ligand conjugated to (a) and/or (b), wherein the targeting ligand provides for targeted binding to a cell surface protein.
74. The kit or composition of any one of claims 69-73, wherein the cell surface protein is CD47.
75. The kit or composition of claim 74, wherein the targeting ligand is a SIRP α protein mimetic.
76. The kit or composition of any one of claims 69-75, wherein the delivery vehicle further comprises an endocytosis-triggering ligand.
77. The kit or composition of any one of claims 66-70, wherein the delivery vehicle includes a targeting ligand coated upon a water-oil-water emulsion particle, upon an oil-water emulsion micellar particle, upon a multilamellar water-oil-water emulsion particle, upon a multilayered particle, or upon a DNA origami nanobot.
78. The method of any one of claims 69-74, wherein the targeting ligand is a peptide, an ScFv, a F(ab), a nucleic acid aptamer, or a peptoid.
79. The kit or composition of any one of claims 66-70, wherein the delivery vehicle is non-viral.

Figure 1

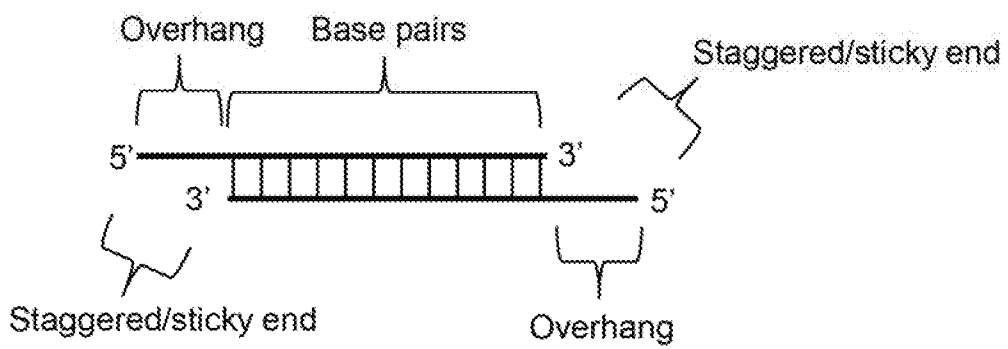
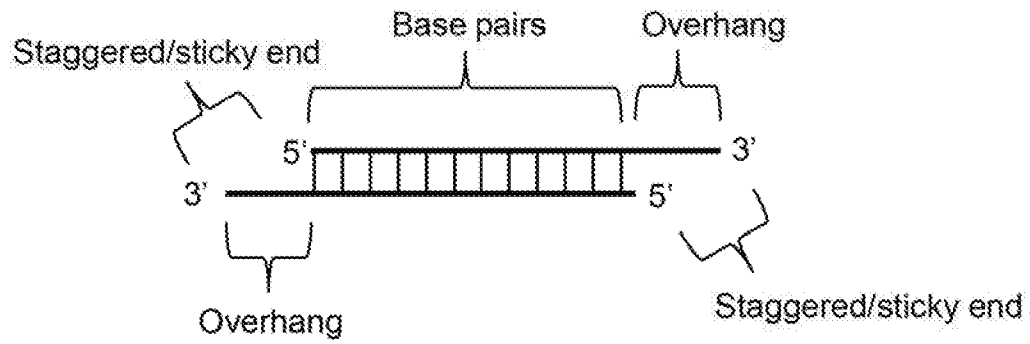


Figure 2

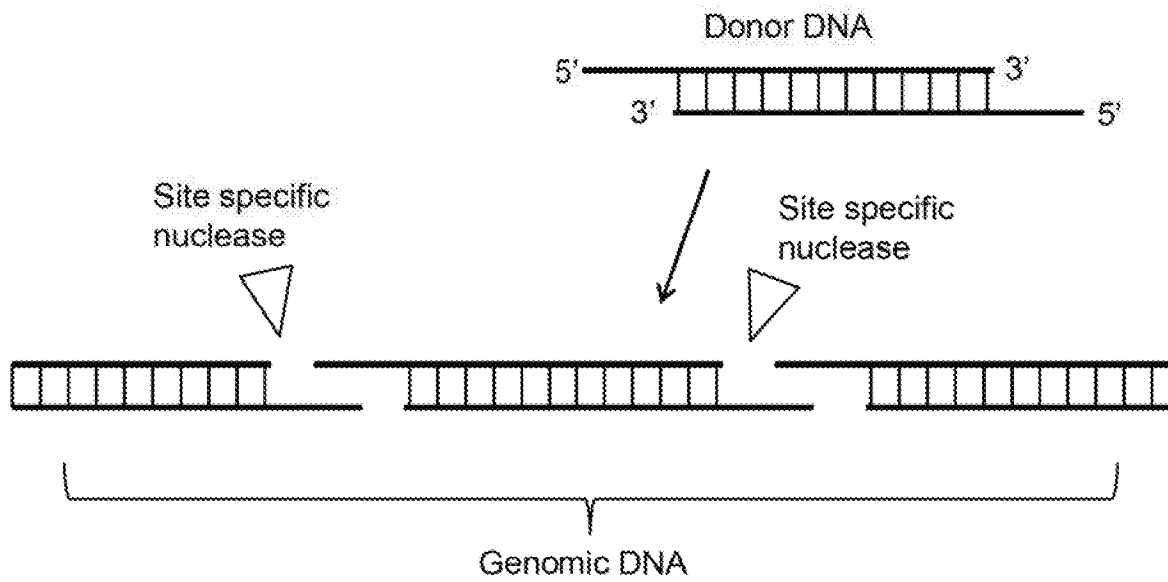


Figure 3

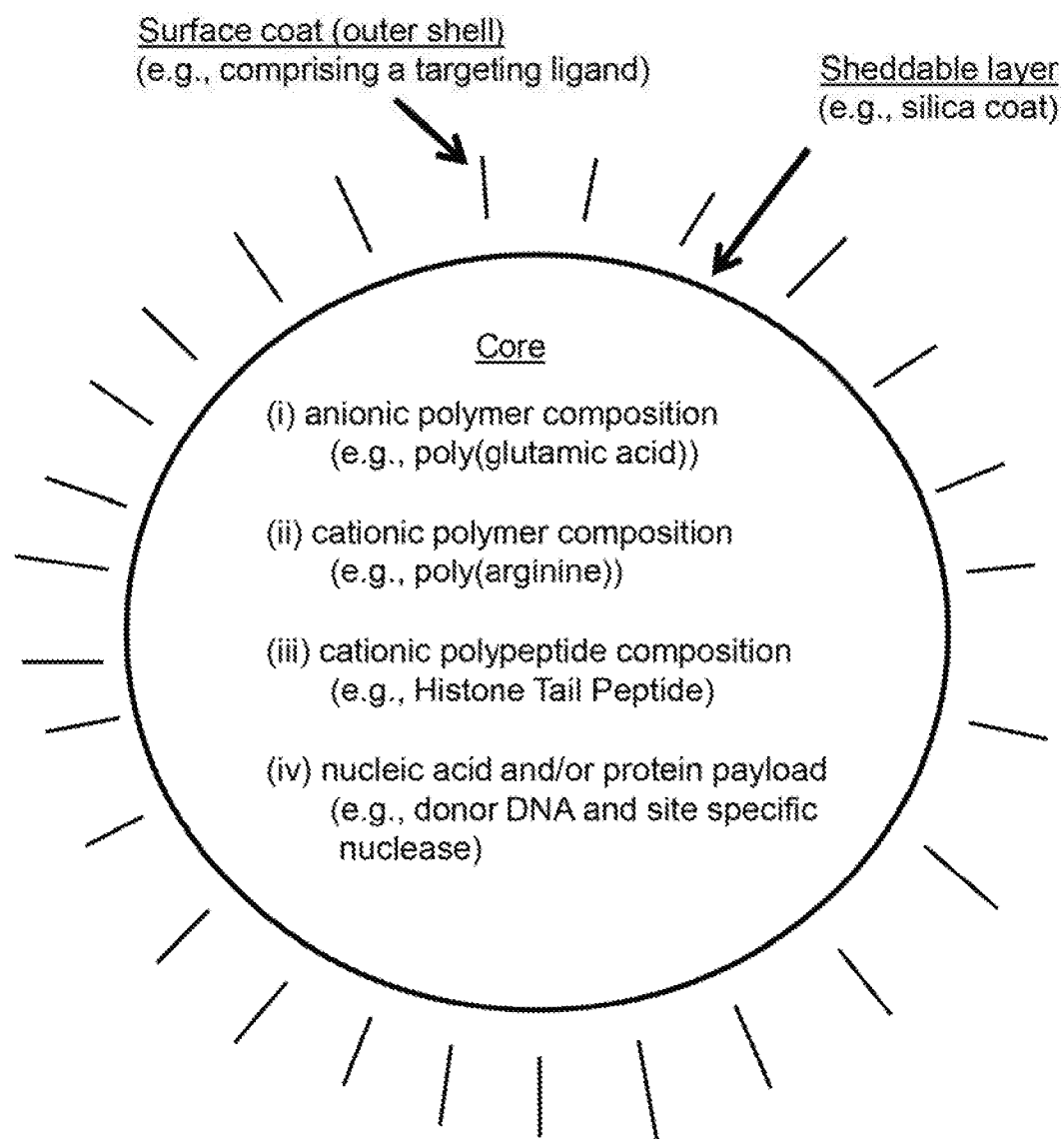


Figure 4

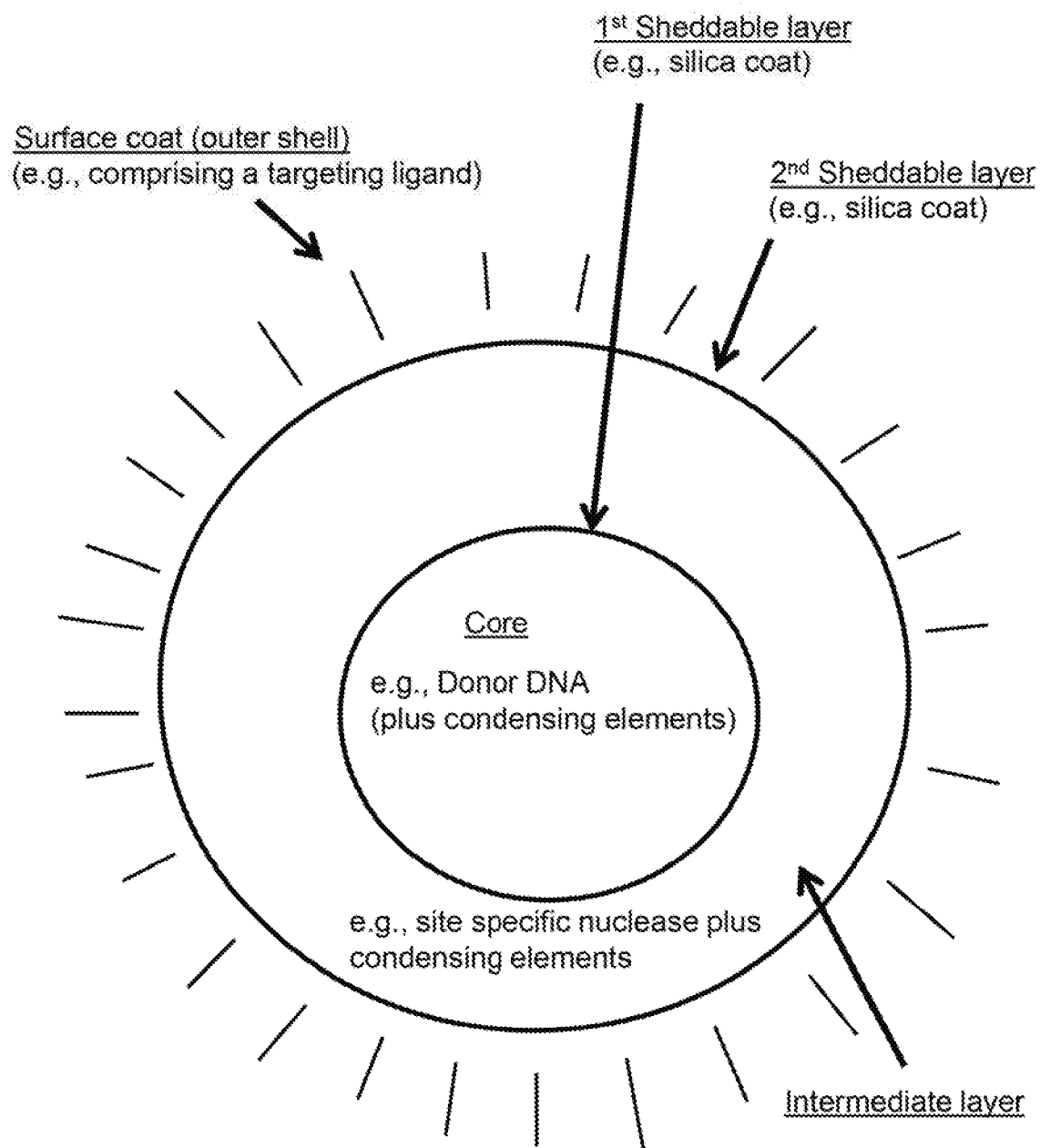
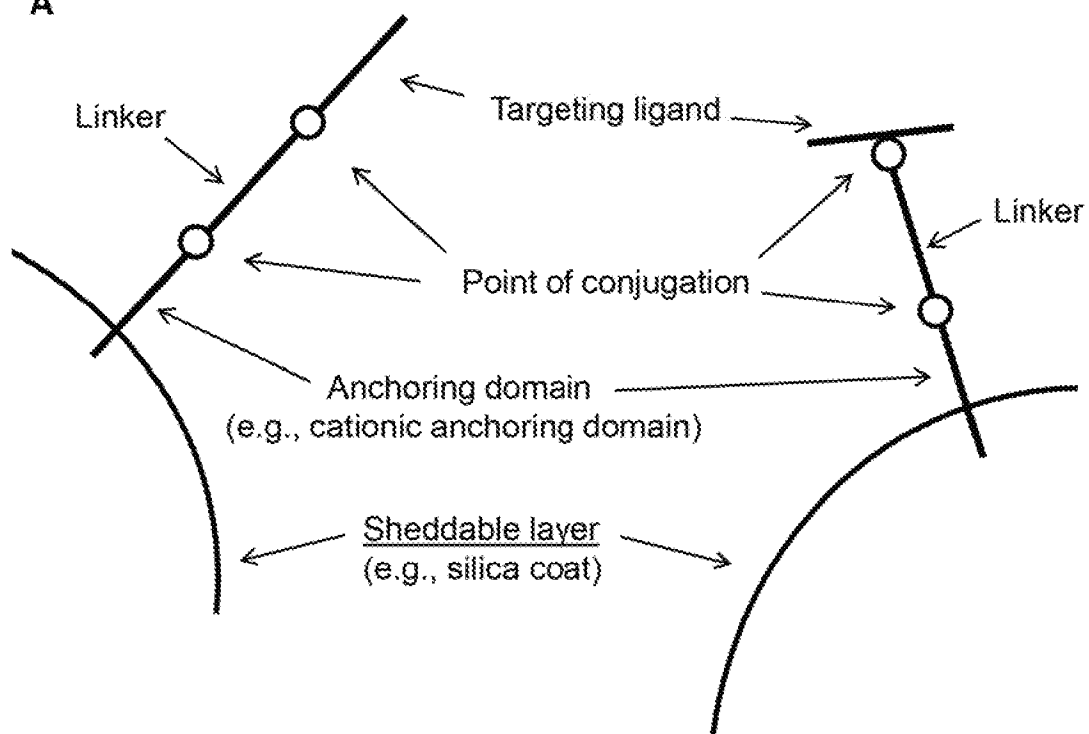


Figure 5

A



B

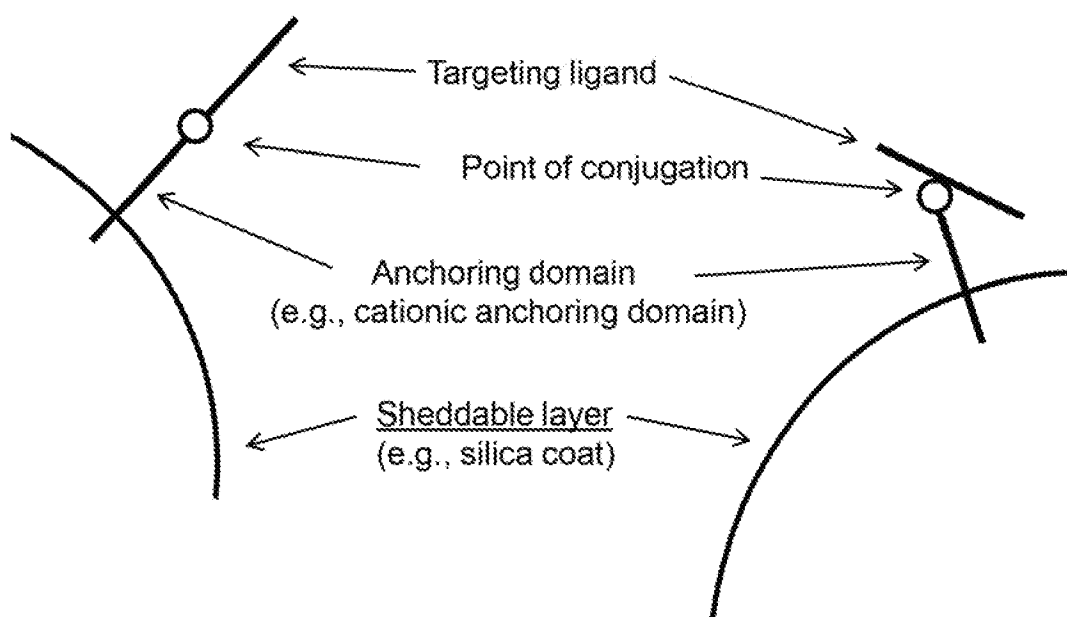
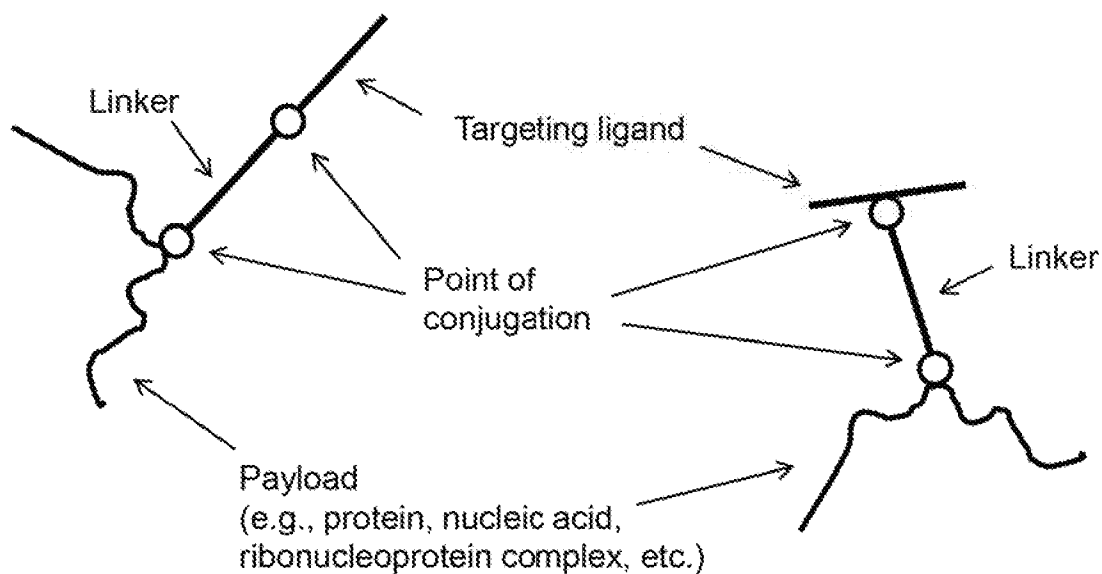


Figure 6

A



B

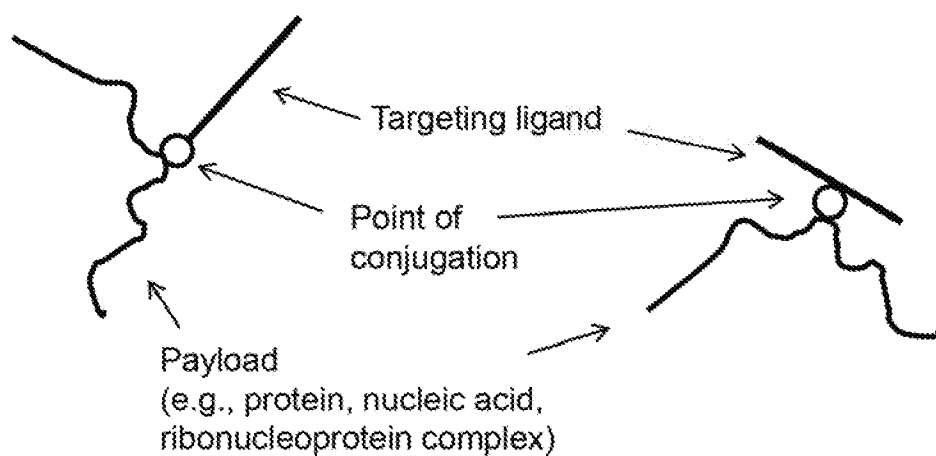
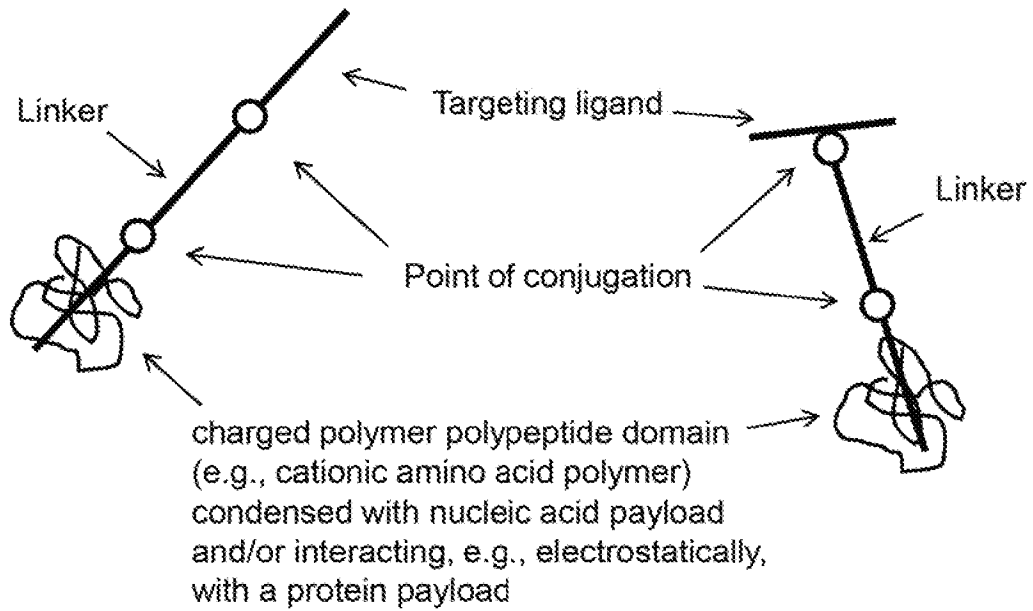


Figure 6 (Cont.)

C



D

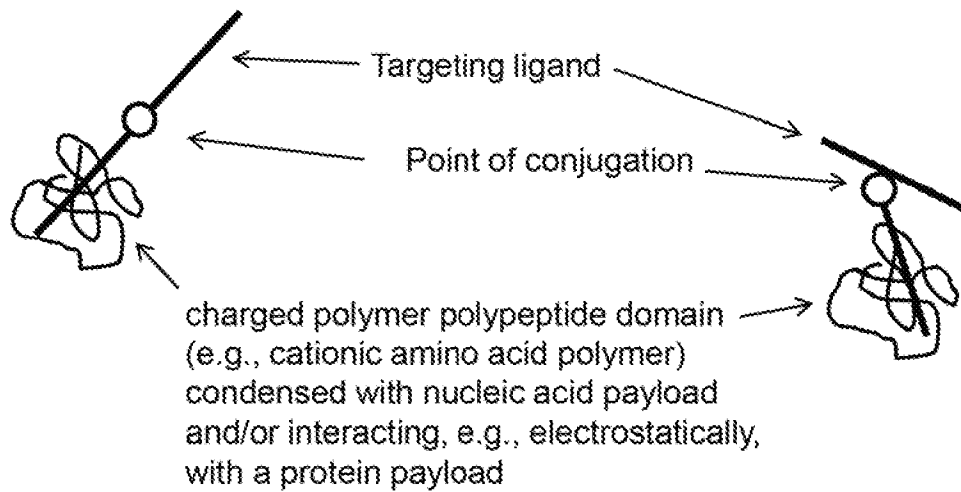


Figure 7

Class 1

LPKKRKFSEISS
 KRKRWENDIP
 KRKRWENNIP
 TGGVMKRKRGSV
 PILFLKRRRGSP
 TYSGVKRKRNVV
 THIGYKRKRDSV
 LSGTKRKRAYFI
 QRRLLKRRKGS
 QIGKKRKRKYLD
 KRGKRKRRLVRPW
 KKGKRKRRLVRPW
 PSRKRKRRESCHI
 PSRKRKRKYAV
 ISRKRKRDLFV
 ITRKRKRDLVFT
 EPNPRKRKRSEL
 TSPSRKRKWDQV
 TLERKRKLAVLY
 RRRKRKRREWEDF
 HRYCGKRKRRTTR

Class 2

SVLGKRSRTWE
 YGRVSKRPRYQF
 RKRGRKRFRSV

Class 4

KRKYAVFLESQN
 KRKYSIYLSQS
 KRKWMAFVMGDP
 KRKCAVFLEGQN
 IPRKRSFAELYD
 RLTPRKRAFSEV

Class 3

KRWSMAFC
 KRTWAQAFTE
 KRPYSIAFPLGQ
 RRRSVLKRSWSVAF
 KRRYSDAFRLPV
 KRRYSDAFGLPV
 IGRKRGYSVAFG
 IGRKRVWAVAFY
 WAGRKRRTWRDAF
 SSHRKRKFSDAF
 PSHRKRKFSDAF
 TAHRKRKFSDAF
 RVQRKRKWSAEF
 RLTRKRKYDCAF
 LVNRKRKYWEAF

Class 5

LGKRYDRDWDYK
 RSSGILGKRKFE
 VHKTVLGKRKYW
 SILGKRKNRDP
 QSVLGKRKSRPF
 TVHLGKRRLRPW
 RVLGKRKTGRSP
 VLGKRKRDDCW
 HGRQVLGKRKR
 SVLGKRKRHPKV
 SVLGKRKRHL
 PVLGKRKRSLSS
 RVLGKRKRREDRP
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 TLGKRKRISCVT
 DTRLGKRKRFPW

Figure 8

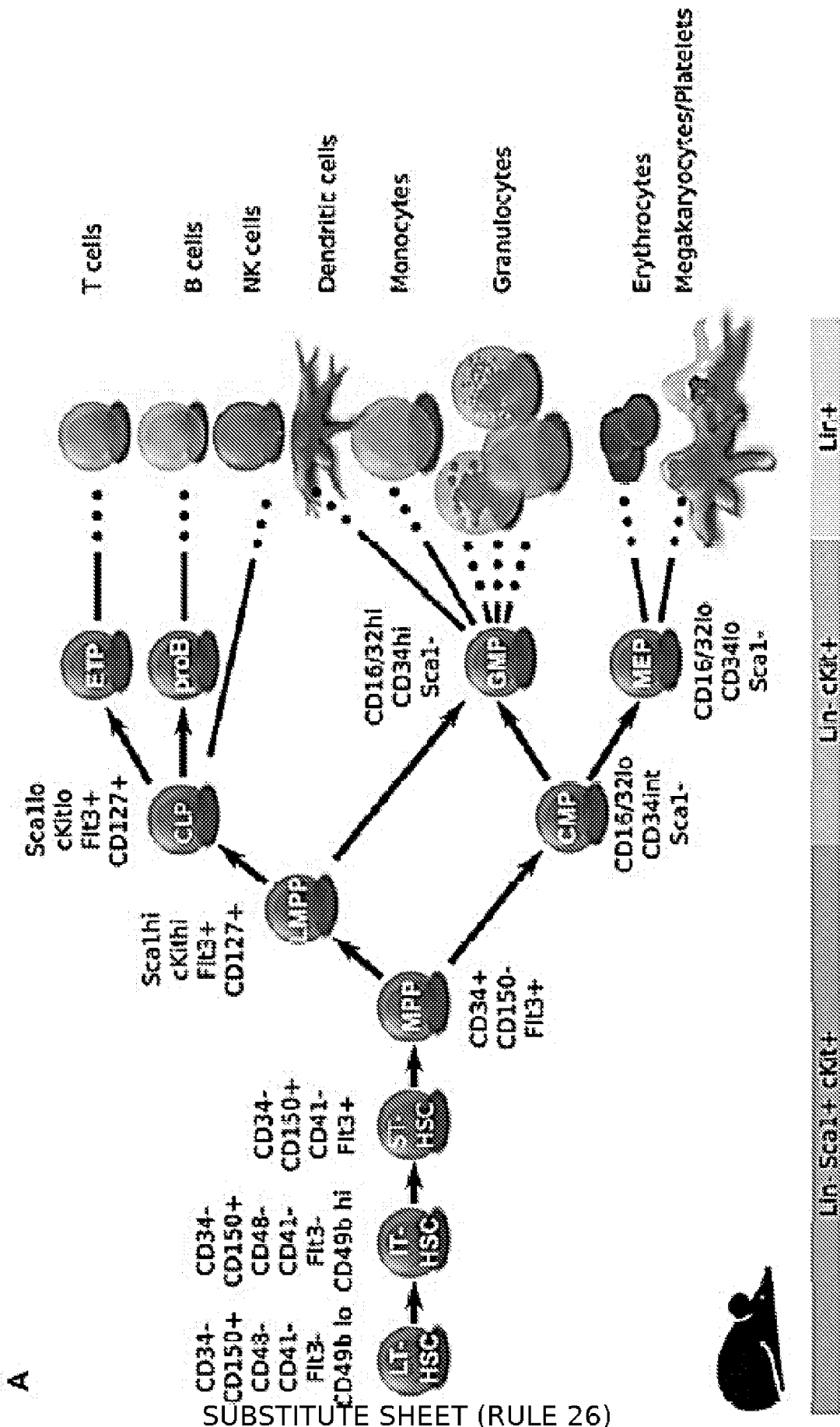


Figure 8 (Cont.)

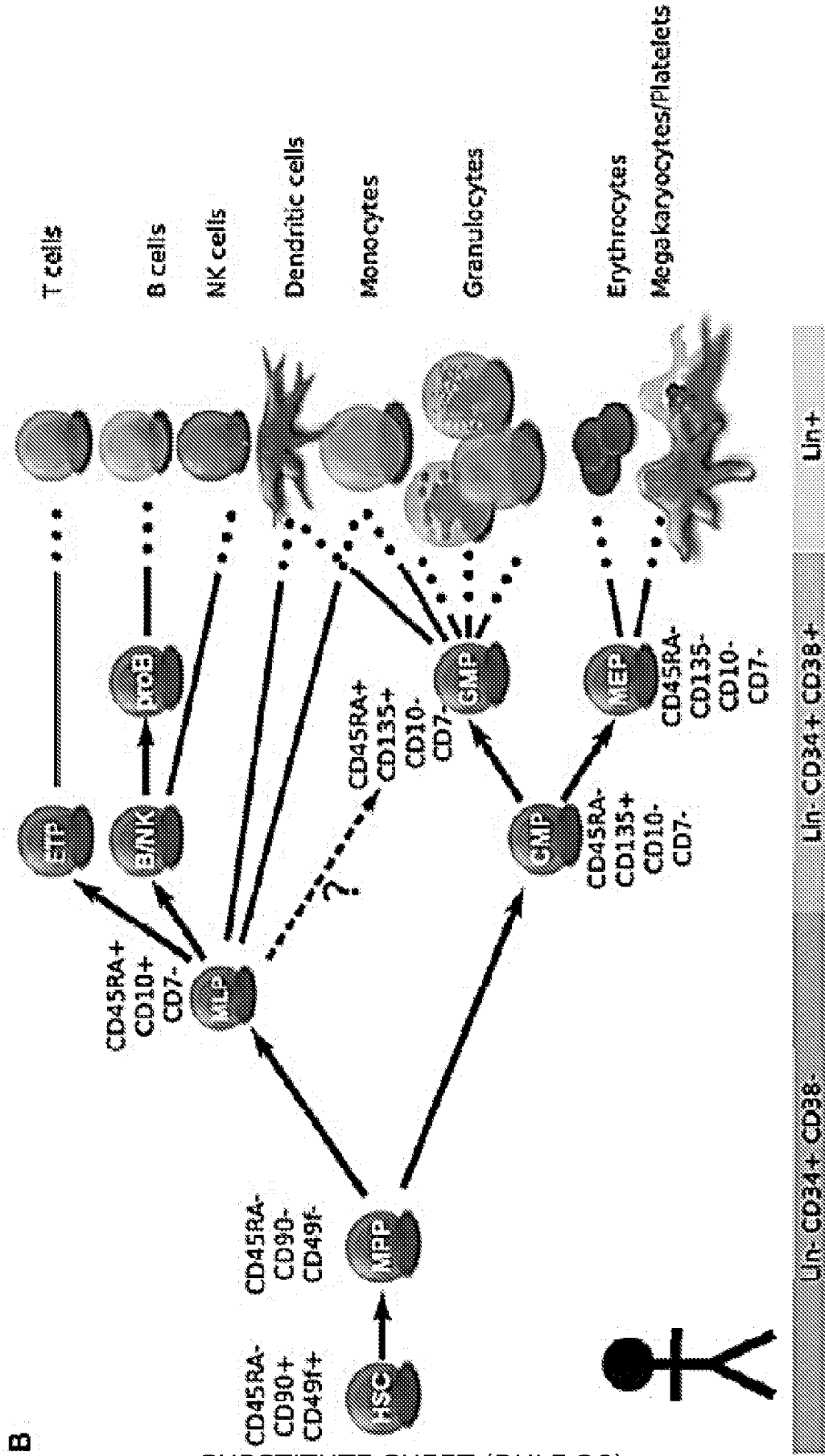


Figure 9

A

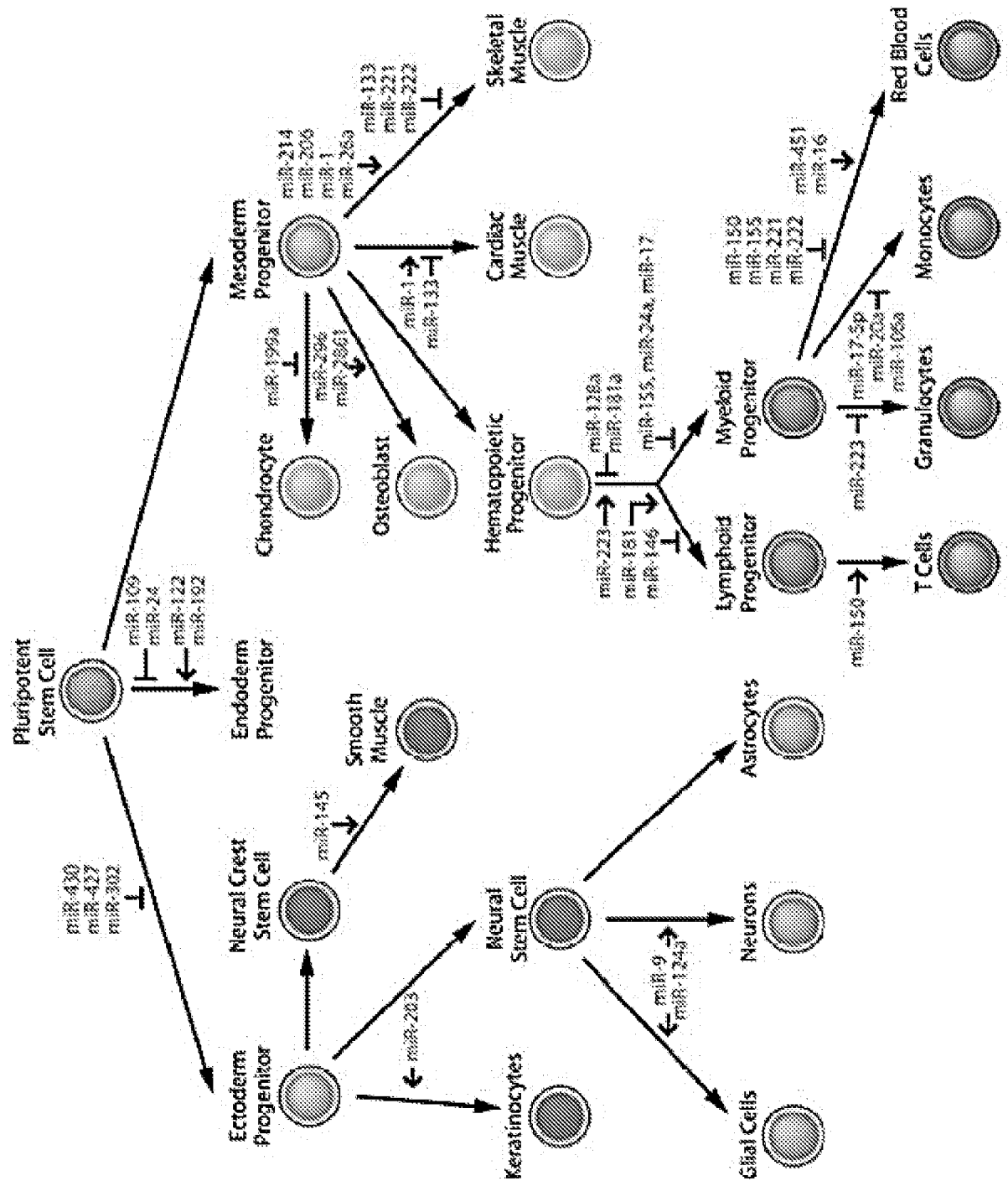
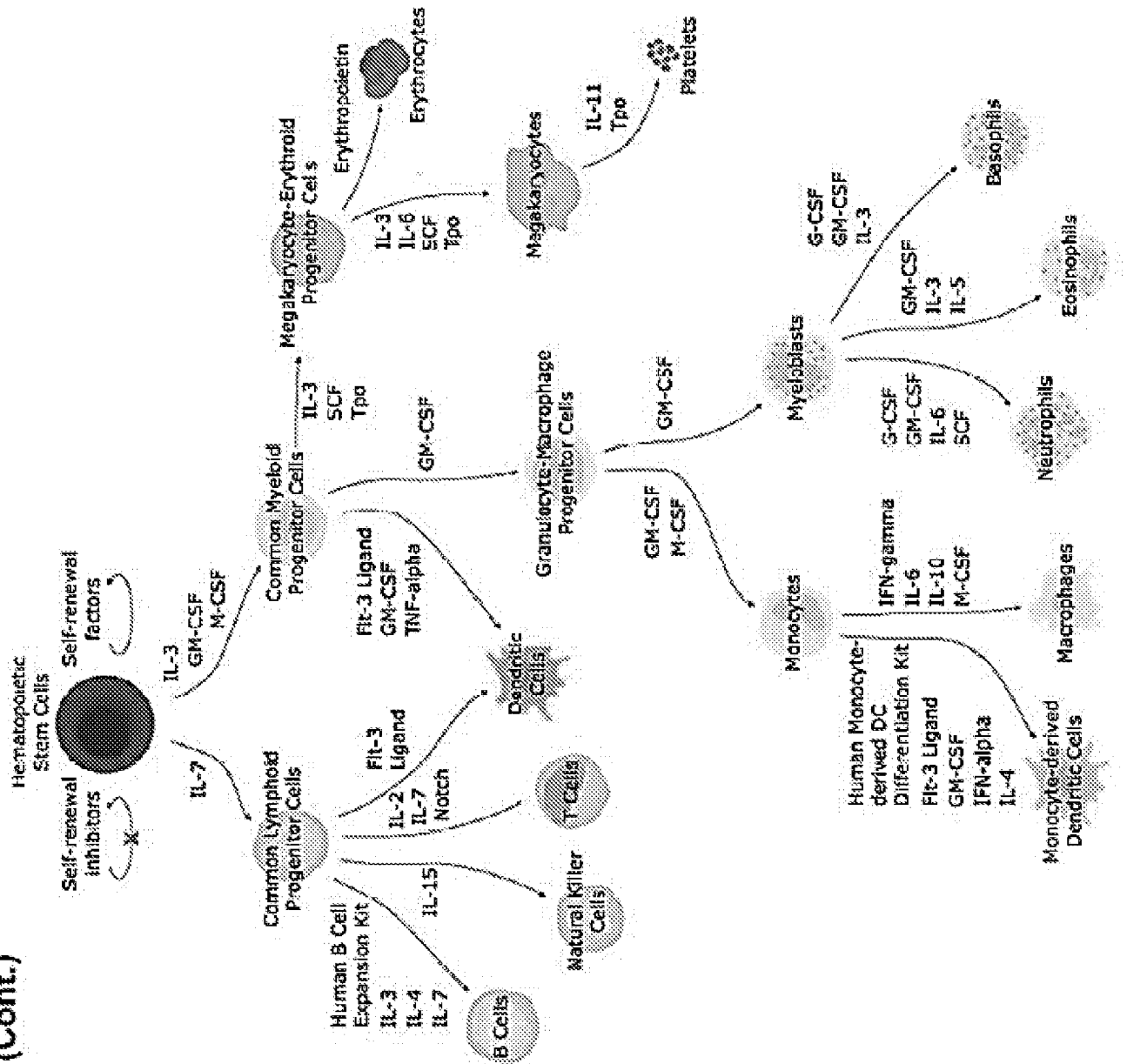


Figure 9 (Cont.)

B



NANOPARTICLE STABILITY EVALUATION

1

Figure 10

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Zeta Potential	Slide 8
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• PDI evaluation: Ligand-modified particles- 72h or Day 7	Slide 20

Figure 11

T-CELL_010.1 (Study A)

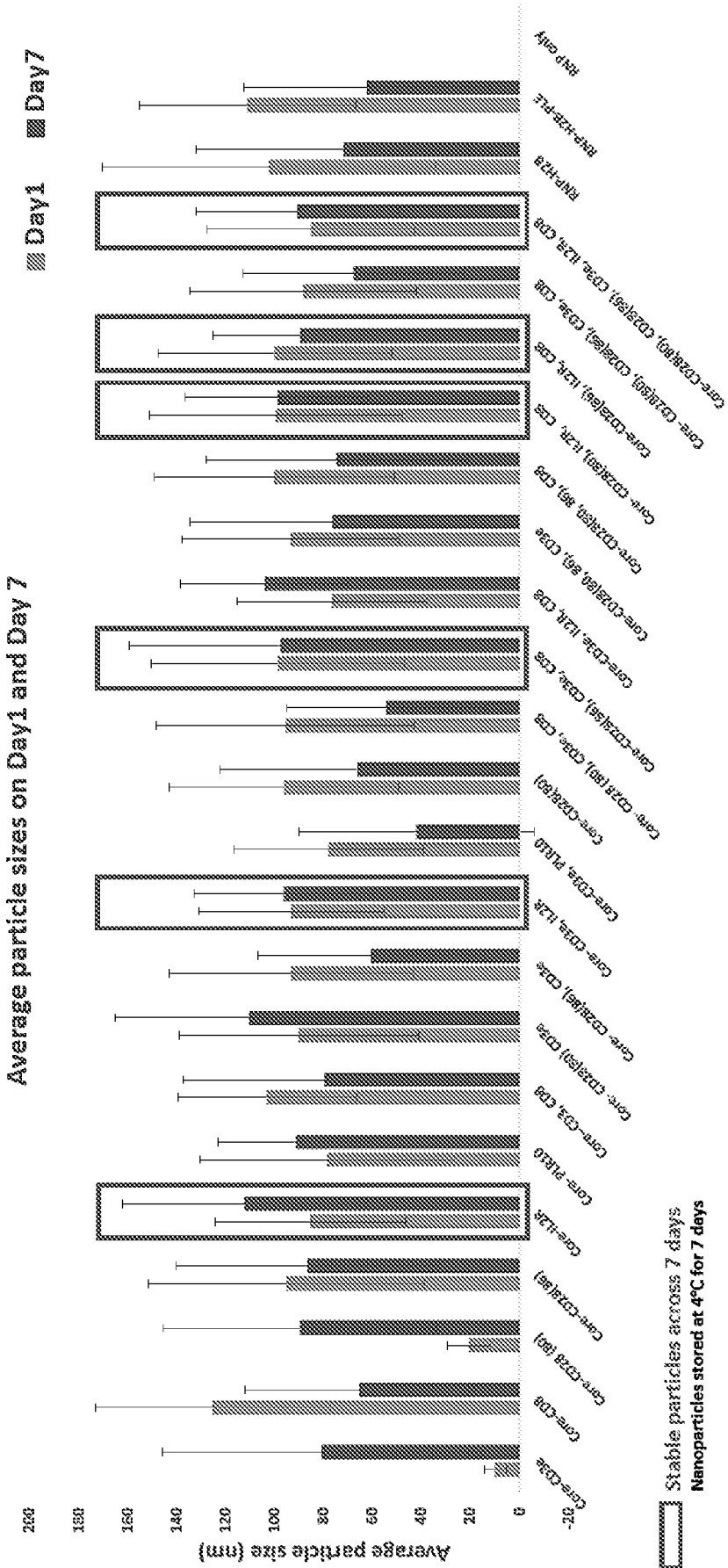


Figure 12

T-CELL_010.1 (Study A)

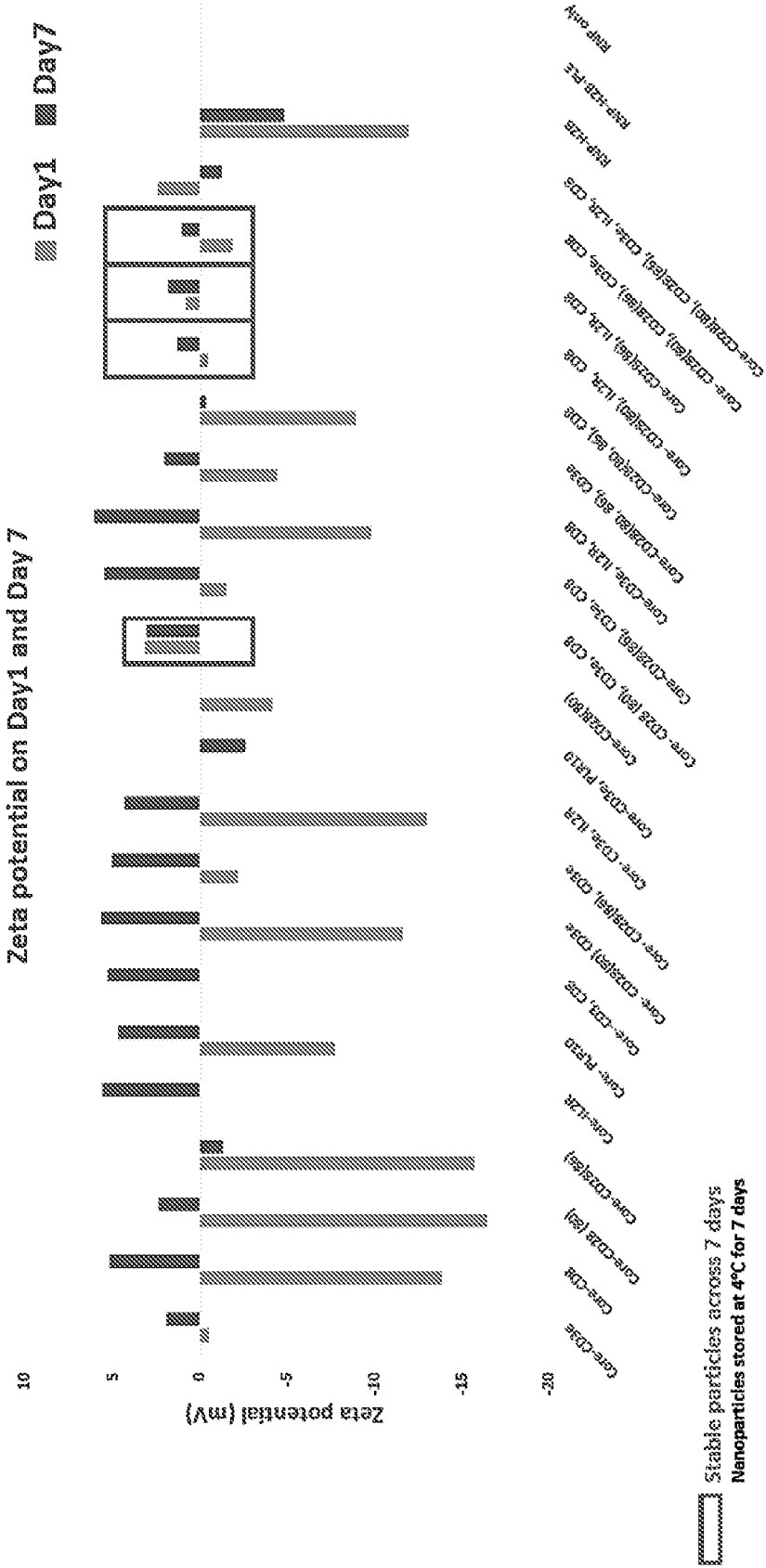


Figure 13

T-CELL_010.1 (Study A)

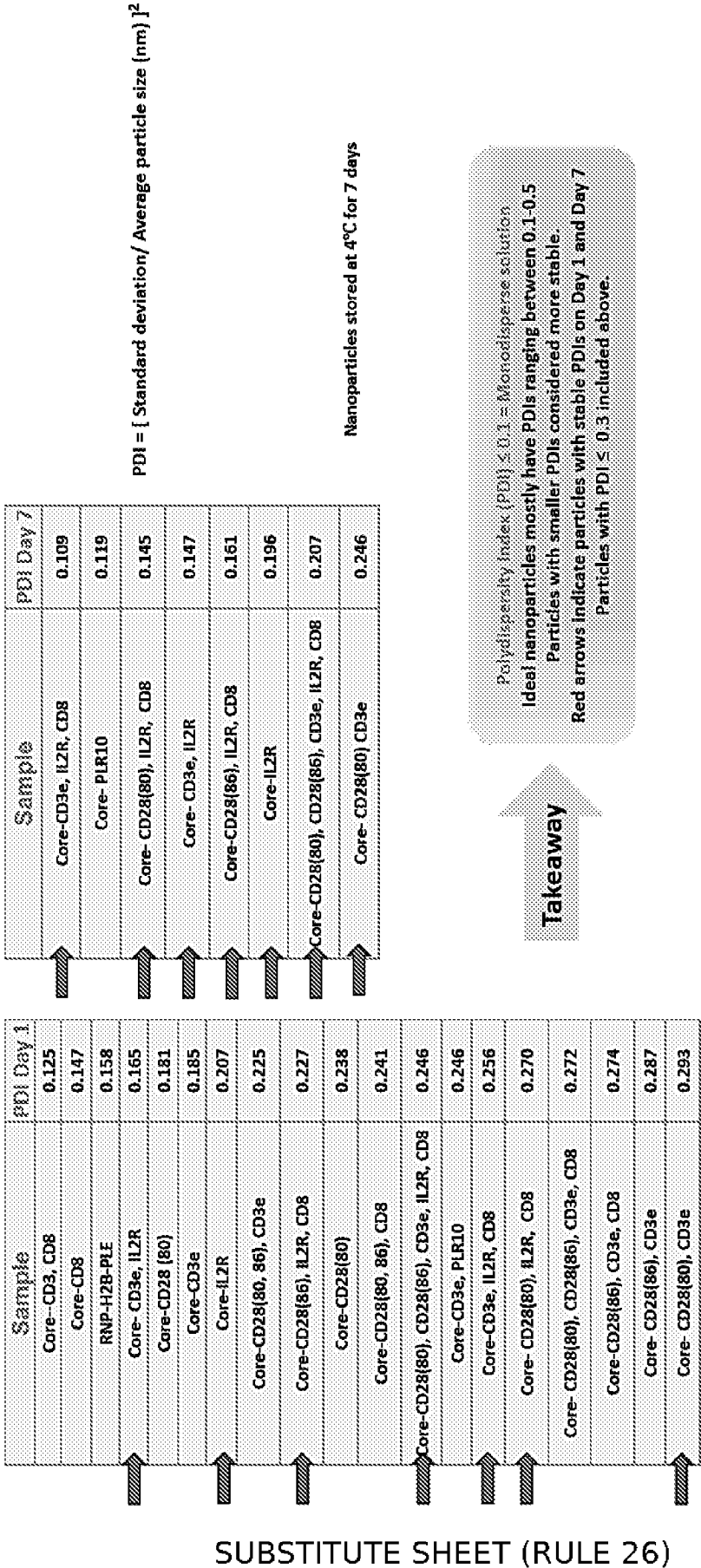


Figure 15

K. Clayton et. al. Physical characterization of nanoparticle size and surface modification using particle scattering diffusiometry.

TC_PBMG_001_2 (Study B)

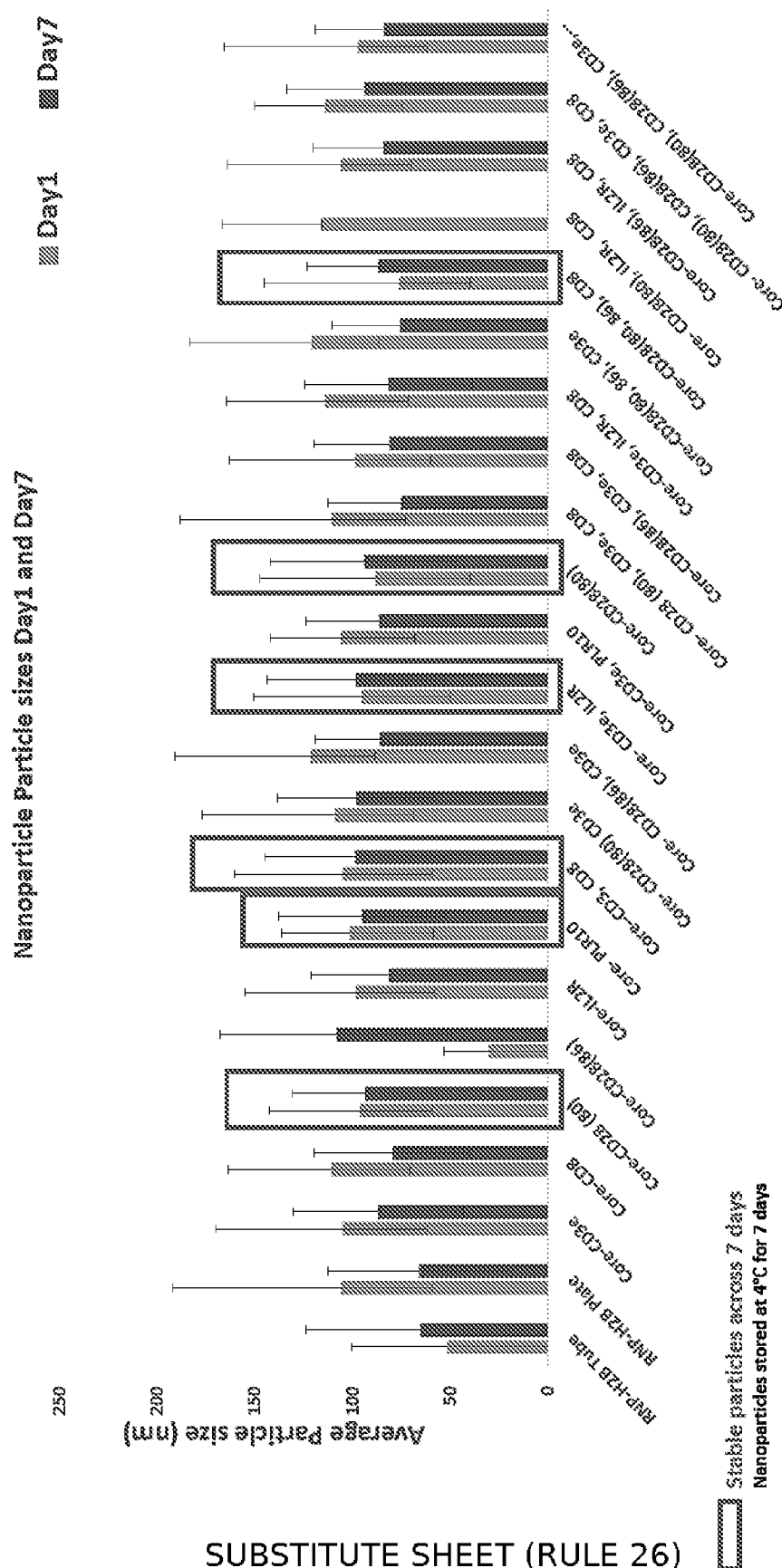


Figure 16

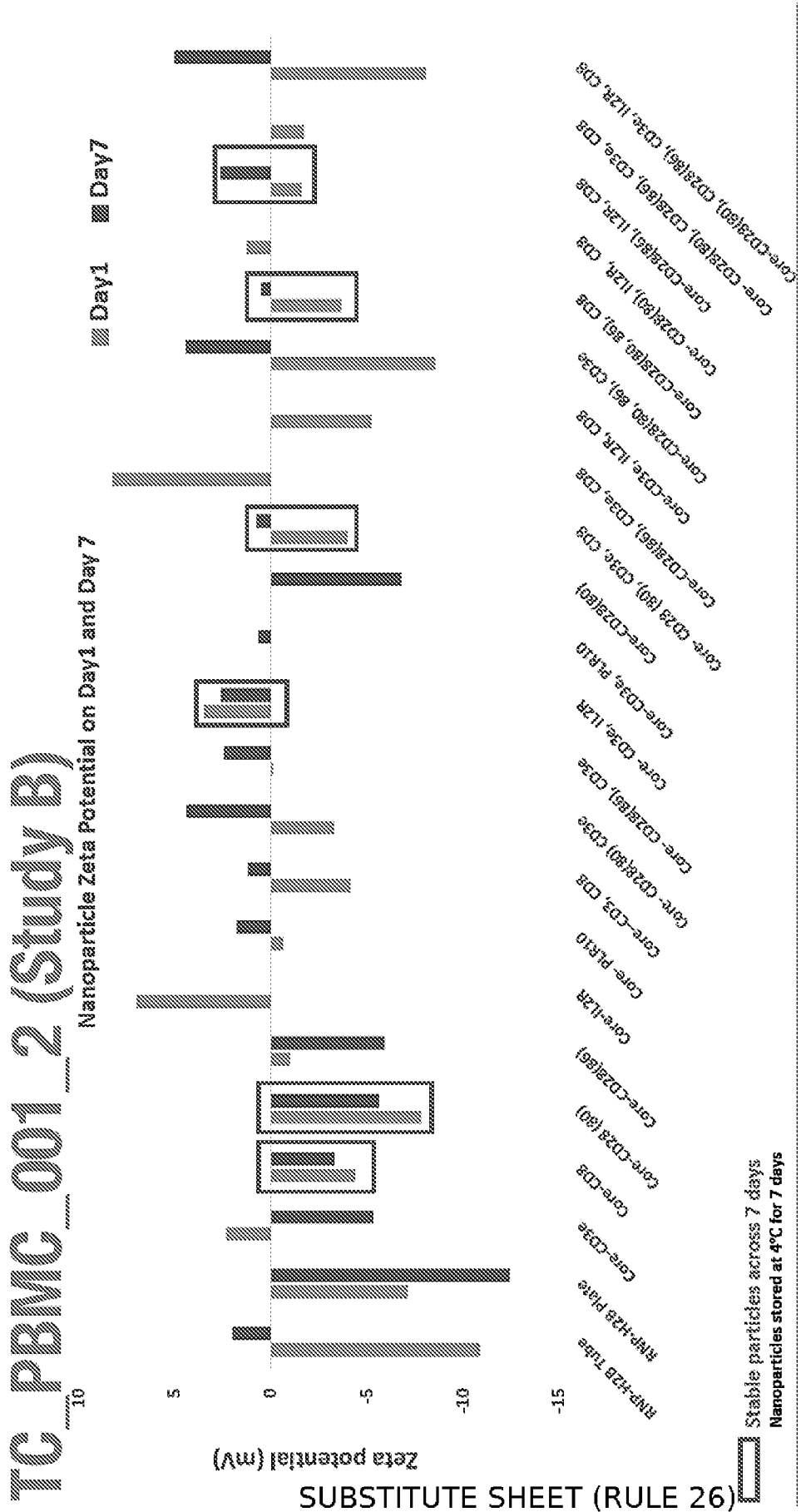


Figure 17

TC_PBMC_001_2 (Study B)

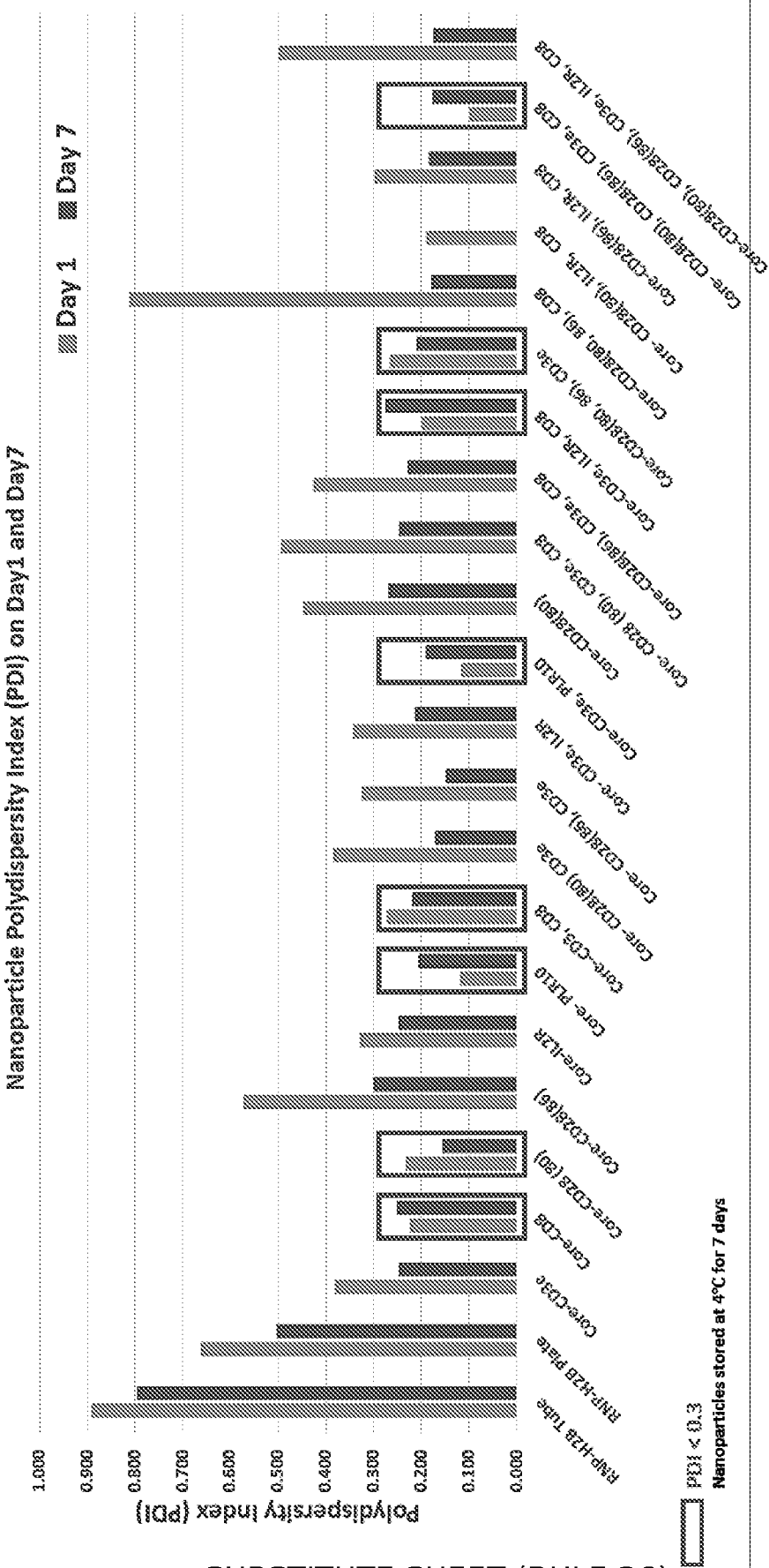


Figure 18

TC_PBM001_2 (Study B)

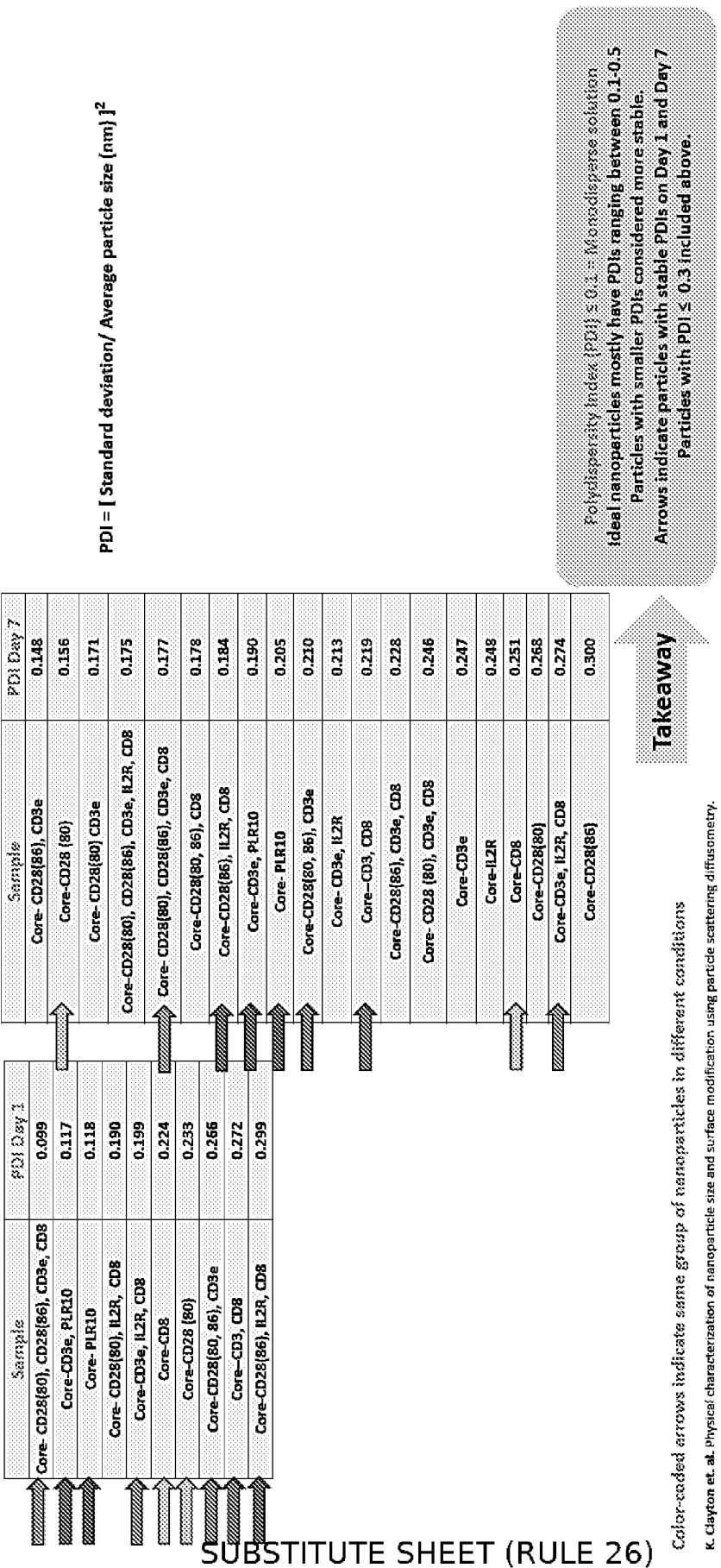
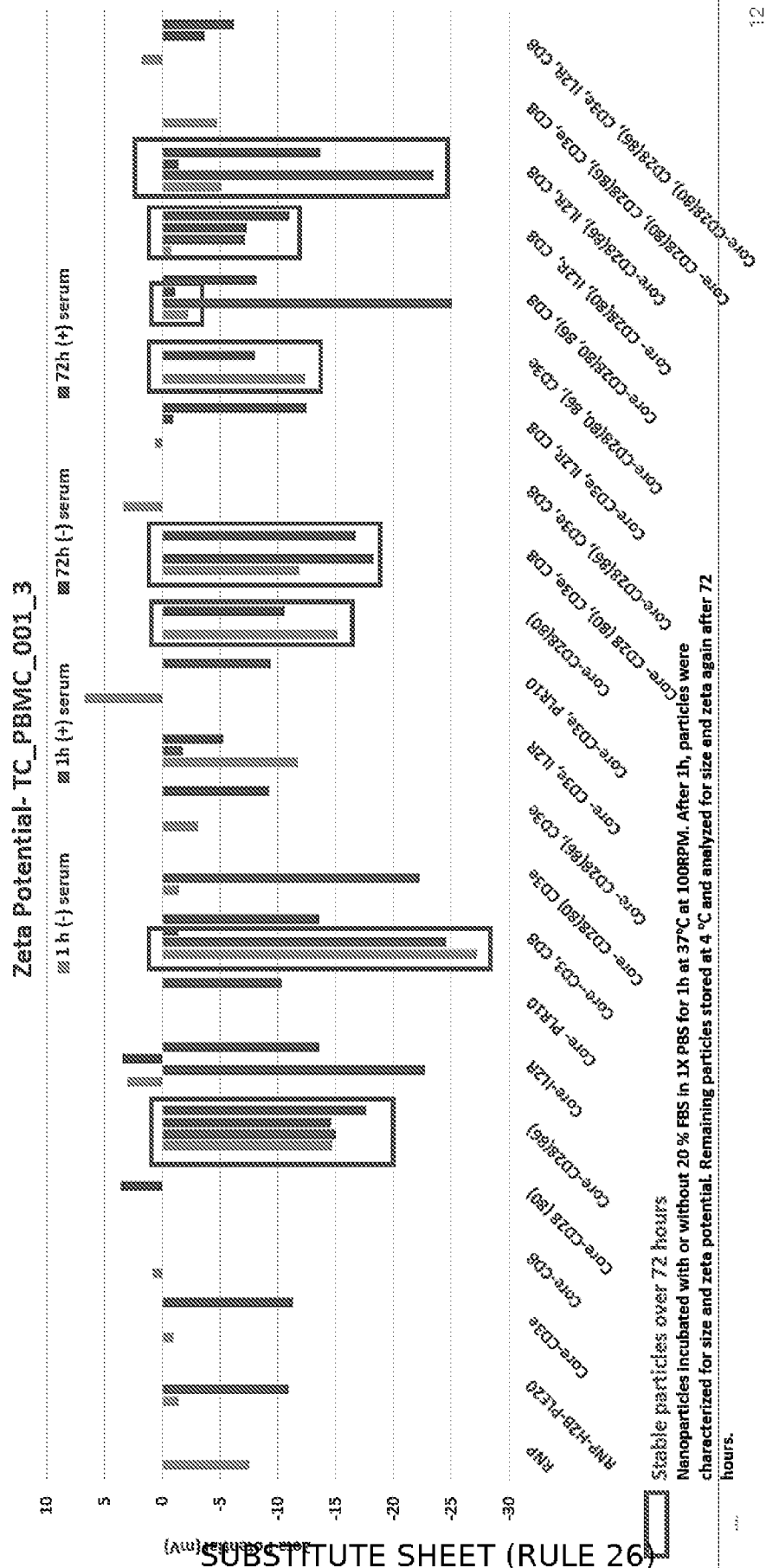


Figure 19

TC_PBMC_001_3 (Study C)



SUBSTITUTE SHEET (RULE 26)

Takeaway

Polydispersity index (PDI) ≤ 0.1 = Monodisperse solution
 Ideal nanoparticles mostly have PDIs ranging between 0.1-0.5
 Particles with smaller PDIs considered more stable.
 Arrows indicate particles with stable PDIs at 1h and 72h timepoints.
 Particles with PDI ≤ 0.3 included above.

44

Figure 23

SYBR GOLD Inclusion assay

- Fluorescence values obtained at 1h more robust than values obtained at 72h
- Condensation of RNPs observed for all nanoparticle groups

[illegible]

Color-coded arrows indicate same group of nanoparticles in different conditions

Fluorescence
Intensity
($\propto$) PMP released
($1/\alpha$)
Nanoparticle
stability

Particles with low SYBR
GOLD fluorescence form
stable complexes with RNPs
and are considered ideal
candidates.

Takeaway

Figure 24

Characterization of RNP-H2B (mini core)

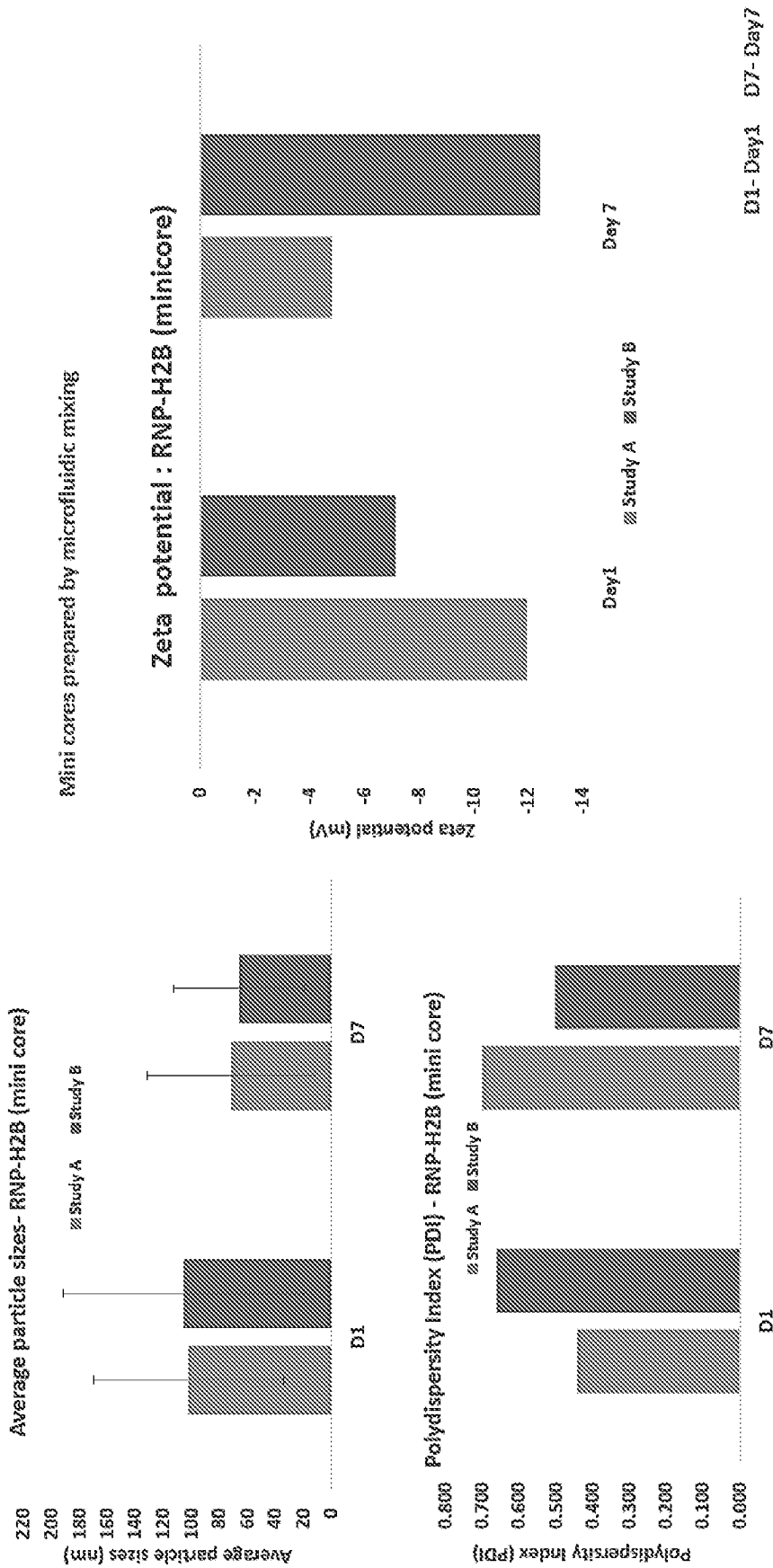


Figure 25

Characterization of RNP-H2B-PLE20:PDE20 (core)

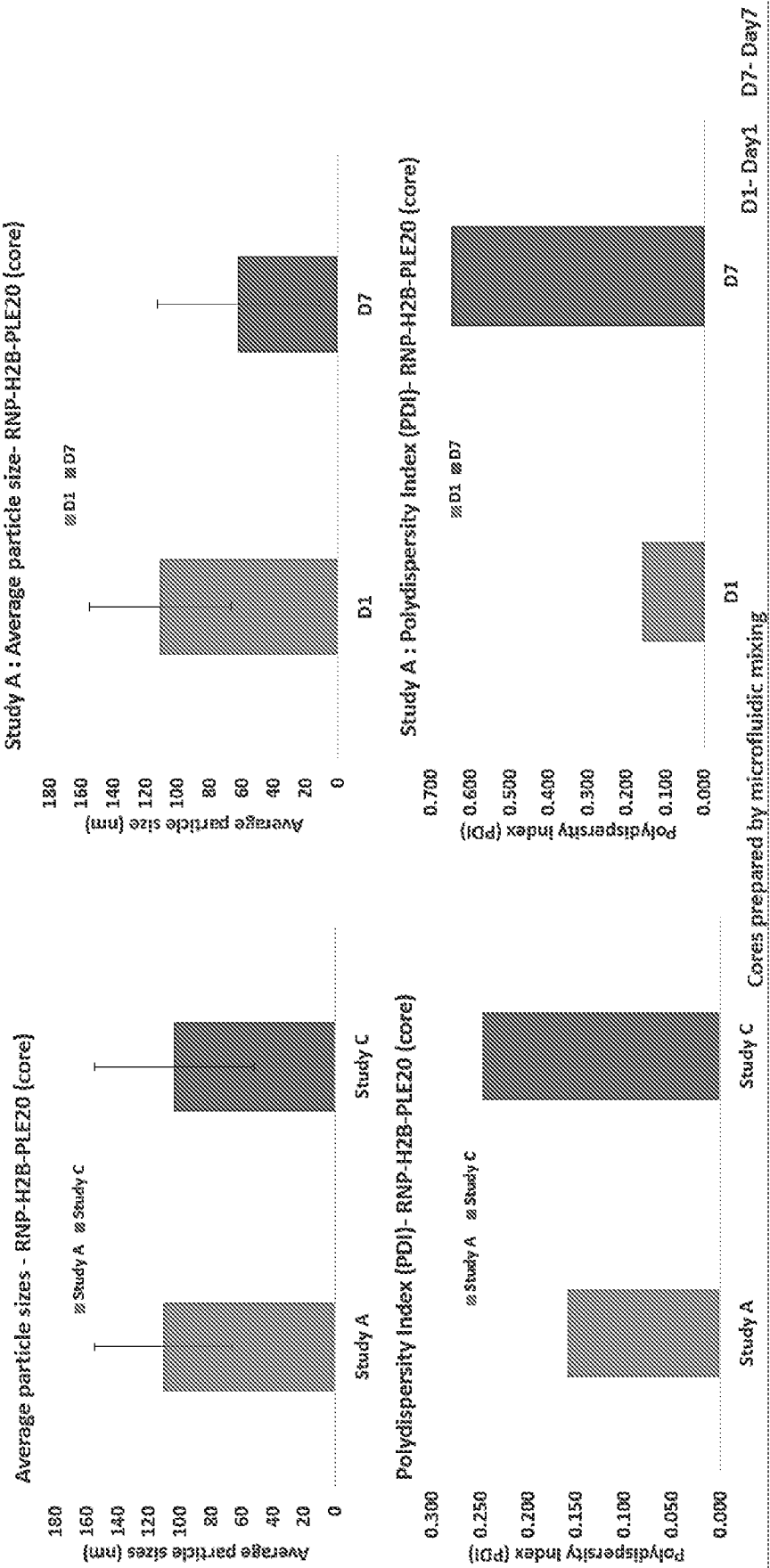


Figure 26

Serum stability of RNP-H2B-PLE20:PDE20 (core)

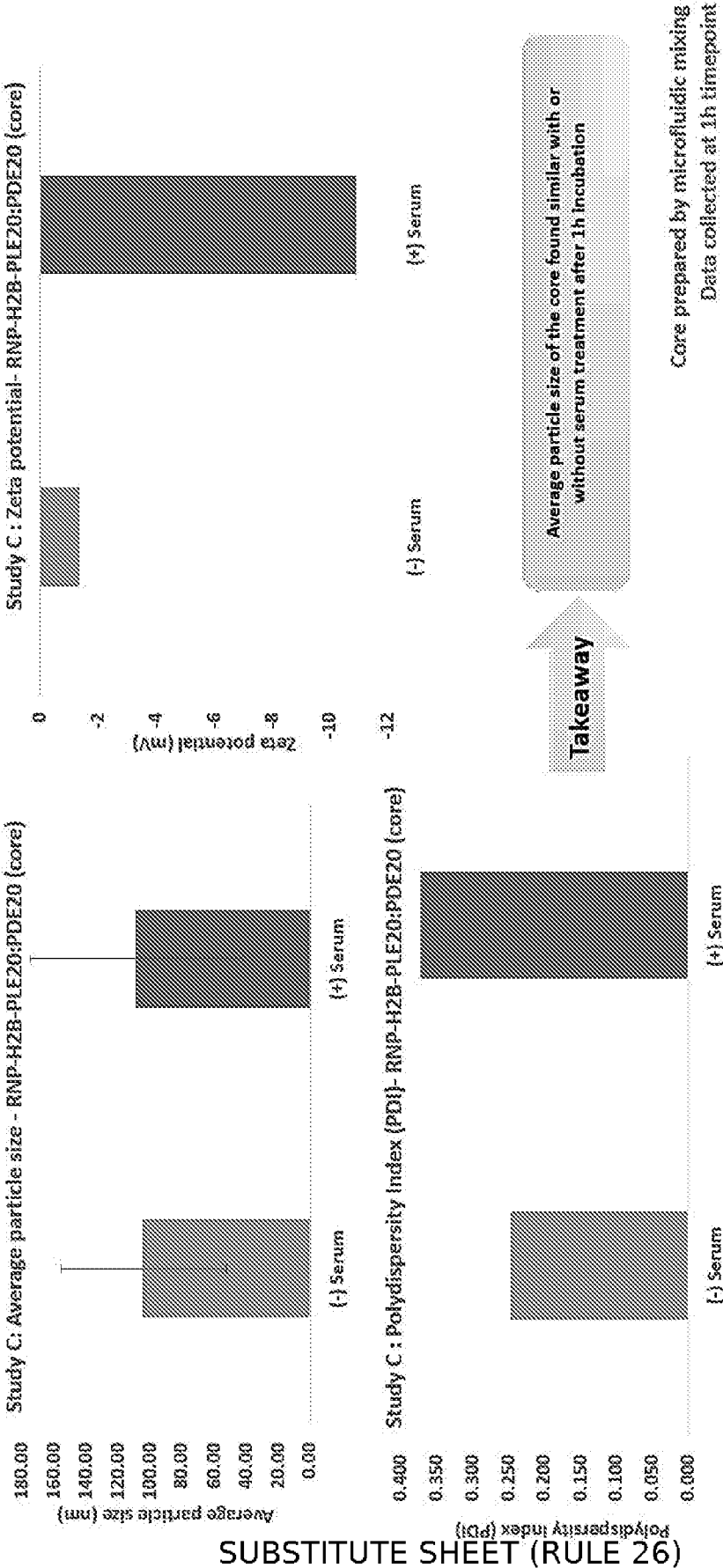


Figure 27

PDI evaluation: Ligand-modified particles- DAY 1

Polydispersity Index (PDI) of ligand-modified particles across studies A, B and C

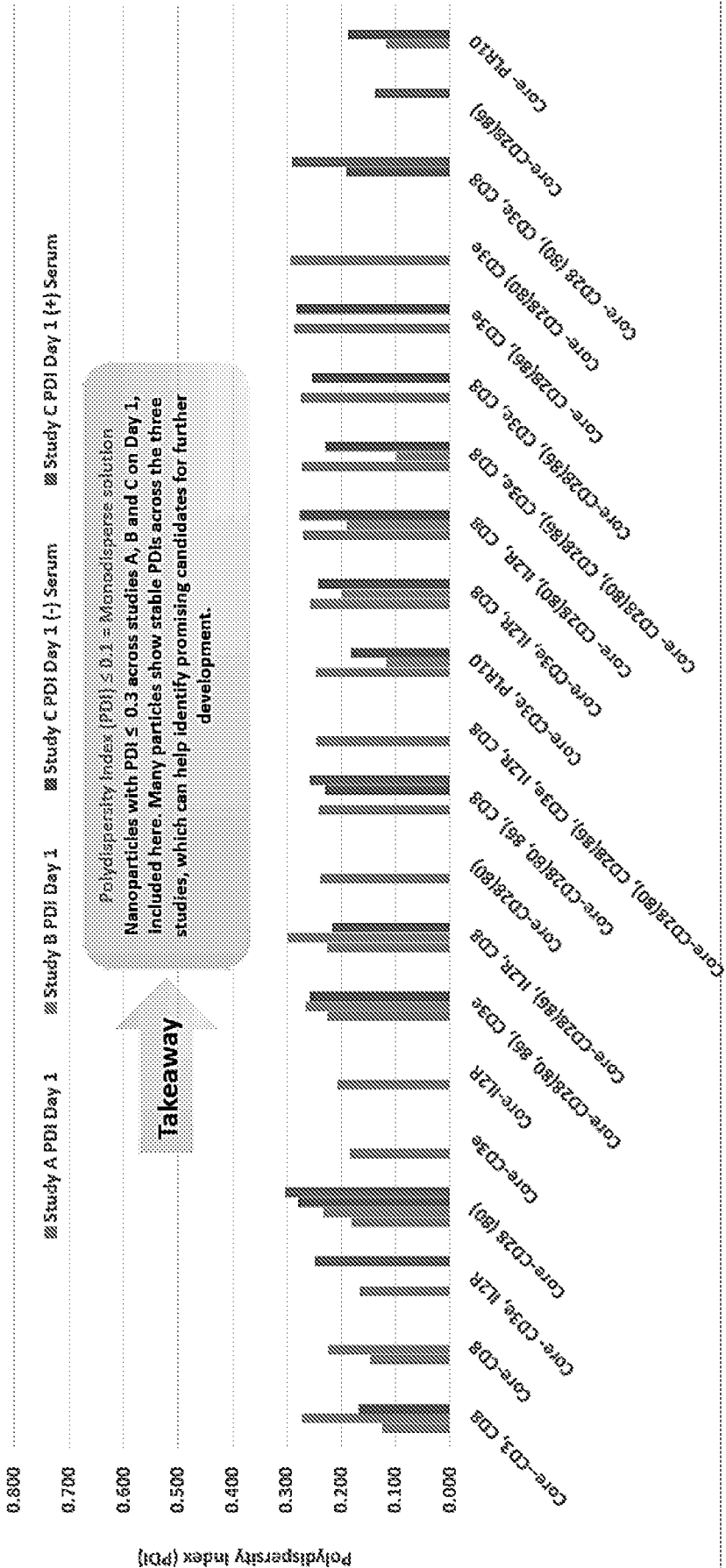


Figure 28

Automated Pipeline Sample Data

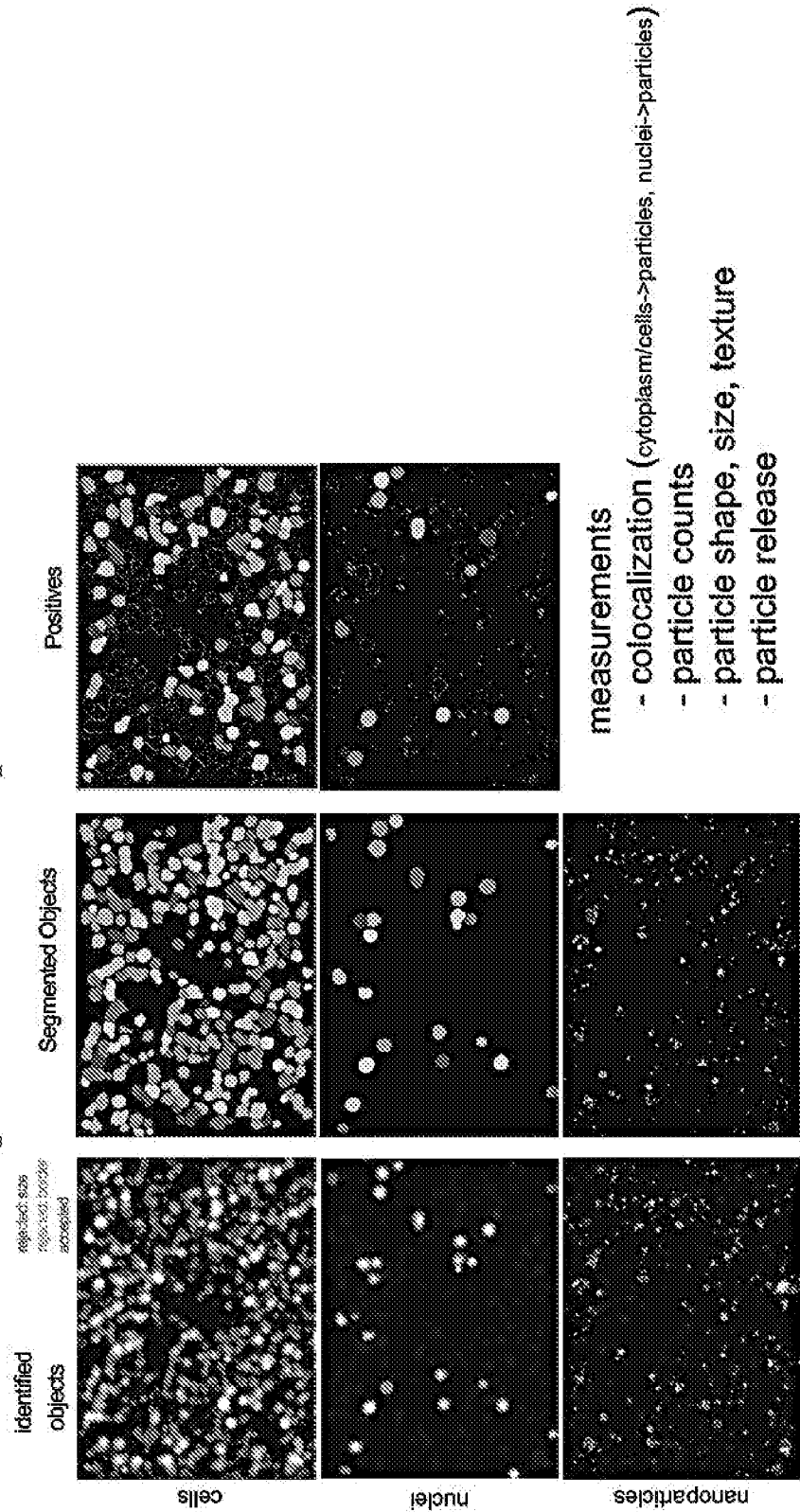


Figure 30

Cellular Particle Colocalization: Data Overview

Treatment	Time (hrs)								
	0.5	2.5	4.5	6.5	8.5	10.5	12.5	14.5	
LPO	28.66%	7.81%	23.40%	24.44%	7.96%	22.40%	22.01%	6.59%	
CORE ONLY	30.83%	30.45%	28.52%	26.71%	28.52%	25.61%	39.51%	20.42%	
RNP ONLY	16.41%	16.04%	9.18%	16.25%	14.49%	8.09%	12.32%	9.88%	
PLR10	23.01%	54.76%	19.10%	44.55%	18.41%	49.45%	53.09%	38.74%	
CD28_mCD80	66.35%	69.92%	52.41%	53.98%	68.29%	55.74%	70.59%	64.44%	
CD28_mCD86	29.68%	33.20%	46.26%	30.80%	29.09%	35.90%	32.03%	29.93%	
CD3	32.68%	37.22%	46.41%	45.14%	43.78%	36.29%	40.00%	46.74%	
CD8	93.10%	94.50%	96.28%	94.76%	98.90%	94.83%	97.96%	97.42%	
IL2R	43.07%	47.46%	53.98%	44.85%	50.28%	49.71%	37.77%	45.86%	
CD3 X CD8	10.93%	11.86%	18.66%	18.94%	8.99%	7.11%	7.09%	8.67%	
CD3 X CD28_mCD80	44.16%	51.34%	52.38%	41.12%	59.69%	48.68%	51.97%	49.70%	
CD3 X PLR10	51.64%	64.49%	74.80%	69.23%	63.98%	61.19%	71.17%	67.61%	
CD3 X IL2R	91.07%	94.44%	96.30%	92.34%	94.21%	92.19%	95.64%	91.70%	
CD28_mCD80 X CD28_mCD86	34.75%	34.64%	25.90%	30.07%	27.21%	33.06%	31.43%	33.33%	
CD3 X CD8 X CD28_mCD80	97.67%	97.28%	97.99%	94.31%	97.49%	98.85%	98.06%	96.46%	
CD3 X CD28_mCD80 X CD28_mCD86	98.69%	96.78%	97.40%	100.00%	97.34%	96.95%	97.96%	97.85%	
CD3 X CD8 X CD28_mCD86	22.58%	24.10%	20.37%	14.56%	21.34%	13.71%	20.00%	21.96%	
CD3 X CD8 X IL2R	34.34%	48.59%	27.43%	30.07%	31.97%	52.24%	31.00%	27.44%	
CD8 X CD28_mCD80 X CD28_mCD86	95.48%	97.55%	96.47%	96.62%	98.57%	97.23%	96.15%	98.71%	
CD8 X CD28_mCD86 X IL2R	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	
CD8 X CD28_mCD80 X IL2R	96.05%	96.60%	97.47%	98.47%	99.56%	96.09%	98.00%	97.30%	
CD3 X CD8 X CD28_mCD80 X CD28_mCD86	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	
CD3_X CD8 X CD28_mCD80 X CD28_mCD86 X IL2R	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	

Figure 31

Cellular Particle Colocalization: Data Overview

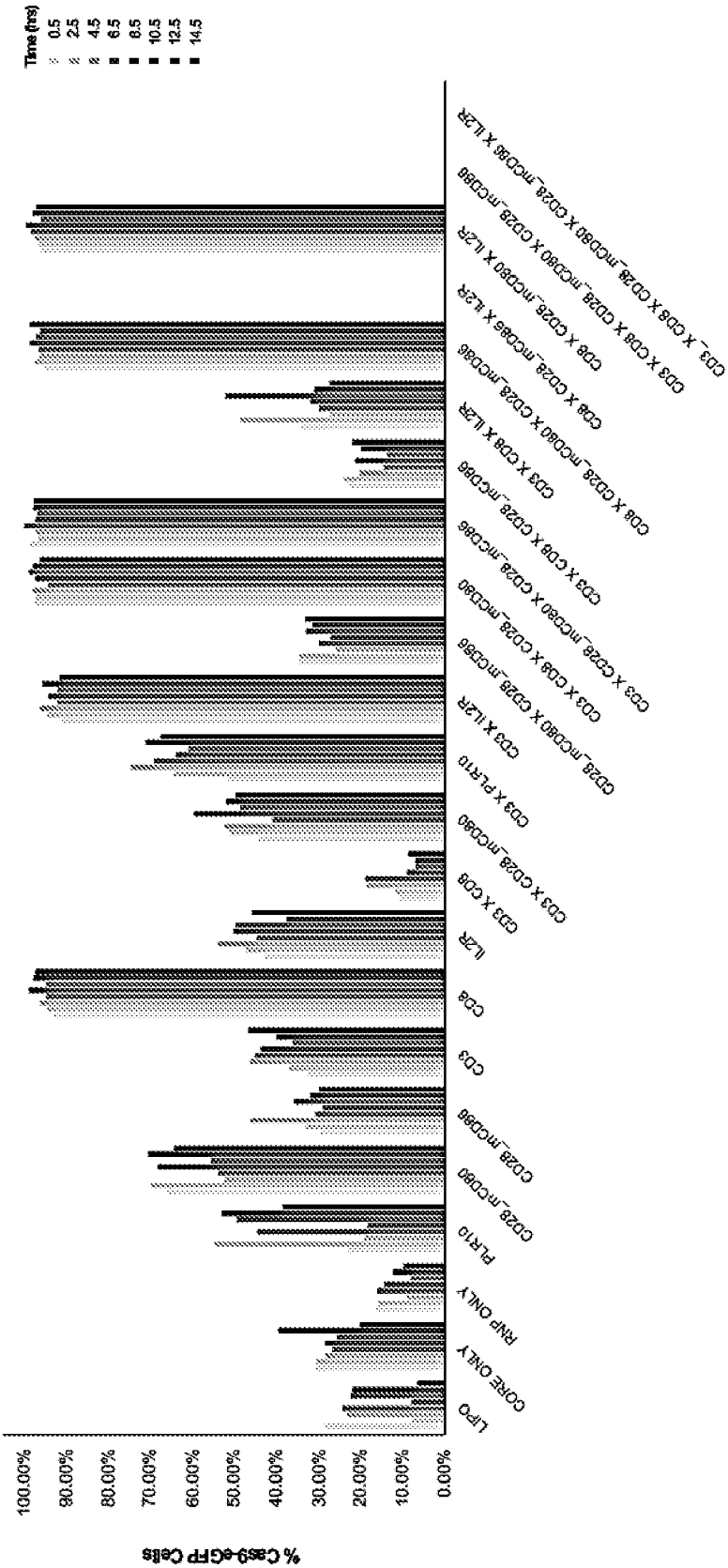


Figure 32

Nuclear Particle Integration: Data Overview

Treatment	Time (hrs)							
	0.5	2.5	4.5	6.5	8.5	10.5	12.5	14.5
LIPO	35.71%	5.88%	14.29%	13.33%	6.25%	14.29%	14.29%	0.00%
CORE ONLY	40.71%	40.15%	38.30%	36.55%	36.88%	35.81%	47.73%	25.81%
RNP ONLY	13.21%	12.50%	7.84%	8.00%	43.75%	10.20%	11.76%	33.33%
PLR10	22.22%	45.26%	21.62%	46.59%	53.41%	43.12%	24.11%	50.00%
CD28_mCD80	58.33%	58.62%	48.28%	50.00%	50.00%	48.28%	60.00%	51.72%
CD28_mCD86	46.74%	42.70%	45.31%	38.64%	35.96%	44.05%	36.47%	36.26%
CD3	41.67%	43.48%	50.00%	55.00%	39.13%	40.91%	47.83%	50.00%
CD8	86.36%	82.61%	78.26%	82.61%	86.36%	78.26%	82.61%	82.61%
IL2R	44.83%	55.00%	53.26%	41.41%	48.89%	51.11%	35.79%	40.45%
CD3 × CD8	21.43%	16.67%	25.00%	25.00%	20.00%	18.18%	20.00%	18.18%
CD3 × CD28_mCD80	48.15%	46.15%	50.00%	38.46%	60.00%	45.83%	45.45%	54.55%
CD3 × PLR10	53.19%	68.75%	73.33%	76.67%	57.78%	55.56%	72.97%	75.00%
CD3 × IL2R	88.89%	92.59%	85.19%	85.19%	88.89%	84.62%	88.89%	84.62%
CD28_mCD80 × CD28_mCD86	20.00%	8.33%	15.38%	7.14%	8.33%	7.14%	25.00%	7.14%
CD3 × CD8 × CD28_mCD80	100.00%	91.67%	90.91%	90.91%	100.00%	100.00%	100.00%	100.00%
CD3 × CD28_mCD80 × CD28_mCD86	81.35%	78.26%	92.00%	78.26%	81.82%	87.50%	82.61%	82.61%
CD3 × CD8 × CD28_mCD86	45.45%	29.41%	21.43%	23.53%	21.43%	31.25%	33.33%	53.33%
CD3 × CD8 × IL2R	42.95%	46.67%	32.26%	34.36%	36.71%	47.76%	36.24%	32.34%
CD8 × CD28_mCD80 × CD28_mCD86	83.33%	71.31%	86.29%	67.50%	89.34%	88.14%	84.17%	77.87%
CD8 × CD28_mCD86 × IL2R	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
CD8 × CD28_mCD80 × IL2R	83.33%	87.88%	78.95%	83.33%	86.75%	87.50%	85.37%	82.09%
CD3 × CD8 × CD28_mCD80 × CD28_mCD86	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
CD3_ × CD8 × CD28_mCD80 × CD28_mCD86 × IL2R	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Figure 33

Nuclear Particle Integration: Data Overview

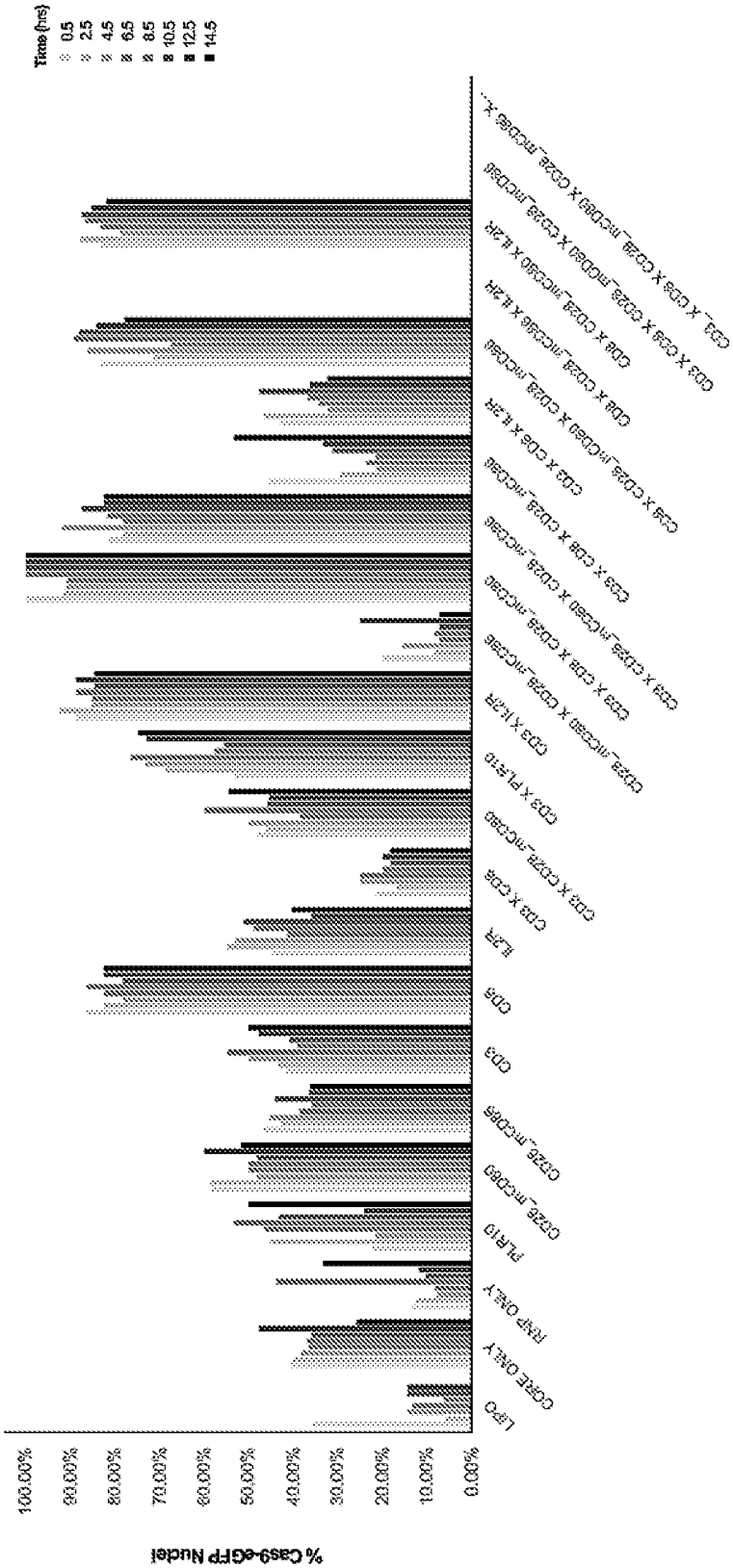
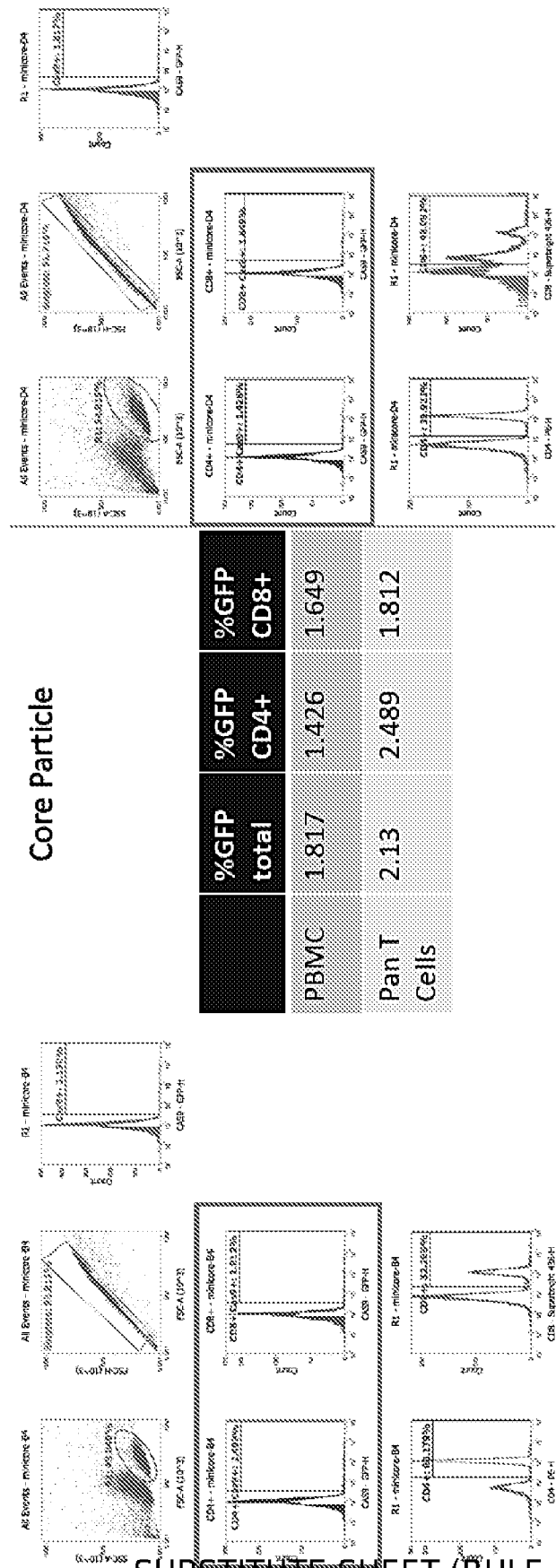


Figure 34

T-CELL AND PBMC TARGETING



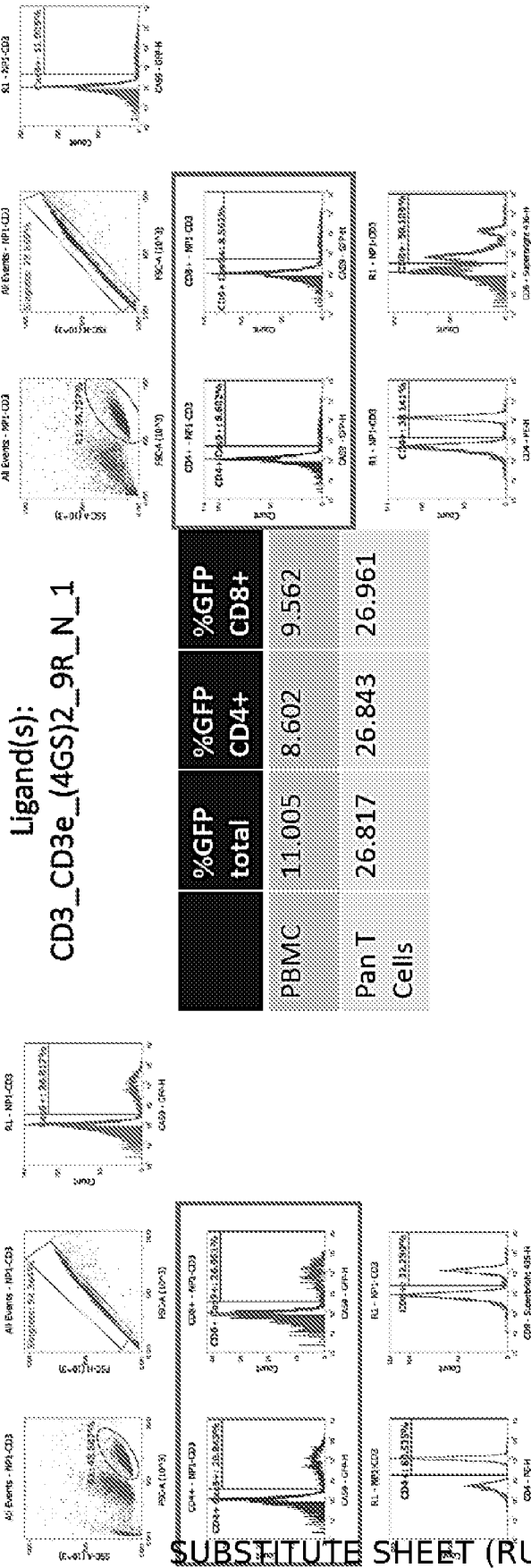
- Does not contain targeting moieties

Human Primary PBMCs

Human Primary Pan T-Cells

Figure 36

T-CELL AND PBMC TARGETING



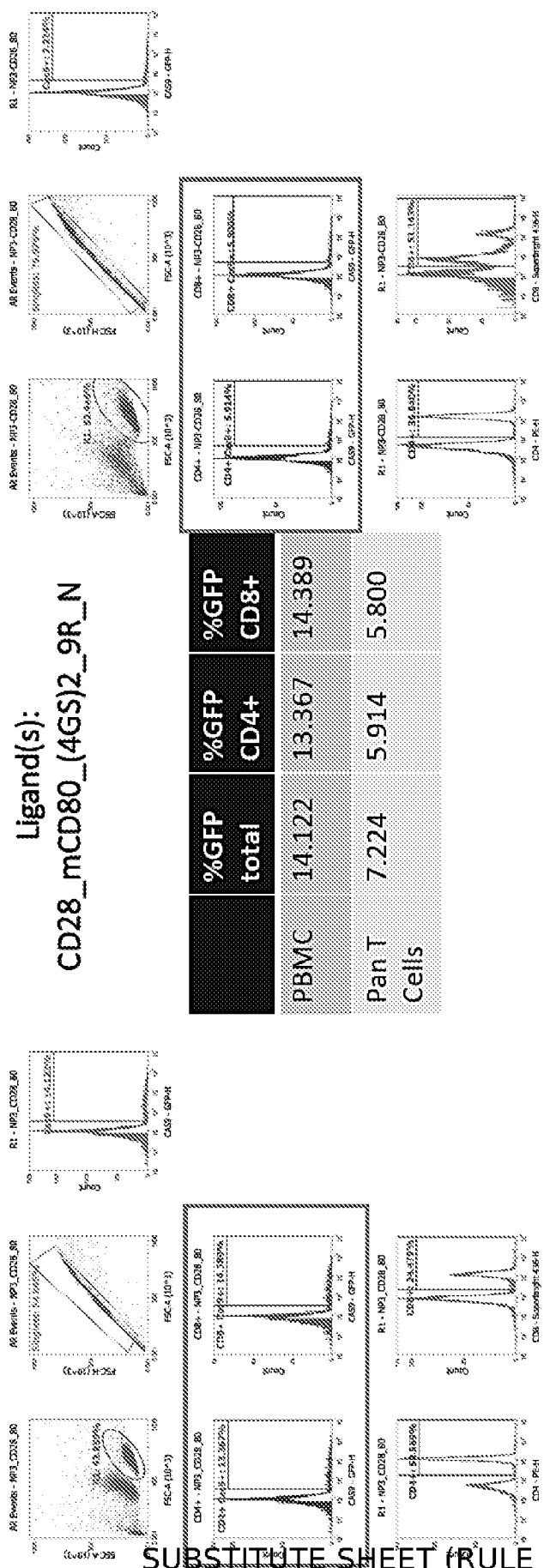
Human Primary PBMCs

- Monovalent surface targeting CD3
- Broad T cell/Thymocyte specificity

Human Primary Pan T-Cells

Figure 38

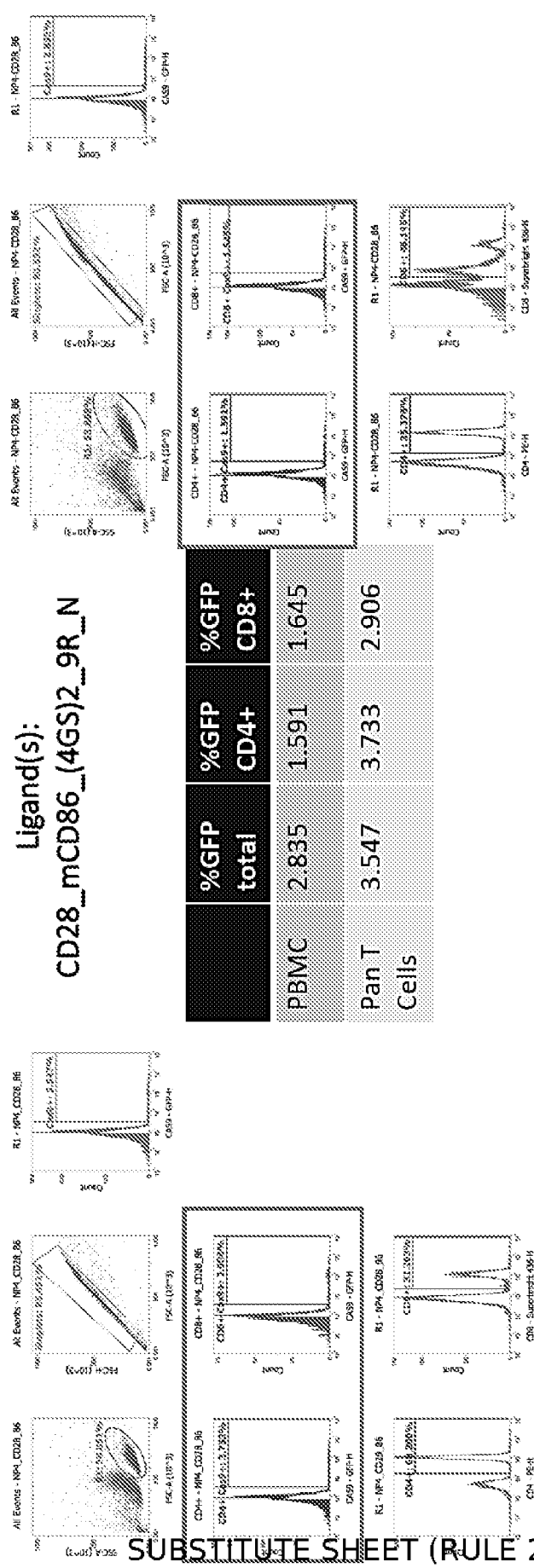
T-CELL AND PBMC TARGETING



- Targets CD28, a T-cell marker
- Ligand mimics CD80 on antigen-presenting cells
- Modest uptake in T-cells

Figure 40

T-CELL AND PBMC TARGETING



	%GFP total	%GFP CD4+	%GFP CD8+
PBMC	2.835	1.591	1.645
Pan T Cells	3.547	3.733	2.906

Human Primary Pan T-Cells

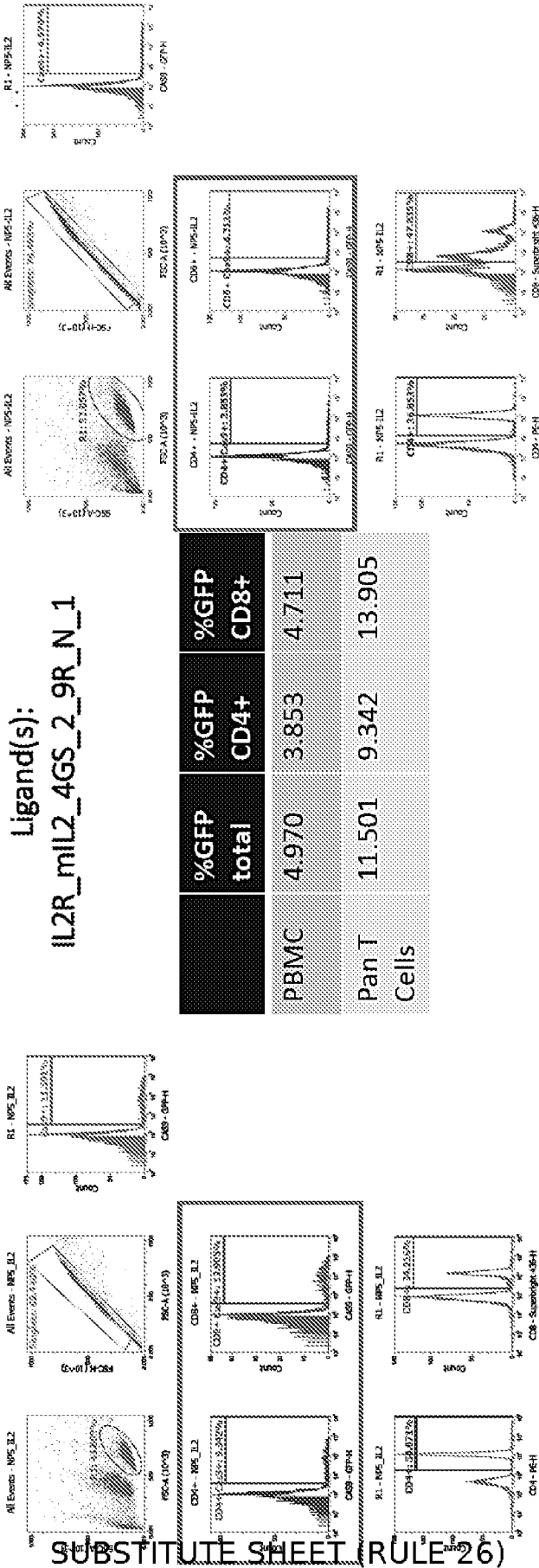
Human Primary PBMCs

- Targets CD28, a T-cell marker
- Ligand mimics CD86 on antigen-presenting cells
- No uptake in T-cells

Figure 41

T-CELL AND PBMC TARGETING

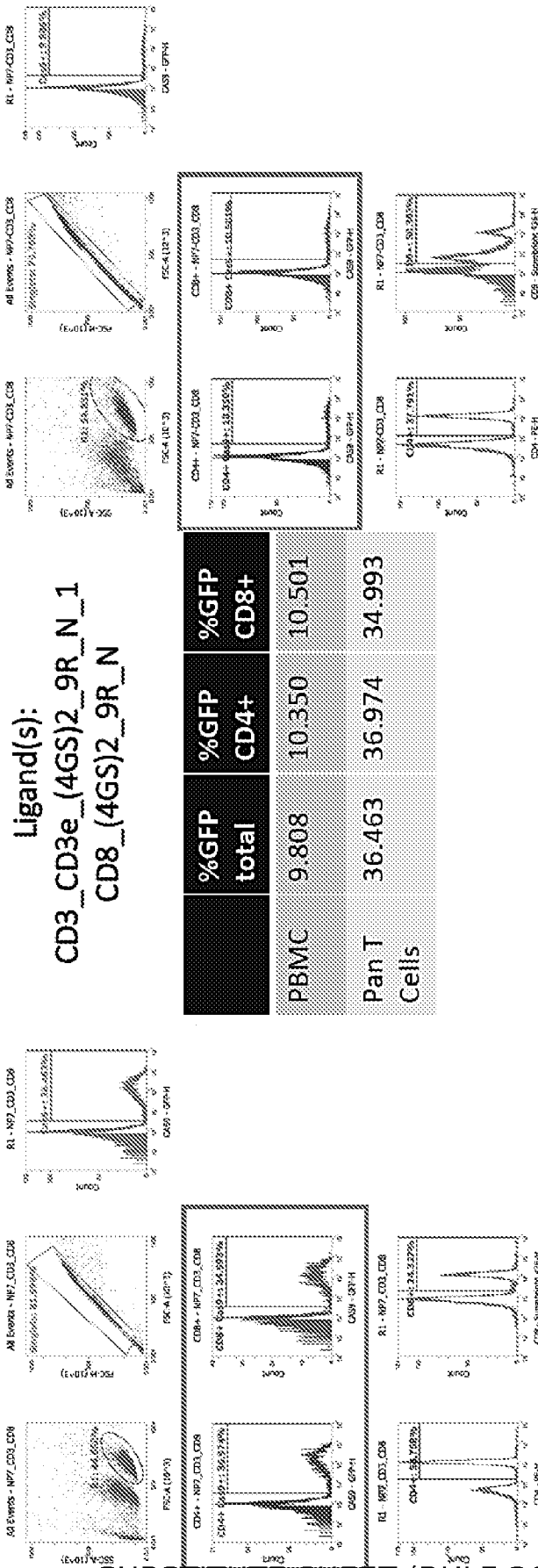
Ligand(s):
IL2R_mIL2_4GS_2_9R_N_1



- Monovalent surface targeting IL2R
- Modest uptake in T-cells

Figure 42

T-CELL AND PBMC TARGETING

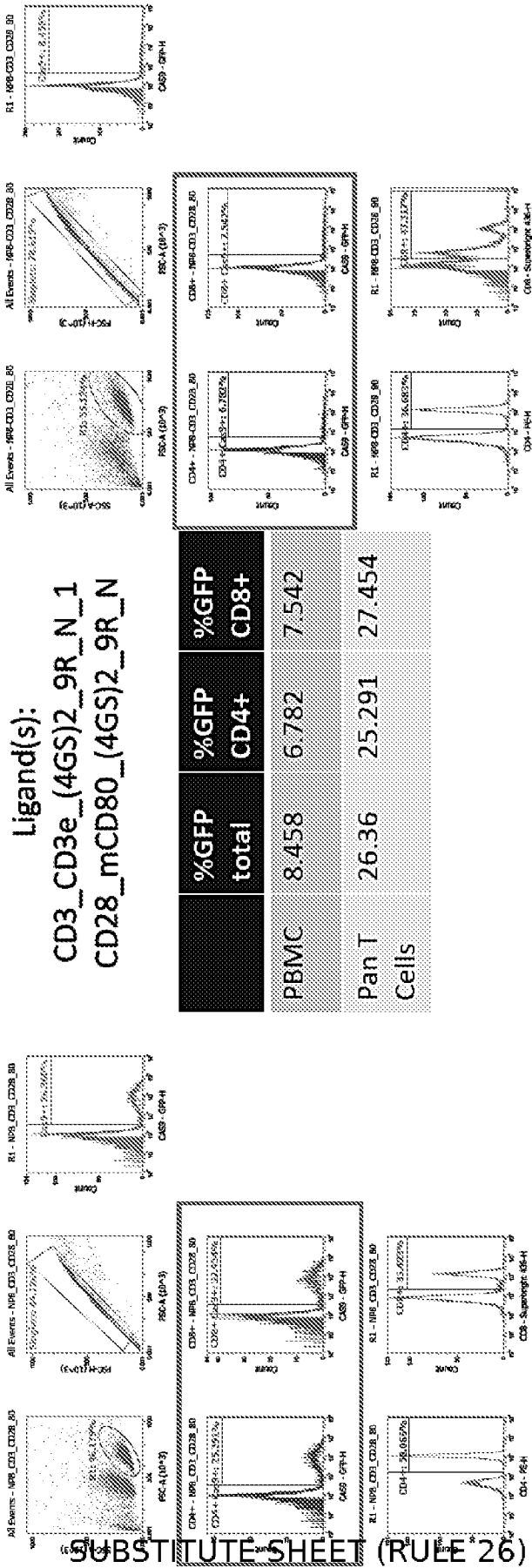


Human Primary Pan T-Cells Human Primary PBMCs

• Heterodivalent combination of ligands targeting CD3 and CD8

Figure 43

T-CELL AND PBMC TARGETING



• Heterodivalent combination of ligands targeting CD3 and CD28 (derived from CD80)

Human Primary PBMCs

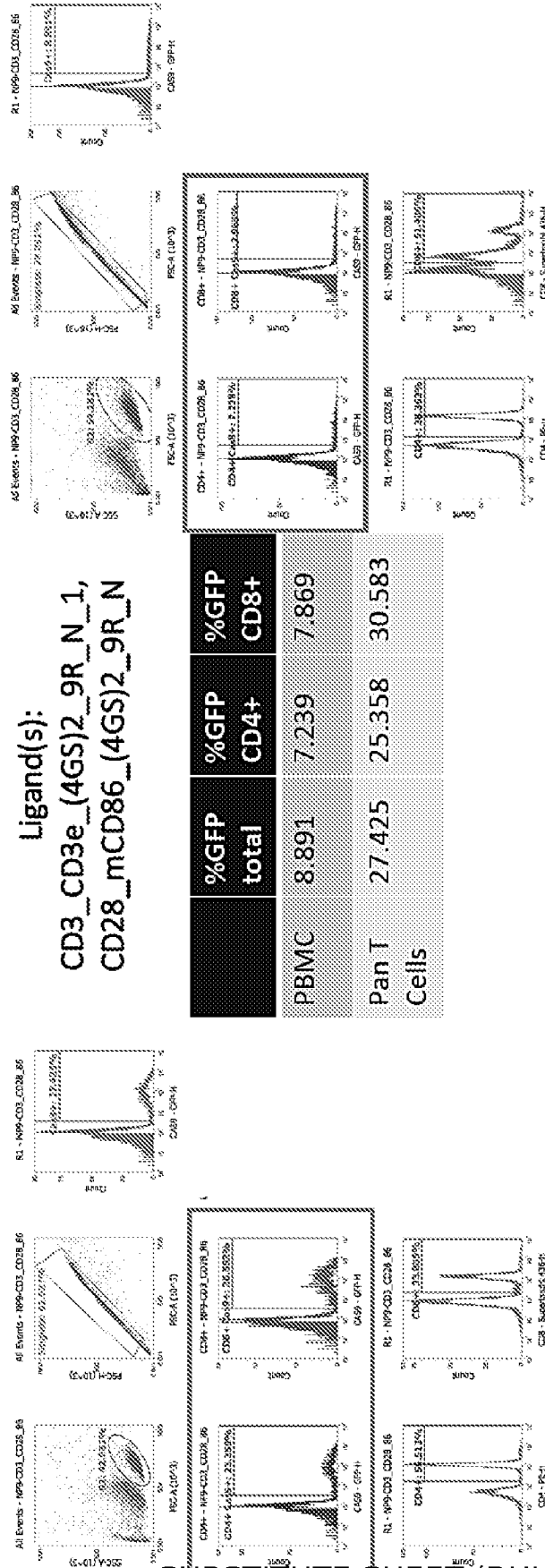
Human Primary Pan T-Cells

Figure 44

T-CELL AND PBMC TARGETING

Ligand(s):

CD3_CD3e_(4GS)2_9R_N_1,
CD28_mCD86_(4GS)2_9R_N



Human Primary Pan T-Cells

- Heterodivalent combination of ligands targeting CD3 and CD28 {derived from CD86}

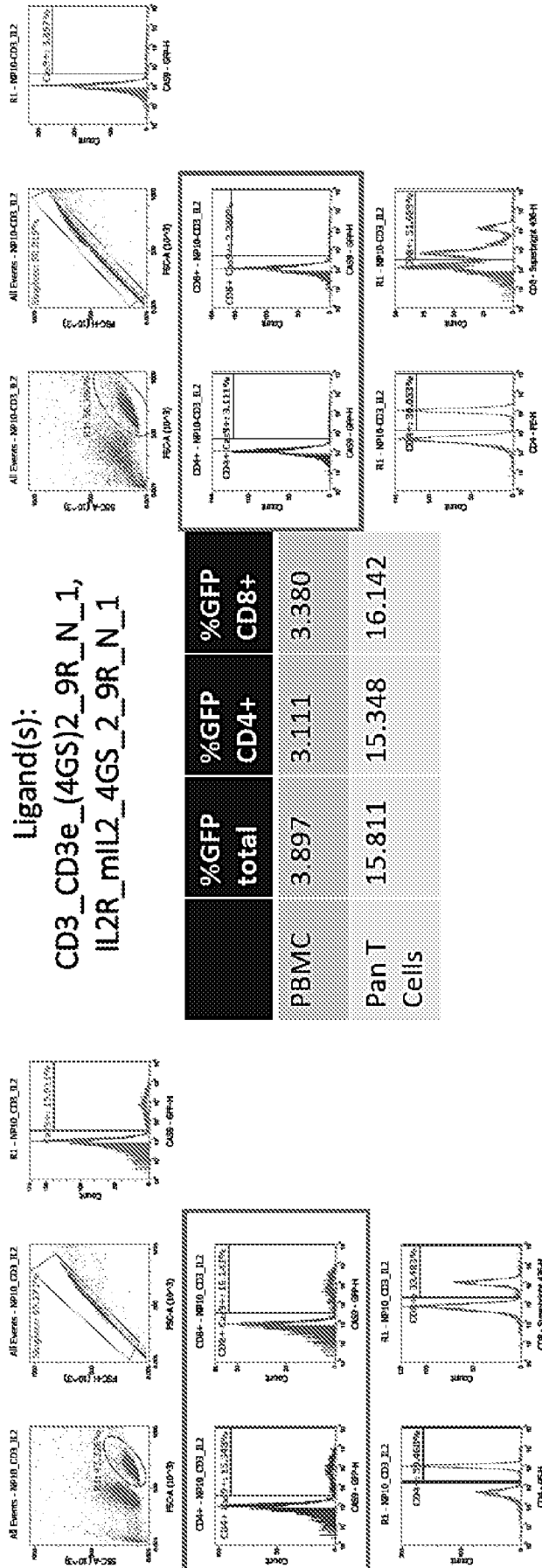
Human Primary PBMCs

Figure 45

T-CELL AND PBMC TARGETING

Ligand(s):

CD3_CD3e_(4GS)2_9R_N_1,
IL2R_mIL2_4GS_2_9R_N_1

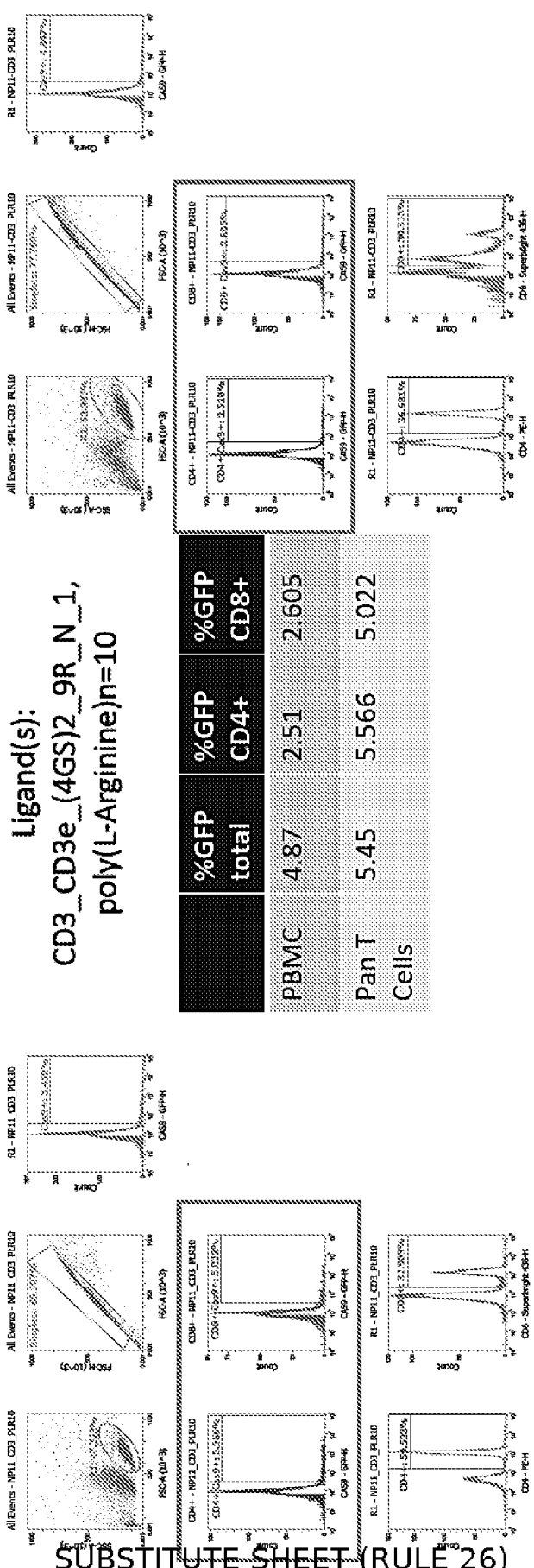


	%GFP total	%GFP CD4+	%GFP CD8+
PBMC	3.897	3.111	3.380
Pan T Cells	15.811	15.348	16.142

- Heterodivalent combination of ligands targeting CD3 and IL2R

Figure 46

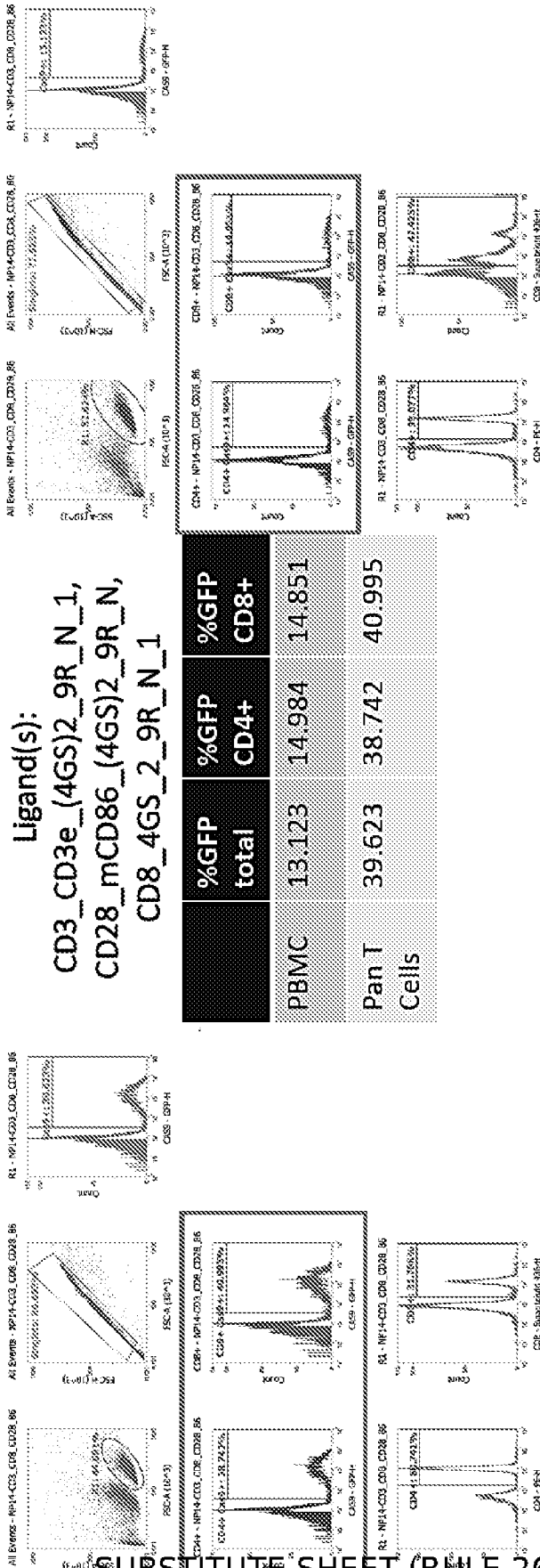
T-CELL AND PBMC TARGETING



- Human Primary Pan T-Cells
- Human Primary PBMCs
- Poly(L-Arginine) coating along with CD3 ligand greatly reduces efficacy from 26%

Figure 47

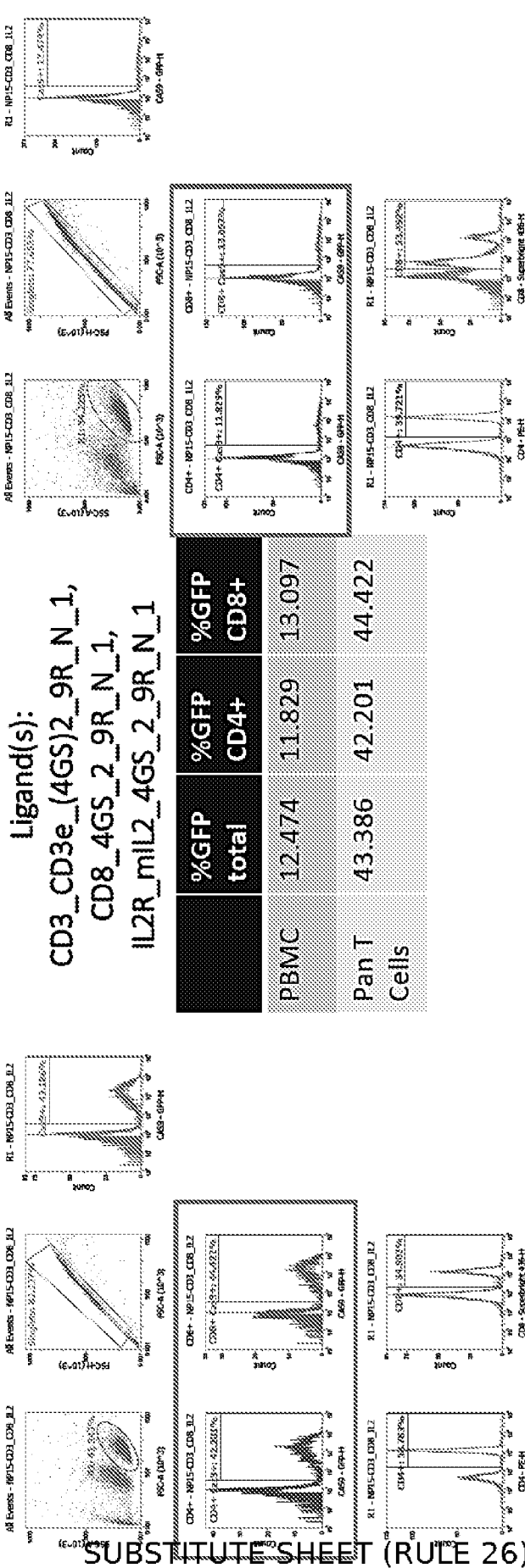
T-CELL AND PBMC TARGETING



- Heterotrivalent surface targeting CD3, CD28 and CD8
- Slight bias of CD8+ T-cell targeting

Figure 49

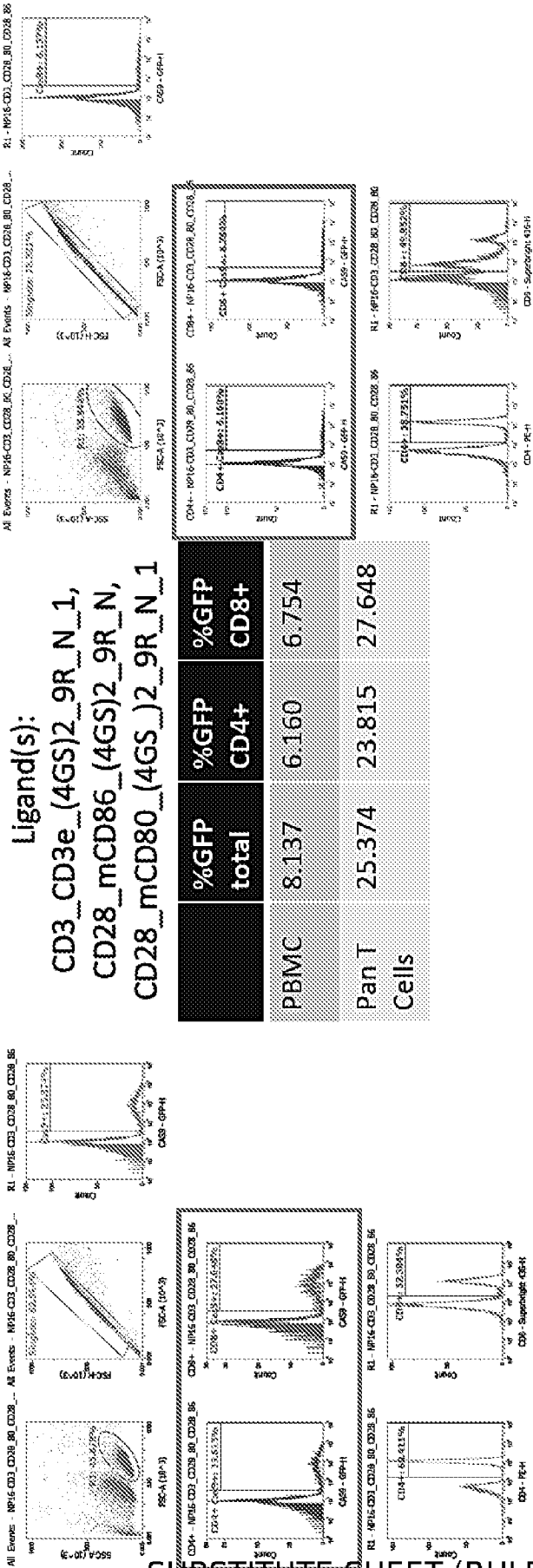
T-CELL AND PBMC TARGETING



- Heterotrivalent surface targeting CD3, CD8, and IL2R
- Slight bias of CD8+ T-cell targeting
- ~44.4% efficient CD8+ T Cell targeting

Figure 50

T-CELL AND PBMC TARGETING



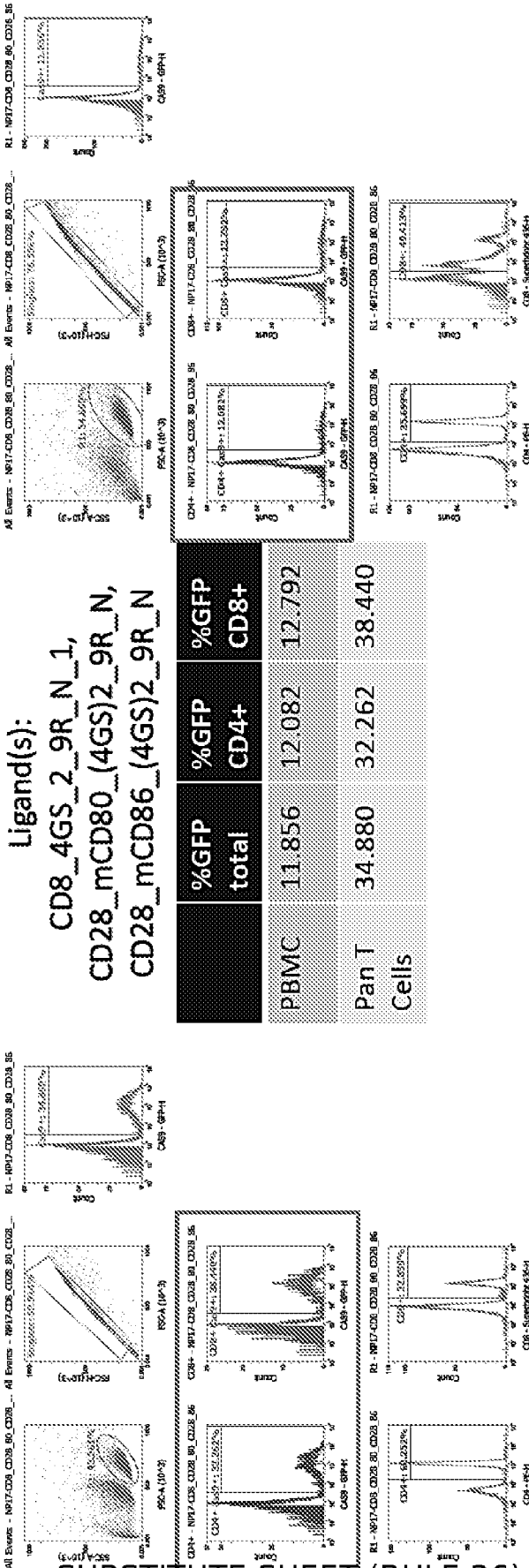
Human Primary PBMCs

- Heterotrivalent surface targeting CD3 and CD28 (mimicking CD80 and CD86 co-presentation)
- Reduction in uptake vs. CD8-containing heterotrivalent surface without CD28(86)
- ~4% bias in targeting CD8+ vs. CD4+ T-cells

Human Primary Pan T-Cells

Figure 52

T-CELL AND PBMC TARGETING



SUBSTITUTE SHEET (RULE 26)

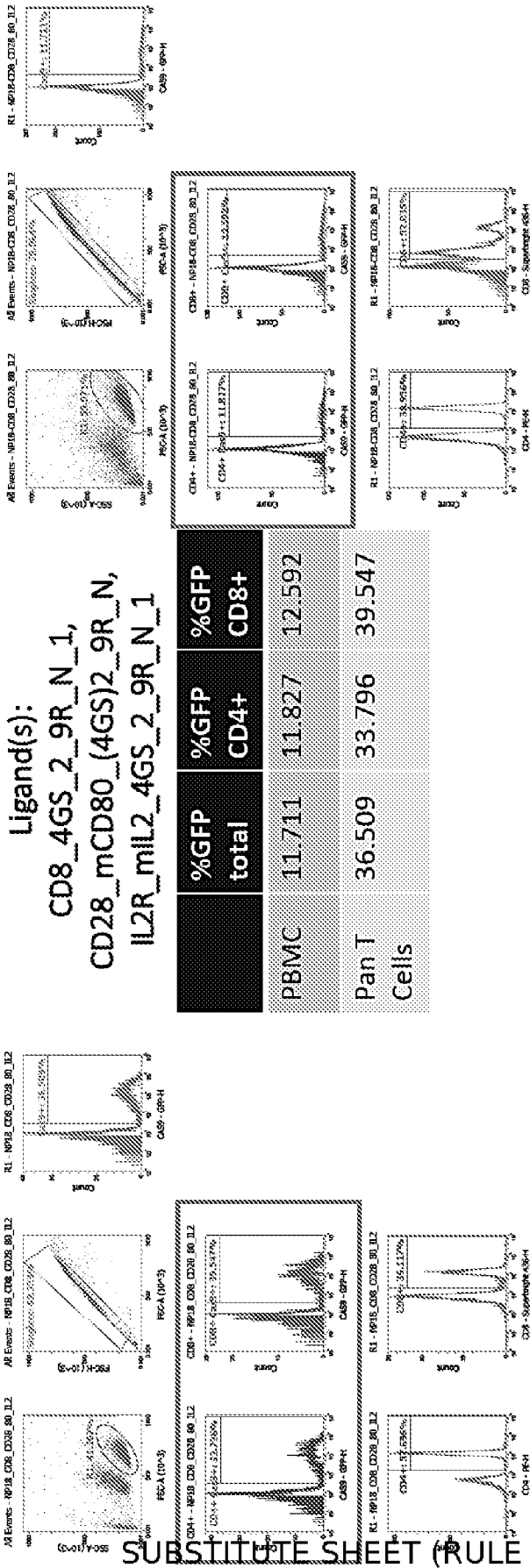
Human Primary PBMCs

- Heterotrivalent surface targeting CD8 and CD28 (mimicking CD80 and CD86 co-presentation)
- Efficient CD8+ T-cell targeting
- ~6% bias in targeting CD8+ vs. CD4+ T-cells

Human Primary Pan T-Cells

Figure 53

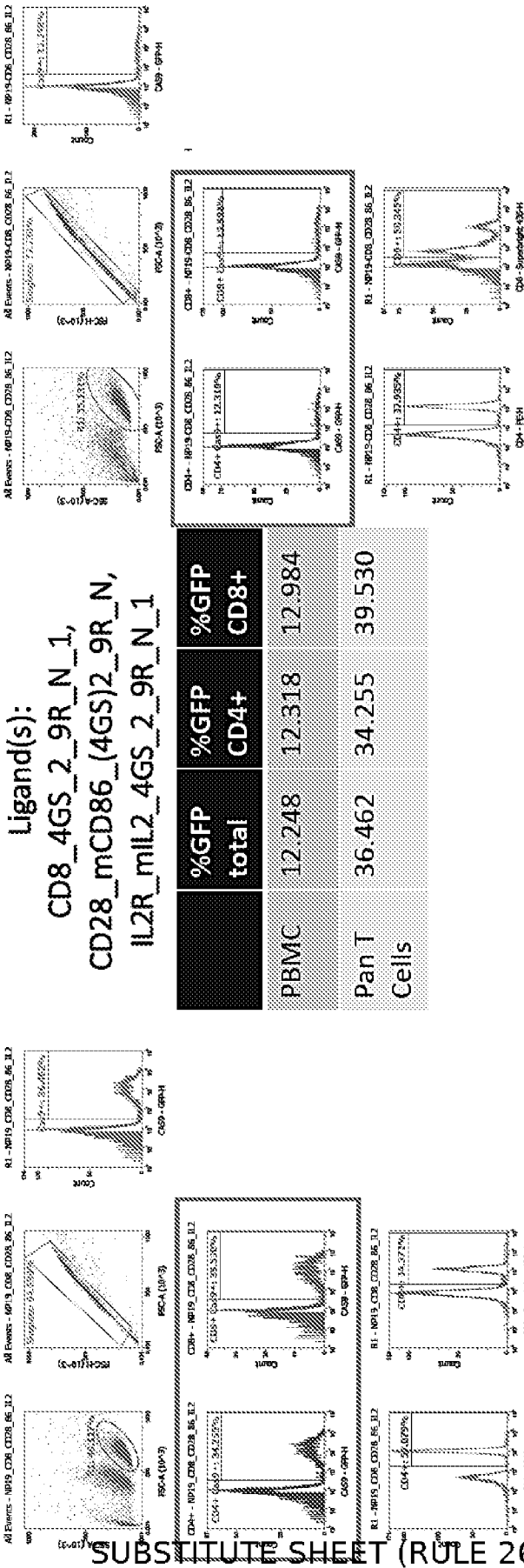
T-CELL AND PBMC TARGETING



- Heterotrivalent surface targeting CD8, CD28(80) and IL2R
- Efficient CD8+ T-cell targeting
- ~6% bias in targeting CD8+ vs. CD4+ T-cells

Figure 54

T-CELL AND PBMC TARGETING



Human Primary PBMCs

- Heterotrivalent surface targeting CD8, CD28(86) and IL2R
- Efficient CD8+ T-cell targeting
- ~6% bias in targeting CD8+ vs. CD4+ T-cells

Human Primary Pan T-Cells

Figure 55

RESULTS COMPILATION

Ligand	Imaging Analysis (1h)		Pan-T Cell Flow Analysis (24h)			PBMC Flow Analysis (24h)		
	%Total T Cell	%Total PBMC	%Total	%CD4	%CD8	%Total	%CD4	%CD8
Poly(L-Arginine)n-10	92	26	4.881	5.926	2.466	5.935	2.466	2.841
CD3_CD3e_(4GS)2_9R_N_1	3	4	26.817	26.843	26.961	11.005	8.602	9.562
CD8_(4GS)2_9R_N	57	39	36.391	39.041	33.179	26.613	24.63	26.002
CD28_mCD80_(4GS)2_9R_N	60	3	7.224	5.914	5.800	14.122	13.367	14.389
CD28_mCD86_(4GS)2_9R_N	11	11	3.547	3.733	2.906	2.835	1.591	1.645
IL2R_mIL2_4GS_2_9R_N_1	39	24	11.501	9.342	13.905	4.970	3.853	4.711
CD3_CD3e_(4GS)2_9R_N_1 CD8_(4GS)2_9R_N	63	37	36.463	36.974	34.993	9.808	10.350	10.501
CD3_CD3e_(4GS)2_9R_N_1 CD28_mCD80_(4GS)2_9R_N	25	23	26.36	25.291	27.454	8.458	6.782	7.542
CD3_CD3e_(4GS)2_9R_N_1 CD28_mCD86_(4GS)2_9R_N	90	46	27.425	25.358	30.583	8.891	7.239	7.869
CD3_CD3e_(4GS)2_9R_N_1 IL2R_mIL2_4GS_2_9R_N_1	6	6	15.811	15.348	16.142	3.897	3.111	3.380
CD3_CD3e_(4GS)2_9R_N_1, poly(L-Arginine)n-10	-	-	5.45	5.566	5.022	4.87	2.51	2.605

Figure 56

RESULTS COMPILATION

Ligand	Imaging Analysis (1h)		Pan-T Cell Flow Analysis (24h)			PBMC Flow Analysis (24h)		
	%Total T Cell	%Total PBMC	%Total	%CD4	%CD8	%Total	%CD4	%CD8
CD28_mCD80_4GS)2_9R_N, CD28_mCD86_4GS)2_9R_N	22	90	12.461	12.008	13.156	7.020	5.914	5.696
CD3_CD3e_4GS)2_9R_N_1, CD28_mCD86_4GS)2_9R_N, CD8_4GS_2_9R_N_1	21	21	39.623	38.742	40.995	13.123	14.984	14.851
CD3_CD3e_4GS)2_9R_N_1, CD8_4GS_2_9R_N_1, IL2R_mIL2_4GS_2_9R_N_1	11	21	43.386	42.201	44.422	12.474	11.829	13.097
CD3_CD3e_4GS)2_9R_N_1, CD28_mCD80_4GS)2_9R_N, CD8_4GS_2_9R_N_1	7	6	41.285	39.209	43.922	13.476	12.624	13.330
CD3_CD3e_4GS)2_9R_N_1, CD28_mCD86_4GS)2_9R_N, CD28_mCD80_4GS)2_9R_N_1	35	36	25.374	23.815	27.648	8.137	6.160	6.754
CD8_4GS_2_9R_N_1, CD28_mCD80_4GS)2_9R_N, CD28_mCD86_4GS)2_9R_N	39	21	34.880	32.262	38.440	11.856	12.082	12.792
CD8_4GS_2_9R_N_1, CD28_mCD80_4GS)2_9R_N, IL2R_mIL2_4GS_2_9R_N_1	42	43	36.509	33.796	39.547	11.711	11.827	12.592
CD8_4GS_2_9R_N_1, CD28_mCD86_4GS)2_9R_N, IL2R_mIL2_4GS_2_9R_N_1	27	27	36.462	34.255	39.530	12.248	12.318	12.984

SUBSTITUTE SHEET (RULE 26)

Figure 57

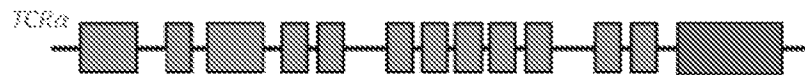
Figure 58 : Target Loci for TCR Engineering

Target Loci

TCR α promoter



TCR α constant region



TCR β promoter



TCR β constant region

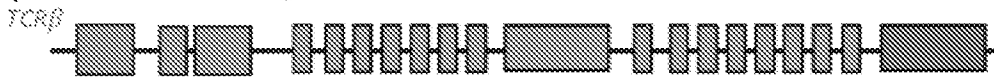
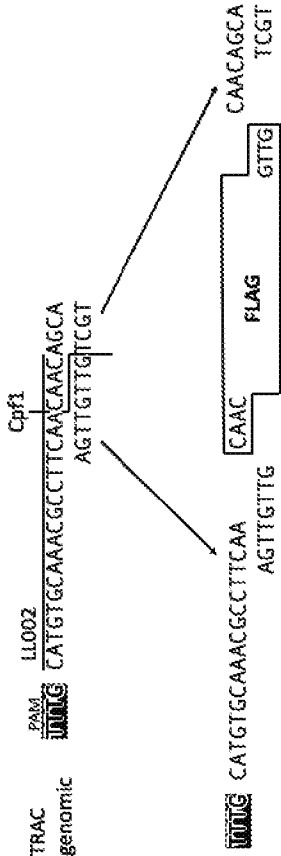


Figure 59: TCR Specific Guide RNA and TALEN Sequences

Sequence #	Sequence	Details
1	CATGTGCAAACGCCTTCAACAACA	TRAC exon1 Cpf1 guide
2	cttctctatgtttccatgaagatg	TRAC promoter Cpf1 guide
3	GGTGTGGGAGATCTCTGCTTCTGA	TRAB exon1 Cpf1 guide
4	CAGATGGGCTGAAGTCTCCACTGT	TRAB promoter Cpf1 guide
5	gagaatcaaaatcggatgaat	TRAC exon1 Cas9 gRNA1
6	aacaaatgtgtcacaagta	TRAC exon1 Cas9 gRNA2
7	GAGCCACTGTAGTCTGCAGA	TRA promoter Cas9 gRNA1
8	GGAACCGGGGATGCAGTGCC	TRA promoter Cas9 gRNA2
9	CAAACACAGCGACCTCGGGT	TRBC exon1 Cas9 gRNA1
10	AGAGATCTCCACACCCAAA	TRBC exon1 Cas9 gRNA2
11	CCCTGAGACAGGGGCTGCTT	TRB promoter Cas9 gRNA1
12	GGAAGCACACCCAGACGACA	TRB promoter Cas9 gRNA2
13	TGCCGTGTACCAGCTGAGA	TRAC exon1 left TALEN
14	TCGGTGAATAGGCAGACAG	TRAC exon1 right TALEN
15	tggagatagggacctcac	TRAC promoter left TALEN
16	tgaggccaggaactggag	TRAC promoter right TALEN
17	TGAACAAGGTGTTCCAC	TRAB exon1 left TALEN
18	tctgcttctgatggctca	TRAB exon1 right TALEN
19	TGTCTCAGGGCCAGGGAA	TRAB promoter left TALEN
20	TCCCTGCTCTGTGTCCTT	TRAB promoter right TALEN
21	agtcaccaagtgtggtctaataataaatcctgtgttcctgaggtca tgagattgagagaggaagtgatgtcactgtgggaacttccgtgt aaggacggggcgctccctcctcctgctcctgctcacagtgatcct gatctggtaagagctccatcctgcctgaccctgcc	Ligandal 147bp TCR beta promoter

Figure 60
TCR genome engineering with for Cpf1 + tetrakisDNA

Cpf1 sgRNA	guide sequence	Target Locus	ssDNA 1	ssDNA 2
LL002	CATGTGCAAAACGCCCTTCAAC	TRAC	GTTGcttatcgtcatcgtcttcttgaatc	CAACgattacaaagacgatgacgataag
LL003	GGTGTGGGAGATCTCTGCTTCTGA	TRBC1 & TRBC2	ICIGcttatcgtcatcgtcttcttgaatc	CAGAgattacaaagacgatgacgataag
LL032	TTTGAGAATCAAAATCGGTG	TRAC	GAAIcttatcgtcatcgtcttcttgaatc	ATTcGattacaaagacgatgacgataag



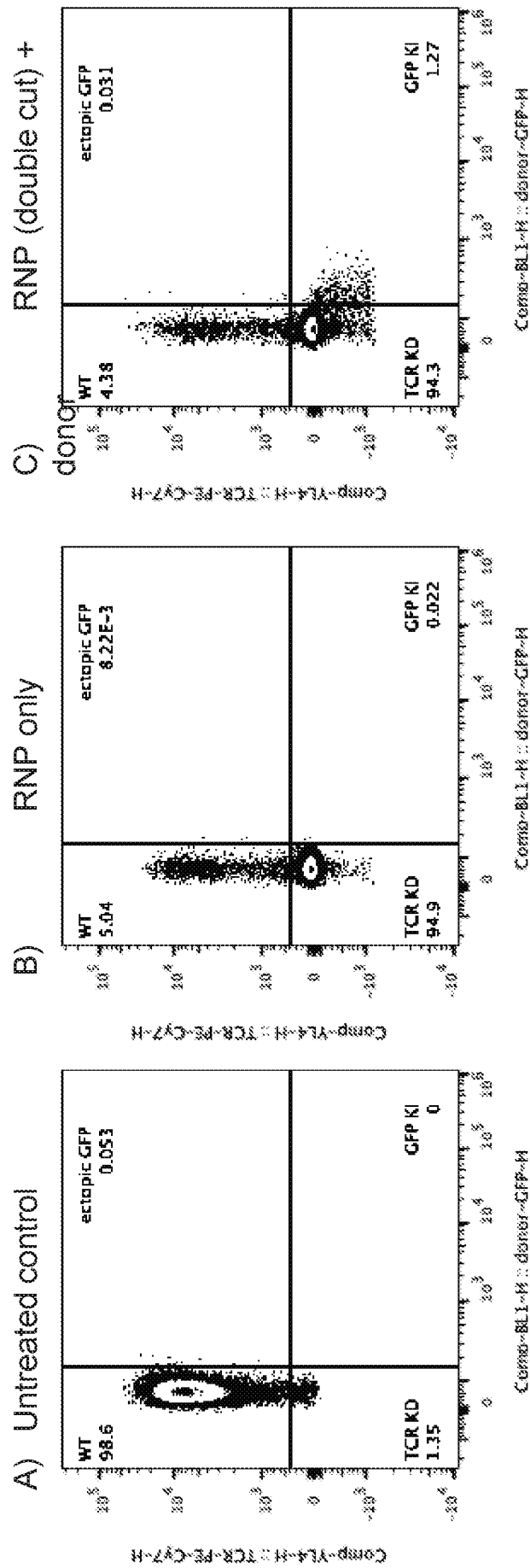


Figure 61

Figure 62

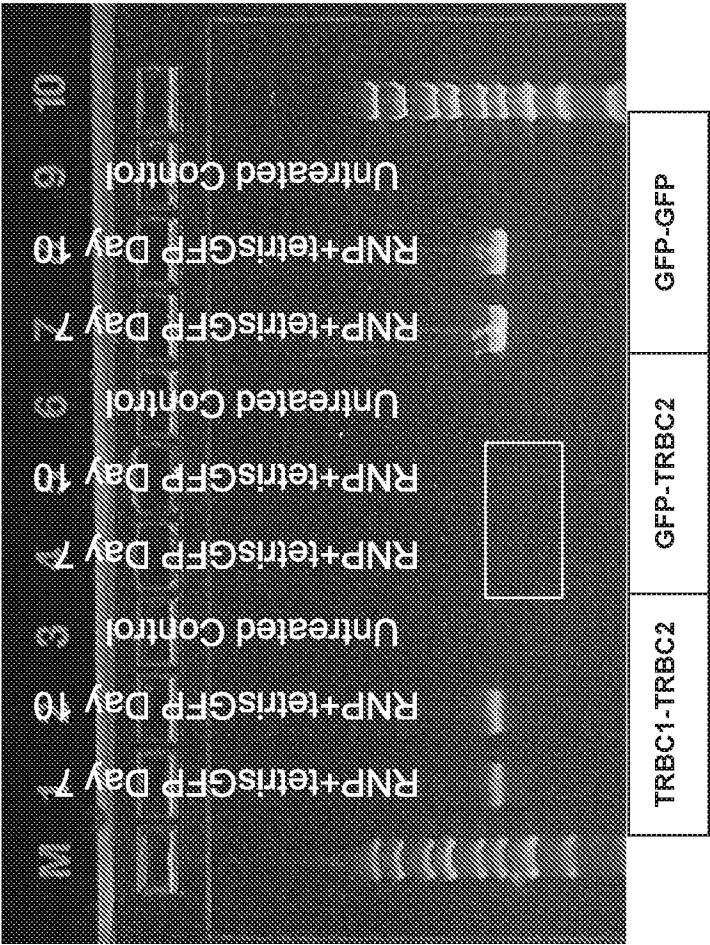


Figure 63

Primer:	TRBC1-TRBC2				GFP-TRBC2			GFP-GFP		
	Deletion				Knock-In			Donor GFP		
Lane:	1	2	3	4	5	6	7	8	9	
TRBC1- TRBC2 or GFP-GFP	+	+	-	-	-	-	+	+	-	
GFP-TRBC2	-	-	-	+	+	-	-	-	-	

A (Control TRBC1/2 trace plots)

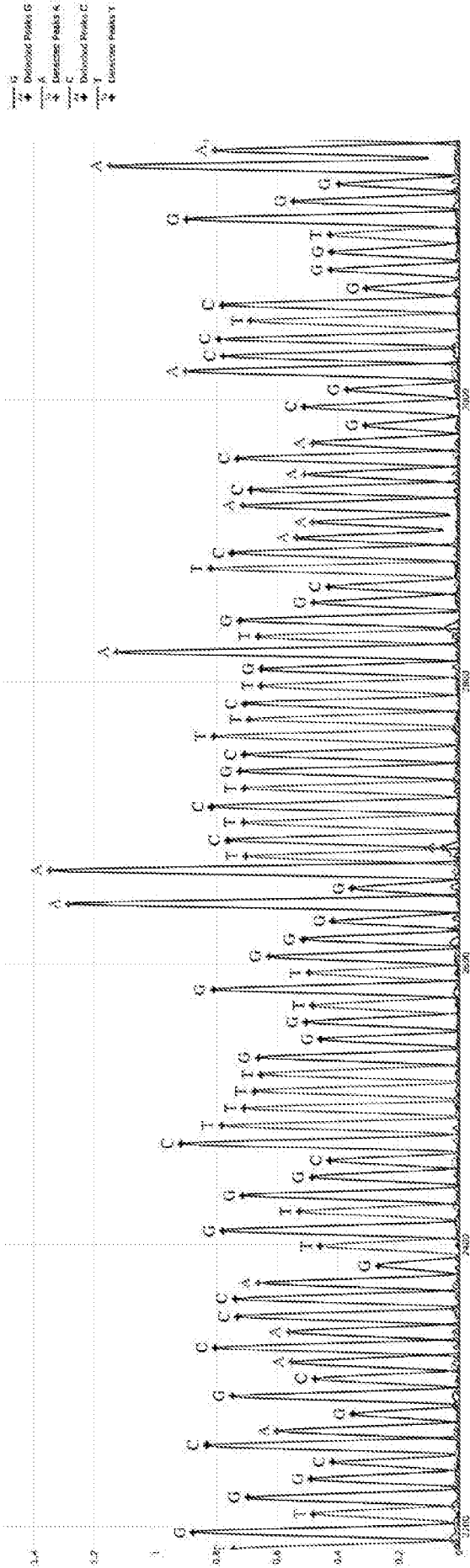


Figure 64

B (Cpf1 + TRBC1/C2 gRNA trace plots)

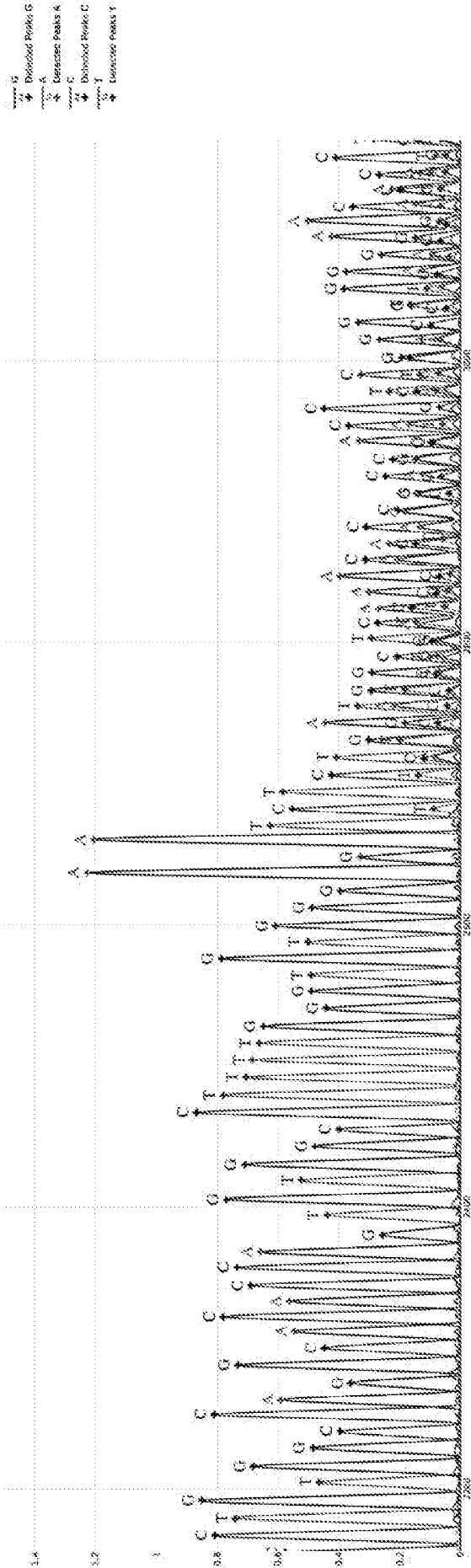


Figure 65

C (Cpf1 + TRBC1/C2 gRNA + tetrisFLAG trace plots)

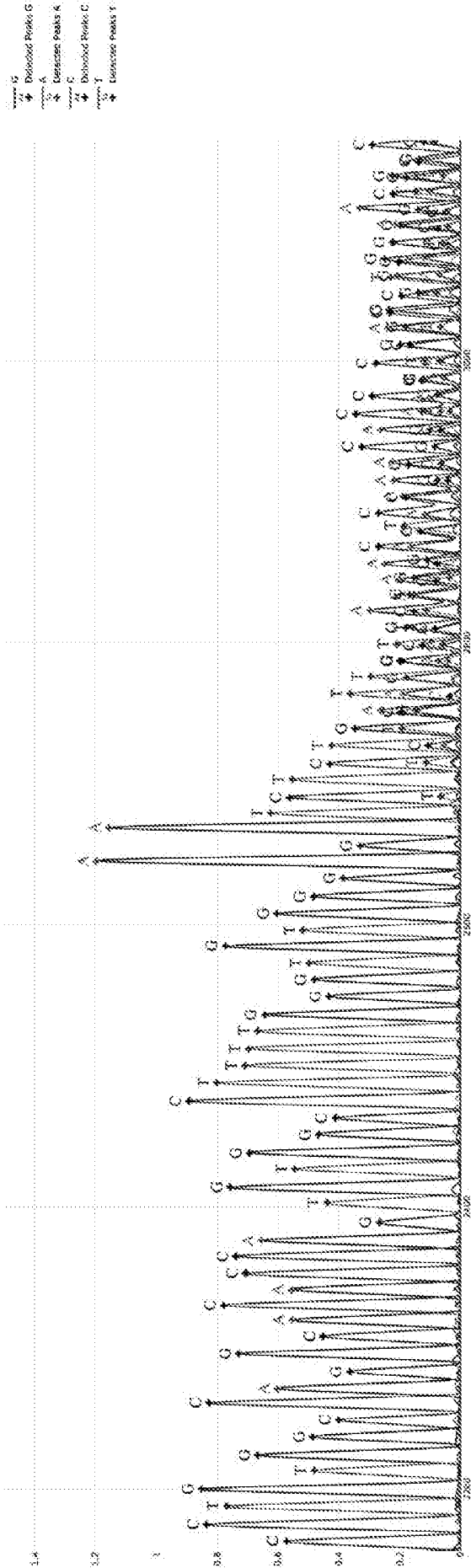


Figure 66

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/28004

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 15/09, 15/10, 15/64, 15/66, 15/90 (2019.01)

CPC - C12N 15/09, 15/10, 15/64, 15/66, 15/90

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2018/013990 A1 (ZYMERGEN INC.) 18 January 2018; paragraphs [0002], [0027], [0046], [0096], [0115], [0149], [0152]	1-2, 3/1-2, 66 ----- 67, 69/67
Y	US 2016/0230189 A1 (RENSSELAER POLYTECHNIC INSTITUTE) 11 August 2016; paragraphs [0006], [0027], [0056], [0057]	67, 69/67
A	(LEE, K et al.) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nature Biomedical Engineering. 2 October 2017, Vol. 1; pages 889-901; page 3, 2nd paragraph, 3rd paragraph; DOI: 10.1038/s41551-017-0137-2	68, 69/68
A	US 2018/0002708 A1 (REGENERON PHARMACEUTICALS, INC.) 4 January 2018; entire document	1
A	WO 2018/064226 A1 (UBIOME, INC.) 5 April 2018; entire document	1

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 July 2019 (19.07.2019)

Date of mailing of the international search report

01 AUG 2019

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P.O. Box 1450, Alexandria, Virginia 22313-1450

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Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/28004

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-65 and 70-79
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.