

SUMMARY

[0005] In one aspect, provided herein is a composition for modifying a genomic target nucleic acid, comprising:

- (a) an active targetable nuclease protein; and
 - (b) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and
 wherein each of the first and/or the second CDL target sequences are capable of cleavage by the active targetable nuclease protein or a complex comprising the active targetable nuclease protein, and
- wherein the composition does not comprise a viral vector.

[0005a] Provided herein is a composition for modifying a target nucleic acid, comprising: (a) a targetable nuclease protein; and (b) a plasmid donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein each of the first and the second CDL target sequences are capable of cleavage by the targetable nuclease protein or a complex comprising the targetable nuclease protein, and wherein the composition is formulated for non-viral delivery into a cell.

[0006] In another aspect, provided herein is a composition for modifying a genomic target nucleic acid, comprising:

- (a) an active CRISPR-CAS RNA-guided nuclease;
- (b) a donor guide RNA (gRNA); and
- (c) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and

(iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, wherein each of the first and the second CDL target sequences are capable of cleavage by the active CRISPR-CAS RNA-guided nuclease, and

wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and/or the second CDL target sequences, and (2) each of the first and/or the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences, and

wherein the composition does not comprise a viral vector.

[0006a] Also provided herein is A composition for modifying a target nucleic acid, comprising: (a) a CRISPR-CAS RNA-guided nuclease; (b) a donor guide RNA (gRNA); and (c) a plasmid donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences, and wherein the composition is formulated for non-viral delivery into a cell.

[0007] Also provided herein is A method for modifying a target nucleic acid of a cell, the method comprising: providing the cell, and introducing or having introduced into the cell a composition formulated for non-viral delivery, the composition comprising: (a) a targetable nuclease protein; and (b) a plasmid donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein each of the first and the second CDL target sequences are capable of cleavage by the targetable nuclease protein or a complex comprising the targetable nuclease protein.

[0008] Also provided for herein is a method for modifying a target nucleic acid of a cell, the method comprising: providing the cell, and introducing or having introduced into the cell a composition formulated for non-viral delivery, the composition comprising: (a) a CRISPR-

CAS RNA-guided nuclease; (b) a donor guide RNA (gRNA); and (c) a plasmid donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences.

[0009] Also provided herein is a method for modifying a genomic target sequence of a cell, the method comprising: providing the cell, and introducing or having introduced into the cell a composition formulated for non-viral delivery, the composition comprising: (a) a CRISPR-CAS RNA-guided nuclease; (b) a donor guide RNA (gRNA); and (c) a plasmid donor template comprising: (i) a homology directed repair (HDR) template comprising a nucleic acid for insertion flanked by homology arms; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences, wherein the donor gRNA comprises at least 17 nucleotides that are complementary to the genomic target sequence of the cell, and wherein the homology arms are complementary to nucleic acid sequences flanking the genomic target sequence of the cell, wherein the nucleic acid for insertion is configured for insertion into the genomic target sequence of the cell.

[0010] In some examples, the targetable nuclease protein is an RNA-guided nuclease. In some examples, the composition further comprises an RNA comprising at least 17 nucleotides that are complementary to the CDL target sequence. In some examples, the RNA-guided nuclease is a Cas protein.

[0011] In some examples, the composition further comprises a donor guide RNA (gRNA) configured to form the complex comprising the targetable nuclease protein, and wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target

sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences. In some examples, the composition further comprises a first donor guide RNA (gRNA) comprising at least 17 nucleotides that are complementary to the first CDL target sequence, a second donor gRNA comprising at least 17 nucleotides that are complementary to the second CDL target sequence, wherein each donor gRNA is configured to form a distinct complex comprising the targetable nuclease protein, and wherein each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences. In some examples, one or more of the donor gRNAs comprises at least 17 nucleotides that are complementary to a genomic target sequence of the cell. In some examples, the HDR template comprises homology arms that are complementary to nucleic acid sequences flanking the genomic target sequence of the cell. In some examples, the homology arms are each independently selected from a length of at least 400bp, at least 500bp, at least 600bp, at least 700bp, at least 800bp, at least 900bp, 1000bp, at least 1100bp, at least 1200bp, at least 1300bp, 1400bp, at least 1500bp, at least 1600bp, at least 1700bp, at least 1800bp, at least 1900bp, or at least 2000bp in length.

[0012] In some examples: (a) the targetable nuclease comprises an RNA-guided nuclease, wherein the RNA-guided nuclease comprises CRISPR-CAS; (b) the composition further comprises a donor guide RNA (gRNA) configured to form the complex comprising the targetable nuclease protein; and (c) wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences.**[0013]** In some examples, the composition further comprises a second targetable nuclease protein, wherein the second targetable nuclease protein or a complex comprising the second targetable nuclease protein is capable of cleaving a genomic target sequence of the cell. In some examples, the second targetable nuclease protein is an RNA-guided nuclease. In some examples, the composition further comprises a second RNA comprising at least 17 nucleotides that are complementary to the genomic target sequence. In some examples, the RNA-guided nuclease is a Cas protein. In some examples, the composition further comprises a target guide RNA (gRNA) configured to form the complex comprising the second targetable nuclease protein, and wherein (1) the target gRNA comprises at least 17 nucleotides that are complementary to the genomic target sequence, and (2) the genomic target sequence is operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the genomic target sequence.

[0014] In some examples, the first CDL target sequence, the second CDL target sequence, and the genomic target sequence comprise the same nucleic acid sequence.

[0015] In some examples, the genomic target sequence comprises a safe-harbor nucleic acid sequence. In some examples, the safe-harbor nucleic acid sequence comprises the nucleic acid sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2). In some examples, one, both, or neither of the PAM sequences are encoded between the CDL target sequences and the HDR template.

[0016] In some examples, the targetable nuclease and each of the gRNAs are in a molar ratio of between 1:10 and 2:1, respectively. In some examples, the targetable nuclease and the donor template are in a molar ratio of between 10:1 and 1000:1, respectively.

[0017] In some examples, the targetable nuclease protein and/or the second targetable nuclease protein comprises a transcription activator-like (TAL) effector DNA-binding protein and a nuclease.

[0018] In some examples, the targetable nuclease protein and/or the second targetable nuclease protein comprises a zinc finger DNA-binding protein and a nuclease.

[0019] In some examples, the targetable nuclease protein and/or the second targetable nuclease protein is fused to a nuclear localization signal (NLS) sequence.

[0020] In some examples, the targetable nuclease protein and/or the second targetable nuclease protein is a Cas9 protein.

[0021] Also provided herein is a method for modifying the target nucleic acid in the cell, comprising introducing or having non-virally introduced into the cell any of the compositions described herein, wherein the HDR template is integrated into the target nucleic acid. In some examples, the introducing comprises electroporation. In some examples, the cell is a primary cell. In some examples, the primary cell is a primary T cell.

[0022] Also provided for herein is a ribonucleoprotein complex for modifying a target nucleic acid, comprising any of the compositions described herein.

[0023] In another aspect, provided herein is a ribonucleoprotein complex for modifying a genomic target nucleic acid,

- (a) an active CRISPR-CAS RNA-guided nuclease; and
 - (b) a donor guide RNA (gRNA), wherein the donor gRNA comprises at least 17 nucleotides that are complementary to a co-delivery linearization (CDL) target sequence comprising the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2), and
- wherein the composition is formulated for non-viral delivery into a cell.

[0023a] Also provided for herein is a ribonucleoprotein complex for modifying a target nucleic acid, (a) a CRISPR-CAS RNA-guided nuclease; and (b) a donor guide RNA (gRNA), wherein the donor gRNA comprises at least 17 nucleotides that are complementary to a co-delivery linearization (CDL) target sequence, and wherein the composition is formulated for non-viral delivery into a cell. In some examples, the composition further comprises a plasmid

donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences.

[0024] Also provided for herein is a method for modifying the target nucleic acid in the cell, comprising introducing into the cell any of the ribonucleoprotein complex described herein. In some examples, the introducing comprises electroporation. In some examples, the cell is a primary cell. In some examples, the primary cell is a primary T cell. In some examples, the method is performed in vivo, in vitro, or ex vivo.

[0025] In another aspect, provided herein is a method of forming a ribonucleoprotein (RNP) complex, comprising incubating or having incubated (a) an active CRISPR-CAS RNA-guided nuclease; and (b) a donor guide RNA (gRNA), wherein the donor gRNA comprises at least 17 nucleotides that are complementary to a co-delivery linearization (CDL) target sequence comprising the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2).

[0025a] Also provided for herein is a method of forming a ribonucleoprotein (RNP) complex, comprising incubating or having incubated (a) a CRISPR-CAS RNA-guided nuclease; and (b) a donor guide RNA (gRNA), wherein the donor gRNA comprises at least 17 nucleotides that are complementary to a co-delivery linearization (CDL) target sequence. In some examples, the Cas protein and the gRNA are incubated together at 37 °C for at least 17 minutes. In some examples, the molar ratio of gRNA:Cas protein is between 0.25:1 and 4:1. In some examples, the RNP complex has a size that is less than 100 nm. In some examples, the RNP complex has a size that is between 20 nm and 90 nm.

[0026] Also provided herein is a composition comprising a plasmid donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL

target sequence is operably linked 3' of the HDR template, and wherein each of the first and the second CDL target sequences are capable of cleavage by a targetable nuclease protein or a complex comprising the targetable nuclease protein. In some examples, the composition further comprises a donor guide RNA (gRNA) configured to form the complex comprising the targetable nuclease protein. In some examples, the composition comprises the targetable nuclease protein.

[0027] In some examples, the donor template comprises from 5' to 3' the sequence: P1a-N1-P2b-H-P3c-N2-P4d, and wherein: (1) P1, P2, P3, and P4 are PAM sequences; (2) N1 is the first CDL target sequence and N2 is the second CDL target sequence; (3) H is the HDR template; (4) a is 0 and b is 1, or a is 1 and b is 0; and (5) c is 0 and d is 1; or c is 1 and d is 0.

[0028] In certain examples, described herein is a method for modifying a target nucleic acid of a cell, the method comprising: providing the cell, and introducing or having introduced into the cell a composition formulated for non-viral delivery, the composition comprising: (a) a targetable nuclease protein; and (b) a plasmid donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein each of the first and the second CDL target sequences are capable of cleavage by the targetable nuclease protein or a complex comprising the targetable nuclease protein. In certain examples, described herein is a method for modifying a target nucleic acid of a cell, the method comprising: providing the cell, and introducing or having introduced into the cell a composition formulated for non-viral delivery, the composition comprising: (a) a CRISPR-CAS RNA-guided nuclease; (b) a donor guide RNA (gRNA); and (c) a plasmid donor template comprising: (i) a homology directed repair (HDR) template; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences. In certain examples, described herein is a method for modifying a genomic target sequence of a cell, the method comprising: providing the cell, and introducing or having introduced into the cell a composition formulated for non-viral delivery, the composition comprising: (a) a CRISPR-CAS RNA-guided nuclease; (b) a donor guide RNA (gRNA); and (c) a plasmid donor template comprising: (i) a homology directed repair (HDR) template comprising a nucleic acid for insertion flanked by homology arms; (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template; and (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and wherein (1)

the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences wherein the donor gRNA comprises at least 17 nucleotides that are complementary to the genomic target sequence of the cell, and wherein the homology arms are complementary to nucleic acid sequences flanking the genomic target sequence of the cell, wherein the nucleic acid for insertion is configured for insertion into the genomic target sequence of the cell. In certain examples, the cell is a human cell. In certain examples, the cell is an immune cell. In certain examples, the immune cell is a T cell. In certain examples, the T cell is a primary T cell. In certain examples, the introducing the composition formulated for non-viral delivery comprises electroporation. In certain examples, the amount of donor template is at least about 80, 10-120, 10, 20, 30, 40, 50, 60, 70, 90, 100, 110, or 120mg. In certain examples, the number of cells for an individual electroporation reaction is at least about 5, 1-10, 1, 2, 3, 4, 6, 7, 8, 9, or 10 e7. In certain examples, the total number of cells provided is at least greater than 10 e7 and greater than one electroporation reaction is performed. In certain examples, the total volume of cell suspension is about 1 mL. In certain examples, the method results in increased template insertion in the genomic target sequence of the cells relative to an otherwise identical control composition but lacking the CDL target sequences, optionally wherein the template insertion increases by at least about 1-5, 1, 2, 3, 4, or 5 fold relative to the control.

[0028a] In another aspect, provided herein is an in-vitro or ex-vivo method for modifying a genomic target nucleic acid in a cell, comprising non-virally introducing or having non-virally introduced into the cell any one of the compositions as described herein, wherein the HDR template is integrated into the target nucleic acid.

[0028b] In another aspect, provided herein is the use of any one of the compositions as described herein in the manufacture of a medicament for modifying a target nucleic acid in a cell of a subject in need of cancer immunotherapy, wherein the HDR template is to be integrated into the target nucleic acid.

[0028c] In another aspect, provided herein is a composition for modifying a genomic target nucleic acid comprising a plasmid donor template comprising:

- (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and
- wherein each of the first and the second CDL target sequences are capable of cleavage by a targetable nuclease protein or a complex comprising the targetable nuclease protein.

[0028d] In another aspect, provided herein is an *in-vitro* or *ex-vivo* method for modifying a genomic target nucleic acid of a cell, the method comprising:

- providing the cell, and
 - non-virally introducing or having non-virally introduced into the cell a composition, the composition comprising:
 - (a) an active targetable nuclease protein; and
 - (b) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and
- wherein each of the first and the second CDL target sequences are capable of cleavage by the active targetable nuclease protein or a complex comprising the active targetable nuclease protein.

[0028e] In yet another aspect, provided herein is an *in-vitro* or *ex-vivo* method for modifying a genomic target nucleic acid of a cell, the method comprising:

- providing the cell, and
- non-virally introducing or having non-virally introduced into the cell a composition, the composition comprising:
 - (a) an active CRISPR-CAS RNA-guided nuclease;
 - (b) a donor guide RNA (gRNA); and

(c) a plasmid donor template comprising:

- (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, wherein each of the first and the second CDL target sequences are capable of cleavage by the active CRISPR-CAS RNA-guided nuclease, and
- wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences.

[0028f] In yet another aspect, provided herein is an *in-vitro* or *ex-vivo* method for modifying a genomic target sequence of a cell, the method comprising:

- providing the cell, and
 - non-virally introducing or having non-virally introduced into the cell a composition, the composition comprising:
 - (a) an active CRISPR-CAS RNA-guided nuclease;
 - (b) a donor guide RNA (gRNA); and
 - (c) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template comprising a nucleic acid for insertion flanked by homology arms;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, wherein each of the first and the second CDL target sequences are capable of cleavage by the active CRISPR-CAS RNA-guided nuclease, and
- wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each

of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences, wherein the donor gRNA comprises at least 17 nucleotides that are complementary to the genomic target sequence of the cell, and wherein the homology arms are complementary to nucleic acid sequences flanking the genomic target sequence of the cell, wherein the nucleic acid for insertion is configured for insertion into the genomic target sequence of the cell.

Definitions

[0029] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0030] As used herein, the "CRISPR-Cas" system refers to a class of bacterial systems for defense against foreign nucleic acid. CRISPR-Cas systems are found in a wide range of eubacterial and archaeal organisms. CRISPR-Cas systems include type I, II, and III sub-types. Wild-type type II CRISPR-Cas systems utilize an RNA-mediated nuclease, for example, Cas9 protein, in complex with guide and activating RNA (*e.g.*, single-guide RNA or sgRNA) to recognize and cleave foreign nucleic acids, *e.g.*, foreign nucleic acids including natural or modified nucleotides.

[0031] As used herein, the term “targetable nuclease” refers to a protein that can recognize a sequence of a cognate nucleic acid sequence (*e.g.*, a target gene within a genome and/or a CDL target sequence) and bind to the cognate nucleic acid sequence. In some examples, the targetable nuclease can modify cognate nucleic acid sequence. In some examples, a targetable nuclease can be an RNA-guided nuclease, *e.g.*, a Cas protein. In other examples, a targetable nuclease can be a fusion protein that includes a protein that can bind to a cognate nucleic acid sequence (*e.g.*, a transcription activator-like (TAL) effector DNA-binding protein or a zinc finger DNA-binding protein) and a protein that can modify a cognate nucleic acid sequence (*e.g.*, a nuclease, a transcription activator or repressor). In some examples, a targetable nuclease has nuclease activity. In other examples, a targetable nuclease does not have nuclease activity. In some examples, a targetable nuclease can modify a cognate nucleic acid sequence by cleaving the target nucleic acid. The cleaved target nucleic acid can then undergo homologous recombination with a nearby a homology directed repair (HDR) template, such as through homology directed repair or homology mediated end joining (HMEJ). In other examples, a targetable nuclease (*e.g.*, a targetable nuclease without any nuclease activity) can regulate the expression of cognate nucleic acid sequence. For example, a targetable nuclease can be a fusion protein containing a TAL effector DNA-binding protein and a transcription activator.

[0032] As used herein, the term “plasmid donor template” refers to a polynucleotide that includes a homology directed repair (HDR) template and CDL target sequences. An HDR template can include a 5’ homology arm, a nucleotide insert (*e.g.*, an exogenous sequence and/or a sequence that encodes a heterologous protein or fragment thereof), and a 3’ homology arm (for example, *see* FIG. 1). As described further herein, pre-incubation of the RNP complex containing a targetable nuclease (*e.g.*, a Cas protein) and donor gRNA and a plasmid donor template prior to electroporation improves in knock-in yield, such as through improved knock-in efficiency and/or reduced cytotoxicity.

[0033] As used herein, the term “co-delivery linearization (CDL) target sequence” refers to a nucleotide sequence that is recognized and bound by a targetable nuclease. In some examples, a targetable nuclease, *e.g.*, a transcription activator-like (TAL) effector DNA-binding protein or zinc finger DNA-binding protein, can directly recognize and bind a CDL target sequence. In other examples, a targetable nuclease, *e.g.*, an RNA-guided nuclease, can indirectly recognize and bind a CDL target sequence via a donor gRNA. A RNA-guided

nuclease binds to the donor gRNA, while the donor gRNA hybridizes to a CDL target sequence. In some examples, a CDL target sequence is a portion of genomic target nucleic acid.

[0034] As used herein, the “RNA-guided nuclease” refers to a nuclease that binds to a guide RNA (gRNA) and utilizes the gRNA to selectively bind regions within a DNA polynucleotide. In general, an RNA-guided nuclease can selectively bind nearly any sequence within a DNA polynucleotide that is complementary to the gRNA. In some examples, a RNA-guided nuclease has nuclease activity and can cleave the linkage (*e.g.*, phosphodiester bonds) between nucleotides in the DNA polynucleotide. In other examples, a RNA-guided nuclease does not have nuclease activity and can be used to selectively bind and/or localize other proteins (*e.g.*, transcriptional activator or repressors) that are fused to the RNA-guided nuclease to the region of interest within the DNA polynucleotide.

[0035] As used herein, the term “guide RNA” or “gRNA” refers to a DNA-targeting RNA that can guide an RNA-guided nuclease (*e.g.*, a Cas protein) to a cognate nucleic acid sequence by hybridizing to the cognate nucleic acid sequence. In some examples, a guide RNA can be a single-guide RNA (sgRNA), which contains (1) a guide sequence (*e.g.*, crRNA equivalent portion of the single-guide RNA) that guides a RNA-guided nuclease to a cognate nucleic acid sequence and (2) a scaffold sequence (*e.g.*, tracrRNA equivalent portion of the single-guide RNA) that interacts with the RNA-guided nuclease. In other examples, a guide RNA can contain two components, (1) a guide sequence (*e.g.*, crRNA equivalent portion of the single-guide RNA) that guides a RNA-guided nuclease to cognate nucleic acid sequence and (2) a scaffold sequence (*e.g.*, tracrRNA equivalent portion of the single-guide RNA) that interacts with the RNA-guided nuclease. A portion of the guide sequence can hybridize to a portion of the scaffold sequence to form the two-component guide RNA.

[0036] As used herein, the term “target guide RNA” or “target gRNA” refers to a gRNA that can hybridize to a cognate nucleic acid sequence to be modified, *e.g.*, at a location in a DNA polynucleotide where integration of an HDR template is desired, such as the genome of a T cell and/or safe-harbor genomic locations.

[0037] As used herein, the term “donor guide RNA” or “donor gRNA” refers to a gRNA that can hybridize a CDL target sequence within a plasmid donor template. In some examples, a CDL target sequence can be complementary (*e.g.*, partially complementary or completely complementary) to an equal length portion of the sequence of a donor gRNA.

[0038] As used herein, the term “single-guide RNA” or “sgRNA” refers to a DNA-targeting RNA including (1) a guide sequence (*e.g.*, crRNA equivalent portion of the single-guide RNA) that targets a Cas protein to a cognate nucleic acid sequence and (2) a scaffold sequence (*e.g.*, tracrRNA equivalent portion of the single-guide RNA) that interacts with a Cas protein.

[0039] As used herein, the term “complementary” or “complementarity” refers to the capacity for base pairing between nucleobases, nucleosides, or nucleotides, as well as the capacity for base pairing between one polynucleotide to another polynucleotide. In some examples, one polynucleotide can have “complete complementarity,” or be “completely complementary,” to another polynucleotide, which means that when the two polynucleotides are optionally aligned, each nucleotide in one polynucleotide can engage in Watson-Crick base pairing with its corresponding nucleotide in the other polynucleotide. In other examples, one polynucleotide can have “partial complementarity,” or be “partially complementary,” to another polynucleotide, which means that when the two polynucleotides are optionally aligned, at least 60% (*e.g.*, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 97%) but less than 100% of the nucleotides in one polynucleotide can engage in Watson-Crick base pairing with their corresponding nucleotides in the other polynucleotide. In other words, there is at least one (*e.g.*, one, two, three, four, five, six, seven, eight, nine, or ten) mismatched nucleotide base pair when the two polynucleotides are hybridized. Pairs of nucleotides that engage in Watson-Crick base pairing includes, *e.g.*, adenine and thymine, cytosine and guanine, and adenine and uracil, which all pair through the formation of hydrogen bonds. Examples of mismatched bases include a guanine and uracil, guanine and thymine, and adenine and cytosine pairing.

[0040] As used herein, the term “Cas protein” refers to a Clustered Regularly Interspaced Short Palindromic Repeats-associated protein or nuclease. A Cas protein can be a wild-type Cas protein or a Cas protein variant. Cas9 protein is an example of a Cas protein that belongs in the type II CRISPR-Cas system (*e.g.*, Rath et al., *Biochimie* 117:119, 2015). Other examples of Cas proteins are described in detail further herein. A naturally-occurring type II Cas protein generally requires both a crRNA and a tracrRNA for site-specific DNA recognition and cleavage. The crRNA associates, through a region of partial complementarity, with the tracrRNA to guide the Cas protein to a region homologous to the crRNA in the target DNA called a “protospacer”. A naturally-occurring type II Cas protein cleaves DNA to generate blunt ends at the double-strand break at sites specified by a guide sequence contained within a crRNA transcript. In some examples of the compositions and methods described herein, a Cas protein associates with a target gRNA or a donor gRNA to form a ribonucleoprotein (RNP)

complex. In some examples of the compositions and methods described herein, the Cas protein has nuclease activity. In other examples, the Cas protein does not have nuclease activity.

[0041] As used herein, the term “Cas protein variant” refers to a Cas protein that has at least one amino acid substitution (*e.g.*, one, two, three, four, five, six, seven, eight, nine, ten, or more amino acid substitutions) relative to the sequence of a wild-type Cas protein and/or is a truncated version or fragment of a wild-type Cas protein. In some examples, a Cas protein variant has at least 75% sequence identity (*e.g.*, at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity) to the sequence of a wild-type Cas protein. In some examples, a Cas protein variant is a fragment of a wild-type Cas protein and has at least one amino acid substitution relative to the sequence of the wild-type Cas protein. A Cas protein variant can be a Cas9 protein variant. In some examples, a Cas protein variant has nuclease activity. In other examples, a Cas protein variant does not have nuclease activity.

[0042] As used herein, the term “ribonucleoprotein complex” or “RNP complex” refers to a complex comprising a Cas protein or variant (*e.g.*, a Cas9 protein or variant) and a gRNA.

[0043] As used herein, the term “modifying” in the context of modifying a target nucleic acid in the genome of a cell refers to inducing a change (*e.g.*, cleavage) in the target nucleic acid. In some examples, the change can be a structural change in the sequence of the target nucleic acid. For example, the modifying can take the form of inserting a nucleotide sequence into the target nucleic acid. For example, an exogenous nucleotide sequence can be inserted into the target nucleic acid. The target nucleic acid can also be excised and replaced with an exogenous nucleotide sequence. In another example, the modifying can take the form of cleaving the target nucleic acid without inserting a nucleotide sequence into the target nucleic acid. For example, the target nucleic acid can be cleaved and excised. Such modifying can be performed, for example, by inducing a double stranded break within the target nucleic acid, or a pair of single stranded nicks on opposite strands and flanking the target nucleic acid. Methods for inducing single or double stranded breaks at or within a target nucleic acid include the use of a targetable nuclease (*e.g.*, a Cas protein) as described herein directed to the target nucleic acid. In other examples, modifying a target nucleic acid includes targeting another protein to the target nucleic acid and does not include cleaving the target nucleic acid.

WHAT IS CLAIMED IS:

1. A composition for modifying a genomic target nucleic acid, comprising:
 - (a) an active targetable nuclease protein; and
 - (b) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and
 wherein each of the first and/or the second CDL target sequences are capable of cleavage by the active targetable nuclease protein or a complex comprising the active targetable nuclease protein, and
 wherein the composition does not comprise a viral vector.
2. The composition of claim 1, wherein the active targetable nuclease protein is an RNA-guided nuclease, optionally wherein the RNA-guided nuclease is a Cas protein, optionally wherein the composition further comprises an RNA comprising at least 17 nucleotides that are complementary to each of the first and/or the second CDL target sequence.
3. The composition of claim 2, wherein the composition further comprises a donor guide RNA (gRNA) configured to form the complex comprising the active targetable nuclease protein, and wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and/or the second CDL target sequences, and (2) each of the first and/or the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences.
4. The composition of claim 2, wherein the composition further comprises a first donor guide RNA (gRNA) comprising at least 17 nucleotides that are complementary to the first CDL target sequence, a second donor gRNA comprising at least 17 nucleotides that are complementary to the second CDL target sequence, wherein each donor gRNA is configured to form a distinct complex comprising the active targetable nuclease protein,

and wherein each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences.

5. The composition of claim 1-4, wherein the active targetable nuclease protein or a complex comprising the active targetable nuclease protein is capable of modifying a genomic target sequence.
6. The composition of any one of claims 3-5, wherein one or more of the donor gRNAs comprises at least 17 nucleotides that are complementary to a genomic target sequence of the cell.
7. The composition of claim 5 or 6, wherein the genomic target sequence comprises the nucleic acid sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2).
8. A composition for modifying a genomic target nucleic acid, comprising:
 - (a) an active CRISPR-CAS RNA-guided nuclease;
 - (b) a donor guide RNA (gRNA); and
 - (c) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, wherein each of the first and the second CDL target sequences are capable of cleavage by the active CRISPR-CAS RNA-guided nuclease, and

wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and/or the second CDL target sequences, and (2) each of the first and/or the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences, and

wherein the composition does not comprise a viral vector.

9. The composition of any one of claims 5-8, wherein the first CDL target sequence, the second CDL target sequence, and the genomic target sequence comprise the same nucleic acid sequence.
10. The composition of claim 9, wherein the genomic target sequence comprises the nucleic acid sequence GAGCCATGCTTGGCTTACGA.
11. The composition of any one of claims 3 to 9, wherein one or both of the PAM sequences are encoded between the CDL target sequences and the HDR template.
12. The composition of any one of claims 1 to 7 or 9 to 10, wherein the active targetable nuclease protein is a Cas9 protein or comprises a transcription activator-like (TAL) effector DNA-binding protein and a nuclease or a zinc finger DNA-binding protein and a nuclease.
13. An in-vitro or ex-vivo method for modifying a genomic target nucleic acid in a cell, comprising non-virally introducing or having non-virally introduced into the cell the composition of any one of claims 1 to 12, wherein the HDR template is integrated into the target nucleic acid.
14. The method of claim 13, wherein the introducing comprises electroporation.
15. The method of claim 13 or 14, wherein the cell is a primary cell or a primary T cell.
16. A ribonucleoprotein complex for modifying a target nucleic acid, comprising the composition of any one of claims 1 to 12.
17. A ribonucleoprotein complex for modifying a genomic target nucleic acid,
 - (a) an active CRISPR-CAS RNA-guided nuclease; and
 - (b) a donor guide RNA (gRNA), wherein the donor gRNA comprises at least 17 nucleotides that are complementary to a co-delivery linearization (CDL) target sequence comprising the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2), andwherein the composition is formulated for non-viral delivery into a cell.

18. Use of the composition of any one of claims 1 – 12 in the manufacture of a medicament for modifying a target nucleic acid in a cell of a subject in need of cancer immunotherapy, wherein the HDR template is to be integrated into the target nucleic acid.
19. The use of claim 18, wherein the composition is to be introduced into the cell via electroporation.
20. The use of claim 18 or claim 19, wherein the cell is a primary cell or a primary T-cell.
21. A method of forming a ribonucleoprotein (RNP) complex, comprising incubating or having incubated (a) an active CRISPR-CAS RNA-guided nuclease; and (b) a donor guide RNA (gRNA), wherein the donor gRNA comprises at least 17 nucleotides that are complementary to a co-delivery linearization (CDL) target sequence comprising the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2).
22. A composition for modifying a genomic target nucleic acid comprising a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, andwherein each of the first and the second CDL target sequences are capable of cleavage by a targetable nuclease protein or a complex comprising the targetable nuclease protein.
23. An *in-vitro* or *ex-vivo* method for modifying a genomic target nucleic acid of a cell, the method comprising:
 - providing the cell, and
 - non-virally introducing or having non-virally introduced into the cell a composition, the composition comprising:
 - (a) an active targetable nuclease protein; and
 - (b) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;

(ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and

(iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, and

wherein each of the first and the second CDL target sequences are capable of cleavage by the active targetable nuclease protein or a complex comprising the active targetable nuclease protein.

24. An *in-vitro* or *ex-vivo* method for modifying a genomic target nucleic acid of a cell, the method comprising:

- providing the cell, and
- non-virally introducing or having non-virally introduced into the cell a composition, the composition comprising:
 - (a) an active CRISPR-CAS RNA-guided nuclease;
 - (b) a donor guide RNA (gRNA); and
 - (c) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, wherein each of the first and the second CDL target sequences are capable of cleavage by the active CRISPR-CAS RNA-guided nuclease, and

wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences.

25. An *in-vitro* or *ex-vivo* method for modifying a genomic target sequence of a cell, the method comprising:

- providing the cell, and

- non-virally introducing or having non-virally introduced into the cell a composition, the composition comprising:

- (a) an active CRISPR-CAS RNA-guided nuclease;
- (b) a donor guide RNA (gRNA); and
- (c) a plasmid donor template comprising:
 - (i) a homology directed repair (HDR) template comprising a nucleic acid for insertion flanked by homology arms;
 - (ii) a first co-delivery linearization (CDL) target sequence, wherein the first CDL target sequence is operably linked 5' of the HDR template and comprises the sequence GAGCCATGCTTGGCTTACGA (SEQ ID NO: 2); and
 - (iii) a second CDL target sequence, wherein the second CDL target sequence is operably linked 3' of the HDR template, wherein each of the first and the second CDL target sequences are capable of cleavage by the active CRISPR-CAS RNA-guided nuclease, and
 wherein (1) the donor gRNA comprises at least 17 nucleotides that are complementary to each of the first and the second CDL target sequences, and (2) each of the first and the second CDL target sequences are operably linked to a 3-base pair protospacer adjacent motif (PAM) located 3' of the CDL target sequences,

wherein the donor gRNA comprises at least 17 nucleotides that are complementary to the genomic target sequence of the cell, and

wherein the homology arms are complementary to nucleic acid sequences flanking the genomic target sequence of the cell, wherein the nucleic acid for insertion is configured for insertion into the genomic target sequence of the cell.