# SYSTEMS FOR MODIFYING AT LEAST ONE TARGET DNA AND METHODS OF USING THE SAME

## CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority to provisional patent application US 63/549,564, filed February 4, 2024. The entire contents of the aforementioned application(s) are hereby incorporated by reference.

## REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0002] The content of the electronic sequence listing (GEBL-002_01WO_SeqList_ST26.xml; Size: 65,219 bytes and Date of Creation: February 3, 2025) is herein incorporated by reference in its entirety.

## FIELD OF INVENTION

[0003] This application relates to systems and methods for modifying at least one target DNA, in particular systems and methods for modifying at least one target DNA sequence using two or more endonucleases and one or more donor DNA.

## BACKGROUND OF INVENTION

[0004] Gene editing technologies are powerful tools to manipulate genetic materials in target cells, tissues, or organisms. Clustered regularly interspaced short palindromic repeats (CRISPR)-related technologies are among the most promising gene editing tools available. However, the existing gene editing technologies suffer from several drawbacks, such as a low rate of desired homology directed repair precise (HDR)-mediated editing, a less versatile editing format that limited to single bases, a less satisfactory off-target rate, and a high translocation rate and its resulting mutagenesis. There is a need for improved systems and methods for gene editing.

## SUMMARY OF INVENTION

[0005] In some embodiments, provided is a system for modifying at least one target

DNA, including: two or more endonucleases, wherein each endonuclease nicks one target DNA strand at a target nick and at least one donor DNA including a sequence of interest to be incorporated to a target DNA site.

[0006] In some embodiments, the target nicks of the two or more endonucleases are on the same DNA strand. In some embodiments, the target nicks of the two or more endonucleases are on different DNA strands.

[0007] In some embodiments, the nicking event results in a 3'-overhang at the target DNA site. In some other embodiments, the nicking event results in a 5'-overhang at the target DNA site. In yet other embodiments, the nicking event does not produce any overhang; In some embodiments, the nicking event results in a blunt end at the target DNA site. In some embodiment, the nicking event may lead to the formation of a single-strand flap; In some embodiment, the nicking event may lead to the formation of a single-stranded gap; In some cases, the nicking event may result in a large fragment deletion or replacement; in some embodiments, the nicking event may directly result in a double-strand break. In some embodiment, the nicking event may lead to a strand displacement. In some embodiment, the complementary strand or the nick strand may be displaced to form a single-stranded region, or the formation of a gap between two nearby nicks, which may be filled by DNA polymerase and sealed by DNA ligase. These outcomes may depend on the location and distance between the nicks, as well as the cellular repair mechanisms involved, and have specific applications in DNA modification, such as gene knockout, insertion, deletion, or repair.

[0008] In some embodiments, the donor DNA is a single-strand DNA. In some embodiments, the donor DNA is a double-strand DNA. In some embodiments, the donor DNA includes one or more portions of single-strand DNA and one or more portions of double-strand DNA. In some embodiments, the single-strand DNA is a circularized DNA. In some embodiments, the double-strand DNA is a circularized DNA. In some embodiments, the length of the donor DNA may be optimized.

[0009] In some embodiments, the single-strand DNA is preferably 6-nucleotide to 100,000-nucleotide long. In some embodiments, the single-strand DNA is 6-nucleotide to

10-nucleotide in length. In some embodiments, the single-strand DNA is 6-nucleotide to 10-nucleotide in length. In some embodiments, the single-strand DNA is 10-nucleotide to 15-nucleotide in length. In some embodiments, the single-strand DNA is 15-nucleotide to 20-nucleotide in length. In some embodiments, the single-strand DNA is 20-nucleotide to 25-nucleotide in length. In some embodiments, the single-strand DNA is 25-nucleotide to 30-nucleotide in length. In some embodiments, the single-strand DNA is 30-nucleotide to 40-nucleotide in length. In some embodiments, the single-strand DNA is 40-nucleotide to 50-nucleotide in length. In some embodiments, the single-strand DNA is 50-nucleotide to 75-nucleotide in length. In some embodiments, the single-strand DNA is 75-nucleotide to 100-nucleotide in length. In some embodiments, the single-strand DNA is 100-nucleotide to 150-nucleotide in length. In some embodiments, the single-strand DNA is 150-nucleotide to 200-nucleotide in length. In some embodiments, the single-strand DNA is 200-nucleotide to 300-nucleotide in length. In some embodiments, the single-strand DNA is 300-nucleotide to 400-nucleotide in length. In some embodiments, the single-strand DNA is 400-nucleotide to 500-nucleotide in length. In some embodiments, the single-strand DNA is 500-nucleotide to 1,000-nucleotide in length. In some embodiments, the single-strand DNA is 1,000-nucleotide to 5,000-nucleotide in length. In some embodiments, the single-strand DNA is 5,000-nucleotide to 10,000-nucleotide in length. In some embodiments, the single-strand DNA is 10,000-nucleotide to 50,000-nucleotide in length. In some embodiments, the single-strand DNA is 50,000-nucleotide to 100,000-nucleotide in length.

[0010] In some embodiments, the double-strand DNA is preferably 6-base pair to 100,000-base pair long. In some embodiments, the double-strand DNA is 6-base pair to 10-base pair in length. In some embodiments, the double-strand DNA is 10-base pair to 15-base pair in length. In some embodiments, the double-strand DNA is 15-base pair to 20-base pair in length. In some embodiments, the double-strand DNA is 20-base pair to 25-base pair in length. In some embodiments, the double-strand DNA is 25-base pair to 30-base pair in length. In some embodiments, the double-strand DNA is 30-base pair to 40-base pair in length. In some embodiments, the double-strand DNA is 40-base pair to 50-base pair in length. In some embodiments, the double-strand DNA is 50-base pair to 75-base pair in length. In some embodiments, the double-strand DNA is 75-base pair to

100-base pair in length. In some embodiments, the double-strand DNA is 100-base pair to 150-base pair in length. In some embodiments, the double-strand DNA is 150-base pair to 200-base pair in length. In some embodiments, the double-strand DNA is 200-base pair to 300-base pair in length. In some embodiments, the double-strand DNA is 300-base pair to 400-base pair in length. In some embodiments, the double-strand DNA is 400-base pair to 500-base pair in length. In some embodiments, the double-strand DNA is 500-base pair to 1,000-base pair in length. In some embodiments, the double-strand DNA is 1,000-base pair to 5,000-base pair in length. In some embodiments, the double-strand DNA is 5,000-base pair to 10,000-base pair in length. In some embodiments, the double-strand DNA is 10,000-base pair to 50,000-base pair in length. In some embodiments, the double-strand DNA is 50,000-base pair to 100,000-base pair in length.

[0011] In some embodiments, the single-strand and double-strand mixed donor DNA preferably has a total length of 6-nucleotide to 100,000-nucleotide long. In some embodiments, the mixed donor DNA has a total length of 6 nucleotides to 10 nucleotides. In some embodiments, the mixed donor DNA has a total length of 10 nucleotides to 15 nucleotides. In some embodiments, the mixed donor DNA has a total length of 15 nucleotides to 20 nucleotides. In some embodiments, the mixed donor DNA has a total length of 20 nucleotides to 25 nucleotides. In some embodiments, the mixed donor DNA has a total length of 25 nucleotides to 30 nucleotides. In some embodiments, the mixed donor DNA has a total length of 30 nucleotides to 40 nucleotides. In some embodiments, the mixed donor DNA has a total length of 40 nucleotides to 50 nucleotides. In some embodiments, the mixed donor DNA has a total length of 50 nucleotides to 75 nucleotides. In some embodiments, the mixed donor DNA has a total length of 75 nucleotides to 100 nucleotides. In some embodiments, the mixed donor DNA has a total length of 100 nucleotides to 150 nucleotides. In some embodiments, the mixed donor DNA has a total length of 150 nucleotides to 200 nucleotides. In some embodiments, the mixed donor DNA has a total length of 200 nucleotides to 300 nucleotides. In some embodiments, the mixed donor DNA has a total length of 300 nucleotides to 400 nucleotides. In some embodiments, the mixed donor DNA has a total length of 400

nucleotides to 500 nucleotides. In some embodiments, the mixed donor DNA has a total length of 500 nucleotides to 1,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 1,000 nucleotides to 5,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 5,000 nucleotides to 10,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 10,000 nucleotides to 50,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 50,000 nucleotides to 100,000 nucleotides.

[0012] In some embodiments, two target nicks produced by the two or more endonucleases are separated by 1 to 3,000,000 bases. In some embodiments, the two target nicks are separated by 1 to 100,000 bases. In some embodiments, the two target nicks are separated by 1 to 10,000 bases. In some embodiments, the two target nicks are separated by 1 to 1,000 bases. In some embodiments, the two target nicks are separated by 1 to 200 bases. In some embodiments, the two target nicks are separated by 1 to 100 bases. In some embodiments, the two target nicks are separated by 1 to 60 bases. In some embodiments, the two target nicks are separated by 1 to 50 bases. In some embodiments, the two target nicks are separated by 1 to 40 bases. In some embodiments, the two target nicks are separated by 1 to 30 bases. In some embodiments, the two target nicks are separated by 10 to 100,000 bases. In some embodiments, the two target nicks are separated by 10 to 10,000 bases. In some embodiments, the two target nicks are separated by 10 to 1,000 bases. In some embodiments, the two target nicks are separated by 10 to 200 bases. In some embodiments, the two target nicks are separated by 10 to 100 bases. In some embodiments, the two target nicks are separated by 10 to 60 bases. In some embodiments, the two target nicks are separated by 10 to 50 bases. In some embodiments, the two target nicks are separated by 10 to 40 bases. In some embodiments, the two target nicks are separated by 10 to 30 bases. In some embodiments, the two target nicks are separated by 20 to 100,000 bases. In some embodiments, the two target nicks are separated by 20 to 10,000 bases. In some embodiments, the two target nicks are separated by 20 to 1,000 bases. In some embodiments, the two target nicks are separated by 20 to 200 bases. In some embodiments, the two target nicks are separated by 20 to 100 bases. In some embodiments, the two target nicks are separated by 20 to 60 bases. In some

embodiments, the two target nicks are separated by 20 to 50 bases. In some embodiments, the two target nicks are separated by 20 to 40 bases. In some embodiments, the two target nicks are separated by 20 to 30 bases. In some embodiments, the two target nicks are separated by 30 to 100,000 bases. In some embodiments, the two target nicks are separated by 30 to 10,000 bases. In some embodiments, the two target nicks are separated by 30 to 1,000 bases. In some embodiments, the two target nicks are separated by 30 to 200 bases. In some embodiments, the two target nicks are separated by 30 to 100 bases. In some embodiments, the two target nicks are separated by 30 to 60 bases. In some embodiments, the two target nicks are separated by 30 to 50 bases. In some embodiments, the two target nicks are separated by 30 to 40 bases. In some embodiments, the two target nicks are separated by 30 to 35 bases. In some embodiments, the two target nicks are separated by 35 to 50 bases. In some embodiments, the two target nicks are separated by 35 to 45 bases. In some embodiments, the two target nicks are separated by 35 to 40 bases. In some embodiments, the two target nicks are separated by 40 to 100,000 bases. In some embodiments, the two target nicks are separated by 40 to 10,000 bases. In some embodiments, the two target nicks are separated by 40 to 1,000 bases. In some embodiments, the two target nicks are separated by 40 to 200 bases. In some embodiments, the two target nicks are separated by 40 to 100 bases. In some embodiments, the two target nicks are separated by 40 to 60 bases. In some embodiments, the two target nicks are separated by 40 to 50 bases. In some embodiments, the two target nicks are separated by 50 to 100,000 bases. In some embodiments, the two target nicks are separated by 50 to 10,000 bases. In some embodiments, the two target nicks are separated by 50 to 1,000 bases. In some embodiments, the two target nicks are separated by 50 to 200 bases. In some embodiments, the two target nicks are separated by 50 to 100 bases. In some embodiments, the two target nicks are separated by 50 to 60 bases. In some embodiments, the two target nicks are separated by 60 to 100,000 bases. In some embodiments, the two target nicks are separated by 60 to 10,000 bases. In some embodiments, the two target nicks are separated by 60 to 1,000 bases. In some

embodiments, the two target nicks are separated by 60 to 200 bases. In some embodiments, the two target nicks are separated by 60 to 100 bases. In some embodiments, the two target nicks are separated by 100 to 100,000 bases. In some embodiments, the two target nicks are separated by 100 to 10,000 bases. In some embodiments, the two target nicks are separated by 100 to 2,000 bases. In some embodiments, the two target nicks are separated by 100 to 1,000 bases. In some embodiments, the two target nicks are separated by 100 to 200 bases. In some embodiments, the two target nicks are separated by 200 to 100,000 bases. In some embodiments, the two target nicks are separated by 200 to 10,000 bases. In some embodiments, the two target nicks are separated by 200 to 1,000 bases. In some embodiments, the two target nicks are separated by 1,000 to 100,000 bases. In some embodiments, the two target nicks are separated by 1,000 to 10,000 bases. In some embodiments, the two target nicks are separated by 10,000 to 100,000 bases. In some embodiments, the two target nicks are separated by 100,000 to 3,000,000 bases.

[0013] In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1 to 3,000,000 or more bases, or on two different chromosomes. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1 base pair to 10 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 10 base pairs to 50 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 50 base pairs to 100 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 100 base pairs to 200 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 200 base pairs to 300 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 300 base pairs to 400 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 400 base pairs to 500 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 500 base pairs to 1,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1,000 base pairs to 2,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 2,000 base pairs to 3,000 base pairs. In

some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 3,000 base pairs to 4,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 4,000 base pairs to 5,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 5,000 base pairs to 10,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 10,000 base pairs to 20,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 20,000 base pairs to 30,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 30,000 base pairs to 40,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 40,000 base pairs to 50,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 50,000 base pairs to 100,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 100,000 base pairs to 200,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 200,000 base pairs to 300,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 300,000 base pairs to 400,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 400,000 base pairs to 500,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 500,000 base pairs to 1,000,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1,000,000 base pairs to 2,000,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 2,000,000 base pairs to 3,000,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are located on two different chromosomes.

[0014] In some embodiments, the endonuclease targeting sites on the target genomic DNA are on two different chromosomes.

[0015] In the system, each endonuclease nicks one target DNA strand at a target nick (nick site). In some embodiments, the distance of each endonuclease targeting site may

vary relative to the distance between the two nick sites. For example, in some cases, the distance of an endonuclease targeting site may be shorter than the distance between the two nick sites. In other cases, the distance of an endonuclease targeting site may be longer than the distance between the two nick sites. This variability allows for flexibility in the design and application of the system, enabling precise targeting of genomic regions regardless of the spatial relationship between the nick sites. Additionally, the relative distances may depend on factors such as the size of the target DNA, the specific endonucleases used, and the desired outcome of the DNA modification. In some embodiments, the distant between two nick sites are 1 to 3,000,000 base pairs in length. In some embodiments, the distance between two nick sites has a length of 1 base pair to 3,000,000 base pairs. In some embodiments, the distance between two nick sites has a length of 1 base pair to 10 base pairs. In some embodiments, the distance between two nick sites has a length of 10 base pairs to 50 base pairs. In some embodiments, the distance between two nick sites has a length of 50 base pairs to 100 base pairs. In some embodiments, the distance between two nick sites has a length of 100 base pairs to 200 base pairs. In some embodiments, the distance between two nick sites has a length of 200 base pairs to 300 base pairs. In some embodiments, the distance between two nick sites has a length of 300 base pairs to 400 base pairs. In some embodiments, the distance between two nick sites has a length of 400 base pairs to 500 base pairs. In some embodiments, the distance between two nick sites has a length of 500 base pairs to 1,000 base pairs. In some embodiments, the distance between two nick sites has a length of 1,000 base pairs to 2,000 base pairs. In some embodiments, the distance between two nick sites has a length of 2,000 base pairs to 3,000 base pairs. In some embodiments, the distance between two nick sites has a length of 3,000 base pairs to 4,000 base pairs. In some embodiments, the distance between two nick sites has a length of 4,000 base pairs to 5,000 base pairs. In some embodiments, the distance between two nick sites has a length of 5,000 base pairs to 10,000 base pairs. In some embodiments, the distance between two nick sites has a length of 10,000 base pairs to 20,000 base pairs. In some embodiments, the distance between two nick sites has a length of 20,000 base pairs to 30,000 base pairs. In some embodiments, the distance between two nick sites has a length of 30,000 base pairs to 40,000 base pairs. In some embodiments, the distance between

two nick sites has a length of 40,000 base pairs to 50,000 base pairs. In some embodiments, the distance between two nick sites has a length of 50,000 base pairs to 100,000 base pairs. In some embodiments, the distance between two nick sites has a length of 100,000 base pairs to 200,000 base pairs. In some embodiments, the distance between two nick sites has a length of 200,000 base pairs to 300,000 base pairs. In some embodiments, the distance between two nick sites has a length of 300,000 base pairs to 400,000 base pairs. In some embodiments, the distance between two nick sites has a length of 400,000 base pairs to 500,000 base pairs. In some embodiments, the distance between two nick sites has a length of 500,000 base pairs to 1,000,000 base pairs. In some embodiments, the distance between two nick sites has a length of 1,000,000 base pairs to 2,000,000 base pairs. In some embodiments, the distance between two nick sites has a length of 2,000,000 base pairs to 3,000,000 base pairs.

[0016] In some embodiments, a donor DNA comprising a sequence of interest serves as a template for repair or recombination processes, enabling the integration of the desired sequence into the target DNA at the site modified by the endonucleases. In some embodiments, the donor DNA molecule contains a specific nucleotide sequence intended to be introduced or incorporated into the target DNA. This sequence of interest may include, but is not limited to, functional genes, regulatory elements, mutations, or other genetic modifications designed to alter or enhance the properties of the target DNA.

[0017] In some embodiments, the donor sequence may comprise at least one nucleotide change relative to the target site of the genomic locus. As such, the donor sequence has substantial sequence identity to the target region in the genomic locus of interest. In some embodiments, depending upon the length of the target site, the donor sequence may be flanked by sequences or homologous portions having substantial sequence identity to sequences located upstream and downstream of the target site. In some embodiments, the donor DNA including one or more homologous portions of sequences homologous to the one or more endonuclease targeting sites.

[0018] In some embodiments, the DNA donor has two homology arms (homologous portions), each of which is homologous/sufficiently identical to enable a sequence

modification (e.g., replacement). In some embodiments, the DNA donor sequence comprises a sequence unrelated to the targeting sequence located adjacent to or between (flanked by) homology arm(s) (homologous portion(s)) with sufficient sequence identity to a region of the sequence to be modified to enable sequence modification (e.g., replacement).

[0019] In some embodiments, one DNA donor including a homologous portion with sequence homologous to one targeting site and a second DNA donor including a homologous portion with sequence homologous to a second targeting site.

[0020] The length of the homologous portions may vary depending on the specific application and the target locus. For example, in some embodiments, the homologous portions may range from 10 to 500 base pairs in length, although longer or shorter homologous sequences may also be utilized. Additionally, these homologous portions may be positioned at the 5' end, the 3' end, or both ends of the DNA donor molecule. The use of homologous portions at the 5' and/or 3' ends of the DNA donor ensures that the donor molecule is accurately positioned and integrated into the genome, minimizing off-target effects and maximizing the efficiency of the desired genetic modification. The lengths of the homologous portions at the 5' and 3' ends may be similar or may vary significantly. For example, in some cases, one homologous portion may be substantially longer than the other to optimize alignment and integration efficiency. In some embodiments, only one homologous portion (either at the 5' end or the 3' end) may be present, while the other end lacks a homologous portion. This configuration may be utilized in specific applications where single-ended homology is sufficient for targeted integration or repair. The design of the homologous portions, whether symmetric, asymmetric, or single-ended, can be tailored to the specific requirements of the DNA modification process.

[0021] In some embodiments, the 5' homologous portion may range in length from 6 base pairs to 100,000 base pairs. In some embodiments, the 5' homologous portion has a length of 6 base pairs to 10 base pairs. In some embodiments, the 5' homologous portion has a length of 10 base pairs to 20 base pairs. In some embodiments, the 5' homologous

portion has a length of 20 base pairs to 30 base pairs. In some embodiments, the 5' homologous portion has a length of 30 base pairs to 40 base pairs. In some embodiments, the 5' homologous portion has a length of 40 base pairs to 50 base pairs. In some embodiments, the 5' homologous portion has a length of 50 base pairs to 75 base pairs. In some embodiments, the 5' homologous portion has a length of 75 base pairs to 100 base pairs. In some embodiments, the 5' homologous portion has a length of 100 base pairs to 150 base pairs. In some embodiments, the 5' homologous portion has a length of 150 base pairs to 200 base pairs. In some embodiments, the 5' homologous portion has a length of 200 base pairs to 300 base pairs. In some embodiments, the 5' homologous portion has a length of 300 base pairs to 400 base pairs. In some embodiments, the 5' homologous portion has a length of 400 base pairs to 500 base pairs. In some embodiments, the 5' homologous portion has a length of 500 base pairs to 1,000 base pairs. In some embodiments, the 5' homologous portion has a length of 1,000 base pairs to 2,000 base pairs. In some embodiments, the 5' homologous portion has a length of 2,000 base pairs to 5,000 base pairs. In some embodiments, the 5' homologous portion has a length of 5,000 base pairs to 10,000 base pairs. In some embodiments, the 5' homologous portion has a length of 10,000 base pairs to 20,000 base pairs. In some embodiments, the 5' homologous portion has a length of 20,000 base pairs to 50,000 base pairs. In some embodiments, the 5' homologous portion has a length of 50,000 base pairs to 100,000 base pairs.

[0022] In some embodiments, the 3' homologous portion may range in length from 6 base pairs to 100,000 base pairs. In some embodiments, the 3' homologous portion has a length of 6 base pairs to 10 base pairs. In some embodiments, the 3' homologous portion has a length of 10 base pairs to 20 base pairs. In some embodiments, the 3' homologous portion has a length of 20 base pairs to 30 base pairs. In some embodiments, the 3' homologous portion has a length of 30 base pairs to 40 base pairs. In some embodiments, the 3' homologous portion has a length of 40 base pairs to 50 base pairs. In some embodiments, the 3' homologous portion has a length of 50 base pairs to 75 base pairs. In some embodiments, the 3' homologous portion has a length of 75 base pairs to 100 base pairs. In some embodiments, the 3' homologous portion has a length of 100 base pairs to 150 base pairs. In some embodiments, the 3' homologous portion has a length of 150 base

pairs to 200 base pairs. In some embodiments, the 3' homologous portion has a length of 200 base pairs to 300 base pairs. In some embodiments, the 3' homologous portion has a length of 300 base pairs to 400 base pairs. In some embodiments, the 3' homologous portion has a length of 400 base pairs to 500 base pairs. In some embodiments, the 3' homologous portion has a length of 500 base pairs to 1,000 base pairs. In some embodiments, the 3' homologous portion has a length of 1,000 base pairs to 2,000 base pairs. In some embodiments, the 3' homologous portion has a length of 2,000 base pairs to 5,000 base pairs. In some embodiments, the 3' homologous portion has a length of 5,000 base pairs to 10,000 base pairs. In some embodiments, the 3' homologous portion has a length of 10,000 base pairs to 20,000 base pairs. In some embodiments, the 3' homologous portion has a length of 20,000 base pairs to 50,000 base pairs. In some embodiments, the 3' homologous portion has a length of 50,000 base pairs to 100,000 base pairs.

[0023] In some embodiments, the homologous portion of the donor DNA is configured capable of base-pairing the target DNA site.

[0024] In some embodiments, the homologous portion of the donor DNA is configured to be filled in the single-strand portion of the target DNA site. In some specific embodiments, the single-strand portion serves as a template or binding site for the homologous portion of the donor DNA to facilitate the filling of the donor DNA.

[0025] In some embodiments, the homologous portion of the donor DNA is configured to be capable of stranding in and displacing the nicked target DNA. The donor DNA integrates into the nicked target DNA by pairing with the single-stranded region. This process may involve strand invasion, where the donor DNA displaces the nicked strand and pairs with the complementary strand. Alternatively, it may occur through other mechanisms such as branch migration, where the donor DNA dynamically reconfigures the target DNA structure to integrate itself; single-strand annealing (SSA), where the donor DNA anneals directly to the single-stranded region of the target DNA; or strand displacement synthesis, where DNA polymerase synthesizes a new strand while displacing the existing single-stranded DNA. Additionally, engineered DNA modification

tools (e.g., CRISPR-Cas systems) or chemical ligation methods may facilitate the integration of the donor DNA into the target DNA. In general, any mechanism that allows the donor DNA to interact with and integrate into the target DNA, whether through complementary base pairing, enzymatic activity, or chemical methods, falls within the scope of this process.

[0026] In some embodiments, the donor DNA may replace or displace the nicked strand, either partially or completely, through mechanisms such as branch migration or DNA repair processes. This displacement can result in the incorporation of the donor DNA sequence into the target DNA.

[0027] In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand. In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand at its 5' end. In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand at its 3' end.

[0028] In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand and spanning the nicked position. The homologous portion of the donor DNA is designed to base-pair with the complementary strand of the target DNA, extending across the site of the nick.

[0029] In some embodiments, the donor DNA includes a modified nucleoside. In some embodiments, the modified nucleoside includes an inverted nucleoside, a locked nucleic acid, a 2'fluoro, a 2' O-alkyl, a methylated cytosine, or a combination thereof. In some embodiments, the donor DNA includes modified inter-nucleoside linkage including a phosphorothioate linkage.

[0030] In some embodiments, the endonuclease is an RNA-guided nickase and an associated guide RNA. In some embodiments, the system comprises at least two associated guide RNAs comprising a first guide RNA capable of hybridizing to a first

target sequence in the DNA, and a second guide RNA capable of hybridizing to a second target sequence in the DNA. In some embodiments, the guide RNAs bind to different strands of the target DNA. In some embodiments, the guide RNAs bind to the same strand of the target DNA.

[0031] In some embodiments, the first guide RNA and the second guide RNA are configured capable of being positioned with their PAM sites oriented towards each other (PAM-facing); In some embodiments, the first guide RNA and the second guide RNA are configured capable of being positioned with their PAM sites oriented apart from each other (PAM-backing). In some embodiments, the first guide RNA and the second guide RNA are configured capable of being positioned with their PAM sites co-directional each other (PAM-same-directional).

[0032] In some embodiments, the gRNA includes a modified nucleoside. In some embodiments, the modified nucleoside includes an inverted nucleoside, a locked nucleic acid, a 2'fluoro, a 2' O-alkyl, a methylated cytosine, or a combination thereof. In some embodiments, the gRNA includes modified inter-nucleoside linkage including a phosphorothioate linkage.

[0033] In some embodiments, the system comprises two or more endonucleases. In some embodiments, each endonuclease is a CRISPR Cas nickase. CRISPR Cas nickases are derived from CRISPR nucleases by inactivation of one of the nuclease domains. In specific embodiments, the CRISPR nickase can be derived from a type II CRISPR nuclease. For example, the type II CRISPR nuclease can be a Cas9 protein. Suitable Cas9 nucleases include, Streptococcus pyogenes Cas9 (SpCas9), Francisella novicida Cas9 (FnCas9), Staphylococcus aureus (SaCas9), Streptococcus thermophilus Cas9 (StCas9); Streptococcus pasteurianus (SpaCas9), Campylobacter jejuni Cas9 (CjCas9), Neisseria meningitis Cas9 (NmCas9), or Neisseria cinerea Cas9 (NcCas9); in some other embodiments, the nickase can be derived from a type V CRISPR nuclease, such as a Cpf1 nuclease. Suitable Cpf1 nucleases include Francisella novicida Cpf1 (FnCpf1), Acidaminococcus sp. Cpf1 (AsCpf1), or Lachnospiraceae bacterium ND2006 Cpf1(LbCpf1).

[0034] In some embodiments, each endonuclease is a class II CRISPR Cas nickase. In some specific embodiments, each CRISPR nickase can be individually a type II CRISPR nickase, or a type V CRISPR nickase. In some embodiments, each endonuclease is individually selected from a Cas9 nickase or a Cpf1 nickase. In some embodiments, each endonuclease is individually a Cas9 nickase. In some embodiments, each endonuclease is individually selected from a SpCas9 nickase, a FnCas9 nickase, a SaCas9 nickase, a StCas9 nickase, a SpaCas9 nickase, a CjCas9 nickase, a NmCas9 nickase, or a NcCas9 nickase. In some embodiments, each endonuclease can be individually a Cas9-D10A nickase or a Cas9-H840A nickase. In some embodiments, each endonuclease is a SpCas9-D10A nickase. In some embodiments, each endonuclease is a SpCas9-H840A nickase. In some embodiments, at least two endonucleases are the same. In some embodiments, at least two endonucleases are different; In some embodiments, at least one endonuclease is SpCas9-D10A nickase and at least one endonuclease is SpCas9-D10A nickase. In some embodiments, the system comprises two endonucleases, which one is SpCas9-D10A nickase and another is SpCas9-D10A nickase.

[0035] In some embodiments, each endonuclease may be individually coupled with a nuclear export domain, a nuclear localization domain an affinity tag and/or Flag. In some embodiments, each endonuclease is individually coupled to a DNA ligase. In some embodiments, each endonuclease is individually coupled to a DNA polymerase. In some embodiments, each endonuclease is individually coupled to a DNA exonuclease. In some embodiments, each endonuclease is individually coupled to a HUH endonuclease. In some embodiments, the coupling is covalent. In some embodiments, the coupling forms a fusion protein with a linker. In some embodiments, the coupling is non-covalent. In some embodiments, the guide RNA includes a portion that binds the endonuclease and a MS2 hairpin portion, wherein the endonuclease contains a MS2 binding motif.

[0036] In some embodiments, at least two endonucleases are coupled with each other.

[0037] In some embodiments, the donor DNA is coupled to the endonuclease. In some embodiments, the coupling is covalent. In some embodiments, the coupling is formed by bioconjugation ability of an HUH endonuclease to covalently tether the donor DNA. In

some embodiments, the coupling is non-covalent. In some embodiments, the coupling is via complementary pairing of a portion of the guide RNA and a portion of the donor DNA.

[0038] In some embodiments, the target DNA is on the genomic DNA within a cell.

[0039] In some embodiments, the system is complexed in vitro and delivered into a target cell. In some embodiments, the system is packaged in vivo in a producer cell and delivered into a target cell.

[0040] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for modifying at least one target DNA.

[0041] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for replacing a target DNA sequence with a desired sequence of 1 to 10 bases, 11 to 50 bases, 51 to 200 bases, 201 to 1,000 bases, 1,001 to 10,000 bases, or 10,001 to 100,000 bases.

[0042] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for replacing a target DNA sequence of 1 to 3,000,000 genomic bases with a desired sequence of 1 to 10 bases, 11 to 50 bases, 51 to 200 bases, 201 to 1,000 bases, 1,001 to 10,000 bases, or 10,001 to 100,000 bases.

[0043] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for large-size DNA insertion by using a donor DNA comprising partially double-strand DNA and two partially single-strand DNAs, wherein each of the single-strand portion is homologous to one target nick site, and the two nick sites are aparted for a large distance on a genomic DNA.

[0044] In some embodiments, the method includes the steps of: (i) complexing the system in vitro; and (ii) delivering the system into a target cell. In some embodiments, the system is packaged in a delivery vehicle in vitro to enhance stability, cellular uptake, and targeted delivery. The delivery vehicle may be selected from the group consisting of lipid nanoparticles, polymeric nanoparticles.

[0045] In some embodiments, the method includes the steps of: (i) packaging the system in vivo in a producer cell; and (ii) delivering the system into a target cell. In some embodiments, the system is packaged within a delivery vehicle for efficient and targeted delivery to the target cell. In some embodiments, the delivery vehicle may be selected from the group consisting of viral vectors (e.g., lentivirus, adenovirus, or AAV), virus-like particles (VLPs) or exosomes.

[0046] In another aspects, the system described herein for use in the treatment of genetic disease is provided.

[0047] In another aspect, a pharmaceutical composition comprising a system described herein and a pharmaceutically acceptable carrier is provided.

[0048] In another aspect, a kit for gene editing comprising the system described herein, a delivery vehicle, and instructions for use is provided.

[0049] In another aspect, a use of the system described herein in the manufacture of a medicament for treating a genetic disorder is provided.

[0050] In some embodiments, provided is a method of using the system as described for modifying at least one target DNA.

[0051] In another aspect, the disclosure provides an engineered cell produced by the method of disclosure. In some embodiments, the engineered cell is an in vitro cell. In some embodiments, the engineered cell is an in vivo cell. In some embodiments, the sequence of interest is incorporated into the target DNA of the engineered cell.

[0052] In another aspect, the disclosure provides a population of cells derived from the engineered cell of the disclosure.

[0053] Other example embodiments will be described below.

## BRIEF DESCRIPTION OF FIGURES

[0054] FIGs. 1A and 1B are schematic diagrams showing different combinations of

two SpCas9 nickases (H840A and D10A) according to example embodiments.

[0055] FIG. 2 shows the effect of relative nicking positions on desired editing efficiency (CTT insertion) based on dual H840A SpCas9 nickases, on HEK3 site, in HEK293T cells according to an example embodiment.

[0056] FIG. 3 shows the editing outcome visualization by the CRISPResso2 software for the condition number 4 as shown in FIG. 2.

[0057] FIG. 4 shows the editing outcome visualization by the CRISPResso2 software for the condition number 15 as shown in FIG. 2.

[0058] FIG. 5 shows the editing outcome visualization by the CRISPResso2 software for the condition number 26 as shown in FIG. 2.

[0059] FIG. 6 is a schematic diagram showing four different combinations of the two SpCas9 nickases (H840A and D10A) nicking two sites at the optimal distance as shown in FIG. 2, namely -38 and 0, and the "dig-and-fill" mode of action.

[0060] FIG. 7 shows the editing efficiencies of the four different combinations SpCas9 nickases (H840A and D10A), nicking two sites at position -38 and 0, by editing outcome types: all indels (including desired and non-desired; Left) and desired edits (Right) among all reads according to an example embodiment.

[0061] FIG. 8 shows the editing outcome visualization by the CRISPResso2 software for condition HD: H840A (HEK -38) +D10A (HEK 0) as shown in FIG. 7.

[0062] FIG. 9 shows the editing outcome visualization by the CRISPResso2 software for condition DH: D10A (HEK -38) +H840A (HEK 0) as shown in FIG. 7.

[0063] FIG. 10 shows the editing outcome visualization by the CRISPResso2 software for condition DD: D10A (HEK -38) +D10A (HEK 0) as shown in FIG. 7.

[0064] FIG. 11 shows the editing outcome visualization by the CRISPResso2 software for condition HH: H840A (HEK -38) +H840A (HEK 0) as shown in FIG. 7.

[0065] FIG. 12 shows the exemplary positional relationships of the PAM sites relative to the targeting sequences.

## DETAILED DESCRIPTION

[0066] Disclosed herein are systems for modifying DNA sequences and comprising two or more endonuclease designs and one or more donor DNA oligos, wherein each of the endonuclease design comprising one endonuclease and one guide RNA, and wherein the donor DNA contains sequence of interest. Further disclosed herein are methods of using the systems described herein.

[0067] In some embodiments, provided is a system for modifying at least one target DNA, including: two or more endonucleases, wherein each endonuclease nicks one target DNA strand at a target nick and at least one donor DNA including a sequence of interest to be incorporated to a target DNA site. The term "two or more endonucleases" refers to a system comprising at least two endonucleases, which, in some embodiments, may include: (i) different types of endonucleases, such as SpCas9-D10A and SpCas9-H840A, each capable of introducing single-strand nicks at specific target sites on the DNA; or (ii) same type of endonuclease with different performance characteristics, such as two SpCas9-D10A endonucleases that, through interactions with other molecules (e.g., distinct guide RNAs (gRNAs)), exhibit different targeting activities or other functional variations. In this configuration, the endonucleases may be identical in their protein sequence and function but are directed to different genomic loci by unique gRNAs, thereby enabling multiple targeted modifications within the same system.

[0068] In some embodiments, the term "endonucleases" may be interpreted differently depending on its usage, as described below: In some embodiments, the term "endonuclease(s)" may refer to endonuclease design(s), which comprise both the endonuclease protein and its associated molecule such as a gRNA (e.g., when "endonucleases" appears alone in a specific embodiment). In other embodiments, the term "endonucleases" may refer specifically to the endonuclease protein molecule itself, particularly when it is described in association with other molecules such as guide RNAs

(gRNAs); In such cases, the endonuclease protein and the gRNA together form part of an endonuclease design. In some embodiments, the term "endonucleases" may refer specifically to the endonuclease protein molecule itself, regardless of the context in which it appears (alone or in association with other molecules). A person skilled in the art shall understand its meaning based on the context. Different interpretations may lead to different embodiments, all of which are included in the embodiments of the present disclosure and are considered to be disclosed and documented herein.

[0069] As described in this disclosure, the expressions "each endonuclease nicks one target DNA strand at a target nick", "each endonuclease is capable of nicking one target DNA strand at a target nick", and "each endonuclease nicks one strand of the target DNA at a target nick" (or similar formulations) describe the functionality of the endonucleases within the system. Specifically, each endonuclease is designed to introduce a single-strand break (nick) at a specific site (target nick) on one strand of the target DNA. This capability enables precise DNA modifications, such as facilitating homology-directed repair (HDR) or other targeted editing processes. Whether describing the actual behavior of the endonucleases (i.e., each endonuclease actively nicking the DNA) or their inherent capability (i.e., each endonuclease having the potential to nick the DNA), these expressions emphasize that the endonucleases are functionally equipped to create targeted nicks, which are essential for the system's DNA modification purposes.

[0070] In the system, each endonuclease is designed to introduce a single-strand break (nick) at a specific site (target nick) on one strand of the target DNA. Depending on the system's design, the endonucleases can operate in different possible configurations: Multiple nicks on the same strand: two or more endonucleases can introduce nicks at different positions on the same strand of the target DNA; Or nicks on different strands: Each endonuclease can introduce a nick on a separate strand of the target DNA (e.g., one on the forward strand and one on the reverse strand). For example, in a target DNA molecule: Endonuclease A nicks the forward strand at position 1, and endonuclease B nicks the forward strand at position 2; Or endonuclease A nicks the forward strand at position 1, and endonuclease B nicks the reverse strand at position 2. In some embodiment, the introduction of nicks can lead to various structural outcomes. In some

embodiment, the nicks are introduced on opposite strands and a single-stranded overhang (5' or 3') may be generated; In some embodiments, the nicks are introduced on the same strand and a single-stranded flap may be formed. In some embodiments, the DNA segment between two nicks on the same strand is removed, a single-stranded gap may be created.

[0071] "Target nick" or "nick site", or "nicked position" refers to the specific site on a target DNA molecule where a single-strand break (nick) is introduced by an endonuclease (e.g., a nickase). The nick is made on one strand of the double-stranded DNA, while the complementary strand remains intact. The location of the target nick is determined by the recognition sequence of the endonuclease and its associated guide molecule (e.g., guide RNA).

[0072] As used in this disclosure, in some embodiments, the term "site" refers to a specific location or region on a DNA molecule that is functionally relevant in the context of DNA modification. In some embodiments, a "site" may include: (i) Endonuclease-related sites: A specific point: The exact position on the DNA where an endonuclease introduces a single-strand nick or double-strand break. A sequence region: The entire DNA sequence recognized by an endonuclease, including the target sequence and any adjacent motifs (e.g., PAM sequences) required for binding and cleavage; gRNA-related sites: A gRNA binding region: A sequence on the DNA that is complementary to a guide RNA (gRNA), which directs the endonuclease to the target location. (ii) Donor-related sites: A complementary region: A sequence on the target DNA that is complementary to a portion of the donor DNA, facilitating repair process or other integration mechanisms. (iii) Functional units: A region of interest: A segment of DNA targeted for modification, which may include one or more cutting points, recognition sequences, or complementary regions. Other relevant sites: Any functionally relevant region: Any other region on the DNA that plays a role in the DNA modification process, as dictated by the specific application or context. The interpretation of "site" as a point, a sequence region, a complementary region, or a functional unit depends on the specific context in which the term is used.

[0073] As used herein, the term "target DNA site" refers to a specific region or site on the double-stranded target DNA that, in some embodiments, encompasses: (1) the precise location(s) where the endonuclease introduces single-strand breaks (nicks); (2) the sequence between the nicks (if multiple nicks are introduced), and/or (3) the adjacent regions that may extend beyond the nicks on one or both sides. The target DNA site is the region where the donor DNA's sequence of interest is incorporated, which may include the sequence between the nicks, the sequence spanning one or more nicks, or the sequence extending beyond the nicks on one or both sides. This definition applies regardless of the number of nicks introduced (e.g., two, three, or more). In some embodiments, the term "target DNA site" may be used interchangeably with "target DNA" when "target DNA" refers specifically to the site or region.

[0074] In some embodiments, the target nicks of the two or more endonucleases are on the same DNA strand. In some embodiments, the target nicks of the two or more endonucleases are on different DNA strands. "the target nicks of the two or more endonucleases" means the target nicks produced by the two or more endonucleases.

[0075] In some embodiments, the nicking event results in a 3'-overhang at the target DNA site. In some other embodiments, the nicking event results in a 5'-overhang at the target DNA site. In yet other embodiments, the nicking event does not produce any overhang; In some embodiments, the nicking event results in a blunt end at the target DNA site; In some embodiment, the nicking event may lead to the formation of a single-strand flap; In some embodiment, the nicking event may lead to the formation of a single-stranded gap; In some cases, the nicking event may result in a large fragment deletion or replacement; In some embodiments, the nicking event may directly result in a double-strand break. In some embodiment, the nicking event may lead to a strand displacement. In some embodiment, the complementary strand may be displaced to form a single-stranded region, or the formation of a gap between two nearby nicks, which may be filled by DNA polymerase and sealed by DNA ligase. These outcomes depend on the location and distance between the nicks, as well as the cellular repair mechanisms involved, and have specific applications in DNA modification, such as gene knockout, insertion, deletion, or repair.

[0076] In some embodiments, the donor DNA is a single-strand DNA. In some embodiments, the donor DNA is a double-strand DNA. In some embodiments, the donor DNA includes one or more portions of single-strand DNA and one or more portions of double-strand DNA. In some embodiments, the single-strand DNA is a circularized DNA. In some embodiments, the double-strand DNA is a circularized DNA. The length of the donor DNA may be optimized.

[0077] In some embodiments, the single-strand DNA is preferably 6-nucleotide to 100,000-nucleotide long. In some embodiments, the single-strand DNA is 6-nucleotide to 10-nucleotide in length. In some embodiments, the single-strand DNA is 10-nucleotide to 15-nucleotide in length. In some embodiments, the single-strand DNA is 15-nucleotide to 20-nucleotide in length. In some embodiments, the single-strand DNA is 20-nucleotide to 25-nucleotide in length. In some embodiments, the single-strand DNA is 25-nucleotide to 30-nucleotide in length. In some embodiments, the single-strand DNA is 30-nucleotide to 40-nucleotide in length. In some embodiments, the single-strand DNA is 40-nucleotide to 50-nucleotide in length. In some embodiments, the single-strand DNA is 50-nucleotide to 75-nucleotide in length. In some embodiments, the single-strand DNA is 75-nucleotide to 100-nucleotide in length. In some embodiments, the single-strand DNA is 100-nucleotide to 150-nucleotide in length. In some embodiments, the single-strand DNA is 150-nucleotide to 200-nucleotide in length. In some embodiments, the single-strand DNA is 200-nucleotide to 300-nucleotide in length. In some embodiments, the single-strand DNA is 300-nucleotide to 400-nucleotide in length. In some embodiments, the single-strand DNA is 400-nucleotide to 500-nucleotide in length. In some embodiments, the single-strand DNA is 500-nucleotide to 1,000-nucleotide in length. In some embodiments, the single-strand DNA is 1,000-nucleotide to 5,000-nucleotide in length. In some embodiments, the single-strand DNA is 5,000-nucleotide to 10,000-nucleotide in length. In some embodiments, the single-strand DNA is 10,000-nucleotide to 50,000-nucleotide in length. In some embodiments, the single-strand DNA is 50,000-nucleotide to 100,000-nucleotide in length.

[0078] In some embodiments, the double-strand DNA is preferably 6-base pair to 100,000-base pair long. In some embodiments, the double-strand DNA is 6-base pair to

10-base pair in length. In some embodiments, the double-strand DNA is 10-base pair to 15-base pair in length. In some embodiments, the double-strand DNA is 15-base pair to 20-base pair in length. In some embodiments, the double-strand DNA is 20-base pair to 25-base pair in length. In some embodiments, the double-strand DNA is 25-base pair to 30-base pair in length. In some embodiments, the double-strand DNA is 30-base pair to 40-base pair in length. In some embodiments, the double-strand DNA is 40-base pair to 50-base pair in length. In some embodiments, the double-strand DNA is 50-base pair to 75-base pair in length. In some embodiments, the double-strand DNA is 75-base pair to 100-base pair in length. In some embodiments, the double-strand DNA is 100-base pair to 150-base pair in length. In some embodiments, the double-strand DNA is 150-base pair to 200-base pair in length. In some embodiments, the double-strand DNA is 200-base pair to 300-base pair in length. In some embodiments, the double-strand DNA is 300-base pair to 400-base pair in length. In some embodiments, the double-strand DNA is 400-base pair to 500-base pair in length. In some embodiments, the double-strand DNA is 500-base pair to 1,000-base pair in length. In some embodiments, the double-strand DNA is 1,000-base pair to 5,000-base pair in length. In some embodiments, the double-strand DNA is 5,000-base pair to 10,000-base pair in length. In some embodiments, the double-strand DNA is 10,000-base pair to 50,000-base pair in length. In some embodiments, the double-strand DNA is 50,000-base pair to 100,000-base pair in length.

[0079] In some embodiments, the single-strand and double-strand mixed donor DNA preferably has a total length of 6-nucleotide to 100,000-nucleotide long. In some embodiments, the mixed donor DNA has a total length of 6 nucleotides to 10 nucleotides. In some embodiments, the mixed donor DNA has a total length of 10 nucleotides to 15 nucleotides. In some embodiments, the mixed donor DNA has a total length of 15 nucleotides to 20 nucleotides. In some embodiments, the mixed donor DNA has a total length of 20 nucleotides to 25 nucleotides. In some embodiments, the mixed donor DNA has a total length of 25 nucleotides to 30 nucleotides. In some embodiments, the mixed donor DNA has a total length of 30 nucleotides to 40 nucleotides. In some embodiments, the mixed donor DNA has a total length of 40 nucleotides to 50 nucleotides. In some embodiments, the mixed donor DNA has a total length of 50 nucleotides to 75

nucleotides. In some embodiments, the mixed donor DNA has a total length of 75 nucleotides to 100 nucleotides. In some embodiments, the mixed donor DNA has a total length of 100 nucleotides to 150 nucleotides. In some embodiments, the mixed donor DNA has a total length of 150 nucleotides to 200 nucleotides. In some embodiments, the mixed donor DNA has a total length of 200 nucleotides to 300 nucleotides. In some embodiments, the mixed donor DNA has a total length of 300 nucleotides to 400 nucleotides. In some embodiments, the mixed donor DNA has a total length of 400 nucleotides to 500 nucleotides. In some embodiments, the mixed donor DNA has a total length of 500 nucleotides to 1,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 1,000 nucleotides to 5,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 5,000 nucleotides to 10,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 10,000 nucleotides to 50,000 nucleotides. In some embodiments, the mixed donor DNA has a total length of 50,000 nucleotides to 100,000 nucleotides.

[0080] In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1 to 3,000,000 or more bases. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1 base pair to 10 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 10 base pairs to 50 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 50 base pairs to 100 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 100 base pairs to 200 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 200 base pairs to 300 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 300 base pairs to 400 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 400 base pairs to 500 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 500 base pairs to 1,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1,000 base pairs to 2,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 2,000 base pairs to 3,000 base pairs. In some

embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 3,000 base pairs to 4,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 4,000 base pairs to 5,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 5,000 base pairs to 10,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 10,000 base pairs to 20,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 20,000 base pairs to 30,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 30,000 base pairs to 40,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 40,000 base pairs to 50,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 50,000 base pairs to 100,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 100,000 base pairs to 200,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 200,000 base pairs to 300,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 300,000 base pairs to 400,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 400,000 base pairs to 500,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 500,000 base pairs to 1,000,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 1,000,000 base pairs to 2,000,000 base pairs. In some embodiments, the endonuclease targeting sites on the target genomic DNA are distant for 2,000,000 base pairs to 3,000,000 base pairs.

[0081] In some embodiments, the endonuclease targeting sites on the target genomic DNA are located on two different chromosomes.

[0082] In the system, each endonuclease nicks one target DNA strand at a target nick (nick site). In some embodiments, the distance of each endonuclease targeting site may vary relative to the distance between the two nick sites. For example, in some cases, the distance of an endonuclease targeting site may be shorter than the distance between the

two nick sites. In other cases, the distance of an endonuclease targeting site may be longer than the distance between the two nick sites. This variability allows for flexibility in the design and application of the system, enabling precise targeting of genomic regions regardless of the spatial relationship between the nick sites. Additionally, the relative distances may depend on factors such as the size of the target DNA, the specific endonucleases used, and the desired outcome of the DNA modification. In some embodiments, the distant between two nick sites are 1 to 3,000,000 base pairs in length. In some embodiments, the distance between two nick sites has a length of 1 base pair to 3,000,000 base pairs. In some embodiments, the distance between two nick sites has a length of 1 base pair to 10 base pairs. In some embodiments, the distance between two nick sites has a length of 10 base pairs to 50 base pairs. In some embodiments, the distance between two nick sites has a length of 50 base pairs to 100 base pairs. In some embodiments, the distance between two nick sites has a length of 100 base pairs to 200 base pairs. In some embodiments, the distance between two nick sites has a length of 200 base pairs to 300 base pairs. In some embodiments, the distance between two nick sites has a length of 300 base pairs to 400 base pairs. In some embodiments, the distance between two nick sites has a length of 400 base pairs to 500 base pairs. In some embodiments, the distance between two nick sites has a length of 500 base pairs to 1,000 base pairs. In some embodiments, the distance between two nick sites has a length of 1,000 base pairs to 2,000 base pairs. In some embodiments, the distance between two nick sites has a length of 2,000 base pairs to 3,000 base pairs. In some embodiments, the distance between two nick sites has a length of 3,000 base pairs to 4,000 base pairs. In some embodiments, the distance between two nick sites has a length of 4,000 base pairs to 5,000 base pairs. In some embodiments, the distance between two nick sites has a length of 5,000 base pairs to 10,000 base pairs. In some embodiments, the distance between two nick sites has a length of 10,000 base pairs to 20,000 base pairs. In some embodiments, the distance between two nick sites has a length of 20,000 base pairs to 30,000 base pairs. In some embodiments, the distance between two nick sites has a length of 30,000 base pairs to 40,000 base pairs. In some embodiments, the distance between two nick sites has a length of 40,000 base pairs to 50,000 base pairs. In some embodiments, the distance between two nick sites has a length of 50,000 base pairs to

100,000 base pairs. In some embodiments, the distance between two nick sites has a length of 100,000 base pairs to 200,000 base pairs. In some embodiments, the distance between two nick sites has a length of 200,000 base pairs to 300,000 base pairs. In some embodiments, the distance between two nick sites has a length of 300,000 base pairs to 400,000 base pairs. In some embodiments, the distance between two nick sites has a length of 400,000 base pairs to 500,000 base pairs. In some embodiments, the distance between two nick sites has a length of 500,000 base pairs to 1,000,000 base pairs. In some embodiments, the distance between two nick sites has a length of 1,000,000 base pairs to 2,000,000 base pairs. In some embodiments, the distance between two nick sites has a length of 2,000,000 base pairs to 3,000,000 base pairs.

[0083] In some embodiments, a donor DNA comprising a sequence of interest serves as a template for repair or recombination processes, enabling the integration of the desired sequence into the target DNA at the site modified by the endonucleases. In some embodiments, the donor DNA molecule contains a specific nucleotide sequence intended to be introduced or incorporated into the target DNA. This sequence of interest may include, but is not limited to, functional genes, regulatory elements, mutations, or other genetic modifications designed to alter or enhance the properties of the target DNA.

[0084] In some embodiments, the donor sequence may comprise at least one nucleotide change relative to the target site of the genomic locus. As such, the donor sequence has substantial sequence identity to the target region in the genomic locus of interest. In some embodiments, depending upon the length of the target site, the donor sequence may be flanked by sequences or homologous portions having substantial sequence identity to sequences located upstream and downstream of the target site. In some embodiments, the donor DNA including one or more homologous portions of sequences homologous to the one or more endonuclease targeting sites. The term "substantial sequence identity" or "homologous portion" refers to sequences having at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81 %, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91 %, at least about 92%, at

least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% sequence, or 100% identity to the referred sequence. In some embodiments, the homologous portion is understood to be a sequence of at least 20bp, 30bp, 40bp, 50bp, 60bp, 70bp, 80bp, 90bp, 100 bp, 150 bp, 200 bp, 250 bp, 300 bp, 350 bp, 400 bp, 450 bp, 500 bp, 600bp,700 bp, 800bp, 900bp, or 1000 bp or more, with a sequence identity to a region within the DNA sequence sufficient to mediated modification, such as at least 90%, preferably 95%, 96%, 97%, 98%, 99%, most preferably 100% sequence identity to a region within the DNA sequence to be modified. In some embodiments, the homology arm has a sequence identity sufficient to enable hybridization of the homologous portion (homology arm) to the corresponding region of the sequence to be modified. Preferably, the homology arms share this sequence identity with a region located at the end of the end sequence to be modified.

[0085] In some embodiments, the DNA donor has two homology arms (homologous portions), each of which is homologous/sufficiently identical to enable a sequence modification (e.g., replacement). In some embodiments, the DNA donor sequence comprises a sequence of interest which is located adjacent to or between (flanked by) the homology arm(s) (homologous portion(s)) with sufficient sequence identity to a region of the sequence to be modified to enable sequence modification (e.g., replacement).

[0086] In some embodiments, one DNA donor including a homologous portion with sequence homologous to one targeting site and a second DNA donor including a homologous portion with sequence homologous to a second targeting site.

[0087] As used herein, the "endonuclease targeting sites" or "target site" or "targeting site" refers to the target DNA site or portion of target DNA site.

[0088] The length of the homologous portions may vary depending on the specific application and the target locus. For example, in some embodiments, the homologous portions may range from 10 to 500 base pairs in length, although longer or shorter homologous sequences may also be utilized. Additionally, these homologous portions may be positioned at the 5' end, the 3' end, or both ends of the DNA donor molecule. The

use of homologous portions at the 5' and/or 3' ends of the DNA donor ensures that the donor molecule is accurately positioned and integrated into the genome, minimizing off-target effects and maximizing the efficiency of the desired genetic modification. The lengths of the homologous portions at the 5' and 3' ends may be similar or may vary significantly. For example, in some cases, one homologous portion may be substantially longer than the other to optimize alignment and integration efficiency. In some embodiments, only one homologous portion (either at the 5' end or the 3' end) may be present, while the other end lacks a homologous portion. This configuration may be utilized in specific applications where single-ended homology is sufficient for targeted integration or repair. The design of the homologous portions, whether symmetric, asymmetric, or single-ended, can be tailored to the specific requirements of the DNA modification process.

[0089] In some embodiments, the 5' homologous portion may range in length from 6 to 100,000 nucleotides. In some embodiments, the 5' homologous portion may range in length from 6 to 10 nucleotides. In some embodiments, the 5' homologous portion may range in length from 10 to 20 nucleotides. In some embodiments, the 5' homologous portion may range in length from 20 to 30 nucleotides. In some embodiments, the 5' homologous portion may range in length from 30 to 40 nucleotides. In some embodiments, the 5' homologous portion may range in length from 40 to 50 nucleotides. In some embodiments, the 5' homologous portion may range in length from 50 to 75 nucleotides. In some embodiments, the 5' homologous portion may range in length from 75 to 100 nucleotides. In some embodiments, the 5' homologous portion may range in length from 100 to 150 nucleotides. In some embodiments, the 5' homologous portion may range in length from 150 to 200 nucleotides. In some embodiments, the 5' homologous portion may range in length from 200 to 300 nucleotides. In some embodiments, the 5' homologous portion may range in length from 300 to 400 nucleotides. In some embodiments, the 5' homologous portion may range in length from 400 to 500 nucleotides. In some embodiments, the 5' homologous portion may range in length from 500 to 1,000 nucleotides. In some embodiments, the 5' homologous portion may range in length from 1,000 to 2,000 nucleotides. In some embodiments, the 5' homologous portion may range in length from 2,000 to 5,000 nucleotides. In some

embodiments, the 5' homologous portion may range in length from 5,000 to 10,000 nucleotides. In some embodiments, the 5' homologous portion may range in length from 10,000 to 20,000 nucleotides. In some embodiments, the 5' homologous portion may range in length from 20,000 to 50,000 nucleotides. In some embodiments, the 5' homologous portion may range in length from 50,000 to 100,000 nucleotides.

[0090] In some embodiments, the 3' homologous portion may range in length from 6 to 100,000 nucleotides. In some embodiments, the 3' homologous portion may range in length from 6 to 10 nucleotides. In some embodiments, the 3' homologous portion may range in length from 10 to 20 nucleotides. In some embodiments, the 3' homologous portion may range in length from 20 to 30 nucleotides. In some embodiments, the 3' homologous portion may range in length from 30 to 40 nucleotides. In some embodiments, the 3' homologous portion may range in length from 40 to 50 nucleotides. In some embodiments, the 3' homologous portion may range in length from 50 to 75 nucleotides. In some embodiments, the 3' homologous portion may range in length from 75 to 100 nucleotides. In some embodiments, the 3' homologous portion may range in length from 100 to 150 nucleotides. In some embodiments, the 3' homologous portion may range in length from 150 to 200 nucleotides. In some embodiments, the 3' homologous portion may range in length from 200 to 300 nucleotides. In some embodiments, the 3' homologous portion may range in length from 300 to 400 nucleotides. In some embodiments, the 3' homologous portion may range in length from 400 to 500 nucleotides. In some embodiments, the 3' homologous portion may range in length from 500 to 1,000 nucleotides. In some embodiments, the 3' homologous portion may range in length from 1,000 to 2,000 nucleotides. In some embodiments, the 3' homologous portion may range in length from 2,000 to 5,000 nucleotides. In some embodiments, the 3' homologous portion may range in length from 5,000 to 10,000 nucleotides. In some embodiments, the 3' homologous portion may range in length from 10,000 to 20,000 nucleotides. In some embodiments, the 3' homologous portion may range in length from 20,000 to 50,000 nucleotides. In some embodiments, the 3' homologous portion may range in length from 50,000 to 100,000 nucleotides.

[0091] In the context of describing the length of DNA or RNA sequences, the terms

"nucleotide(s)" (nt), "base(s)", "base pair(s)" (bp), and "bp" may be used interchangeably when referring to sequence length, regardless of whether the sequence is single-stranded or double-stranded. All the terms "nucleotide(s)" (nt), "base(s)", "base pair(s)", "base pair(s)" (bp), and "bp" can be used to describe the length of both single-stranded and double-stranded DNA or RNA sequences.

[0092] In some embodiments, the homologous portion of the donor DNA is configured capable of base-pairing the target DNA site.

[0093] The term "base-pairing" or "base pair", when used to describe complementary interactions between nucleic acid sequences, refers to refers to the formation of hydrogen bonds between complementary nucleotide bases (e.g., A-T, C-G, or A-U in RNA) in two nucleic acid strands. Base-pairing is a fundamental mechanism in molecular biology that enables the specific interaction between nucleic acid sequences. Importantly, base-pairing does not require all bases in the sequences to be complementary; partial complementarity is sufficient to achieve stable hybridization. In some embodiments, base-pairing enables the homologous portion of the donor DNA to interact with the target DNA site, facilitating precise genetic modifications.

[0094] In some embodiments, the homologous portion of the donor DNA is configured to be filled in the single-strand portion of the target DNA site. The term "single-strand portion" refers to a region of single-stranded DNA generated at the target DNA site as a result of nicking activity. In some embodiments, this single-stranded region may include: (i) The nicked strand: The DNA strand that is directly cleaved by the endonuclease, resulting in a single-strand break, flap or gap; (ii) The non-nicked strand: The complementary DNA strand that may become single-stranded during DNA repair, processing or any other mechanism. In some specific embodiments, the single-strand portion serves as a template or binding site for the homologous portion of the donor DNA to facilitate the filling of the donor DNA. The term "fill", "filled" or "filling" refers to the process of incorporating a donor DNA sequence into a single-stranded portion of a target DNA site, thereby restoring or modifying the DNA structure through various ways. This process may rely on complementary base pairing, cellular repair mechanisms, NHEJ,

MMEJ, SSA, strand displacement synthesis, engineered tools, transposon-mediated integration, chemical ligation or any other applicable mechanisms.

[0095] In some embodiments, the homologous portion of the donor DNA is configured to be capable of stranding in and displacing the nicked target DNA. The donor DNA integrates into the nicked target DNA by pairing with the single-stranded region. This process may involve strand invasion, where the donor DNA displaces the nicked strand and pairs with the complementary strand. Alternatively, it may occur through other mechanisms such as branch migration, where the donor DNA dynamically reconfigures the target DNA structure to integrate itself; single-strand annealing (SSA), where the donor DNA anneals directly to the single-stranded region of the target DNA; or strand displacement synthesis, where DNA polymerase synthesizes a new strand while displacing the existing single-stranded DNA. Additionally, engineered DNA modification tools (e.g., CRISPR-Cas systems) or chemical ligation methods may facilitate the integration of the donor DNA into the target DNA. In general, any mechanism that allows the donor DNA to interact with and integrate into the target DNA, whether through complementary base pairing, enzymatic activity, or chemical methods, falls within the scope of this process.

[0096] In some embodiments, the donor DNA may replace or displace the nicked strand, either partially or completely, through mechanisms such as branch migration or DNA repair processes. This displacement can result in the incorporation of the donor DNA sequence into the target DNA.

[0097] In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand. In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand at its 5' end. In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand at its 3' end. To clarify, the nicked strand refers to the strand of the target DNA that has been cleaved or nicked, resulting in a break with a 5' terminus and a 3' terminus at the site of the nick. The

homologous portion of the donor DNA is designed to align with the complementary strand at a specific region that corresponds to either the 5' end or the 3' end of the nicked strand. This alignment is achieved through base-pairing between the homologous portion of the donor DNA and the complementary strand of the target DNA. Thus, it should be understood that the phrases "at its 5' end" or "at its 3' end" denote a positional relationship, indicating that the base-pairing event occurs on the complementary strand at a region corresponding to the 5' end or 3' end of the nicked strand, respectively.

[0098] In some embodiments, the homologous portion of the donor DNA is configured to base-pair the target DNA on the complementary strand to the nicked strand and spanning the nicked position (target nick). The homologous portion of the donor DNA is designed to base-pair with the complementary strand of the target DNA, extending across the site of the nick. This means that the donor DNA's homologous region covers the area where the nick is located, even though the nick itself is on the opposite strand. For example: Donor DNA homologous region: 5' ---A T G C T A C G--- 3; Target DNA on the complementary strand to the nicked strand: 3' ---T A C G A T G C--- 5'; Nicked strand: 5' ---A T G C*T A C G--- 3' (* = nick). The donor DNA's homologous region base-pairs with the complementary strand, spanning the nicked position (target nick, indicated by *).

[0099] In some embodiments, the donor DNA includes a modified nucleoside.

[00100] In some embodiments, the modified nucleoside includes an inverted nucleoside, a locked nucleic acid, a 2'fluoro, a 2' O-alkyl, a methylated cytosine, or a combination thereof. In some embodiments, the donor DNA includes modified inter-nucleoside linkage including a phosphorothioate linkage.

[00101] In some embodiments, the endonuclease is an RNA-guided nickase and an associated guide RNA. "the endonuclease is an RNA-guided nickase and an associated guide RNA" describes a system comprising two key components: (i) An RNA-guided nickase: This refers to an endonuclease (e.g., a Cas9 nickase) that is directed to a specific DNA target by a guide RNA. The nickase introduces a single-strand break (nick) in the DNA at the target site. (ii) An associated guide RNA: This is the RNA molecule that

guides the nickase to the target DNA sequence. The guide RNA contains a sequence complementary to the target DNA, enabling precise targeting. The phrase "and an associated guide RNA" indicates that the guide RNA is functionally linked to the RNA-guided nickase, forming a complex (e.g., a ribonucleoprotein, RNP) that enables targeted DNA nicking. To clarify, the endonuclease (e.g., nickase) and the guide RNA are distinct molecular entities that work together as part of a functional system. The guide RNA directs the nickase to the target DNA, and the nickase performs the nicking activity. In some embodiments, "the endonuclease is an RNA-guided nickase and an associated guide RNA" may suggest the endonuclease is an RNA-guided nickase that capable of forming a complex with an associated guide RNA. In some embodiments, the system comprises at least two associated guide RNAs comprising a first guide RNA capable of hybridizing to a first target sequence in the DNA, and a second guide RNA capable of hybridizing to a second target sequence in the DNA. The term "target sequence" as used herein, is a nucleotide sequence in the dsDNA molecule that is recognized by the guide RNA that is associated with the RNA guided nickase due to the target specific sequence (spacer sequence) comprised by the guide RNA.

[00102] In some embodiments, the first guide RNA and the second guide RNA are configured capable of being positioned with their PAM sites oriented towards each other (PAM-facing); In some embodiments, the first guide RNA and the second guide RNA are configured capable of being positioned with their PAM sites oriented apart from each other (PAM-backing). PAM-facing refers to the arrangement where the PAM sites compared to target sequence targeted by the spacers of the guide RNAs (gRNAs) are oriented towards each other; the PAM sites of the two gRNAs face each other, creating a "face-to-face" orientation. PAM-backing refers to the arrangement where the PAM sites compared to target sequence targeted by the spacers of the guide RNAs (gRNAs) are oriented away from each other; The PAM sites of the two gRNAs face away from each other, creating a "back-to-back" orientation. The PAM- Co-directional refers to the arrangement where the PAM sites, compared to target sequence targeted by the spacers of the guide RNAs (gRNAs), are oriented in the same direction; the PAM sites of the two gRNAs face the same direction (either both towards the 5' end or both towards

the 3' end), creating a "co-directional" or "same-directional" orientation. For example, in some specific embodiments, exemplary positional relationships of their PAM sites can be described as presented in Figure 12 (FIG. 12): (A) The PAM-facing arrangement; (B) The PAM-backing arrangement; and (C) The PAM-same-directional arrangement.

[00103] In some embodiments, the gRNA includes a modified nucleoside.

[00104] In some embodiments, the modified nucleoside includes an inverted nucleoside, a locked nucleic acid, a 2'fluoro, a 2' O-alkyl, a methylated cytosine, or a combination thereof. In some embodiments, the gRNA includes modified inter-nucleoside linkage including a phosphorothioate linkage.

[00105] In some embodiments, the system comprising two or more endonucleases. In some embodiments, each endonuclease is a CRISPR Cas nickase. CRISPR Cas nickases are derived from CRISPR nucleases by inactivation of one of the nuclease domains. In specific embodiments, the CRISPR nickase can be derived from a type II CRISPR nuclease. For example, the type II CRISPR nuclease can be a Cas9 protein. Suitable Cas9 nucleases include, Streptococcus pyogenes Cas9 (SpCas9), Francisella novicida Cas9 (FnCas9), Staphylococcus aureus (SaCas9), Streptococcus thermophilus Cas9 (StCas9); Streptococcus pasteurianus (SpaCas9), Campylobacter jejuni Cas9 (CjCas9), Neisseria meningitis Cas9 (NmCas9), or Neisseria cinerea Cas9 (NcCas9); in some other embodiments, the nickase can be derived from a type V CRISPR nuclease, such as a Cpf1 nuclease. Suitable Cpf1 nucleases include Francisella novicida Cpf1 (FnCpf1), Acidaminococcus sp. Cpf1 (AsCpf1), or Lachnospiraceae bacterium ND2006 Cpf1 (LbCpf1).

[00106] In some embodiments, at least one endonuclease is a CRISPR-Cas nickase; In some embodiments, at least one endonuclease is a Class II CRISPR-Cas nickase. In some embodiments, each endonuclease is a class II CRISPR Cas nickase. In some specific embodiments, each CRISPR nickase can be individually a type II CRISPR nickase or a type V CRISPR nickase. In some embodiments, each endonuclease is individually selected from a Cas9 nickase or a Cpf1 nickase. In some embodiments, each endonuclease is individually a Cas9 nickase. In some embodiments, each endonuclease is

individually selected from a SpCas9 nickase, a FnCas9 nickase, a SaCas9 nickase, a StCas9 nickase, a SpaCas9 nickase, a CjCas9 nickase, a NmCas9 nickase, or a NcCas9 nickase. In some embodiments, each endonuclease can be individually a Cas9-D10A nickase or a Cas9-H840A nickase. In some embodiments, each endonuclease is a SpCas9-D10A nickase. In some embodiments, each endonuclease is a SpCas9-H840A nickase. In some embodiments, at least two endonucleases are the same. In some embodiments, at least two endonucleases are different; In some embodiments, at least one endonuclease is SpCas9-D10A nickase and at least one endonuclease is SpCas9-D10A nickase. In some embodiments, the system comprises two endonucleases; In some embodiments, the system comprises two endonucleases, which one is SpCas9-D10A nickase and another is SpCas9-H840A nickase.

[00107] In some embodiments, the SpCas9 nickase comprises an amino acid sequence that is at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to SEQ ID NO: 3. In some embodiments, the SpCas9-D10A nickase comprises an amino acid sequence that is at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to SEQ ID NO: 4, with an alanine at the position corresponding to A10. In some embodiments, the SpCas9-D10A nickase comprises an amino acid sequence that is at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to SEQ ID NO: 7, with an alanine at the position corresponding to A10. In some embodiments, the SpCas9-H840A nickase comprises an amino acid sequence that is at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to SEQ ID NO: 5, with an alanine at the position corresponding to A840. In some embodiments, the SpCas9-H840A nickase comprises an amino acid sequence that is at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to SEQ ID NO: 6, with an alanine at the position

corresponding to A840.

[00108] In some embodiments, the RNA-guided nickases and the guide RNAs are arranged according to any one of the Embodiments in Table 1.

**Table 1.** Non-limiting Examples of the Arrangement of RNA-guided Nickases and the Guide RNAs

| **Embodiment** | **Guide RNA PAM Orientation** | **Guide RNA Binding Sites** | **The First Endonuclease** | **The Second Endonuclease** |
|---|---|---|---|---|
| 1 | PAM-backing | On different strands of the target DNA | SpCas9-D10A nickase | SpCas9-D10A nickase |
| 2 | PAM-backing | On different strands of the target DNA | SpCas9-D10A nickase | SpCas9-H840A nickase |
| 3 | PAM-backing | On different strands of the target DNA | SpCas9-H840A nickase | SpCas9-H840A nickase |
| 4 | PAM-backing | On different strands of the target DNA | SpCas9-H840A nickase | SpCas9-D10A nickase |
| 5 | PAM-backing | On the same strand of the target DNA | SpCas9-D10A nickase | SpCas9-D10A nickase |
| 6 | PAM-backing | On the same strand of the target DNA | SpCas9-D10A nickase | SpCas9-H840A nickase |
| 7 | PAM-backing | On the same strand of the target DNA | SpCas9-H840A nickase | SpCas9-H840A nickase |
| 8 | PAM-backing | On the same strand of the target DNA | SpCas9-H840A nickase | SpCas9-D10A nickase |

| | | | | |
|---|---|---|---|---|
| 9 | PAM-facing | On different strands of the target DNA | SpCas9-D10A nickase | SpCas9-D10A nickase |
| 10 | PAM-facing | On different strands of the target DNA | SpCas9-D10A nickase | SpCas9-H840A nickase |
| 11 | PAM-facing | On different strands of the target DNA | SpCas9-H840A nickase | SpCas9-H840A nickase |
| 12 | PAM-facing | On different strands of the target DNA | SpCas9-H840A nickase | SpCas9-D10A nickase |
| 13 | PAM-facing | On the same strand of the target DNA | SpCas9-D10A nickase | SpCas9-D10A nickase |
| 14 | PAM-facing | On the same strand of the target DNA | SpCas9-D10A nickase | SpCas9-H840A nickase |
| 15 | PAM-facing | On the same strand of the target DNA | SpCas9-H840A nickase | SpCas9-H840A nickase |
| 16 | PAM-facing | On the same strand of the target DNA | SpCas9-H840A nickase | SpCas9-D10A nickase |
| 17 | PAM-same-directional | On different strands of the target DNA | SpCas9-D10A nickase | SpCas9-D10A nickase |
| 18 | PAM-same-directional | On different strands of the target DNA | SpCas9-D10A nickase | SpCas9-H840A nickase |
| 19 | PAM-same-directional | On different strands of the target DNA | SpCas9-H840A nickase | SpCas9-H840A nickase |

| | | | | |
|---|---|---|---|---|
| 20 | PAM-same-directional | On different strands of the target DNA | SpCas9-H840A nickase | SpCas9-D10A nickase |
| 21 | PAM-same-directional | On the same strand of the target DNA | SpCas9-D10A nickase | SpCas9-D10A nickase |
| 22 | PAM-same-directional | On the same strand of the target DNA | SpCas9-D10A nickase | SpCas9-H840A |
| 23 | PAM-same-directional | On the same strand of the target DNA | SpCas9-H840A nickase | SpCas9-H840A nickase |
| 24 | PAM-same-directional | On the same strand of the target DNA | SpCas9-H840A nickase | SpCas9-D10A nickase |

[00109] In some embodiments, each endonuclease may be individually coupled with a nuclear export domain, a nuclear localization domain an affinity tag and/or Flag. In some embodiments, each endonuclease is individually coupled to a DNA ligase. In some embodiments, each endonuclease is individually coupled to a DNA polymerase. In some embodiments, each endonuclease is individually coupled to a DNA exonuclease. In some embodiments, each endonuclease is individually coupled to a HUH endonuclease. In some embodiments, at least two endonucleases are coupled with each other.

[00110] In some embodiments, the coupling is covalent. In some embodiments, the coupling forms a fusion protein with a linker. In some embodiments, the coupling is non-covalent. In some embodiments, the guide RNA includes a portion that binds the endonuclease and a MS2 hairpin portion, wherein the endonuclease contains a MS2 binding motif.

[00111] In some embodiments, at least two endonucleases are coupled with each other via a linker. In some embodiments, at least two endonucleases are coupled with each other via a linker. For example, the two endonucleases may form a fusion protein,

wherein the endonucleases are connected by a peptide linker. The length of the linker may range from 10 to 200 amino acids (10aa-200aa), providing sufficient flexibility and stability for the fusion protein to function effectively. In some embodiments, the length of the linker may range from 10 to 15 amino acids. In some embodiments, the length of the linker may range from 16 to 20 amino acids. In some embodiments, the length of the linker may range from 21 to 30 amino acids. In some embodiments, the length of the linker may range from 31 to 40 amino acids. In some embodiments, the length of the linker may range from 41 to 50 amino acids. In some embodiments, the length of the linker may range from 51 to 60 amino acids. In some embodiments, the length of the linker may range from 61 to 70 amino acids. In some embodiments, the length of the linker may range from 71 to 80 amino acids. In some embodiments, the length of the linker may range from 81 to 90 amino acids. In some embodiments, the length of the linker may range from 91 to 100 amino acids. In some embodiments, the length of the linker may range from 101 to 120 amino acids. In some embodiments, the length of the linker may range from 121 to 140 amino acids. In some embodiments, the length of the linker may range from 141 to 160 amino acids. In some embodiments, the length of the linker may range from 161 to 180 amino acids. In some embodiments, the length of the linker may range from 181 to 200 amino acids.

[00112] In some embodiments, at least two endonucleases are coupled with each other via a mediated nucleic acid through base paring.

[00113] In some embodiments, the donor DNA is coupled to the endonuclease. In some embodiments, the coupling is covalent. In some embodiments, the coupling is formed by bioconjugation ability of an HUH endonuclease to covalently tether the donor DNA. In some embodiments, the coupling is non-covalent. In some embodiments, the coupling is via complementary pairing of a portion of the guide RNA and a portion of the donor DNA.

[00114] As presented in this disclosure, the term "couple", "coupled" or "coupling", as used herein, refers to the association or connection between two or more molecules, wherein the connection can be either covalent or non-covalent, and is achieved through

direct or indirect interactions. The association may involve proteins, nucleic acids, or combinations thereof, and includes cases where an intermediary molecule (e.g., guide RNA) facilitates the connection. The coupling results in a functional association, enabling the coupled entities to cooperate in a biological or biochemical process. The association may involve: (i) proteins (e.g., endonucleases, DNA ligases, DNA polymerases, DNA exonucleases, HUH endonucleases); (ii) nucleic acids (e.g., donor DNA, guide RNA), or (iii) combinations thereof (e.g., a protein coupled to a nucleic acid, or a nucleic acid mediating the connection between a protein and another nucleic acid). The coupling may involve direct physical contact between the coupled entities or indirect association mediated by an intermediate molecule (e.g., guide RNA in an RNP complex facilitating the interaction between endonuclease and donor DNA). In some embodiments, the coupling may also include cases where RNA and donor DNA are present on a single nucleic acid strand, either covalently linked or non-covalently associated (e.g., through hybridization or other intermolecular interactions). For example, the guide RNA and donor DNA may be designed as part of a continuous nucleic acid strand, enabling coordinated function in gene editing or recombination processes. In some embodiments, the coupling is formed by the bioconjugation ability of an HUH endonuclease to covalently tether the donor DNA. For instance, the HUH endonuclease may recognize and bind to a specific sequence or structure in the donor DNA, forming a covalent linkage that stabilizes the association between the endonuclease and the donor DNA.

[00115] In some embodiments, the target DNA is on the genomic DNA within a cell.

[00116] In some embodiments, the system is complexed in vitro and delivered into a target cell. In some embodiments, the system is packaged in vivo in a producer cell and delivered into a target cell.

[00117] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for modifying at least one target DNA.

[00118] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for replacing a target DNA sequence with a desired

sequence of 1 to 10 bases, 11 to 50 bases, 51 to 200 bases, 201 to 1,000 bases, 1,001 to 10,000 bases, or 10,001 to 100,000 bases. In some embodiments, desired sequence has a length of 1 to 10 bases, 11 to 20 bases, 21 to 30 bases, 31 to 40 bases, 41 to 50 bases, 51 to 60 bases, 61 to 70 bases, 71 to 80 bases, 81 to 90 bases, 91 to 100 bases, 101 to 150 bases, 151 to 200 bases, 201 to 300 bases, 301 to 400 bases, 401 to 500 bases, 501 to 600 bases, 601 to 700 bases, 701 to 800 bases, 801 to 900 bases, 901 to 1,000 bases, 1,001 to 2,000 bases, 2,001 to 3,000 bases, 3,001 to 4,000 bases, 4,001 to 5,000 bases, 5,001 to 6,000 bases, 6,001 to 7,000 bases, 7,001 to 8,000 bases, 8,001 to 9,000 bases, 9,001 to 10,000 bases, 10,001 to 20,000 bases, 20,001 to 30,000 bases, 30,001 to 40,000 bases, 40,001 to 50,000 bases, 50,001 to 60,000 bases, 60,001 to 70,000 bases, 70,001 to 80,000 bases, 80,001 to 90,000 bases, or 90,001 to 100,000 bases.

[00119] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for replacing a target DNA sequence of 1 to 3,000,000 genomic bases with a desired sequence of 1 to 10 bases, 11 to 50 bases, 51 to 200 bases, 201 to 1,000 bases, 1,001 to 10,000 bases, or 10,001 to 100,000 bases. In some embodiments, the desired sequence has a length of 1 to 10 bases, 11 to 20 bases, 21 to 30 bases, 31 to 40 bases, 41 to 50 bases, 51 to 60 bases, 61 to 70 bases, 71 to 80 bases, 81 to 90 bases, 91 to 100 bases, 101 to 150 bases, 151 to 200 bases, 201 to 300 bases, 301 to 400 bases, 401 to 500 bases, 501 to 600 bases, 601 to 700 bases, 701 to 800 bases, 801 to 900 bases, 901 to 1,000 bases, 1,001 to 2,000 bases, 2,001 to 3,000 bases, 3,001 to 4,000 bases, 4,001 to 5,000 bases, 5,001 to 6,000 bases, 6,001 to 7,000 bases, 7,001 to 8,000 bases, 8,001 to 9,000 bases, 9,001 to 10,000 bases, 10,001 to 20,000 bases, 20,001 to 30,000 bases, 30,001 to 40,000 bases, 40,001 to 50,000 bases, 50,001 to 60,000 bases, 60,001 to 70,000 bases, 70,001 to 80,000 bases, 80,001 to 90,000 bases, or 90,001 to 100,000 bases.

[00120] In some embodiments, provided is a method of using the system as described in any one of the embodiments above for large-size DNA insertion by using a donor DNA comprising partially double-strand DNA and two partially single-strand DNAs, wherein each of the single-strand portion is homologous to one target nick site, and the two nick sites are a parted for a large distance on a genomic DNA.

[00121] In some embodiments, the method includes the steps of: (i) complexing the system in vitro; and (ii) delivering the system into a target cell. In some embodiments, the system is packaged in a delivery vehicle in vitro to enhance stability, cellular uptake, and targeted delivery. The delivery vehicle may be selected from the group consisting of lipid nanoparticles, polymeric nanoparticles.

[00122] In some embodiments, the method includes the steps of: (i) packaging the system in vivo in a producer cell; and (ii) delivering the system into a target cell. In some embodiments, the system is packaged within a delivery vehicle for efficient and targeted delivery to the target cell. In some embodiments, the delivery vehicle may be selected from the group consisting of viral vectors (e.g., lentivirus, adenovirus, or AAV), virus-like particles (VLPs) or exosomes.

[00123] In another aspects, the system described herein for use in the treatment of genetic disease is provided.

[00124] In another aspect, a pharmaceutical composition comprising a system described herein and a pharmaceutically acceptable carrier is provided.

[00125] In another aspect, a kit for gene editing comprising the system described herein, a delivery vehicle, and instructions for use is provided.

[00126] In another aspect, a use of the system described herein in the manufacture of a medicament for treating a genetic disorder is provided.

[00127] Other definitions:

[00128] It must be noted that as used herein and in the appended claims, the singular forms or the terms “a”, “an”, “the” and similar terms used in the context of the present disclosure (especially in the context of the claims) are to be construed to cover both the singular and plural unless otherwise indicated herein or clearly contradicted by the context. In some embodiments, the above terms can be reasonably comprehended as “one” or “one or more”. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00129] Unless otherwise indicated, all singular and plural terms used herein are intended to encompass their respective singular and plural forms. Additionally, terms used in the present tense are intended to encompass their past and future tense forms, and vice versa, as appropriate depending on the context of the description. This interpretation applies to all terms used in the specification, claims, and drawings, unless explicitly stated otherwise. For example, the term 'cell' may refer to a single cell or multiple cells, and the term 'delivers' may refer to the action of delivering in the past, present, or future, as understood from the context.

[00130] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and the term "group consisting of" and the like refers to a specific set or collection of elements, components, or features. It may include one or more of the specified elements, components, or features. For example, a group consisting of: A, B, or C may refer to a set that includes any one or more of the specified elements A, B, or C. The claim encompasses the possibility of having any single element (A, B, or C) individually, any two elements combined (A and B, A and C, or B and C), or all three elements together (A, B, and C). This phrase defines the invention in terms of its variability within the specified options, allowing for different combinations of the listed elements while still maintaining the claimed scope.

[00131] When "t" or "T" appears in a sequence in this invention as a nucleotide of an RNA sequence, it should be understood as "u" or "U".

[00132] The term "identity" in the context of two or more nucleic acids or polypeptide sequences refers to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same as measured using a BLAST or BLAST 2.0 or FASTA etc. sequence comparison algorithms with default parameters described below.

[00133] The term "exemplary" is used herein to mean serving as an example, instance, or illustration. Any aspect or design described herein as "exemplary" is not necessarily to

be construed as preferred or advantageous over other aspects, embodiments, or designs.

[00134] As used herein, the term “optional” or “optionally” means that the subsequent described event, circumstance or substituent may or may not occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

[00135] The use of "or" or "/" is inclusive and means "and/or" unless stated otherwise; or it could be interpreted differently based on the context. The term “and/or” as used herein a phrase such as "A and/or B" is intended to include both A and B; A or B; A (alone); and B (alone). Likewise, the term "and/or" as used herein a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[00136] The terms "about" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, are meant to encompass variations of and from the specified value It is to be understood that the value to which the modifier "about" refers is itself also specifically, and preferably, disclosed.

[00137] Various embodiments are described herein. It should be noted that these specific embodiments are not intended to be exhaustive or to limit the broader aspects discussed in this disclosure. Any aspect described in connection with a particular embodiment is not necessarily limited to that embodiment and may be applied to other embodiments. Throughout this specification, references to 'in some embodiments,' 'in certain embodiments,' 'in some specific embodiments,' 'in specific embodiments,' 'in some cases,' or similar expressions mean that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present disclosure. Additionally, particular features, structures, or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art, in one or more embodiments. Furthermore, while some embodiments described herein may include certain features but not others, combinations of features from different embodiments are intended to fall within the scope of this disclosure. For example, in the

appended claims, any of the claimed embodiments may be used in any combination. The embodiments described herein are provided to enable a person skilled in the art to make and use the invention, and are not intended to limit the scope of the disclosure. Any combination of features described in the specification, whether explicitly stated or implied, is within the scope of the present disclosure.

**Further Numbered Embodiments**

[00138] 1. A system for modifying at least one target DNA, comprising: two or more endonucleases, or one or more nucleic acid(s) encoding said endonucleases, wherein each endonuclease nicks one strand of the target DNA at a target nick within an endonuclease targeting site, and at least one donor DNA comprising a sequence of interest to be incorporated to the target DNA.

[00139] 2. The system of embodiment 1, wherein the target DNA is a double-stranded DNA.

[00140] 3. The system of embodiment 1 or 2, wherein the target nicks produced by the two or more endonucleases are on the same DNA strand.

[00141] 4. The system of embodiment 1 or 2, wherein the target nicks produced by the two or more endonucleases are on different DNA strands.

[00142] 5. The system of any one of embodiments 1-4, wherein the donor DNA is a single-stranded DNA.

[00143] 6. The system of any one of embodiments 1-4, wherein the donor DNA is a double-stranded DNA.

[00144] 7. The system of any one of embodiments 1-4, wherein the donor DNA comprises one or more portions of single-stranded DNA and one or more portions of double-stranded DNA.

[00145] 8. The system of any one of embodiments 1-7, wherein the donor DNA is a circularized DNA.

[00146] 9. The system of any one of embodiments 1-7, wherein the donor DNA is 6-nucleotide to 100,000-nucleotide long; preferably, the donor DNA is 50-nucleotide to 4,000-nucleotide long.

[00147] 10. The system of any one of embodiments 1-7, wherein the single-stranded and double-stranded mixed donor DNA has a total length of 6-nucleotide to 100,000-nucleotide long; preferably, the single-stranded and double-stranded mixed donor DNA has a total length of 50-nucleotide to 4,000-nucleotide long.

[00148] 11. The system of any one of embodiments 1-10, where two of target nicks are separated by 1 to 3,000,000 bases;

preferably, 10 to 2,000 bases; more preferably, 10 to 200 bases; more preferably, 10 to 100 bases; more preferably, 20 to 60 bases; more preferably, 30 to 50 bases; more preferably, 35 to 40 bases; or

preferably 100 to 2,000 bases.

[00149] 12. The system of any one of embodiments 1-10, wherein the endonuclease targeting sites on the target DNA are separated by 1 to 3,000,000 bases;

preferably, the endonuclease targeting sites on the target DNA are separated by 10 to 2,000 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 10 to 200 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 10 to 100 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 20 to 60 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 30 to 50 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 35 to 40 bases; or

preferably, the endonuclease targeting sites on the target DNA are separated by 100 to 2,000 bases;

preferably, the target DNA is a genomic DNA.

[00150] 13. The system of any one of embodiments 1-10, wherein the endonuclease targeting sites on the target DNA are on two different chromosomes, wherein the target DNA is a genomic DNA.

[00151] 14. The system of any one of embodiments 1-13, wherein the donor DNA comprises one or more homologous portions, each having a sequence homologous to the one or more endonuclease targeting sites.

[00152] 15. The system of any one of embodiments 1-13, wherein a first donor DNA comprises a homologous portion with a sequence homologous to a first targeting site; and a second donor DNA comprises a homologous portion with a sequence homologous to a second targeting site.

[00153] 16. The system of embodiment 14 or 15, wherein the homologous portion has 20-1,000 nucleotides in length, preferably, the homologous portion has 20-800 nucleotides in length.

[00154] 17. The system of any one of embodiments 14-16, wherein the homologous portion is located at the 5' end of the donor DNA and/or 3' end of the donor DNA.

[00155] 18. The system of any one of embodiments 14-17, wherein the homologous portion of the donor DNA is configured capable of base-pairing with the target DNA.

[00156] 19. The system of any one of embodiments 14-18, wherein the homologous portion of the donor DNA is configured to fill in the single-stranded portion of the target DNA after the target nicks are produced by the endonucleases.

[00157] 20. The system of any one of embodiments 14-19, wherein the homologous portion of the donor DNA is configured to be capable of stranding in and displacing a portion of the nicked target DNA.

[00158] 21. The system of any one of embodiments 14-20, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand.

[00159] 22. The system of any one of embodiments 14-21, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand at its 5' end.

[00160] 23. The system of any one of embodiments 14-22, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand at its 3' end.

[00161] 24. The system of any one of embodiments 14-23, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand and spanning the target nicks.

[00162] 25. The system of any one of embodiments 1-24, wherein the endonuclease comprises an RNA-guided nickase; optionally, the endonuclease further comprises an associated guide RNA.

[00163] 26. The system of any one of embodiments 1-25, wherein the system comprises (a) at least two associated guide RNAs: a first guide RNA capable of hybridizing to a first target sequence in the target DNA, and a second guide RNA capable of hybridizing to a second target sequence in the target DNA, or (b) one or more nucleic acid(s) encoding said associated guide RNAs.

[00164] 27. The system of embodiment 26, wherein the first guide RNA and the second guide RNA are configured to be positioned with their PAM sites oriented towards each other (PAM-facing) on the target DNA; optionally, the first and second guide RNAs bind to different strands of the target DNA.

[00165] 28. The system of embodiment 26, wherein the first guide RNA and the second guide RNA are configured to be positioned with their PAM sites oriented away from each other (PAM-backing) on the target DNA; optionally, the guide RNAs bind to different strands of the target DNA.

[00166] 29. The system of embodiment 26, wherein the first guide RNA and the second guide RNA are configured to be positioned with their PAM sites oriented in the same

direction (PAM-same-directional); optionally, the guide RNAs bind to the same strand of the target DNA.

[00167] 30. The system of any one of embodiments 25-29, wherein each RNA-guided nickase is a CRISPR Cas nickase; preferably, each RNA-guided nickase is a class II CRISPR Cas nickase.

[00168] 31. The system of any one of embodiments 25-29, wherein each RNA-guided nickase is individually a Cas9 nickase; preferably, each Cas9 nickase is individually SpCas9-D10A nickase or SpCas9-H840A nickase.

[00169] 32. The system of any one of embodiments 25-31, wherein at least one RNA-guided nickase is SpCas9-D10A nickase and at least one RNA-guided nickase is SpCas9-H840A nickase.

[00170] 33. The system of any of embodiments 25-32, wherein the RNA-guided nickases and the guide RNAs are arranged according to any one of the Embodiments in Table 1.

[00171] 34. The system of any one of embodiments 1-33, wherein each endonuclease is individually coupled to a DNA ligase, a DNA polymerase, a DNA exonuclease, a HUH endonuclease or a MS2 binding motif.

[00172] 35. The system of any one of embodiments 1-34, wherein at least two endonucleases are coupled with each other.

[00173] 36. The system of embodiment 34 or 35, wherein the coupling is covalent; optionally, the coupling forms a fusion protein with a linker.

[00174] 37. The system of embodiment 34 or 35, wherein the coupling is non-covalent.

[00175] 38. The system of any one of embodiments 25-37, wherein the guide RNA comprises a portion that binds the RNA-guided nickase and an MS2 hairpin portion, wherein the endonuclease contains a MS2 binding motif.

[00176] 39. The system of any one of embodiments 1-38, wherein the donor DNA is coupled to at least one of the endonucleases.

[00177] 40. The system of embodiment 39, wherein the coupling is covalent.

[00178] 41. The system of embodiment 40, wherein the coupling is formed by bioconjugation ability of an HUH endonuclease to covalently tether the donor DNA.

[00179] 42. The system of embodiment 39, wherein the coupling is non-covalent.

[00180] 43. The system of embodiment 42, wherein the coupling is via complementary pairing of a portion of the guide RNA and a portion of the donor DNA.

[00181] 44. The system of any one of embodiments 1 to 43, wherein the donor DNA comprises a modified nucleoside.

[00182] 45. The system of any one of embodiments 25 to 44, wherein the guide RNA comprises a modified nucleoside.

[00183] 46. The system of any one of embodiments 44 to 45, wherein the modified nucleoside comprises an inverted nucleoside, a locked nucleic acid, a 2' fluoro, a 2' O-alkyl, a methylated cytosine, or a combination thereof.

[00184] 47. The system of any one of embodiments 1 to 46, wherein the donor DNA comprises a modified inter-nucleoside linkage; preferably, the modified inter-nucleoside linkage is a phosphorothioate linkage.

[00185] 48. The system of any one of embodiments 1 to 47, wherein the target DNA is a genomic DNA; optionally, the target DNA is part of the DNA genome within a cell.

[00186] 49. The system of any one of embodiments 1 to 48, wherein the system is complexed in vitro; optionally, the system is for delivery into target cells.

[00187] 50. The system of any one of embodiments 1 to 48, wherein the system is packaged in vivo in a producer cell; optionally, the system is for delivery into target cells.

[00188] 51. A method of using the system of any one of embodiments 1 to 50 for modifying at least one target DNA in a target cell; optionally, the target cell is in vitro.

[00189] 52. The method of embodiment 51, comprising the steps of: (i) forming the system in vitro; and (ii) delivering the system into the target cell.

[00190] 53. The method of embodiment 51, comprising the steps of: (i) packaging the system in vivo in a producer cell; and (ii) delivering the system into the target cell.

[00191] 54. The method of any one of embodiments 51-53, wherein the method is for treating a genetic disease in a subject; optionally, the target cell is in the subject.

[00192] 55. An engineered cell produced by the method of any one of embodiments 51-54; optionally, the engineered cell is an in vitro cell.

[00193] 56. The engineered cell of embodiment 55, wherein the sequence of interest is incorporated into the target DNA of the engineered cell.

[00194] 57. A population of cells derived from the engineered cell of embodiment 55 or 56.

[00195] 58. The system of any one of embodiments 1 to 47 for use in the treatment of a genetic disease.

[00196] Although the description referred to particular embodiments and aspects, the disclosure should not be construed as limited to the embodiments set forth herein.

## EXAMPLES

[00197] Provided herein are examples that describe in more detail certain embodiments of the present disclosure. The examples provided herein are merely for illustrative purposes and are not meant to limit the scope of the invention in any way. All references given below and elsewhere in the present application are hereby included by reference.

Example 1

[00198] Different combinations of two SpCas9 nickases (H840A and D10A) are shown in Figure 1 (FIG. 1), including using two SpCas9-H840A nickases (Fig. 1A: A, B, and C), two SpCas9-D10A nickases (Fig. 1A: D, E, and F), one SpCas9-H840A nickase and one SpCas9-D10A nickase (Fig. 1B: G, H, I, J), or two SpCas9-H840A nickases and one SpCas9-D10A nickase (Fig. 1B: K).

Example 2

[00199] To evaluate the effect of relative nicking positions on desired editing efficiency based on dual H840A SpCas9 nickases, we performed an editing experiment to insert a 'CTT' to the target site *HEK3* in HEK293T cells. A total of 38 different experiment and replicates using two SpCas9-H840A nickases were included, as listed in Figure 2 (FIG. 2) table, where 'guides' indicated targeting positions (e.g. -108 and 0 for No. 1; the position 0 refers to the corresponding nicking site when using the spacer 'GGCCCAGACTGAGCACGTGA' with SpCas9. A negative position refers to using a spacer that nicks in the upstream relative to the position 0 on the forward strand, and a positive position refers to the downstream relative to the position 0 on the forward strand (All nick positions are defined according to the forward strand. Nicks on the reverse strand are referred to their opposing positions on the forward strand). And 'ODN' (oligodeoxynucleotide) indicated the donor DNA (D36P36: see SEQ ID NO: 2; or D20P36: see SEQ ID NO: 1; or 'no ODN' as control). The HEK293T cells were seeded in a 24-well plate one day before transfection. First, guide RNAs were assembled with SpCas9-H840A (with NLS) protein to form a ribonucleoprotein complex (RNP). Next, ODN was added to form an RNP-ODN mixture. The RNP-ODN mixture was transfected into the cells using lipofectamine. DNA from the cells were extracted 2-3 days after transfection. Amplicon sequencing library was generated with 2-step PCR using target site specific primers and index primers. The library was sequenced by Illumina Sequencing System. The editing efficiencies were analyzed by using the CRISPResso2 software. Figure 2 (FIG. 2) showed the total editing efficiency for all indels ('Indel (%)', including both desired and non-desired) and the desired insertion 'CTT/ALL (%)'. Figure 3 (FIG.3) showed the detailed editing outcome visualization by the CRISPResso2 software for the condition No. 4 in Figure 2 (FIG.2). Figure 4 (FIG.4) showed the

detailed editing outcome visualization by the CRISPResso2 software for condition No. 15 in Figure 2 (FIG. 2). Figure 5 (FIG. 5) showed the detailed editing outcome visualization by the CRISPResso2 software for condition No. 26 in Figure 2 (FIG.2). The data demonstrates that the efficiency of desired editing is the highest when the two targeting positions are separated by about 37 bases and the two nickases are in PAM-backing direction in this experimental setup.

Example 3

[00200] After determining the highest efficiency of the desired editing on HEK3 when targeting positions -38 and 0, we then experimented using the engineered virus-like particle (eVLP) to deliver the editing system disclosed herein and used two SpCas9 nickases, different combinations of H (H840A) and D (D10A) on the positions -38 and 0 (Fig. 6). The experimental model and details are designed in accordance with common methods (e.g.: Cell 185, 250–265, January 20, 2022, WO2019228117A1 etc.). Briefly, eVLPs were made by co-transfecting the donor DNA and a group of plasmids into the same producer cell line Gesicle HEK293T. The plasmids included an expression cassette encoding the viral structural protein "MLV gag-pol", an expression cassette encoding the viral structural protein "Gag" and the Cas-derived protein that are fused via a short peptide linker, an expression cassette encoding gene-targeting single-guide RNA, and an expression cassette encoding the viral glycoprotein conferring cell/ tissue tropism to eVLPs. Figure 7 (FIG. 7) showed the total editing efficiency for all indels ('Indel (%)', including both desired and non-desired) and the desired insertion 'CTT/ALL (%)' for the above different conditions. For the H or D nickase combinations, the first letter refers to the nickase used at the position -38, and the second letter refers to the nickase used at the position 0. Figure 8 (FIG. 8) showed the editing outcome visualization by the CRISPResso2 software for the condition HD: H840A (HEK -38) + D10A (HEK 0). Figure 9 (FIG. 9) showed the editing outcome visualization by the CRISPResso2 software for the condition DH: D10A (HEK -38) + H840A (HEK 0). Figure 10 (FIG. 10) showed the editing outcome visualization by the CRISPResso2 software for the condition DD: D10A (HEK -38) + D10A (HEK 0). Figure 11 (FIG. 11) showed the editing outcome visualization by the CRISPResso2 software for the condition HH: H840A (HEK

-38) + H840A (HEK 0). Figure 7 (FIG.7) (Right) showed an explanatory 'dig-and-fill' mode of action (which was the most obvious for the 'HD' condition, as in Fig. 8, when used with the donor DNA D20P36), where the target DNA fragment to be replaced is detached and displaced by the donor DNA following cellular endogenous reparation mechanisms.

[00201] The sequences used in the examples described herein are shown in Table 2 below

**Table 2: The oligo sequences (ODN) used in the examples.**

| SEQ ID | Name | Sequence |
|---|---|---|
| 1 | D20P36 | c*t*ccaaaaaaaaaaactccatctggaggaagcagggcttcctttcctctgccatcaaag cgtgctcagtctgggccc*c*a |
| 2 | D36P36 | c*t*ccaaaaaaaaaaactccatctggaggaagcagggcttcctttcctctgccatcaaag cgtgctcagtctgggccccaaggattgacccag*g*c*c |

"*" indicates a phosphorothioate linkage between nucleotides.

**Table 3: Additional Sequences of the Disclosure**

| SEQ ID NO | Sequence Name | Type |
|---|---|---|
| 3 | SpCas9 | Protein |
| 4 | SpCas9-D10A | Protein |
| 5 | SpCas9-H840A | Protein |
| 6 | SpCas9-H840A (with NLS) | Protein |
| 7 | SpCas9-D10A (with NLS) | Protein |
| 8 | Gag-SpCas9-H840A | Protein |
| 9 | Gag-SpCas9-D10A | Protein |
| 10 | MLV gag-pol | Protein |
| 11 | glycoprotein (VSVG) | Protein |
| 12 | Gag-SpCas9-H840A (DNA) | DNA |
| 13 | Gag-SpCas9-D10A (DNA) | DNA |
| 14 | MLV gag-pol (DNA) | DNA |
| 15 | Glycoprotein VSVG (DNA) | DNA |
| 16 | HEK_-108 [spacer] | DNA |

| SEQ ID NO | Sequence Name | Type |
|---|---|---|
| 17 | HEK_-38 [spacer] | DNA |
| 18 | HEK_0 [spacer] | DNA |
| 19 | HEK_+26 [spacer] | DNA |
| 20 | HEK_+37 [spacer] | DNA |
| 21 | HEK_+63 [spacer] | DNA |
| 22 | HEK_+90 [spacer] | DNA |
| 23 | scaffold | DNA |
| 24 | scaffold with terminal signal | DNA |
| 25 | scaffold [guide RNA] | RNA |
| 26 | scaffold with terminal signal [guide RNA] | RNA |
| 27 | HEK_0 [guide RNA] | RNA |
| 28 | HEK_-108 [guide RNA] | RNA |
| 29 | HEK_-38 [guide RNA] | RNA |
| 30 | HEK_+26 [guide RNA] | RNA |
| 31 | HEK_+37 [guide RNA] | RNA |
| 32 | HEK_+63 [guide RNA] | RNA |
| 33 | HEK_+90 [guide RNA] | RNA |

[00202] The exemplary embodiments of the present invention are thus fully described. Although the description referred to particular embodiments, it will be clear to one skilled in the art that the present invention may be practiced with variation of these specific details. Hence this invention should not be construed as limited to the embodiments set forth herein.

## CLAIMS

What is claimed is:

1. A system for modifying at least one target DNA, comprising:
two or more endonucleases, or one or more nucleic acid(s) encoding said endonucleases, wherein each endonuclease nicks one strand of the target DNA at a target nick, and at least one donor DNA comprising a sequence of interest to be incorporated to the target DNA site.
2. The system of claim 1, wherein the target DNA is a double-stranded DNA.
3. The system of claim 1 or 2, wherein the target nicks of the two or more endonucleases are on the same DNA strand.
4. The system of claim 1 or 2, wherein the target nicks of the two or more endonucleases are on different DNA strands.
5. The system of any one of claims 1-4, wherein the donor DNA is a single-stranded DNA.
6. The system of any one of claims 1-4, wherein the donor DNA is a double-stranded DNA.
7. The system of any one of claims 1-4, wherein the donor DNA comprises one or more portions of single-stranded DNA and one or more portions of double-stranded DNA.
8. The system of any one of claims 1-7, wherein the donor DNA is a circularized DNA.
9. The system of any one of claims 1-7, wherein the donor DNA is 6-nucleotide to 100,000-nucleotide long; preferably, the donor DNA is 50-nucleotide to 4,000-nucleotide long.
10. The system of any one of claims 1-7, wherein the single-stranded and double-stranded mixed donor DNA has a total length of 6-nucleotide to 100,000-nucleotide long; preferably, the single-stranded and double-stranded mixed donor DNA has a total length of 50-nucleotide to 4,000-nucleotide long.
11. The system of any one of claims 1-10, where two of the target nicks are separated by 1 to 3,000,000 bases;
preferably, 10 to 2,000 bases; more preferably, 10 to 200 bases; more preferably, 10 to 100 bases; more preferably, 20 to 60 bases; more preferably, 30 to 50 bases; more preferably, 35 to 40 bases; or
preferably, 100 to 2,000 bases.
12. The system of any one of claims 1-10, wherein the endonuclease targeting sites comprising the target nicks on the target DNA are distant for 1 to 3,000,000 bases;
preferably, the endonuclease targeting sites comprising the target nicks on the target DNA are distant for 10 to 2,000 bases; more preferably, the endonuclease targeting sites comprising the target nicks on the target DNA are distant for 10 to

200 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 10 to 100 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 20 to 60 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 30 to 50 bases; more preferably, the endonuclease targeting sites on the target DNA are separated by 35 to 40 bases; or

preferably, the endonuclease targeting sites on the target DNA are separated by 100 to 2,000 bases;

preferably, the target DNA is a genomic DNA.

13. The system of any one of claims 1-10, wherein the endonuclease targeting sites comprising the target nicks on the target DNA are on two different chromosomes, wherein the target DNA is a genomic DNA.
14. The system of any one of claims 1-13, wherein the donor DNA comprises one or more homologous portions, each having a sequence homologous to the one or more endonuclease targeting sites comprising the target nicks.
15. The system of any one of claims 1-13, wherein a first donor DNA comprises a homologous portion with a sequence homologous to a first targeting site; and a second donor DNA comprises a homologous portion with a sequence homologous to a second targeting site.
16. The system of claim 14 or 15, wherein the homologous portion has 20-1,000 nucleotides in length, preferably, the homologous portion has 20-800 nucleotides in length.
17. The system of any one of claims 14-16, wherein the homologous portion is located at the 5' end of the donor DNA and/or 3'end of the donor DNA.
18. The system of any one of claims 14-17, wherein the homologous portion of the donor DNA is configured capable of base-pairing with the target DNA site.
19. The system of any one of claims 14-18, wherein the homologous portion of the donor DNA is configured to be filled in the single-stranded portion of the target DNA site.
20. The system of any one of claims 14-19, wherein the homologous portion of the donor DNA is configured to be capable of stranding in and displacing the nicked target DNA.
21. The system of any one of claims 14-20, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand.
22. The system of any one of claims 14-21, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand at its 5' end.

23. The system of any one of claims 14-22, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand at its 3' end.
24. The system of any one of claims 14-23, wherein the homologous portion of the donor DNA is configured to base-pair with the target DNA on the complementary strand to the nicked strand and spanning the target nicks.
25. The system of any one of claims 1-24, wherein the endonuclease is an RNA-guided nickase and an associated guide RNA.
26. The system of any one of claims 1-25, wherein the system comprises **(a)** at least two associated guide RNAs: a first guide RNA capable of hybridizing to a first target sequence in the target DNA, and a second guide RNA capable of hybridizing to a second target sequence in the target DNA, or **(b)** one or more nucleic acid(s) encoding said associated guide RNAs.
27. The system of claim 26, wherein the first guide RNA and the second guide RNA are configured to be positioned with their PAM sites oriented towards each other (PAM-facing) on the target DNA; optionally, the first and second guide RNAs bind to different strands of the target DNA.
28. The system of claim 26, wherein the first guide RNA and the second guide RNA are configured to be positioned with their PAM sites oriented away from each other (PAM-backing) on the target DNA; optionally, the guide RNAs bind to different strands of the target DNA.
29. The system of claim 26, wherein the first guide RNA and the second guide RNA are configured to be positioned with their PAM sites oriented in the same direction (PAM-same-directional); optionally, the guide RNAs bind to the same strand of the target DNA.
30. The system of any one of claims 25-29, wherein each RNA-guided nickase is a CRISPR Cas nickase; preferably, each RNA-guided nickase is a class II CRISPR Cas nickase.
31. The system of any one of claims 25-29, wherein each RNA-guided nickase is individually a Cas9 nickase; preferably, each Cas9 nickase is individually SpCas9-D10A nickase or SpCas9-H840A nickase.
32. The system of any one of claims 25-31, wherein at least one RNA-guided nickase is SpCas9-D10A nickase and at least one RNA-guided nickase is SpCas9-H840A nickase.
33. The system of any of claims 25-32, wherein the RNA-guided nickases and the guide RNAs are arranged according to any one of the Embodiments in Table 1.
34. The system of any one of claims 1-33, wherein each endonuclease is individually coupled to a DNA ligase, a DNA polymerase, a DNA exonuclease, a HUH endonuclease or a MS2 binding motif.

35. The system of any one of claims 1-34, wherein at least two endonucleases are coupled with each other.
36. The system of claim 34 or 35, wherein the coupling is covalent; optionally, the coupling forms a fusion protein with a linker.
37. The system of claim 34 or 35, wherein the coupling is non-covalent.
38. The system of any one of claims 25-37, wherein the guide RNA comprises a portion that binds the RNA-guided nickase and an MS2 hairpin portion, wherein the endonuclease contains a MS2 binding motif.
39. The system of any one of claims 1-38, wherein the donor DNA is coupled to at least one of the endonucleases.
40. The system of claim 39, wherein the coupling is covalent.
41. The system of claim 40, wherein the coupling is formed by bioconjugation ability of an HUH endonuclease to covalently tether the donor DNA.
42. The system of claim 39, wherein the coupling is non-covalent.
43. The system of claim 42, wherein the coupling is via complementary pairing of a portion of the guide RNA and a portion of the donor DNA.
44. The system of any one of claims 1 to 43, wherein the donor DNA comprises a modified nucleoside.
45. The system of any one of claims 25 to 44, wherein the guide RNA comprises a modified nucleoside.
46. The system of any one of claims 44 to 45, wherein the modified nucleoside comprises an inverted nucleoside, a locked nucleic acid, a 2' fluoro, a 2' O-alkyl, a methylated cytosine, or a combination thereof.
47. The system of any one of claims 1 to 46, wherein the donor DNA comprises a modified inter-nucleoside linkage; preferably, the modified inter-nucleoside linkage is a phosphorothioate linkage.
48. The system of any one of claims 1 to 47, wherein the target DNA is a genomic DNA; optionally, the target DNA is part of the DNA genome within a cell.
49. The system of any one of claims 1 to 48, wherein the system is complexed in vitro; optionally, the system is for delivery into target cells.
50. The system of any one of claims 1 to 48, wherein the system is packaged in vivo in a producer cell; optionally, the system is for delivery into target cells.
51. A method of using the system of any one of claims 1 to 50 for modifying at least one target DNA.
52. The method of claim 51, comprising the steps of: (i) complexing the system in vitro; and (ii) delivering the system into the target cell.
53. The method of claim 51, comprising the steps of: (i) packaging the system in vivo in a producer cell; and (ii) delivering the system into the target cell.
54. The method of any one of claims 51-53, wherein the method is for treating a genetic disease in a subject; optionally, the target cell is in the subject.

55. An engineered cell produced by the method of any one of claims 51-54; optionally, the engineered cell is an in vitro cell.
56. The engineered cell of claim 55, wherein the sequence of interest is incorporated into the target DNA of the engineered cell.
57. A population of cells derived from the engineered cell of claim 55 or 56.
58. The system of any one of claims 1 to 50 for use in the treatment of a genetic disease.