

## SUMMARY

This summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This summary is not intended to identify key features of the claimed subject matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter.

In one example, the disclosure provides a method of editing a double stranded DNA (dsDNA) molecule with a sense strand and antisense strand. The method comprises contacting the dsDNA molecule with a first editing complex specific for a first target sequence on the sense strand of the dsDNA molecule and a second editing complex specific for a second target sequence on the antisense strand of the dsDNA molecule. The first editing complex and the second editing complex each comprise a fusion editor protein and an extended guide RNA molecule associated therewith. The fusion editors each comprise a functional nickase domain and a functional reverse transcriptase domain. The extended guide RNA molecule of the first editing complex comprises a first guide domain with a first sequence that hybridizes to the first target sequence and a first extended domain at the 3' end. The extended guide RNA molecule of the second editing complex comprises a second guide domain with a second sequence that hybridizes to the second target sequence and a second extended domain at the 3' end. The method further comprises permitting the functional nickase domain of the first editing complex and the functional nickase domain of the second editing complex to create a first single-stranded break and a second single-stranded break in opposite strands of the dsDNA molecule at the first target sequence and second target sequence, respectively. Next, the method comprises permitting the functional reverse transcriptase domain of the first editing complex to generate a first 3' overhang from the first single-stranded break using the first extended domain as template, and permitting the functional reverse transcriptase domain of the second editing complex to generate a second 3' overhang from the second single-stranded break using the second extended domain as template. Finally, the method comprises repairing the dsDNA molecule by excising the portion of the dsDNA originally disposed between the first single-stranded break and second single stranded break and incorporating the first 3' overhang and second 3' overhang into the repaired dsDNA molecule.

In one aspect, the disclosure provides a method of editing a double stranded DNA (dsDNA) molecule with a sense strand and antisense strand, comprising:

contacting the dsDNA molecule with a first editing complex specific for a first target sequence on the sense strand of the dsDNA molecule and a second editing complex specific for a second target sequence on the antisense strand of the dsDNA molecule;

wherein the first editing complex and the second editing complex each comprise a fusion editor protein and an extended guide RNA molecule associated therewith, wherein the fusion editors each comprise a functional nickase domain and a functional reverse transcriptase domain;

wherein the extended guide RNA molecule of the first editing complex comprises a first guide domain with a first sequence that hybridizes to the first target sequence and a first extended domain at the 3' end; and

wherein the extended guide RNA molecule of the second editing complex comprises a second guide domain with a second sequence that hybridizes to the second target sequence and a second extended domain at the 3' end;

permitting the functional nickase domain of the first editing complex and the functional nickase domain of the second editing complex to create a first single-stranded break and a second single-stranded break in opposite strands of the dsDNA molecule at the first target sequence and second target sequence, respectively;

permitting the functional reverse transcriptase domain of the first editing complex to generate a first 3' overhang from the first single-stranded break using the first extended domain as template, and permitting the functional reverse transcriptase domain of the second editing complex to generate a second 3' overhang from the second single-stranded break using the second extended domain as template;

wherein the first 3' overhang comprises a first repair domain with a sequence that is complementary to a sequence immediately 5' to the second 3' overhang in the antisense strand, and wherein the second 3' overhang comprises a second repair domain with a sequence that is complementary to a sequence immediately 5' to the first 3' overhang in the sense strand; and

repairing the dsDNA molecule by excising the portion of the dsDNA originally disposed between the first single-stranded break and second single stranded break and incorporating the first 3' overhang and second 3' overhang into the repaired dsDNA molecule.

In some examples, the functional nickase domain of the first editing complex and the functional nickase domain of the second editing complex are independently CRISPR-associated (Cas) enzyme, *Pyrococcus furiosus* Argonaute, and the like, or a

functional nickase domain derived therefrom. In some examples, the Cas is Cas9, Cas12, Cas13, Cas3, CasΦ, and the like. In some examples, the functional reverse transcriptase domain of the first editing complex and the functional reverse transcriptase domain of the second editing complex are independently M-MLV RT, HIV RT, group II intron RT (TGIRT), superscript IV, and the like, or a functional domain thereof.

In some examples, the first target sequence is disposed in a more 5' location in the sense strand than the reverse complement of the second target sequence. In some examples, the first target sequence is disposed in a more 3' location in the sense strand than the reverse complement of the second target sequence. In some examples, the first 3' overhang and the second 3' overhang are reverse complements of each other and hybridize in the repairing step.

In some examples, the first 3' overhang comprises a first repair domain with a sequence that corresponds to a sequence immediately 5' to the second 3' overhang in the antisense strand, and wherein the second 3' overhang comprises a second repair domain with a sequence that corresponds to sequence immediately 5' to the first 3' overhang in the sense strand. In some examples, the first 3' overhang further comprises an insertion sequence 5' to the first repair domain, and wherein the second 3' overhang comprises a reverse complement sequence of the insertion sequence 5' to the second repair domain.

In some examples, the first 3' overhang comprises a first repair domain with a sequence that corresponds to a sequence immediately 3' to the second single stranded break, and wherein the second 3' overhang comprises a second repair domain with a sequence that corresponds to a sequence immediately 3' to the first single stranded break, whereby the repairing step results in an inversion of the sequence corresponding to the portion of the dsDNA originally disposed between the first single-stranded break and second single stranded break.

In some examples, the first 3' overhang comprises a first repair domain with a sequence that corresponds to a first end domain of an insertion DNA fragment, wherein the second 3' overhang comprises a second repair domain with a sequence that corresponds to a second end domain of the insertion DNA fragment, and wherein the first end domain and second end domain are at opposite ends of the insertion DNA fragment or are at distinct sites within a larger dsDNA molecule.

In some examples, the portion of the dsDNA molecule originally disposed between the first single-stranded break and second single stranded break that is excised is

at least 5 nucleotides long. In some examples, the portion of the dsDNA molecule originally disposed between the first single-stranded break and second single stranded break that is excised is between about 10 nucleotides and 1,000,000 nucleotides long.

5 In some examples, the first editing complex and/or the second editing complex comprise(s) an additional functional domain configured to enhance the efficiency of 3'-overhang generation. In some examples, the fusion editor protein of the first editing complex and/or the second editing complex comprise(s) an additional functional domain configured to enhance the efficiency of DNA repair using generated 3' overhangs.

10 In some examples, the first guide domain and second guide domain are independently between about 20 and about 200 nucleotides long. In some examples, the first guide domain and second guide domain are independently between about 25 and 100 nucleotides long, between about 25 and 50 nucleotides long, or between about 25 and 40 nucleotides long.

15 In some examples, the first guide domain and the second guide domain are configured to be compatible with the first editing complex and the second editing complex, respectively, and/or one or more nucleotide residues in the first guide domain and/or the second guide domain are modified with 2'-O-methylation, locked nucleic acids, peptide nucleic acids, or a similar functionally modified nucleic acid moiety.

20 In some examples, the first extended domain and the second extended domain are independently at least about 10 nucleotides long. In some examples, the first extended domain and the second extended domain are independently about 10 nucleotides to about 40 nucleotides long.

25 In some examples, the method is performed in a cell *in vitro*. In some examples, the method is performed in a cell *in vivo*. In some examples, the method is a therapeutic method comprising deletion of a genomic sequence, inverting a genomic sequence, interchromosomal rearrangement, and/or inserting a new sequence into a target region or site of the genome.

30 In some examples, the method is expanded to encompass multiple pairs of first and second editing complexes to implement edits at multiple locations in the dsDNA molecule. The method can comprise contacting the dsDNA with multiple pairs of first and second editing complexes, wherein each pair of first and second editing complexes targets different pairs of first and second target sequences within the dsDNA.

In some examples, the method comprises pooling a plurality of pegRNAs or a plurality of nucleic acid molecules encoding the pegRNAs, and contacting a cell comprising the dsDNA molecule with the pool of the plurality of pegRNAs or a plurality of nucleic acid molecules encoding the pegRNAs. In some examples, the method also  
5 comprises contacting the cell with one or more fusion editor proteins or one or more nucleic acid molecules encoding the one or more fusion editor proteins, and permitting the fusion editor proteins to express and/or complex within the cell.

In another example, the disclosure provides a method of editing one or more double stranded DNA (dsDNA) molecules in a cell. The method comprises contacting the cell with  
10 one or more pairs of first and second editing complexes, or one or more nucleic acids encoding components of the one or more pairs of first and second complexes and permitting the components to be expressed and assembled in the cell. For each pair of the one or more pairs first and second editing complexes, the following applies:

the first editing complex is specific for a first target sequence on the sense strand of  
15 the dsDNA molecule and the second editing complex specific for a second target sequence on the antisense strand of the dsDNA molecule;

the first editing complex and the second editing complex each comprise a fusion editor protein and an extended guide RNA molecule associated therewith, wherein the fusion editors each comprise a functional nickase domain and a functional reverse  
20 transcriptase domain;

the extended guide RNA molecule of the first editing complex comprises a first guide domain with a first sequence that hybridizes to the first target sequence and a first extended domain at the 3' end; and

the extended guide RNA molecule of the second editing complex comprises a  
25 second guide domain with a second sequence that hybridizes to the second target sequence and a second extended domain at the 3' end.

The method comprises (for each pair of first and second editing complexes) permitting the functional nickase domain of the first editing complex and the functional nickase domain of the second editing complex to create a first single-stranded break and a  
30 second single-stranded break in opposite strands of the dsDNA molecule at the first target sequence and second target sequence, respectively; permitting the functional reverse transcriptase domain of the first editing complex to generate a first 3' overhang from the first single-stranded break using the first extended domain as template, and permitting the

functional reverse transcriptase domain of the second editing complex to generate a second 3' overhang from the second single-stranded break using the second extended domain as template; and repairing the dsDNA molecule by excising the portion of the dsDNA originally disposed between the first single-stranded break and second single stranded break and incorporating the first 3' overhang and second 3' overhang into the repaired dsDNA molecule.

In some examples, the method comprises contacting the cell with a plurality of pairs of first and second editing complexes, or a plurality of nucleic acids encoding components of the plurality of pairs of first and second complexes and permitting the components to be expressed and assembled in the cell. Each pair of first and second editing complexes targets different first and second target sequences on the one or more dsDNA molecules in the cell.

In another example, the disclosure provides a kit comprising a first editing complex and the second editing complex as described herein, wherein the first target sequence on the sense strand and second target sequence on the antisense strand are separated by an intervening sequence. The first editing complex and the second editing complex are configured to delete intervening sequence, to invert the intervening sequence, and/or inserting one or more new sequences at the first and/or second single stranded breaks induced by the first editing complex and the second editing complex in the target dsDNA molecule.

In one aspect, the disclosure provides a kit comprising the first editing complex and the second editing complex as disclosed herein, wherein the first target sequence on the sense strand and second target sequence on the antisense strand are separated by an intervening sequence, and wherein the first editing complex and the second editing complex are configured to delete the intervening sequence and/or insert one or more new sequences at the first and/or second single stranded breaks induced by the first editing complex and the second editing complex in the target dsDNA molecule.

#### DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this disclosure will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

Figures 1A-1H. Precise episomal deletions using *PRIME-Del*. (1A-1C) Schematic of Cas9/paired-sgRNA deletion strategy (1A), PE3 (1B), and *PRIME-Del* (1C). For *PRIME-Del* in 1C, a pair of pegRNAs encodes the sites to be nicked at each end of the intended deletion but on opposing strands, as well as 3' flaps. In the illustrated embodiment, the 3' flaps contain sequence that is complementary to the region targeted by the other pegRNA. Letter designations are imposed indicating how the flaps hybridize with the targeted dsDNA sequence and are integrated into the repaired, edited sequence. (1D) Cartoon representation of deletions programmed within the episomally-encoded *eGFP*