

CLAIMS

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. 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; ~~and~~

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.

2. The method of claim 1, wherein 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.

3. The method of claim 2, wherein the functional nickase domain of the first editing complex and the functional nickase domain of the second editing complex are independently a Cas enzyme selected from the group consisting of ~~is~~ Cas9, Cas12, Cas13, Cas3, CasΦ, ~~and the like.~~

4. The method of one of claim 1 to claim 3, wherein 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.

5. The method of one of claim 1 to claim 4, wherein the first target sequence is disposed in a more 5' location in the sense strand than the reverse complement of the second target sequence.

~~6. The method of one of claim 1 to claim 4, wherein the first target sequence is disposed in a more 3' location in the sense strand than the reverse complement of the second target sequence.~~

~~7. The method of one of any one of claim 1 to claim 6, wherein the first 3' overhang and the second 3' overhang are reverse complements of each other and hybridize in the repairing step.~~

~~8. The method of one of claim 1 to claim 6, wherein 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.~~

~~96.~~ The method of claim ~~81~~, wherein 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.

~~10.~~ The method of claim 1, wherein 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.

~~11.~~ The method of claim 1, wherein 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.

~~127.~~ The method of claim 1, wherein 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.

~~138.~~ The method of claim ~~127~~, wherein 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.

~~149.~~ The method of claim 1, wherein 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.

~~15~~10. The method of claim 1, wherein 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.

~~16~~11. The method of claim 1, wherein the first guide domain and second guide domain are independently between about 20 and about 200 nucleotides long.

~~17. The method of claim 16, wherein 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.~~

~~18~~12. The method of claim 1, wherein ~~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

~~19~~13. The method of claim 1, wherein the first extended domain and the second extended domain are independently at least about 10 nucleotides long.

~~20~~14. The method of claim ~~19~~13, ~~the method of claim 19,~~ wherein the first extended domain and the second extended domain are independently about 10 nucleotides to about 40 nucleotides long.

~~21~~15. The method of claim 1, wherein the method is performed *in vitro* in a cell ~~in vitro~~ that comprises the dsDNA molecule.

~~22. The method of claim 1, wherein the method is performed in a cell in vivo.~~

~~23~~16. The method of claim ~~22~~15, wherein the method ~~is a therapeutic method comprising results in~~ 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.

~~24~~17. The method of one of claim 1 to claim ~~23~~16, wherein the method comprises 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.

~~2518.~~ The method of claim ~~2417~~, wherein the contacting comprises pooling a plurality of pegRNAs or a plurality of nucleic acid molecules encoding the pegRNAs, and contacting ~~a-the~~ cell comprising the dsDNA molecule with the pool of the plurality of pegRNAs or a plurality of nucleic acid molecules encoding the pegRNAs.

~~2619.~~ The method of claim ~~2518~~, further comprising 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.

~~27.—A method of editing one or more double stranded DNA (dsDNA) molecules in a cell, comprising contacting the cell with 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;~~

~~wherein for each pair of the one or more pairs first and second editing complexes:~~

~~the first editing complex is specific for a first target sequence on the sense strand of 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 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 second guide domain with a second sequence that hybridizes to the second target sequence and a second extended domain at the 3' end; and~~

~~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 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.~~

~~28. — The method of claim 27, comprising 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, wherein 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.~~

~~2920.~~ A kit comprising the first editing complex and the second editing complex as recited in one of claim 1 to claim ~~2014~~, 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, ~~to invert the intervening sequence~~, and/or ~~inserting-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.