

CLAIMS

1. A system for simultaneously editing both strands of a double-stranded DNA sequence at a target site to be edited, said system comprising:

(a) a prime editor or one or more polynucleotides encoding the prime editor, wherein the prime editor comprises a nucleic acid programmable DNA binding protein (napDNAbp) and a polypeptide comprising an RNA-dependent DNA polymerase activity, wherein the napDNAbp comprises a RuvC nuclease domain and a HNH nuclease domain, and wherein the HNH nuclease domain comprises one or more mutations that decrease or eliminate its nuclease activity;

(b) a first prime editing guide RNA (first PEGRNA) or one or more polynucleotides encoding the first PEGRNA, wherein the first PEGRNA comprises

(i) a first spacer sequence that is complementary to a first binding site on a first strand of the double-stranded DNA sequence upstream of the target site relative to the second strand,

(ii) a first gRNA core that is capable of complexing with the prime editor,

(iii) a first DNA synthesis template that encodes a first single-stranded DNA sequence, and

(iv) a first primer binding site complementary to a region of the second strand upstream of a first cut site;

and

(c) a second prime editing guide RNA (second PEGRNA) or one or more polynucleotides encoding the second PEGRNA, wherein the second PEGRNA comprises

(i) a second spacer sequence that is complementary to a second binding site on a second strand of the double-stranded DNA sequence downstream of the target site relative to the second strand;

(ii) a second gRNA core that is capable of complexing with the prime editor,

(iii) a second DNA synthesis template that encodes a second single-stranded DNA sequence, and

(iv) a second primer binding site complementary to a region of the first strand upstream of a second cut site;

wherein the prime editor is capable of cleaving the first strand at the second cut site when complexed with the second PEGRNA and cleaving the second strand at the first cut site when complexed with the first PEGRNA,

wherein the first single-stranded DNA sequence and the second single-stranded DNA sequence are reverse complements over a region of complementarity of each single-stranded DNA sequence of at least 5 nucleotides in length, and

wherein the first single-stranded DNA sequence comprises a first edit compared to the second strand of the target site that starts at a position no more than 3 nucleotides from the first cut site.

2. The system of claim 1, wherein the second single-stranded DNA sequence comprises a second edit compared to the first strand of the target site that starts at a position no more than 3 nucleotides from the second cut site.

3. The system of claim 1 or 2, wherein a portion of 3' ends of the first and second single-stranded DNA sequence are reverse complements of each other.

4. The system of claim 1 or 2, wherein the first single-stranded DNA sequence is the reverse complement of the second single-stranded DNA sequence.

5. The system of any one of claims 2-4, wherein the region of complementarity of the first single-stranded DNA sequence and the second single-stranded DNA sequence forms a duplex comprising the first edit and the second edit.

6. The system of any one of claims 1-5, wherein the region of complementarity is at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, at least 50%, at least 45%, at least 40%, at least 35%, at least 30%, at least 25%, at least 20%, at least 15%, or at least 10% of the total length of the first single-stranded DNA sequence or the second single-stranded DNA sequence.

7. The system of any one of claims 1-6, wherein the distance between the first and second binding sites is at least 41 nucleotides, at least 42 nucleotides, at least 43 nucleotides, at least 44 nucleotides, at least 45 nucleotides, at least 46 nucleotides, at least 47 nucleotides, at least 48 nucleotides, at least 49 nucleotides, at least 50 nucleotides, at least 51 nucleotides,

at least 52 nucleotides, at least 53 nucleotides, at least 54 nucleotides, at least 55 nucleotides, at least 56 nucleotides, at least 57 nucleotides, at least 58 nucleotides, at least 59 nucleotides, at least 60 nucleotides, at least 61 nucleotides, at least 62 nucleotides, at least 63 nucleotides, at least 64 nucleotides, at least 65 nucleotides, at least 66 nucleotides, at least 67 nucleotides, at least 68 nucleotides, at least 69 nucleotides, at least 70 nucleotides, at least 71 nucleotides, at least 72 nucleotides, at least 73 nucleotides, at least 74 nucleotides, at least 75 nucleotides, at least 76 nucleotides, at least 77 nucleotides, at least 78 nucleotides, at least 79 nucleotides, at least 80 nucleotides, at least 81 nucleotides, at least 82 nucleotides, at least 83 nucleotides, at least 84 nucleotides, at least 85 nucleotides, at least 86 nucleotides, at least 87 nucleotides, at least 88 nucleotides, at least 89 nucleotides, at least 90 nucleotides, at least 91 nucleotides, at least 92 nucleotides, at least 93 nucleotides, at least 94 nucleotide at least 95 nucleotides, at least 96 nucleotides, at least 97 nucleotides, at least 98 nucleotides, at least 99 nucleotides, at least 100 nucleotides, at least 101 nucleotides, at least 102 nucleotides, at least 103 nucleotides, at least 104 nucleotides, at least 105 nucleotides, at least 106 nucleotides, at least 107 nucleotides, at least 108 nucleotides, at least 109 nucleotides, or at least 110 nucleotides in length.

8. The system of any one of claims 1-7, wherein the first edit comprises an insertion, a deletion, a substitution, or a combination thereof.
9. The system of any one of claims 2-8, wherein the second edit comprises an insertion, a deletion, a substitution, or a combination thereof.
10. The system of any one of claims 1-9, wherein the first single-stranded DNA sequence and the second single-stranded DNA sequence have the same length.
11. The system of any one of claims 1-10, wherein the first single-stranded DNA sequence and the second single-stranded DNA sequence have different lengths.
12. The system of any one of claims 2-11, wherein the first or second edit comprises one or more recombinase sites.
13. The system of claim 12, further comprising a recombinase, or a polynucleotide encoding the recombinase.

14. The system of claim 13, wherein the recombinase is a serine recombinase.
15. The system of claim 14, wherein the recombinase is Bxb1.
16. The system of any one of claims 1-15, wherein the first edit starts at a position no more than 2 nucleotides from the first cut site.
17. The system of any one of claims 2-16, wherein the second edit starts at a position no more than 2 nucleotides from the second cut site.
18. The system of any one of claims 1-17, wherein the first edit starts at a position that is 0 nucleotides from the first cut site.
19. The system of any one of claims 2-18, wherein the second edit starts at a position that is 0 nucleotides from the second cut site.
20. The system of any one of claims 1-19, wherein the first single-stranded DNA sequence and/or the second single-stranded DNA sequence does not comprise sequence homology as compared to the DNA sequence at the target site.
21. The system of any one of claims 1-20, wherein the napDNAbp has a nickase activity.
22. The system of any one of claims 1-21, wherein the napDNAbp comprises an amino acid sequence of any one of SEQ ID NOs: 18, 21, 23, 25-39, 42-61, 75, and 77-88, or an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity with any one of SEQ ID NOs: 18, 21, 23, 25-39, 42-61, 75, and 77-88.
23. The system of any one of claims 1-22, wherein the polypeptide comprising the RNA-dependent DNA polymerase activity is a reverse transcriptase.
24. The system of any one of claims 1-23, wherein the polypeptide comprising the RNA-dependent DNA polymerase activity comprises an amino acid sequence of any one of SEQ ID NOs: 89-100, 106-122, 128, 129, 132, 139, 143, 149, 154, 159, 700-736, 738-742, and

763-766, or an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity with any one of SEQ ID NOs: 89-100, 106-122, 128, 129, 132, 139, 143, 149, 154, 159, 700-736, 738-742, and 763-766.

25. The system of any one of claims 1-24 further comprising a donor template or a polynucleotide encoding the donor template.

26. The system of any one of claims 1-25, wherein the region of complementarity is at least 10 nucleotides in length.

27. The system of any one of claims 1-26, wherein the region of complementarity is from 22 to 38 nucleotides in length.

28. One or more polynucleotides encoding the system of any one of claims 1-27.

29. One or more vectors comprising the one or more polynucleotides of claim 28.

30. An *in vitro* method for simultaneously editing both strands of a double-stranded DNA sequence at a target site comprising contacting the double-stranded DNA sequence with the system of any one of claims 1-27.

31. The system of any one of claims 1-27, the one or more polynucleotides of claim 28, or the one or more vectors of claim 29, for use as a medicament.

32. Use of the system of any one of claims 1-27, the one or more polynucleotides of claim 28, or the one or more vectors of claim 29, in the manufacture of a pharmaceutical composition for treating a disease or a disorder selected from the group consisting of adenosine deaminase (ADA) deficiency, alpha-1 antitrypsin deficiency, cystic fibrosis, Duchenne muscular dystrophy, galactosemia, hemochromatosis, Huntington's disease, maple syrup urine disease, Marfan syndrome, neurofibromatosis type 1, pachyonychia congenital, phenylketonuria, severe combined immunodeficiency, sickle cell disease, Smith-Lemli-Opitz syndrome, Tay-Sachs disease, heart disease, high blood pressure, Alzheimer's disease, arthritis, diabetes, cancer, obesity, prion disease, trinucleotide repeat expansion disease, and cyclin-dependent kinase-like (CDKL) 5 deficiency disorder.