

CLAIMS

1. A system for modifying DNA comprising:

(a) a polypeptide or a nucleic acid encoding the polypeptide, wherein the polypeptide comprises (i) a reverse transcriptase (RT) domain, (ii) a DNA-binding domain (DBD); and (iii) an endonuclease domain, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3225 or 3138, or an amino acid sequence having at least 90% identity thereto; and

(b) a template RNA (or DNA encoding the template RNA) comprising, from 5' to 3', (i) optionally a sequence that binds a target site, (ii) optionally a sequence that binds the polypeptide, (iii) a heterologous object sequence, and (iv) a 3' target homology domain.

2. A polypeptide or a nucleic acid encoding the polypeptide, wherein the polypeptide comprises (i) a reverse transcriptase (RT) domain, (ii) a DNA-binding domain (DBD); and (iii) an endonuclease domain, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3225 or 3138, or an amino acid sequence having at least 90% identity thereto.

3. The system of claim 1, or the polypeptide of claim 2, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3225, or an amino acid sequence having at least 90% identity thereto.

4. The system of claim 1 or 3, or the polypeptide of claim 2 or 3, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3225 or 3138, or an amino acid sequence at least 95% identity thereto.

5. The system of any one of claims 1, 3, or 4, or the polypeptide of any one of claims 2-4, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3225 or 3138, or an amino acid sequence having at least 99% identity thereto.

6. The system of any one of claims 1 or 3-5, or the polypeptide of any one of claims 2-5, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3225.

7. The system of claim 1, or the polypeptide of claim 2, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3138, or an amino acid sequence having at least 90% identity thereto.

8. The system of claim 1 or 7, or the polypeptide of claim 2 or 7, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3138, or an amino acid sequence having at least 95% identity thereto.

9. The system of any one of claims 1, 7, or 8, or the polypeptide of any one of claims 2, 7, or 8, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3138, or an amino acid sequence having at least 99% identity thereto.

10. The system of any one of claims 1 or 7-9, or the polypeptide of any one of claims 2 or 7-9, wherein the RT domain has the amino acid sequence of SEQ ID NO: 3138.

11. The system of any one of claims 1 or 3-10, or the polypeptide of any one of claims 2-10, wherein the DBD comprises a CRISPR/Cas domain.

12. The system of any one of claims 1 or 3-11, or the polypeptide of any one of claims 2-11, wherein the endonuclease domain is a nickase domain, e.g., a Cas9 nickase domain, optionally wherein the Cas9 nickase domain:

- (i) is a SpyCas9 nickase domain, e.g., a SpyCas9(N863A) nickase domain;
- (ii) comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 3269;
- (iii) is an NmeCas9 domain;
- (iv) is a St1Cas9 domain; or
- (v) is a SauCas9 domain.

13. The system of any one of claims 1 or 3-12, or the polypeptide of any one of claims 2-12, wherein the RT domain is positioned C-terminal of the endonuclease domain in the polypeptide.

14. The system of any one of claims 1 or 3-13, or the polypeptide of any one of claims 2-13, wherein the polypeptide comprises a linker positioned between the RT domain and the DBD or between the RT domain and the endonuclease domain, and/or the linker is between 2-50 amino acids in length.

15. The system of any one of claims 1 or 3-14, wherein the 3' target homology domain is complementary to a sequence adjacent to a site to be modified by the system.

16. The system of any one of claims 1 or 3-15, wherein the heterologous object sequence is complementary to a site to be modified by the system except at a position or positions to be modified, and/or wherein the heterologous object sequence inserts at least 1 nucleotide into the target site.

17. The system of any one of claims 1 or 3-16, wherein the sequence that binds the polypeptide comprises a gRNA scaffold.

18. The system of any one of claims 1 or 3-17, wherein the sequence that binds to the target site is at least 18 nucleotides long and/or is complementary to a second strand of the target site.

19. The system of any one of claims 1 or 3-18, further comprising a second strand-targeting gRNA that directs the endonuclease domain to nick a second strand, optionally wherein
(a) the template RNA further comprises the second strand-targeting gRNA, or
(b) the second strand-targeting gRNA is disposed on a separate nucleic acid from the template RNA.

20. The system of any one of claims 1 or 3-19, or the polypeptide of any one of claims 2-14, wherein the polypeptide further comprises a nuclear localization sequence (NLS), optionally wherein:

(i) the NLS is fused to the N-terminus of the endonuclease domain, or the NLS is fused to the C-terminus of the polypeptide;

(ii) the NLS is a monopartite NLS or a bipartite NLS; and/or

(iii) a second linker is disposed between the NLS and the endonuclease domain.

21. The system of any one of claims 1 or 3-20, wherein at least 80% of the template RNA present contains one or more modified nucleotides, and/or wherein the template RNA comprises a 2'-O-methyl modified nucleotide.

22. The system of any one of claims 1 or 3-21, or the polypeptide of any one of claims 2-14 or 20, wherein the nucleic acid encoding the polypeptide comprises 5-methoxyuridine.

23. The system of any one of claims 1 or 3-22, wherein the polypeptide and/or template RNA are packaged into a virus, viral-like particle, virosome, liposome, vesicle, exosome, or LNP.

24. The polypeptide of any one of claims 2-14, 20, or 22, wherein the polypeptide is packaged into a virus, viral-like particle, virosome, liposome, vesicle, exosome, or LNP.

25. The system of any one of claims 1 or 3-23, or the polypeptide of any one of claims 2-14, 20, 22, or 24, which is substantially free of pyrogen, virus, fungus, bacterial pathogen, and/or host cell protein contamination.

26. Use of the system of any one of claim 1, 3-23, or 25, or the polypeptide of any one of claims 2-14, 20, 22, 24, or 25, in the manufacture of a medicament for use in modifying a target site in genomic DNA in a cell.

27. Use of the system of any one of claims 1, 3-23, or 25, or the polypeptide of any one of claims 2-14, 20, 22, 24, or 25, in the manufacture of a medicament for use in treating a subject having a disease or condition associated with a genetic defect.