

CLAIMS

1. A method of site-specifically integrating an exogenous nucleic acid sequence into a mammalian cell genome or intracellular target nucleic acid, the method comprising:

(a) incorporating at least one integration sequence at a specific target site in the cell genome or intracellular target nucleic acid by introducing *ex vivo* into a mammalian cell:

(i) an expressible polynucleotide construct encoding an editing polypeptide, wherein the editing polypeptide comprises a DNA binding nuclease domain linked via a linker to a reverse transcriptase domain, wherein the DNA binding nuclease domain comprises a nickase activity; and

(ii) a guide RNA (gRNA) comprising a targeting sequence, a primer binding sequence, and a complement of the at least one integration sequence,

wherein the gRNA interacts with the expressed editing polypeptide to direct the editing polypeptide to the specific target site of the cell genome or intracellular target nucleic acid,

wherein the DNA binding nuclease domain nicks a strand of the cell genome or intracellular target nucleic acid to form a nicked site, and

wherein the reverse transcriptase domain reverse transcribes the complement of the at least one integration sequence within the gRNA and thereby incorporates the at least one integration sequence into the nicked site, thereby incorporating the at least one integration sequence at the specific target site of the cell genome or intracellular target nucleic acid; and

(b) integrating an exogenous nucleic acid sequence into the cell genome or intracellular target nucleic acid by introducing into the cell:

(i) the exogenous nucleic acid sequence linked to a sequence that is an integration cognate to the site-specifically incorporated-integration sequence; and

(ii) an expressible polynucleotide construct encoding an integration enzyme, wherein the integration enzyme integrates the exogenous nucleic acid sequence into the cell genome or the intracellular target nucleic acid at the at least one site-specifically incorporated integration sequence, thereby site-specifically integrating the exogenous nucleic acid sequence into the cell genome or the intracellular target nucleic acid,

wherein the expressible polynucleotide encoding the editing polypeptide, the gRNA, the expressible polynucleotide construct encoding the integration enzyme, and the exogenous nucleic acid sequence are introduced into the mammalian cell concurrently.

- 5 2. The method of claim 1, wherein the gRNA, the expressible polynucleotide construct encoding the editing polypeptide, and the expressible polynucleotide construct encoding the integration enzyme are introduced into the mammalian cell using a virus, a RNP, an mRNA, a lipid, or a polymeric nanoparticle.
- 10 3. The method of claim 1, wherein the gRNA hybridizes to a strand of the mammalian cell genome.
4. The method of claim 1, wherein the exogenous nucleic acid is introduced into the mammalian cell as an adeno-associated virus (AAV) or an adenovirus (AdV).
- 15 5. The method of claim 1, wherein the exogenous nucleic acid is introduced into the mammalian cell as a minicircle, a plasmid, mRNA₂ or a linear DNA.
6. The method of claim 5, wherein the minicircle does not comprise a sequence of a bacterial origin.
- 20 7. The method of claim 1, wherein the linker is cleavable.
8. The method of claim 1, wherein the linker is non-cleavable.
9. The method of claim 1, wherein the linker is two associating binding domains of the DNA binding
25 nuclease linked to a reverse transcriptase.
10. The method of claim 1, wherein the integration enzyme is selected from the group consisting of Dre, Vika, Bxb1, ϕ C31, RDF, FLP, ϕ BT1, R1, R2, R3, R4, R5, TP901-1, A118, ϕ FC1, ϕ C1, MR11, TG1, ϕ 370.1, W β , BL3, SPBc, K38, Peaches, Veracruz, Rebeuca, Theia, Benedict, KSSJEB, PattyP, 30 Doom, Scowl, Lockley, Switzer, Bob3, Troube, Abrogate, Anglerfish, Sarfire, SkiPole, ConceptII, Museum, Severus, Airmid, Benedict, Hinder, ICleared, Sheen, Mundrea, BxZ2, ϕ RV,

retrotransposases encoded by R2, L1, Tol2 Tc1, Tc3, Mariner Himar 1, Mariner mos-1, and Minos, and any mutants thereof.

11. The method of claim 1, wherein the integration sequence is an attB sequence, an attP sequence,
5 an attL sequence, an attR sequence, a lox71 sequence, a Vox sequence, or a FRT sequence.

12. The method of claim 1, wherein the DNA binding nuclease domain comprising a nickase activity is selected from Cas9-D10A, Cas9-H840A, and Cas12a/b nickase.

10 13. The method of claim 1, wherein the reverse transcriptase domain comprises a mutation relative to a wild-type sequence.

14. The method of claim 1, wherein the reverse transcriptase domain is selected from the group consisting of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase domain, transcription
15 xenopolymerase (RTX), avian myeloblastosis virus reverse transcriptase (AMV-RT), and Eubacterium rectale maturase RT.

15. The method of claim 14, wherein the M-MLV reverse transcriptase domain comprises one or more mutations selected from the group consisting of D200N, T306K, W313F, T330P, and L603W.

20 16. The method of claim 1, further comprising introducing a nicking guide RNA (ngRNA).

17. The method claim 1, wherein:

the exogenous nucleic acid is a reporter gene;

25 the exogenous nucleic acid is a degradation tag for programmable knockdown of proteins in the presence of small molecules;

the exogenous nucleic acid is a T-cell receptor (TCR), a chimeric antigen receptor (CAR), an interleukin, a cytokine, or an immune checkpoint gene and the mammalian cell is a T-cell or natural killer (NK) cell, the exogenous nucleic acid is a beta hemoglobin (HBB) gene and the cell is a
30 hematopoietic stem cell (HSC)

the exogenous nucleic acid is a metabolic gene; or

the exogenous nucleic acid is a gene involved in an inherited disease or an inherited syndrome.

18. The method of claim 17, wherein the reporter gene is a fluorescent protein.

19. The method of claim 1, wherein the mammalian cell is a dividing cell or a non-dividing cell.

20. The method of claim 1, wherein:

the exogenous nucleic acid is between 1000 bp and 36,000 bp;

the exogenous nucleic acid is more than 36,000 bp; [[and/]] or

the exogenous nucleic acid is less than 1000 bp.

21. The method of claim 17 wherein the inherited disease is cystic fibrosis, familial hypercholesterolemia, adenosine deaminase (ADA) deficiency, X-linked SCID (X-SCID), Wiskott-Aldrich syndrome (WAS), hemochromatosis, Tay-Sachs, fragile X syndrome, Huntington's disease, Marfan syndrome, phenylketonuria, or muscular dystrophy.

22. A method of site-specifically integrating an exogenous nucleic acid sequence into a mammalian cell genome or intracellular target nucleic acid, the method comprising:

(a) incorporating at least one integration sequence at a specific target site in the cell genome or intracellular target nucleic acid by introducing *ex vivo* into a mammalian cell:

(i) an expressible polynucleotide construct encoding an editing polypeptide, wherein the editing polypeptide comprises a DNA-binding nuclease domain linked via a linker to a reverse transcriptase domain, wherein the DNA-binding nuclease domain comprises a nickase activity; and

(ii) an expressible polynucleotide construct encoding a guide RNA (gRNA) comprising a targeting sequence, a primer binding sequence, and a complement of the at least one integration sequence,

wherein the gRNA interacts with the expressed editing polypeptide to direct the editing polypeptide to the specific target site of the cell genome or intracellular target nucleic acid, wherein the DNA-binding nuclease domain nicks a strand of the cell genome or intracellular target nucleic acid to form a nicked site, and

wherein the reverse transcriptase domain reverse transcribes the complement of the at least one integration sequence and thereby incorporates the at least one integration sequence into the nicked site, thereby incorporating the at least one integration sequence at the specific target site of the cell genome or intracellular target nucleic acid; and

5 (b) integrating the exogenous nucleic acid sequence into the cell genome or intracellular target nucleic acid by introducing into the mammalian cell:

(i) an exogenous nucleic acid sequence linked to a sequence that is an integration cognate to the site-specifically incorporated integration sequence; and

10 (ii) an expressible polynucleotide construct encoding an integration enzyme, wherein the integration enzyme integrates the exogenous nucleic acid into the cell genome or intracellular target nucleic acid at the at least one site-specifically incorporated integration sequence, thereby site-specifically integrating the exogenous nucleic acid into the cell genome or the intracellular target nucleic acid,

15 wherein the integration sequence is an attB sequence, an attP sequence, an attL sequence, an attR sequence, a lox71 sequence, a Vox sequence, or a FRT sequence,

wherein the integration sequence is longer than 38 basepairs, and

wherein the expressible polynucleotide constructs encoding the editing polypeptide, the gRNA, and the integration enzyme, and the exogenous nucleic acid, are introduced into the mammalian cell concurrently.

20 23. The method of claim 22, wherein the integration sequence is 40, 42, 44, or 46 base pairs.