CLAIMS

1. An *in vitro* method for modifying a genome at a genomic locus of interest in a pluripotent cell, comprising:

introducing into the pluripotent cell a Cas9 protein or a nucleic acid encoding the Cas9 protein, a CRISPR RNA that hybridizes to a CRISPR target sequence at the genomic locus of interest or a DNA encoding the CRISPR RNA, a tracrRNA or a DNA encoding the tracrRNA, and a large targeting vector (LTVEC) that is at least 10 kb in size and comprises an insert nucleic acid flanked by:

(i) a 5' homology arm that is homologous to a 5' target sequence at the genomic locus of interest; and

(ii) a 3' homology arm that is homologous to a 3' target sequence at the genomic locus of interest,

wherein the pluripotent cell is a non-human mammalian pluripotent cell or a human induced pluripotent stem cell, and

wherein following introducing into the pluripotent cell the Cas9 protein or the nucleic acid encoding the Cas9 protein, the CRISPR RNA or the DNA encoding the CRISPR RNA, the tracrRNA or the DNA encoding the tracrRNA, and the LTVEC, the genome of the pluripotent cell is modified to comprise a targeted genetic modification comprising (i) deletion of a region of the genomic locus of interest wherein the deletion is at least 30 kb and/or (ii) insertion of the insert nucleic acid at the genomic locus of interest wherein the insertion is at least 30 kb.

2. The method of claim 1, wherein the CRISPR RNA and the tracrRNA are introduced as a single nucleic acid molecule comprising the CRISPR RNA and the tracrRNA.

3. The method of claim 2, wherein the single nucleic acid molecule comprises the CRISPR RNA and the tracrRNA fused together in the form of a single guide RNA (sgRNA).

4. The method of claim 1, wherein the CRISPR RNA and the tracrRNA are introduced separately.

5. The method of any one of claims 1 to 4, wherein:

(a) the Cas9 protein is introduced in the form of a protein, a messenger RNA (mRNA) encoding the Cas9 protein, or a DNA encoding the Cas9 protein;

(b) the CRISPR RNA is introduced in the form of an RNA or a DNA encoding the CRISPR RNA; and

(c) the tracrRNA is introduced in the form of an RNA or a DNA encoding the tracrRNA.

6. The method of claim 5, wherein the Cas9 protein, the CRISPR RNA, and the tracrRNA are introduced as a protein-RNA complex.

7. The method of claim 5, wherein:

(a) the DNA encoding the Cas9 protein is in the form of a first expression construct comprising a first promoter operably linked to a first nucleic acid encoding the Cas9 protein;

(b) the DNA encoding the CRISPR RNA is in the form of a second expression construct comprising a second promoter operably linked to a second nucleic acid encoding the CRISPR RNA; and

(c) the DNA encoding the tracrRNA is in the form of a third expression construct comprising a third promoter operably linked to a third nucleic acid encoding the tracrRNA;

wherein the first, second, and third promoters are active in the pluripotent cell, and

wherein the first, second, and third expression constructs are on a single nucleic acid molecule or on multiple nucleic acid molecules.

8. The method of claim 5, wherein:

(a) the DNA encoding the Cas9 protein is in the form of a first expression construct comprising a first promoter operably linked to a first nucleic acid encoding the Cas9 protein; and

(b) the DNA encoding the CRISPR RNA and the DNA encoding the

tracrRNA are in the form of a second expression construct comprising a second promoter operably linked to a second nucleic acid encoding a gRNA comprising the CRISPR RNA and the tracrRNA;

wherein the first and second promoters are active in the pluripotent cell, and

wherein the first and second expression constructs are on a single nucleic acid molecule or on separate nucleic acid molecules.

9. The method of any one of claims 1 to 8, wherein the targeted genetic modification comprises simultaneous deletion of an endogenous nucleic acid sequence at the genomic locus of interest and insertion of the insert nucleic acid at the genomic locus of interest.

10. The method of claim 9, wherein the deleted endogenous nucleic acid sequence is from 30 kb to about 110 kb, and the insert nucleic acid is from about 40 kb to about 140 kb.

11. The method of any one of claims 1 to 10, wherein the targeted genetic modification is a biallelic genetic modification.

12. The method of claim 11, wherein the biallelic genetic modification comprises deletion of an endogenous nucleic acid sequence and insertion of the insert nucleic acid at the genomic locus of interest in two homologous chromosomes.

13. The method of claim 11, wherein the modified pluripotent cell is compound heterozygous or hemizygous at the genomic locus of interest.

14. The method of claim 13, wherein the targeted genetic modification at the genomic locus of interest in one chromosome comprises deletion of an endogenous nucleic acid sequence and insertion of the insert nucleic acid.

15. The method of claim 14, wherein the targeted genetic modification comprises: (1) deletion of an endogenous nucleic acid sequence at the genomic locus of interest in first and second homologous chromosomes; and (2) insertion of the insert nucleic acid into the genomic locus of interest in the first homologous chromosome and disruption of the genomic locus of interest in the second homologous chromosome.

16. The method of any one of claims 1 to 15, wherein the LTVEC is at least 40 kb; or

wherein the targeted genetic modification comprises deletion of a region of the genomic locus of interest wherein the deletion is at least 30 kb, and the LTVEC is at least 15 kb.

17. The method of any one of claims 1 to 16, wherein targeted genetic modification comprises insertion of the insert nucleic acid, wherein the insert nucleic acid is at least 40 kb~~; or~~

~~wherein the targeted genetic modification comprises deletion of a region of the genomic locus of interest wherein the deletion is at least 30 kb and insertion of the insert nucleic acid wherein the insert nucleic acid is at least 10 kb~~.

18. The method of any one of claims 1 to 17, wherein the insert nucleic acid is from about 40 kb to about 140 kb.

19. The method of any one of claims 1 to 18, wherein the CRISPR target sequence is immediately flanked by a Protospacer Adjacent Motif (PAM) sequence.

20. The method of any one of claims 1 to 19, wherein the sum total of the 5' and the 3' homology arms of the LTVEC is from 10 kb to 150 kb.

21. The method of claim 20, wherein the sum total of the 5' and the 3' homology arms of the LTVEC is from 30 kb to 150 kb.

22. The method of any one of claims 1 to 21, wherein the LTVEC is from 100 kb to 300 kb in length.

23. The method of any one of claims 1 to 22, wherein the targeted genetic modification comprises:

(a) replacement of an endogenous nucleic acid sequence with a homologous or an orthologous nucleic acid sequence;

(b) deletion of an endogenous nucleic acid sequence;

(c) deletion of an endogenous nucleic acid sequence,

wherein the deletion ranges from ~~at least 30 kb to about 3 Mb~~ about 40 kb to about 60 kb, from about 60 kb to about 80 kb, from about 80 kb to about 100 kb, from about 100 kb to about 150 kb, from about 150 kb to about 200 kb, from about 200 kb to about 300 kb, from about 300 kb to about 400 kb, from about 400 kb to about 500 kb, from about 500 kb to about 1 Mb, from about 1 Mb to about 1.5 Mb, from about 1.5 Mb to about 2 Mb, from about 2 Mb to about 2.5Mb, or from about 2.5 Mb to about 3 Mb;

(d) insertion of an exogenous nucleic acid sequence;

(e) insertion of an exogenous nucleic acid sequence ranging from ~~at least 30 kb to about 400 kb~~ about 40 kb to about 60 kb, from about 60 kb to about 80 kb, from about 80 kb to about 100 kb, from about 100 kb to about 150 kb, from about 150 kb to about 200 kb, from about 200 kb to about 250 kb, from about 250 kb to about 300 kb, from about 300 kb to about 350 kb, or from about 350 kb to about 400 kb;

(f) insertion of an exogenous nucleic acid sequence comprising a homologous or an orthologous nucleic acid sequence;

(g) insertion of a chimeric nucleic acid sequence comprising a human and a non-human nucleic acid sequence;

(h) insertion of a conditional allele flanked by site-specific recombinase target sequences;

(i) insertion of a selectable marker or a reporter gene operably linked to a promoter active in the pluripotent cell; or

(j) a combination thereof.

24. The method of any one of claims 1 to 23, wherein the targeted genetic modification comprises deletion of a region of the genomic locus of interest,

wherein the deletion is at least 40 kb~~; or~~

~~wherein the targeted genetic modification further comprises insertion of the insert nucleic acid at the genomic locus of interest wherein the insertion is at least 30 kb and deletion of a region of the genomic locus of interest wherein the deletion is at least 10 kb~~.

25. The method of any one of claims 1 to 24, wherein the region of the genomic locus being deleted is from about 30 kb to about 110 kb.

26. The method of any one of claims 1 to 25, wherein the targeted genetic modification comprises a deletion of a region of the genomic locus of interest wherein the deletion is at least 30 kb and insertion of the insert nucleic acid at the genomic locus of interest wherein the insertion is at least 30 kb.

27. The method of any one of claims 1 to 26, wherein the LTVEC is from 100 kb to 300 kb, the sum total of the 5' and 3' homology arms is from 30 kb to 150 kb, and the targeted genetic modification comprises deletion of a region of the genomic locus of interest wherein the deletion is from 30 kb to 110 kb and insertion of the insert nucleic acid at the genomic locus of interest wherein the insertion is from 40 kb to 140 kb.

28. The method of any one of claims 1 to 27, wherein the genomic locus of interest is endogenous to the pluripotent cell.

29. The method of any one of claims 1 to 27, wherein the genomic locus of interest comprises a heterologous or exogenous segment of DNA that was integrated into the genome of the pluripotent cell.

30. The method of any one of claims 1 to 29, wherein the genomic locus of interest is an immunoglobulin locus.

31. The method of claim 30, wherein the immunoglobulin locus encodes a mouse immunoglobulin heavy chain variable region amino acid sequence.

32. The method of claim 30, wherein the immunoglobulin locus encodes a mouse immunoglobulin light chain variable region amino acid sequence.

33. The method of claim 32, wherein the immunoglobulin locus comprises an unrearranged mouse λ and/or κ light chain variable region nucleic acid sequence.

34. The method of claim 32, wherein the immunoglobulin locus comprises a rearranged mouse λ and/or κ light chain variable region nucleic acid sequence.

35. The method of any one of claims 1 to 29, wherein the genomic locus of interest is a T cell receptor locus.

36. The method of claim 35, wherein the T cell receptor locus is a T cell receptor alpha locus.

37. The method of any one of claims 1 to 29, wherein the genomic locus of interest comprises an interleukin-2 receptor gamma locus, an *ApoE* locus, a *Rag1* locus, a *Rag2* locus, both of the *Rag1* and the *Rag2* loci, an *Adamts5* locus, a *Trpa1* locus, a *Folh1* locus, an *Erbb4* locus, a *Lrp5* locus, a *C5* (*Hc*) locus, a *Ror1* locus, or a *Dpp4* locus.

38. The method of any one of claims 1 to 37, wherein the insert nucleic acid comprises a genomic nucleic acid sequence that encodes a human immunoglobulin heavy chain variable region amino acid sequence.

39. The method of claim 38, wherein the insert nucleic acid comprises one or more functional human V_H gene segments comprising V_H1-2, V_H1-3, V_H1-8, V_H1-18, V_H1-24, V_H1-45, V_H1-46, V_H1-58, V_H1-69, V_H2-5, V_H2-26, V_H2-70, V_H3-7, V_H3-9, V_H3-11, V_H3-13, V_H3-15, V_H3-16, V_H3-20, V_H3-21, V_H3-23, V_H3-30, V_H3-30-3, V_H3-30-5, V_H3-33, V_H3-35, V_H3-38, V_H3-43, V_H3-48, V_H3-49, V_H3-53, V_H3-64, V_H3-66, V_H3-72, V_H3-73, V_H3-74, V_H4-4, V_H4-28, V_H4-30-1, V_H4-30-2, V_H4-30-4, V_H4-31, V_H4-34, V_H4-39, V_H4-59, V_H4-61, V_H5-51, V_H6-1, V_H7-4-1, V_H7-81, or a combination thereof.

40. The method of claim 38 or 39, wherein the insert nucleic acid comprises one or more functional human D gene segments comprising D1-1, D1-7, D1-14, D1-20, D1-26, D2-2, D2-8, D2-15, D2-21, D3-3, D3-9, D3-10, D3-16, D3-22, D4-4, D4-11, D4-17, D4-23, D5-12, D5-5, D5-18, D5-24, D6-6, D6-13, D6-19, D6-25, D7-27, or a combination thereof.

41. The method of any one of claims 38 to 40, wherein the insert nucleic acid comprises one or more functional J_H gene segments comprising J_H1, J_H2, J_H3, J_H4, J_H5, J_H6, or a combination thereof.

42. The method of any one of claims 1 to 37, wherein the insert nucleic acid comprises a genomic nucleic acid sequence that encodes a human immunoglobulin light chain variable region amino acid sequence.

43. The method of claim 42, wherein the insert nucleic acid comprises one or more human Vκ gene segments comprising Vκ4-1, Vκ5-2, Vκ 7-3, Vκ 2-4, Vκ1-5, Vκ1-6, Vκ3-7, Vκ1-8, Vκ1-9, Vκ2-10, Vκ3-11, Vκ1-12, Vκ1-13, Vκ2-14, Vκ3-15, Vκ1-16, Vκ1-17, Vκ2-18, Vκ2-19, Vκ3-20, Vκ6-21, Vκ1-22, Vκ1-23, Vκ2-24, Vκ3-25, Vκ2-26, Vκ1-27,

Vκ2-28, Vκ2-29, Vκ2-30, Vκ3-31, Vκ1-32, Vκ1-33, Vκ3-34, Vκ1-35, Vκ2-36, Vκ1-37, Vκ2-38, Vκ1-39, Vκ2-40, or a combination thereof.

44. The method of claim 42 or 43, wherein the insert nucleic acid comprises one or more human Vλ gene segments comprising Vλ3-1, Vλ4-3, Vλ2-8, Vλ3-9, Vλ3-10, Vλ2-11, Vλ3-12, Vλ2-14, Vλ3-16, Vλ2-18, Vλ3-19, Vλ3-21, Vλ3-22, Vλ2-23, Vλ3-25, Vλ3-27, or a combination thereof.

45. The method of any one of claims 42 to 44, wherein the insert nucleic acid comprises one or more human Jκ gene segments comprising Jκ1, Jκ2, Jκ3, Jκ4, Jκ5, or a combination thereof.

46. The method of any one of claims 1 to 37, wherein the insert nucleic acid comprises a polynucleotide encoding at least a region of a human T cell receptor.

47. The method of claim 46, wherein the T cell receptor is a T cell receptor alpha.

48. The method of any one of claims 1 to 37, wherein the insert nucleic acid comprises at least one disease allele.

49. The method of claim 48, wherein the insert nucleic acid comprises at least one human disease allele of a human gene.

50. The method of any one of claims 1 to 49, wherein the pluripotent cell is the non-human mammalian pluripotent cell, and wherein the targeted genetic modification results in a humanized genomic locus comprising: (a) an insertion of a homologous or orthologous human nucleic acid sequence; (b) a replacement of an endogenous nucleic acid sequence with a homologous or orthologous nucleic acid sequence; or (c) a combination thereof.

51. The method of claim 50, wherein the pluripotent cell is the non-human mammalian pluripotent cell, and wherein the targeted genetic modification comprises a replacement of an endogenous nucleic acid sequence with a homologous or orthologous human nucleic acid sequence.

52. The method of any one of claims 1 to 51, wherein the CRISPR target sequence is not present in the insert nucleic acid.

53. The method of any one of claims 1 to 52, wherein the CRISPR target sequence is located anywhere between the 5’ target sequence and the 3’ target sequence at the genomic locus of interest.

54. The method of any one of claims 1 to 53, wherein the CRISPR target sequence is immediately adjacent to the 5’ target sequence or the 3’ target sequence at the genomic locus of interest.

55. The method of any one of claims 1 to 54, wherein the CRISPR target sequence is within the 5’ end of the region of the genomic locus of interest targeted for deletion.

~~56. The method of claim 55, wherein the CRISPR target sequence is 50 to 1000 base pairs from the deletion endpoint.~~

~~57~~56. The method of any one of claims 1 to ~~56~~55, wherein the CRISPR target sequence is within the 3’ end of the region of the genomic locus of interest targeted for deletion.

~~58. The method of claim 57, wherein the CRISPR target sequence is 50 to 1000 base pairs from the deletion endpoint.~~

~~59~~57. The method of any one of claims 1 to ~~58~~56, wherein the CRISPR target sequence is located in an intron, an exon, a promoter, an enhancer, a regulatory region, or any non-protein coding region.

~~60~~58. The method of any one of claims 1 to ~~59~~57, wherein the CRISPR target sequence is located within a coding region of a gene or within a regulatory region that influences expression of the gene.

~~61~~59. The method of any one of claims 1 to ~~60~~58, wherein the guide RNA comprises SEQ ID NO: 2, 3, 4, 5, 6, 7, or 8, optionally wherein the guide RNA comprises SEQ ID NO: 3, 4, 5, or 7.

~~62~~60. The method of any one of claims 1 to ~~61~~59, wherein the mammalian

pluripotent cell is a human induced pluripotent stem cell.

~~63~~61. The method of claim ~~62~~60, wherein the human induced pluripotent stem cell is being maintained in a medium comprising a base medium and supplements, wherein the medium comprises:

(a) a leukemia inhibitory factor (LIF) polypeptide;

(b) a glycogen synthase kinase (GSK3) inhibitor; and

(c) a MEK inhibitor;

wherein the base medium has an osmolality of about 180 mOsm/kg to about 250 mOsm/kg.

~~64~~62. The method of any one of claims 1 to ~~63~~61, wherein the pluripotent cell is a non-rat eukaryotic cell.

~~65~~63. The method of any one of claims 1 to ~~61~~59, wherein the mammalian pluripotent cell is a rodent pluripotent cell.

~~66~~64. The method of claim ~~65~~63, wherein the rodent pluripotent cell is a rat embryonic stem (ES) cell or a mouse ES cell.

~~67~~65. The method of claim ~~66~~64, wherein the rodent pluripotent cell is a mouse ES cell.

~~68~~66. The method of claim ~~66~~64, wherein the rodent pluripotent cell is a rat ES cell.

~~69~~67. The method of claim ~~68~~66, wherein the rat ES cell is obtained by culturing blastocyst outgrowths on a feeder cell layer of mitotically inactivated mouse embryonic fibroblasts with the 2i medium set forth in Table 3,

wherein the rat ES cell lacks expression of c-Myc,

wherein the rat ES cell forms spherical, free-floating colonies in culture,

wherein the rat ES cell has a normal karyotype, and

wherein the targeted genetic modification is capable of being transmitted through the germline.

~~70~~68. The method of claim ~~68-~~66 or ~~69~~67, further comprising:

(a) identifying the modified rat ES cell comprising the targeted genetic modification at the genomic locus of interest;

(b) introducing the modified rat ES cell into a rat host embryo; and

(c) gestating the rat host embryo in a surrogate mother,

wherein the surrogate mother produces an F0 generation rat comprising the targeted genetic modification at the genomic locus of interest.

~~71~~69. The method of claim ~~67~~65, further comprising:

(a) identifying the modified mouse ES cell comprising the targeted genetic modification at the genomic locus of interest;

(b) introducing the modified mouse ES cell into a mouse host embryo; and

(c) gestating the mouse host embryo in a surrogate mother,

wherein the surrogate mother produces an F0 generation mouse comprising the targeted genetic modification at the genomic locus of interest.

~~72~~70. The method of any one of claims 1 to ~~71~~69, wherein the Cas9 protein comprises a nuclear localization signal.

~~73~~71. The method of any one of claims 1 to ~~72~~70, wherein the sum total of the 5' and the 3' homology arms of the LTVEC is at least 10 kb.

~~74~~72. The method of any one of claims 1 to ~~73~~71, wherein the CRISPR target sequence is a native sequence that is endogenous to the cell.

~~75~~73. The method of any one of claims 1 to ~~74~~72, wherein the genomic locus of interest is native to the cell.

~~76~~74. The method of any one of claims 1 to ~~75~~73, wherein the Cas9 protein comprises a nuclear localization signal, and

wherein the CRISPR target sequence is located anywhere between the 5’ target sequence and the 3’ target sequence or the CRISPR target sequence is immediately adjacent to the 5’ target sequence or the 3’ target sequence.

~~77~~75. The method of any one of claims 1 to ~~76~~74, wherein the sum total of the 5’ and the 3’ homology arms of the LTVEC is at least 10 kb,

wherein the CRISPR target sequence is a native sequence that is endogenous to the cell, and

wherein the CRISPR target sequence is located anywhere between the 5’ target sequence and the 3’ target sequence or the CRISPR target sequence is immediately adjacent to the 5’ target sequence or the 3’ target sequence.

~~78~~76. The method of any one of claims 1 to ~~77~~75, wherein the sum total of the 5’ and the 3’ homology arms of the LTVEC is at least 10 kb,

wherein the genomic locus of interest is native to the cell, and

wherein the CRISPR target sequence is located anywhere between the 5’ target sequence and the 3’ target sequence or the CRISPR target sequence is immediately adjacent to the 5’ target sequence or the 3’ target sequence.

~~79~~77. The method of any one of claims 1 to ~~78~~76, wherein the targeted genetic modification comprises the insertion of the insert nucleic acid at the genomic locus of interest or wherein the targeted genetic modification comprises the deletion of the region of the genomic locus of interest and insertion of the insert nucleic acid at the genomic locus of interest.

~~80~~78. The method of claim ~~79~~77, wherein the CRISPR target sequence is a native sequence that is endogenous to the cell, and wherein the sum total of the 5’ and the 3’ homology arms of the LTVEC is from 10 kb to 150 kb.