

CLAIMS

1. An engineered *Streptococcus pyogenes* Cas9 (SpCas9) protein variant having an identity of at least 99% identity with SEQ ID NO:1 and comprising the mutations selected from the following group:

K562L-R661L-K855Q;

K562Q-R661L-K855Q;

K652L-R661L-K855Q;

K652Q-R661L-K855Q;

R661L-K855Q-K1003Q;

R661L-K855Q-R1060Q;

R661L-R691L-K855Q;

R661L-R780L-K855Q;

R661L-R780Q-K855Q;

R661L-K810L-K855Q;

R661L-K848L-K855Q;

R661L-K810Q-K855Q;

R661L-K855Q-K1003L; and

R661L-K855Q-R1060L;

with reference to the amino acid numbering in the corresponding position of an unmodified mature, wild-type *Streptococcus pyogenes* Cas9 shown in SEQ ID NO:1.

2. The engineered SpCas9 protein variant of claim 1, wherein the mutations are K652Q-R661L-K855Q.

3. The engineered SpCas9 protein variant of any preceding claim, further comprising one or more heterologous domains fused to the N terminal end, the C terminal end, an internal location, or combination thereof and/or at least one nuclear localization signal fused to the N terminal end, the C terminal end, an internal location, or combination thereof.

4. The engineered SpCas9 protein variant of claim 3, wherein the heterologous domain is selected from a nuclear localization signal, a cell-penetrating domain, a marker or reporter domain which facilitates detection, a chromatin modification domain, an epigenetic modification domain, a transcriptional regulation domain, a DNA or RNA deaminase domain, a uracil-DNA-glycosylase domain, a reverse transcriptase domain, a recombinase domain, an RNA aptamer binding domain, and a non-Cas9 nuclease domain.

5. The engineered SpCas9 protein variant of any preceding claim, further comprising at least one mutation in the RuvC domain, and/or at least one mutation in the HNH domain, optionally wherein the at least one mutation in the RuvC domain, if present, comprises at least one mutation selected from D10A, D8A, E762A, and D986A, and optionally wherein the at least one mutation in the HNH domain, if present, comprises at least one mutation selected from H840A, H559A, N854A, N856A, and N863A.

6. An engineered Cas9 system comprising the engineered SpCas9 protein variant of any preceding claim and at least one engineered guide RNA, wherein the at least one engineered guide RNA is designed to complex with the engineered SpCas9 protein variant.

7. A plurality of nucleic acids encoding the engineered SpCas9 protein variant of any of claims 1-5 or the engineered SpCas9 system of claim 6.

8. The plurality of nucleic acids of claim 7, wherein the plurality of nucleic acids comprises at least one nucleic acid encoding the engineered SpCas9 protein variant, optionally codon optimized for expression in a eukaryotic cell, and at least one nucleic acid encoding the engineered guide RNA.

9. The plurality of nucleic acids of claim 7 or 8, wherein:

(a) at least one nucleic acid is RNA;

(b) at least one nucleic acid is DNA;

(c) the at least one nucleic acid encoding the engineered guide RNA is DNA;

(d) the at least one nucleic acid encoding the engineered SpCas9 protein variant is operably linked to a phage promoter sequence for *in vitro* RNA synthesis or protein expression in a bacterial cell, and the at least one nucleic acid encoding the engineered guide RNA is operably linked to a phage promoter sequence for *in vitro* RNA synthesis; or

(e) the at least one nucleic acid encoding the engineered Cas9 protein variant is operably linked to a eukaryotic promoter sequence for expression in a eukaryotic

cell, and the at least one nucleic acid encoding the engineered guide RNA is operably linked to a eukaryotic promoter sequence for expression in a eukaryotic cell.

10. The plurality of nucleic acids of claim 8 or 9, wherein the eukaryotic cell is a human cell, a non-human mammalian cell, a non-mammalian vertebrate cell, an invertebrate cell, a plant cell, or a single cell eukaryotic organism.

11. At least one vector comprising the plurality of nucleic acids of any one of claims 7-10, which is optionally a plasmid vector, a viral vector, or a self-replicating viral RNA replicon.

12. A eukaryotic cell comprising at least one engineered Cas9 system of claim 6 or the nucleic acid of any of claims 7-10, wherein the cell is optionally a human cell, a non-human mammalian cell, a plant cell, a non-mammalian vertebrate cell, an invertebrate cell, or a single cell eukaryotic organism.

13. The eukaryotic cell of claim 12, which is *ex vivo*, or *in vitro*.

14. The engineered SpCas9 protein variant of any of claims 1-5, which is a Cas9 homologue.

15. A ribonucleoprotein (RNP) complex comprising the engineered SpCas9 protein variant of any of claims 1-5.

16. A fusion protein comprising the engineered SpCas9 protein variant of any of claims 1-5.

17. A pharmaceutical composition comprising the engineered SpCas9 protein variant of any of claims 1-5 and at least one pharmaceutically acceptable excipient.

18. A method for modifying the chromosomal sequence of a eukaryotic cell, the method comprising expressing in the eukaryotic cell the engineered SpCas9 protein variant of any of claims 1-5 together with a guide RNA,

wherein the method is not a process for modifying the germ line genetic identity of human beings and

wherein the method is not a method for treatment of the human or animal body by surgery or therapy.