SauCas9_X according to SEQ ID NO. 1, M-SluCas9_X according to SEQ ID NO. 2, M-SpaCas9_X according to SEQ ID NO. 3, M-ShyCas9_X according to SEQ ID NO. 4, M-SmiCas9_X according to SEQ ID NO. 5, MGib11SpaCas9-3-E410A, according to SEQ ID NO. 8, MGib11Spa-1-M417L according to SEQ ID NO. 133, or any polypeptide sequence that is at least 95% identical to any of the foregoing polypeptides.

In one aspect, the present disclosure provides a M-SmallCas9 polypeptide comprising an amino acid sequence according to SEQ ID NO. 7 (M-SluCas9-R414A). In one example, provided herein is a M-SmallCas9 polypeptide selected from the group: M-SauCas9-R420A, according to SEQ ID NO. 6; M-SluCas9-R414A, according to SEQ ID NO. 7; M-Gib11SpaCas9-3-E410A, according to SEQ ID NO. 8; and M-Gib11Spa-1-M417L according to SEQ ID NO. 133, or any polypeptide sequence that is at least 95% identical to any of the foregoing polypeptides.

In another aspect, provided herein is a composition comprising: (I) a M-SmallCas9 polypeptide disclosed herein; and (II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) that allow the generation of such one or more sgRNA(s) in situ, each sgRNA or DNA encoding an sgRNA comprising (a) an engineered DNA targeting segment that can hybridize to a target sequence in a polynucleotide locus, (b) a tracr mate sequence, and (c) a tracr RNA sequence, wherein the tracr mate sequence can hybridize to the tracr RNA sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation. In some examples, the engineered DNA targeting segment is directly adjacent to the PAM sequence on its 3' end or such PAM sequence is part of the DNA targeting sequence in its 3' portion.

In another aspect, provided herein is a method of targeting, editing, modifying, or manipulating a target DNA at one or more locations in a cell *ex vivo* or *in vitro*, the method comprising (I) introducing a heterologous M-SmallCas9 polypeptide disclosed herein or a nucleic acid encoding a M-SmallCas9 disclosed herein into the cell or into the in vitro environment; and (II) introducing one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) encoding such one or more sgRNA(s) in the cell *ex vivo* or the *in vitro* environment, each sgRNA or DNA encoding the sgRNA comprising: (a) an engineered DNA targeting segment comprising an RNA and capable of hybridizing to a target sequence in a polynucleotide locus, (b) a tracr mate sequence comprised of RNA, and (c) a tracr RNA sequence comprised of RNA, wherein the tracr mate sequence hybridizes to the tracr RNA sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation; and (III) creating one or more nicks or cuts or base edits in the target DNA, wherein the M-SmallCas9 polypeptide is directed to the target DNA by the sgRNA in its processed or unprocessed form.

In one example, provided herein is a method of targeting, editing, modifying, or manipulating a target DNA at one or more locations in a cell or in vitro, the method comprising (I) introducing a heterologous M-SmallCas9 polypeptide disclosed herein or a nucleic acid encoding a M-SmallCas9 disclosed herein into the cell or into the in vitro environment; and (II) introducing one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) encoding such one or more sgRNA(s) in the cell or the in

vitro environment, each sgRNA or DNA encoding the sgRNA comprising: (a) an engineered DNA targeting segment comprising an RNA and capable of hybridizing to a target sequence in a polynucleotide locus,

(b) a tracr mate sequence comprised of RNA, and (c) a tracr RNA sequence comprised of RNA, wherein the tracr mate sequence hybridizes to the tracr sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation; and (III) creating one or more nicks or cuts or base edits in the target DNA, wherein the M-SmallCas9 polypeptide is directed to the target DNA by the sgRNA in its processed or unprocessed form.

In a further aspect, provided herein is use of a composition comprising

(I) a M-SmallCas9 polypeptide as described herein or a nucleic acid encoding the same; and

(II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) suitable for the generation of such one or more sgRNA in situ, each comprising:

(a) an engineered DNA targeting segment comprised of RNA and capable of hybridizing to a target sequence in a polynucleotide locus,

(b) a tracr mate sequence comprised of RNA, and

(c) a tracr RNA sequence comprised of RNA,

wherein the tracr mate sequence hybridizes to the tracr sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation;

for targeting, editing, modifying, or manipulating a target DNA at one or more locations in a cell *ex vivo* or *in vitro*.

In one example, provided herein is use of a composition comprising (I) a M-SmallCas9 polypeptide disclosed herein or a nucleic acid encoding the same; and/or (II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) suitable for the generation of such one or more sgRNA in situ, each comprising: (a) an engineered DNA targeting segment comprised of RNA and capable of hybridizing to such target sequence in a polynucleotide locus, (b) a tracr mate sequence comprised of RNA, and (c) a tracr RNA sequence comprised of RNA, wherein the tracr mate sequence hybridizes to the tracr sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation; for targeting, editing, modifying, or manipulating a target DNA at one or more locations in a cell or in vitro.

In another aspect, provided herein is a cell comprising (I) a M-SmallCas9 polypeptide as disclosed herein, or a nucleic acid encoding a M-SmallCas9 polypeptide disclosed herein; and (II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) suitable for the generation of such one or more sgRNA in situ, each comprising: (a) an engineered DNA targeting segment that can hybridizing to a target sequence in a polynucleotide locus, (b) a tracr mate sequence, and (c) a tracr RNA sequence, wherein the tracr mate sequence that can hybridize to the tracr RNA sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation.

In yet another aspect, provided herein is a kit comprising (I) a nucleic acid sequence encoding a

M-SmallCas9 polypeptide as disclosed herein, wherein the nucleic acid sequence encoding the M-SmallCas9 is operably linked to a promoter; and (II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) suitable for the generation of such one or more sgRNA in situ, each sgRNA comprising: (a) an engineered DNA targeting segment that can hybridize to a target sequence in a polynucleotide locus, (b) a tracr mate sequence, and (c) a tracr RNA sequence, wherein the tracr mate sequence can hybridize to the tracr RNA sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation.

In a further aspect, the present disclsore provides a composition comprising a M-SmallCas9 polypeptide as described herein. In a further aspect, the present disclsore provides the composition as described herein, wherein the pharmaceutical composition is for use in gene therapy. In a further aspect, the present disclsore provides a polynucleotide encoding a M-SmallCas9 polypeptide as described herein.

The entire disclosure of each patent document and scientific article referred to herein, and those patent documents and scientific articles cited thereby, is expressly incorporated by reference herein for all purposes.

Additional features and advantages of the disclosure are more particularly described below.

Brief Description of the Drawings

Figures 1A and 1B are bar graphs depicting the results of experiments testing the relative cleavage activity (y axis) of Cas nucleases as a function a) position of nucleic acid within the target sequence as shown on the x-axis and b) the respective nucleic acid residue in that position, which is visualized by differently shaded boxes that are stacked. The absence of, *e.g.*, the box for "G" in a column means that no cleavage activity was seen in that position when the residue "G" was present. Figure 1A shows these data for SluCas9 according to SEQ ID NO. 9; Figure 1B shows these data for MSluCas9R414A according to SEQ ID NO. 7.

Figure 2 is a bar graph depicting the results of experiments detecting the total specificity values of four different Cas enzymes: SluCas9 (SEQ ID NO: 9), M-SluCas9R414A (SEQ ID NO. 7),

CLAIMS

1. A M-SmallCas9 polypeptide comprising an amino acid sequence according to SEQ ID NO. 7 (M-SluCas9-R414A).

2. A composition comprising
   (I) a M-SmallCas9 polypeptide according to claim 1; and
   (II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) that allow the generation of such one or more sgRNA(s) *in situ*, each sgRNA or DNA encoding an sgRNA comprising:
      (a) an engineered DNA targeting segment that can hybridize to a target sequence in a polynucleotide locus,
      (b) a tracr mate sequence, and
      (c) a tracr RNA sequence,

   wherein the tracr mate sequence can hybridize to the tracr RNA sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation.

3. A method of targeting, editing, modifying, or manipulating a target DNA at one or more locations in a cell *ex vivo* or *in vitro*, the method comprising
   (I) introducing a heterologous M-SmallCas9 polypeptide according to claim 1 or a nucleic acid encoding a M-SmallCas9 of claim 1 into the cell *ex vivo* or into the *in vitro* environment; and
   (II) introducing one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) encoding such one or more sgRNA(s) in the cell *ex vivo* or the *in vitro* environment, each sgRNA or DNA encoding the sgRNA comprising:
      (a) an engineered DNA targeting segment comprising an RNA and capable of hybridizing to a target sequence in a polynucleotide locus,
      (b) a tracr mate sequence comprised of RNA, and
      (c) a tracr RNA sequence comprised of RNA,

   wherein the tracr mate sequence hybridizes to the tracr RNA sequence, and wherein (a), (b), and (b) are arranged in a 5' to 3' orientation; and

(III) creating one or more nicks or cuts or base edits in the target DNA, wherein the M-SmallCas9 polypeptide is directed to the target DNA by the sgRNA in its processed or unprocessed form.

4. Use of a composition comprising

(I) a M-SmallCas9 polypeptide according to claim 1 or a nucleic acid encoding the same; and

(II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) suitable for the generation of such one or more sgRNA in situ, each comprising:

(a) an engineered DNA targeting segment comprised of RNA and capable of hybridizing to a target sequence in a polynucleotide locus,

(b) a tracr mate sequence comprised of RNA, and

(c) a tracr RNA sequence comprised of RNA,

wherein the tracr mate sequence hybridizes to the tracr sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation;

for targeting, editing, modifying, or manipulating a target DNA at one or more locations in a cell *ex vivo* or in vitro.

5. A cell comprising

(I) a M-SmallCas9 polypeptide according to claim 1, or a nucleic acid encoding a M-SmallCas9 polypeptide according to claim 1; and

(II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) suitable for the generation of such one or more sgRNA *in situ*, each comprising:

(a) an engineered DNA targeting segment that can hybridizing to a target sequence in a polynucleotide locus,

(b) a tracr mate sequence, and

(c) a tracr RNA sequence,

wherein the tracr mate sequence that can hybridize to the tracr RNA sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation.

6. A kit comprising

(I) a nucleic acid sequence encoding a M-SmallCas9 polypeptide according to claim 1, wherein the nucleic acid sequence encoding the M-SmallCas9 is operably linked to a promoter; and

(II) one or more single heterologous guide RNA(s) (sgRNA) or DNA(s) suitable for the generation of such one or more sgRNA *in situ*, each sgRNA comprising:

(a) an engineered DNA targeting segment that can hybridize to a target sequence in a polynucleotide locus,

(b) a tracr mate sequence, and

(c) a tracr RNA sequence,

wherein the tracr mate sequence can hybridize to the tracr RNA sequence, and wherein (a), (b), and (c) are arranged in a 5' to 3' orientation.

7. A composition comprising a M-SmallCas9 polypeptide according to claim 1.

8. The composition according to claim 2 or 7, wherein the composition is a pharmaceutical composition.

9. The composition according to claim 8, wherein the pharmaceutical composition is for use in gene therapy.

10. The M-SmallCas9 polypeptide of claim 1, further comprising a nuclear localization signal.

11. A polynucleotide encoding a M-SmallCas9 polypeptide according to claim 1.

12. The polynucleotide of claim 11, wherein the polynucleotide is codon optimized.