

Claims

1. A composition comprising a Type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system for cleavage of a target nucleic acid in a eukaryotic cell, said CRISPR/Cas system comprising (i) a nucleic acid encoding a Cas9 polypeptide and a nuclear localization sequence, and (ii) a nucleic acid encoding a guide RNA that hybridizes to the target nucleic acid in a eukaryotic cell, wherein the guide RNA is a chimeric guide RNA comprising a CRISPR RNA (crRNA) sequence fused to a trans activating crRNA (tracrRNA) sequence, wherein the crRNA sequence is at the 5' end relative to the tracrRNA sequence.
2. The composition of claim 1, wherein said Cas9 polypeptide is a *Streptococcus* Cas9 polypeptide.
3. The composition of claim 2, wherein said Cas9 polypeptide is a *Streptococcus pyogenes* Cas9 polypeptide.
4. The composition of any one of claims 1-3, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in eukaryotic cells.
5. The composition of claim 4, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in mammalian cells.
6. The composition of any one of claims 1-5, wherein said nuclear localization sequence is located at the C terminus or N terminus of the Cas9 polypeptide.
7. The composition of any one of claims 1-6, wherein the target nucleic acid is an endogenous target nucleic acid.
8. The composition of any one of claims 1-7, wherein the guide RNA is in the form of a vector.
9. The composition of any one of claims 1-8, wherein the guide RNA comprises one or more additional nucleic acid residues 5' to the crRNA sequence.
10. The composition of claim 9, wherein the one or more additional nucleic residues are two

nucleic acid residues, and wherein there are no additional nucleic acid residues between the two nucleic acid residues and the crRNA sequence.

11. The composition of claim 10, wherein the two nucleic acid residues at the 5' end are both guanine residues.
12. A composition comprising a Type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system for cleavage of a target nucleic acid in a eukaryotic cell, said CRISPR/Cas system comprising (i) a nucleic acid encoding a Cas9 polypeptide and a nuclear localization sequence, wherein said nucleic acid comprises SEQ ID NO:1, and (ii) a nucleic acid encoding a guide RNA that hybridizes to the target nucleic acid in a eukaryotic cell, wherein the guide RNA is a chimeric guide RNA comprising a CRISPR RNA (crRNA) sequence fused to a trans activating crRNA (tracrRNA) sequence, wherein the crRNA sequence is at the 5' end relative to the tracrRNA sequence.
13. The composition of claim 12, wherein the target nucleic acid sequence comprises a sequence having a trinucleotide protospacer adjacent motif (PAM), wherein the PAM is a trinucleotide of 5'-NGG-3' selected from the group consisting of 5'-AGG-3', 5'-GGG-3', 5'-TGG-3' and 5'-CGG-3'.
14. The composition of claim 12 or 13, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in eukaryotic cells.
15. The composition of claim 14, wherein said nucleic acid encoding a Cas9 polypeptide is codon-optimized for expression in mammalian cells.
16. The composition of any one of claims 13-15, wherein said nuclear localization sequence is located at the C terminus or N terminus of the Cas9 polypeptide.
17. The composition of any one of claims 12-16, wherein the target nucleic acid is an endogenous target nucleic acid.
18. The composition of any one of claims 12-17, wherein the guide RNA is in the form of a vector.

19. The composition of any one of claims 12-18, wherein the guide RNA comprises one or more additional nucleic acid residues 5' to the crRNA sequence.
20. The composition of claim 19, wherein the one or more additional nucleic acid residues are two nucleic acid residues, and wherein there are no additional nucleic acid residues between the two nucleic acid residues and the crRNA sequence.
21. The composition of claim 20, wherein the two nucleic acid residues at the 5' end are both guanine residues.
22. An *in vitro* method of introducing a site-specific, double-stranded break at a target nucleic acid sequence in a eukaryotic cell, the method comprising introducing into the eukaryotic cell a Type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system, wherein the CRISPR/Cas system comprises:
 - a) a nucleic acid encoding a Cas9 polypeptide and a nuclear localization signal, and
 - b) a nucleic acid encoding a guide RNA that hybridizes to the target nucleic acid, wherein the guide RNA is a chimeric guide RNA comprising a CRISPR RNA (crRNA) sequence fused to a trans activating crRNA (tracrRNA) sequence, wherein the crRNA sequence is at the 5' end relative to the tracrRNA sequence; wherein the target nucleic acid sequence comprises a sequence that binds to the crRNA sequence,

and wherein the Cas9 polypeptide and the guide RNA form a Cas9/RNA complex in the eukaryotic cell, whereby a site-specific, double stranded break at the target nucleic acid sequence is introduced.
23. The method of claim 22, wherein the Cas9 polypeptide is a *Streptococcus* Cas9 polypeptide.
24. The method of claim 23, wherein the Cas9 polypeptide is a *Streptococcus pyogenes* Cas9 polypeptide.
25. The method of claim 22 or 23, wherein said nucleic acid encoding a Cas9 polypeptide is

codon-optimized for expression in eukaryotic cells.

26. The method of claim 25, wherein the nucleic acid encoding the Cas 9 polypeptide is codon-optimized for expression in mammalian cells.
27. The method of any one of claims 22-26, wherein the nuclear localization signal is located at the C terminus or N terminus of the Cas9 polypeptide.
28. The method of any one of claims 22-27, wherein the eukaryotic cell is a mammalian cell.
29. The method of claim 28, wherein the mammalian cell is a human cell.
30. The method of any one of claims 22-29, wherein the target nucleic acid sequence is a genomic sequence located at its endogenous site in the genome of the eukaryotic cell.
31. The method of any one of claims 22-30, wherein the nucleic acid encoding the guide RNA is a vector.
32. The method of any one of claims 22-30, wherein the nucleic acid encoding the guide RNA is in vitro transcribed RNA.
33. The method of any one of claims 22-30, wherein the guide RNA comprises one or more additional nucleic acid residues 5' to the crRNA sequence.
34. The method of claim 33, wherein the one or more additional nucleic acid residues are two nucleic acid residues, and wherein there are no additional nucleic acid residues between the two nucleic acid residues and the crRNA sequence.
35. The method of claim 34, wherein the two nucleic acid residues at the 5' end are both guanine residues.
36. The method of any one of claims 22-35, wherein the nucleic acid encoding the Cas9 polypeptide is introduced into the eukaryotic cell before introducing the nucleic acid encoding the guide RNA into the eukaryotic cell.
37. The method of any one of claims 22-35, wherein the nucleic acid encoding the Cas9 polypeptide is co-transfected into the eukaryotic cell with the nucleic acid encoding the

guide RNA.

38. The method of any one of claims 22-37, wherein the eukaryotic cell is a non-human eukaryotic cell.
39. The composition of any one of claims 1-11 or the method of any one of claims 22-38, wherein the target nucleic acid sequence comprises a sequence having a trinucleotide protospacer adjacent motif (PAM), wherein the PAM is a trinucleotide of 5'-NGG-3' selected from the group consisting of 5'-AGG-3', 5'-GGG-3', 5'-TGG-3' and 5'-CGG-3'.
40. The composition of claim 6, wherein said nuclear localization sequence is located at the C terminus of the Cas9 polypeptide.
41. The composition of claim 16, wherein said nuclear localization sequence is located at the C terminus of the Cas9 polypeptide.
42. The method of claim 27, wherein the nuclear localization signal is located at the C terminus of the Cas9 polypeptide.