CLAIMS

WHAT IS CLAIMED IS:

1. A single polynucleotide comprising:

a targeting region comprising deoxyribonucleic acid (DNA);

an activating region adjacent to said targeting region comprising ribonucleic acid (RNA);

wherein said single polynucleotide is for use with a Class 2 CRISPR system.

2. The polynucleotide of claim 1, wherein the activating region comprises DNA.

3. The polynucleotide of claim 1, wherein the targeting region comprises a mixture of DNA and RNA.

4. The polynucleotide of claim 1, wherein the targeting region comprises a mixture of DNA and RNA and the activating region comprises a mixture of DNA and RNA.

5. The polynucleotide of claim 1, wherein said activating region is downstream of said targeting region.

6. The polynucleotide of claim 5, wherein said activating region comprises a structure selected from the group consisting of a lower stem, a bulge, an upper stem, a nexus, and a hairpin.

7. The polynucleotide of claim 1, wherein said activating region is upstream of said targeting region.

8. The polynucleotide of claim 7, wherein said activating region comprises a stem loop structure.

9. The polynucleotide of claim 1, wherein said activating region interacts with a Cas9 protein.

10. The polynucleotide of claim 1, wherein said activating region interacts with a Cpf1 protein.

11. A Class 2 CRISPR system comprising:

a single polynucleotide comprising a targeting region comprising deoxyribonucleic acid (DNA) and configured to hybridize with a target sequence in a nucleic acid;

an activating region adjacent to said targeting region comprising a ribonucleic acid (RNA); and

a site-directed polypeptide.

12. The Class 2 CRISPR system of claim 11, wherein said activating region is downstream of said targeting region and wherein said site-directed polypeptide comprises Cas9.

13. The Class 2 CRISPR system of claim 12, wherein said activating region comprises a structure selected from the group consisting of a lower stem, a bulge, an upper stem, a nexus, and a hairpin.

14. The Class 2 CRISPR system of claim 11, wherein said activating region is upstream of said targeting region and wherein said site-directed polypeptide comprises Cpf1.

15. The Class 2 CRISPR system of claim 14, wherein said activating region comprises a stem loop structure.

16. The Class 2 CRISPR system of claim 11, wherein said site-directed polypeptide interacts with said activating region.

17. The Class 2 CRISPR system of claim 11, wherein said targeting region comprises a mixture of DNA and RNA.

18. The Class 2 CRISPR system of claim 11, wherein said activating region comprises a mixture of DNA and RNA.

19. The Class 2 CRISPR system of claim 11, wherein said targeting region comprises a mixture of DNA and RNA and said activating region comprises a mixture of DNA and RNA.

20. The Class 2 CRISPR system of claim 11, further comprising a donor polynucleotide.

21. A Class 2 CRISPR system comprising:

a first polynucleotide comprising (i) a targeting region comprising deoxyribonucleic acid (DNA) and configured to hybridize with a target sequence in a nucleic acid and (ii) an activating region adjacent to said targeting region comprising ribonucleic acid (RNA);

a second polynucleotide comprising a sequence that is complementary to a sequence in said activating region of said first polynucleotide; and

a site-directed polypeptide.

22. The Class 2 CRISPR system of claim 21, wherein said activating region and said second polynucleotide hybridize to form one or more structures selected from the group consisting of a lower stem, a bulge, an upper stem, a nexus, and a duplex.

23. The Class 2 CRISPR system of claim 21, wherein said site-directed polypeptide is selected from the group consisting of Cas9 and Cpf1.

24. The Class 2 CRISPR system of claim 21, wherein said site-directed polypeptide interacts with said activating region.

25. The Class 2 CRISPR system of claim 21, wherein said activating region further comprises DNA.

26. The Class 2 CRISPR system of claim 21, wherein said second polynucleotide comprises RNA.

27. The Class 2 CRISPR system of claim 26, wherein said second polynucleotide further comprises DNA.

28. Two polynucleotides comprising:

a first polynucleotide comprising (i) a targeting region comprising deoxyribonucleic acid (DNA) and configured to hybridize with a target sequence in a nucleic acid, and (ii) an activating region adjacent to said targeting region comprising ribonucleic acid (RNA);

a second polynucleotide comprising a sequence that is complementary to a sequence in said activating region of said first polynucleotide and wherein said two polynucleotides are for use with a Class 2 CRISPR system.

29. The two polynucleotides of claim 28, wherein said activating region and said second polynucleotide hybridize to form one or more structures selected from the group consisting of a lower stem, a bulge, an upper stem, a nexus, and a duplex.

30. The two polynucleotides of claim 28, wherein said targeting region comprises a mixture of DNA and RNA, said activating region comprises a mixture of DNA and RNA, and said second polynucleotide comprises a mixture of DNA and RNA.

31. A method of modifying a target nucleic acid molecule comprising contacting said target nucleic acid molecule having a target sequence with the Class 2 CRISPR system of any of claims 11-19 or 21-27, wherein said target nucleic acid molecule is cleaved or transcription of at least one gene encoded by said target nucleic acid molecule is modulated.

32. The method of claim 31 wherein said target nucleic acid molecule comprises DNA.

33. The method of claim 31 wherein said target nucleic acid molecule comprises RNA.

34. The method of claim 31 wherein said target nucleic acid molecule comprises a mixture of DNA and RNA.

35. The method of claim 31 further comprising providing a donor polynucleotide.

36. A method for reducing off-target modification using a Class 2 CRISPR system comprising contacting a target nucleic acid molecule having a target sequence with the Class 2 CRISPR system of any of claims 11-19 or 21-27, wherein said target nucleic acid molecule is cleaved or edited at the target sequence more preferentially than at other sequences in the target nucleic acid, thereby reducing off-target modification.

37. The method of claim 36 wherein said target nucleic acid molecule comprises DNA.

38. The method of claim 36 wherein said target nucleic acid molecule comprises RNA.

39. The method of claim 36 wherein said target nucleic acid molecule comprises a mixture of DNA and RNA.

40. The method of claim 36 wherein a targeting region of a polynucleotide for use with the Class 2 CRISPR system is free of uracil.

41. The method of claim 36 further comprising providing a donor polynucleotide.

42. A method for increasing target specific modification using a Class 2 CRISPR system comprising contacting a target nucleic acid molecule having a target sequence with the Class 2 CRISPR system of any of claims 11-19 or 21-27, wherein said target nucleic acid molecule is cleaved or edited at the target sequence more preferentially than at other sequences in the target nucleic acid, thereby increasing target specific modification.

43. The method of claim 42 wherein said target nucleic acid molecule comprises DNA.

44. The method of claim 42 wherein said target nucleic acid molecule comprises RNA.

45. The method of claim 42 wherein said target nucleic acid molecule comprises a mixture of DNA and RNA.

46. The method of claim 42 further comprising providing a donor polynucleotide.

47. A method of introducing a donor polynucleotide into the genome of a cell or organism using a Class 2 CRISPR system comprising contacting a target nucleic acid molecule having a target sequence with the Class 2 CRISPR system of any of claims 11-19 or 21-27, wherein said target nucleic acid molecule is cleaved at, or near the target sequence and providing a donor polynucleotide that is introduced into the genome of the cell or organism at the cleavage site.

48. The method of claim 47 wherein said target nucleic acid molecule comprises DNA.

49. The method of claim 47 wherein said target nucleic acid molecule comprises RNA.

50. The method of claim 47 wherein said target nucleic acid molecule comprises a mixture of DNA and RNA.

51. The method of claim 47 wherein the donor polynucleotide is introduced into the nucleic acid by homologous recombination.

52. The method of claim 47 wherein the donor polynucleotide is introduced into the nucleic acid by non-homologous end joining.

The Claims defining the Invention are as follows:

1. A single Class 2 CRISPR polynucleotide comprising:

a targeting region that comprises deoxyribonucleic acid (DNA) or ribonucleic acid (RNA); and

an activating region adjacent to said targeting region that comprises DNA; wherein said activating region comprises a stem loop structure or comprises a stem and a bulge, and is capable of binding with a site-directed polypeptide selected from the group consisting of Cas9 and Cpf1.

2. The single Class 2 CRISPR polynucleotide of claim 1, wherein the targeting region comprises a mixture of DNA and RNA.

3. The single Class 2 CRISPR polynucleotide of claim 1, wherein the targeting region comprises a mixture of DNA and RNA and the activating region comprises a mixture of DNA and RNA.

4. The single Class 2 CRISPR polynucleotide of claim 1, wherein the targeting region comprises a mixture of DNA and RNA and the activating region comprises a compound selected from the group consisting of phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, alkylphosphonates, 5'-alkylene phosphonates, chiral phosphonates, phosphinates, phosphoramidates, 3'-amino phosphoramidate, amino alkylphosphoramidates, phosphorodiamidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates, and boranophosphates.

5. The single Class 2 CRISPR polynucleotide of claim 1, wherein said activating region comprises a lower stem, a bulge, an upper stem, a nexus, and a hairpin and wherein the site-directed polypeptide is Cas9.

6. The single Class 2 CRISPR polynucleotide of claim 1, wherein said activating region comprises a stem loop structure and wherein the site-directed polypeptide is Cpf1.

7. A Class 2 CRISPR system comprising:

(i) a single polynucleotide that comprises (a) a targeting region comprising deoxyribonucleic acid (DNA) or ribonucleic acid (RNA); and (b) an activating region adjacent to said targeting region comprising DNA; wherein said activating region comprises a stem loop structure or comprises a stem and a bulge, and is capable of binding with a site-directed polypeptide selected from the group consisting of Cas9 and Cpf1; and

(ii) a site-directed polypeptide selected from the group consisting of Cas9 and Cpf1.

8. The Class 2 CRISPR system of claim 7, wherein said targeting region comprises a mixture of DNA and RNA.

9. The Class 2 CRISPR system of claim 7, wherein said activating region comprises a mixture of DNA and RNA.

10. The Class 2 CRISPR system of claim 7, wherein said targeting region comprises a mixture of DNA and RNA and said activating region comprises a mixture of DNA and RNA.

11. The Class 2 CRISPR system of claim 7, further comprising a donor polynucleotide.

12. The Class 2 CRISPR system of claim 7, wherein said targeting region comprises a mixture of DNA and RNA and said activating region comprises a compound selected from the group consisting of phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, alkylphosphonates, 5'-alkylene phosphonates, chiral phosphonates, phosphinates, phosphoramidates, 3'-amino phosphoramidate, amino alkylphosphoramidates, phosphorodiamidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates, and boranophosphates.

13. The Class 2 CRISPR system of claim 7, wherein said activating region comprises a lower stem, a bulge, an upper stem, a nexus, and a hairpin and wherein the site-directed polypeptide is Cas9.

14. The Class 2 CRISPR system of claim 7, wherein said activating region comprises a stem loop structure and wherein the site-directed polypeptide is Cpf1.

15. A method of modifying a target nucleic acid molecule comprising: contacting the target nucleic acid molecule having a target sequence with (i) a single polynucleotide comprising (a) a targeting region that comprises deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and configured to hybridize to the target sequence; and (b) an activating region adjacent to the targeting region that comprises DNA and wherein the activating region comprises a stem loop structure; and (ii) a Cpf1, wherein the Cpf1 binds with the activating region of the single polynucleotide, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs (A) *in vitro*, (B) in a non-human cell, or (C) in an isolated cell and wherein the target nucleic acid molecule is cleaved.

16. The method of claim 15, wherein the target nucleic acid molecule comprises DNA.

17. The method of claim 15, wherein the target nucleic acid molecule comprises RNA.

18. The method of claim 15, wherein the target nucleic acid molecule comprises a mixture of DNA and RNA.

19. The method of claim 15, wherein the targeting region comprises a mixture of DNA and RNA.

20. The method of claim 15, wherein the activating region comprises a mixture of DNA and RNA.

21. The method of claim 15, wherein the targeting region comprises a mixture of DNA and RNA and the activating region comprises a mixture of DNA and RNA.

22. The method of claim 15, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs *in vitro*.

23. The method of claim 15, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs in a non-human cell.

24. The method of claim 15, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs in an isolated cell.

25. The method of claim 24, wherein the cell is selected from the group consisting of a bacterial cell, an archaeal cell, a plant cell, an algal cell, a fungal cell, an invertebrate cell, a vertebrate cell, a mammalian cell and a human cell.

26. The method of claim 15, further comprising providing a donor polynucleotide.

27. A method of modulating transcription of at least one gene within a target nucleic acid molecule comprising: contacting the target nucleic acid molecule having a target sequence with (i) a single polynucleotide comprising (a) a targeting region that comprises deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and configured to hybridize to the target sequence, wherein the target sequence is within the open reading frame sequence of the at least one gene or is within a promoter sequence of the at least one gene; and (b) an activating region adjacent to the targeting region that comprises DNA and wherein the activating region comprises a stem loop structure; and (ii) a Cpf1, wherein the Cpf1 has no nuclease activity, wherein the Cpf1 binds with the activating region of the single polynucleotide, wherein the targeting region of the single polynucleotide hybridizes to the target sequence, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs (A) *in vitro*, (B) in a non-

human cell, or (C) in an isolated cell and wherein the transcription of at least one gene within the target nucleic acid molecule is modulated.

28. The method of claim 27, wherein the targeting region comprises a mixture of DNA and RNA.

29. The method of claim 27, wherein the activating region comprises a mixture of DNA and RNA.

30. The method of claim 27, wherein the targeting region comprises a mixture of DNA and RNA and the activating region comprises a mixture of DNA and RNA.

31. The method of claim 27, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs *in vitro*.

32. The method of claim 27, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs in a non-human cell.

33. The method of claim 27, wherein the contacting of the target nucleic acid molecule with the single polynucleotide and the Cpf1 occurs in an isolated cell.

34. The method of claim 33, wherein the cell is selected from the group consisting of a bacterial cell, an archaeal cell, a plant cell, an algal cell, a fungal cell, an invertebrate cell, a vertebrate cell, a mammalian cell and a human cell.

35. A method of modifying a target nucleic acid molecule in a non-human organism, an isolated cell, or in vitro, wherein said method comprises: introducing into a cell (i) a single polynucleotide comprising (a) a targeting region configured to hybridize to a target sequence within the target nucleic acid, and (b) an activating region adjacent to the targeting region, wherein the activating region comprises deoxyribose nucleic acid (DNA) and wherein the activating region comprises a stem and a bulge; and (ii) a Cas9, wherein the Cas9 binds with the

activating region of the single polynucleotide, wherein the targeting region of the single polynucleotide hybridizes to the target sequence and wherein the target nucleic acid is cleaved.

36. The method of claim 35 wherein the Cas9 is encoded by an expression vector comprising a coding sequence for the Cas9.

37. The method of claim 35 wherein the single polynucleotide and Cas9 form a complex prior to introduction into the cell.

38. The method of claim 35 wherein the Cas9 comprises a nuclear localization signal (NLS).

39. The method of claim 35 wherein the single polynucleotide and Cas9 are introduced into the cell by lipofection, electroporation, nucleofection, microinjection, biolistics, liposomes, immunoliposomes, polycation, lipid:nucleic acid conjugates, or combinations thereof.

40. The method of claim 35 wherein the activating region comprises a compound selected from the group consisting of phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, alkylphosphonates, 5'-alkylene phosphonates, chiral phosphonates, phosphinates, phosphoramidates, 3'-amino phosphoramidate, amino alkylphosphoramidates, phosphorodiamidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates, and boranophosphates.

41. The method of claim 35 wherein the activating region comprises a lower stem, a bulge, an upper stem, a nexus, and a hairpin.

42. The method of claim 35 wherein the cell is selected from the group consisting of a bacterial cell, an archaeal cell, a plant cell, an algal cell, a fungal cell, an invertebrate cell, a vertebrate cell, a mammalian cell, and a human cell.

43. The method of claim 35 wherein the targeting region comprises ribonucleic acid (RNA).

44. The method of claim 35 further comprising introducing a donor polynucleotide into the cell.

45. A method of modulating transcription of at least one gene within a target nucleic acid molecule in a non-human organism, an isolated cell, or in vitro, wherein said method comprises: introducing into a cell (i) a single polynucleotide comprising (a) a targeting region configured to hybridize to a target sequence within the open reading frame of the at least one gene or a target sequence within the promoter sequence of the at least one gene, and (b) an activating region adjacent to the targeting region, wherein the activating region comprises deoxyribose nucleic acid (DNA) and wherein the activating region comprises a stem and a bulge; and (ii) a Cas9, wherein the Cas9 has no nuclease activity, wherein the Cas9 binds with the activating region of the single polynucleotide, wherein the targeting region of the single polynucleotide hybridizes to the target sequence and wherein the transcription of at least one gene within the target nucleic acid molecule is modulated.

46. The method of claim 45 wherein the Cas9 is encoded by an expression vector comprising a coding sequence for the Cas9.

47. The method of claim 45 wherein the single polynucleotide and Cas9 form a complex prior to introduction into the cell.

48. The method of claim 45 wherein the Cas9 comprises a nuclear localization signal (NLS).

49. The method of claim 45 wherein the single polynucleotide and Cas9 are introduced into the cell by lipofection, electroporation, nucleofection, microinjection, biolistics, liposomes, immunoliposomes, polycation, lipid:nucleic acid conjugates or combinations thereof.

50. The method of claim 45 wherein the activating region comprises a compound selected from the group consisting of phosphorothioates, chiral phosphorothioates, phosphorodithioates,

phosphotriesters, aminoalkylphosphotriesters, alkylphosphonates, 5'-alkylene phosphonates, chiral phosphonates, phosphinates, phosphoramidates, 3'-amino phosphoramidate, amino alkylphosphoramidates, phosphorodiamidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates, and boranophosphates.

51. The method of claim 45 wherein the activating region comprises a lower stem, a bulge, an upper stem, a nexus, and a hairpin.

52. The method of claim 45 wherein the cell is selected from the group consisting of a bacterial cell, an archaeal cell, a plant cell, an algal cell, a fungal cell, an invertebrate cell, a vertebrate cell, a mammalian cell, and a human cell.

53. The method of claim 45 wherein the targeting region comprises RNA.