

COMPOSITIONS COMPRISING A CAS12i2 VARIANT POLYPEPTIDE AND USES THEREOF

SEQUENCE LISTING

5 The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 31, 2021, is named 51451-008WO5_Sequence_Listing_03_30_2021_ST25, and is 1,589,401 bytes in size.

BACKGROUND

10 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) genes, collectively known as CRISPR-Cas or CRISPR/Cas systems, are adaptive immune systems in archaea and bacteria that defend particular species against foreign genetic elements.

SUMMARY

15 It is against the above background that the present disclosure provides certain advantages and advancements over the prior art.

 Although the disclosure disclosed herein is not limited to specific advantages or functionalities, in one aspect, the present disclosure provides a variant Cas12i2 polypeptide comprising a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3 to 146 or any one of SEQ ID
20 NOs: 495 to 512.

 In one example of the variant Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises the sequence set forth in SEQ ID NO: 4.

 In another example of the variant Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises the sequence set forth in SEQ ID NO: 5.

25 In another example of the variant Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises the sequence set forth in SEQ ID NO: 495.

 In another example of the variant Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises the sequence set forth in SEQ ID NO: 496.

30 In another example of the variant Cas12i2 polypeptide, the variant Cas12i2 polypeptide further comprises one or more substitution of Table 2.

 In another example of the variant Cas12i2 polypeptide, the variant Cas12i2 polypeptide is a variant of a Cas12i2 polypeptide comprising the sequence set forth in SEQ ID NO: 2.

 In one aspect, the present disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide as described herein, wherein the composition further comprises an RNA guide or a
35 nucleic acid encoding the RNA guide, wherein the RNA guide comprises a direct repeat sequence and a spacer sequence.

In one example of the composition, the direct repeat sequence comprises a nucleotide sequence with at least 95% sequence identity to any one of SEQ ID NO: 492-494.

In another example of the composition, the direct repeat sequence comprises a nucleotide sequence set forth in any one of SEQ ID NO: 492-494.

5 In another example of the composition, the spacer sequence comprises between about 11 and about 50 nucleotides.

In another example of the composition, the spacer sequence comprises between about 15 and about 35 nucleotides.

10 In another example of the composition, the spacer sequence binds to a target nucleic acid sequence, wherein the target nucleic acid sequence is adjacent to a 5'-NTTN-3' sequence.

In another example of the variant Cas12i2 polypeptide or the composition, the variant Cas12i2 polypeptide further comprises at least one nuclear localization signal (NLS), at least one nuclear export signal (NES), or at least one NLS and at least one NES.

15 In another example of the variant Cas12i2 polypeptide or the composition, the variant Cas12i2 polypeptide further comprises a peptide tag, a fluorescent protein, a base-editing domain, a DNA methylation domain, a histone residue modification domain, a localization factor, a transcription modification factor, a light-gated control factor, a chemically inducible factor, or a chromatin visualization factor.

20 In another example of the composition, the composition is present in a delivery system comprising a nanoparticle, a liposome, an exosome, a microvesicle, or a gene-gun.

In one aspect, the present disclosure yet further provides a nucleic acid molecule encoding a variant Cas12i2 polypeptide as described herein.

In one aspect, the present disclosure yet further provides a cell comprising a composition or variant Cas12i2 polypeptide as described herein.

25 In one example of the cell, the cell is a eukaryotic cell or a prokaryotic cell.

In another example of the cell, the cell is a mammalian cell or a plant cell.

In another example of the cell, the cell is a human cell.

In one aspect, the present disclosure yet further provides a composition or formulation comprising a variant Cas12i2 polypeptide as described herein, and optionally an RNA guide and/or a cell.

30 In one aspect, the present disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a complex, and wherein the variant Cas12i2 polypeptide exhibits increased complex formation with the RNA guide as compared to a parent polypeptide.

35 In one aspect, the present disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a complex, and wherein the Cas12i2 variant polypeptide exhibits increased binding affinity to the RNA guide, as compared to a parent polypeptide.

In one aspect, the present disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a complex, and the Cas12i2 variant polypeptide and the RNA guide exhibit increased protein-RNA interactions, as compared to a parent polypeptide and the RNA guide.

5 In one example of the composition, the variant Cas12i2 polypeptide exhibits increased complex formation, increased binding affinity to the RNA guide and/or increased stability over a range of temperatures, e.g., 20°C to 65°C.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased complex formation, increased binding affinity to the RNA guide and/or increased stability over a range of
10 incubation times.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased complex formation, increased binding affinity to the RNA guide and/or increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased
15 complex formation, increased binding affinity to the RNA guide and/or increased stability when a T_m value of the variant binary complex is at least 8°C greater than the T_m value of the parent complex.

In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

In another example of the composition, the variant Cas12i2 polypeptide exhibits equivalent to or
20 greater enzymatic activity than the parent polypeptide.

In another example of the composition, the equivalent to or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased stability and/or protein-RNA interactions.

25 In another example of the composition, the variant Cas12i2 polypeptide further lacks enzymatic activity.

In another example of the composition, the variant Cas12i2 polypeptide further exhibits increased enzymatic activity.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased RNA
30 guide complex formation, RNA guide binding activity and/or RNA guide binding specificity.

In another example of the composition, the variant Cas12i2 polypeptide exhibits altered on-target binding.

In another example of the composition, the variant Cas12i2 polypeptide exhibits altered off-target
binding.

35 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary

complex, and wherein the variant binary complex exhibits decreased complex dissociation than a complex formed by a parent polypeptide and the RNA guide.

The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary
5 complex, and wherein the RNA guide exhibits decreased dissociation from the variant Cas12i2 polypeptide than an RNA guide of a parent complex.

The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits increased stability than a complex formed by a
10 parent polypeptide and the RNA guide.

In one example of the composition, the variant binary complex exhibits increased ternary complex formation, increased binding affinity to the target nucleic acid and/or increased stability over a range of temperatures, e.g., 20°C to 65°C.

In another example of the composition, the variant binary complex exhibits increased stability
15 over a range of incubation times.

In another example of the composition, the variant binary complex exhibits increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation, increased binding affinity to the target nucleic acid and/or increased stability when a T_m
20 value of the variant binary complex is at least 8°C greater than the T_m value of the parent complex.

In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

In another example of the composition, the variant Cas12i2 polypeptide exhibits equivalent or greater enzymatic activity than the parent polypeptide.

25 In another example of the composition, the equivalent or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased stability and/or protein-RNA interactions.

30 In another example of the composition, the variant Cas12i2 polypeptide further lacks enzymatic activity.

In another example of the composition, the variant Cas12i2 polypeptide further exhibits increased enzymatic activity.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased RNA guide complex formation, RNA guide binding activity and/or RNA guide binding specificity.

35 In another example of the composition, the variant Cas12i2 polypeptide exhibits altered on-target binding.

In another example of the composition, the variant Cas12i2 polypeptide exhibits altered off-target binding.

The disclosure yet further provides a method of complexing a variant Cas12i2 polypeptide as described herein with an RNA guide, e.g., RNA guide, as described herein.

5 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, an RNA guide and a target nucleic acid, wherein the variant Cas12i2 polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits increased ternary complex formation with the target nucleic acid as compared to a parent binary complex.

10 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, an RNA guide and a target nucleic acid, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits increased binding affinity to the target nucleic acid, as compared to a parent binary complex.

15 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, an RNA guide and target nucleic acid, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibit increased protein-RNA interactions as compared to a parent binary complex.

20 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, an RNA guide and target nucleic acid, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibit increased protein-DNA interactions as compared to a parent binary complex.

In one example of the composition, the variant binary complex exhibits increased ternary complex formation, increased binding affinity to the target nucleic acid and/or increased stability over a range of temperatures, e.g., 20°C to 65°C.

25 In another example of the composition, the variant binary complex exhibits increased ternary complex formation, increased binding affinity to the target nucleic acid and/or increased stability over a range of incubation times.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation, increased binding affinity to the target nucleic acid and/or increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

30 In another example of the composition, the variant binary complex exhibits increased ternary complex formation, increased binding affinity to the target nucleic acid and/or increased stability when a T_m value of the binary complex is at least 8°C greater than the T_m value of the parent binary complex.

In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

35 In another example of the composition, the variant binary complex exhibits equivalent to or greater enzymatic activity than the parent binary complex.

In another example of the composition, the equivalent to or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

In another example of the composition, the variant binary complex exhibits increased stability and/or protein-RNA interactions.

5 In another example of the composition, the variant binary complex exhibits increased stability and/or protein-DNA interactions.

In another example of the composition, the variant binary complex further lacks enzymatic activity.

10 In another example of the composition, the variant binary complex further exhibits increased enzymatic activity.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased RNA guide complex formation, RNA guide binding activity and/or RNA guide binding specificity.

In another example of the composition, the variant binary complex exhibits increased target nucleic acid complex formation, target nucleic acid activity and/or target nucleic acid specificity.

15 In another example of the composition, the variant binary complex exhibits altered on-target binding.

In another example of the composition, the variant binary complex exhibits altered off-target binding

20 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, an RNA guide and target nucleic acid, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, wherein the variant binary complex and target nucleic acid form a variant ternary complex, and wherein the variant ternary complex exhibits decreased complex dissociation than a parent ternary complex.

25 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, an RNA guide and target nucleic acid, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, wherein the variant binary complex and target nucleic acid form a variant ternary complex, and wherein the target nucleic acid exhibits decreased dissociation from the variant ternary complex than a parent ternary complex.

30 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, an RNA guide and target nucleic acid, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, wherein the variant binary complex and target nucleic acid form a variant ternary complex, and wherein the variant ternary complex exhibits increased stability than a parent ternary complex.

35 In one example of the composition, the variant ternary complex exhibits increased stability over a range of temperatures, e.g., 20°C to 65°C.

In another example of the composition, the variant ternary complex exhibits increased stability over a range of incubation times.

In another example of the composition, the variant ternary complex exhibits increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

In another example of the composition, the variant ternary complex exhibits increased stability when a T_m value of the variant ternary complex is at least 8°C greater than the T_m value of the parent ternary complex.

In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

In another example of the composition, the variant Cas12i2 polypeptide exhibits equivalent or greater enzymatic activity than the parent polypeptide.

In another example of the composition, the equivalent or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased stability and/or protein-RNA interactions.

In another example of the composition, the variant binary complex exhibits increased stability and/or protein-DNA interactions.

In another example of the composition, the variant ternary complex exhibits increased stability.

In another example of the composition, the variant binary complex further lacks enzymatic activity.

In another example of the composition, the variant binary complex further exhibits increased enzymatic activity.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased RNA guide complex formation, RNA guide binding activity and/or RNA guide binding specificity.

In another example of the composition, the variant binary complex exhibits increased target nucleic acid complex formation, target nucleic acid binding activity and/or target nucleic acid binding specificity.

In another example of the composition, the variant binary complex exhibits altered on-target binding.

In another example of the composition, the variant binary complex exhibits altered off-target binding.

The disclosure yet further provides a method of complexing a variant binary complex as described herein with a target nucleic acid, e.g., DNA, as described herein.

The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the variant Cas12i2 polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits increased binding affinity to a target nucleic acid as compared to a parent binary complex.

The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits increased target binding affinity to a target locus of a target nucleic acid as compared to a parent binary complex.

In one example of the composition, the variant binary complex exhibits increased ternary complex formation and/or increased stability over a range of temperatures, e.g., 20°C to 65°C.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation and/or increased stability over a range of incubation times.

5 In another example of the composition, the variant binary complex exhibits increased ternary complex formation and/or increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation and/or increased stability when a T_m value of the binary complex is at least 8°C greater than the T_m value of the parent binary complex.

10 In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

In another example of the composition, the variant binary complex exhibits equivalent to or greater enzymatic activity than the parent binary complex.

15 In another example of the composition, the equivalent to or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

In another example of the composition, the variant binary complex exhibits increased stability and/or protein-RNA interactions.

In another example of the composition, the variant binary complex exhibits increased stability and/or protein-DNA interactions.

20 In another example of the composition, the variant binary complex further lacks enzymatic activity.

In another example of the composition, the variant binary complex further exhibits increased enzymatic activity.

25 In another example of the composition, the variant Cas12i2 polypeptide exhibits increased RNA guide complex formation, RNA guide binding activity and/or RNA guide binding specificity.

In another example of the composition, the variant binary complex exhibits increased target nucleic acid complex formation, target nucleic acid activity and/or target nucleic acid specificity.

In another example of the composition, the variant binary complex exhibits altered on-target binding.

30 In another example of the composition, the variant binary complex exhibits altered off-target binding.

The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the variant binary complexes specifically bind with two or more target loci of a target nucleic acid.

35 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the variant binary

complexes exhibit increased on-target binding of two or more target loci of a target nucleic acid as compared to parent binary complexes.

5 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the variant binary complexes exhibit increased on-target binding with two or more target loci of a target nucleic acid as compared to parent binary complexes.

10 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the variant binary complexes exhibit on-target ternary complex formation with two or more target loci of a target nucleic acid.

15 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the variant binary complexes exhibit increased ternary complex formation with two or more target loci of a target nucleic acid as compared to parent binary complexes.

20 In one example of the composition, the variant binary complex exhibits increased ternary complex formation with the target nucleic acid and/or increased stability over a range of temperatures, e.g., 20°C to 65°C.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation with the target nucleic acid and/or increased stability over a range of incubation times.

25 In another example of the composition, the variant binary complex exhibits increased ternary complex formation with the target nucleic acid and/or increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation with the target nucleic acid and/or increased stability when a T_m value of the binary complex is at least 8°C greater than the T_m value of the parent binary complex.

30 In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

In another example of the composition, the variant Cas12i2 polypeptide exhibits equivalent or greater enzymatic activity than the parent polypeptide.

In another example of the composition, the equivalent or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

35 In another example of the composition, the variant Cas12i2 polypeptide exhibits increased stability and/or protein-RNA interactions.

In another example of the composition, the variant binary complex exhibits increased stability and/or protein-DNA interactions.

In another example of the composition, the variant binary complex further lacks enzymatic activity.

5 In another example of the composition, the variant binary complex further exhibits increased enzymatic activity.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased RNA guide complex formation, RNA guide binding activity and/or RNA guide binding specificity.

10 In another example of the composition, the variant binary complex exhibits increased target nucleic acid ternary complex formation, target nucleic acid binding affinity and/or target nucleic acid binding specificity.

In another example of the composition, the variant binary complex exhibits altered on-target binding.

15 In another example of the composition, the variant binary complex exhibits altered off-target binding.

The disclosure yet further provides a method of complexing a variant binary complex described herein with a target nucleic acid, e.g., DNA, as described herein.

20 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits increased on-target binding affinity to a target locus of a target nucleic acid as compared to a parent binary complex.

25 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the variant Cas12i2 polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits decreased binding affinity to a non-target locus of a target nucleic acid as compared to a parent binary complex.

30 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the variant Cas12i2 polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits increased activity at an on-target locus of a target nucleic acid as compared to a parent binary complex.

The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the variant Cas12i2 polypeptide and the RNA guide form a variant binary complex, and wherein the variant binary complex exhibits decreased activity at a non-target locus of a target nucleic acid as compared to a parent binary complex.

35 In one example of the composition, the variant binary complex exhibits increased ternary complex formation at the target locus and/or increased stability over a range of temperatures, e.g., 20°C to 65°C.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation at the target locus and/or increased stability over a range of incubation times.

In another example of the composition, the variant binary complex exhibits increased ternary complex formation at the target locus and/or increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

5 In another example of the composition, the variant binary complex exhibits increased ternary complex formation at the target locus and/or increased stability when a T_m value of the binary complex is at least 8°C greater than the T_m value of the parent binary complex.

In another example of the composition, the parent binary complex comprises a parent polypeptide that comprises the amino acid sequence of SEQ ID NO: 2.

10 In another example of the composition, the variant Cas12i2 polypeptide exhibits equivalent or greater enzymatic activity than the parent polypeptide.

In another example of the composition, the equivalent or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

In another example of the composition, the variant binary complex exhibits increased stability and/or protein-RNA interactions.

15 In another example of the composition, the variant binary complex exhibits increased stability and/or protein-DNA interactions.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased binary complex formation, RNA guide binding activity and/or RNA guide binding specificity.

20 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form a plurality of variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the plurality of variant binary complexes exhibit increased on-target binding to two or more target loci of a target nucleic acid as compared to a plurality of parent binary complexes.

25 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form a plurality of variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the plurality of variant binary complexes exhibit decreased off-target binding to two or more non-target loci of a target nucleic acid as compared to a plurality of parent binary complexes.

30 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form a plurality of variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the plurality of variant binary complexes exhibit increased on-target activity at two or more target loci of a target nucleic acid as compared to a plurality of parent binary complexes.

35 The disclosure yet further provides a composition comprising a plurality of variant Cas12i2 polypeptides and two or more distinct RNA guides, wherein the distinct RNA guides individually form a plurality of variant binary complexes with separate variant Cas12i2 polypeptides, and wherein the

plurality of variant binary complexes exhibit decreased off-target activity at two or more non-target loci of a target nucleic acid as compared to a plurality of parent binary complexes.

In one example of the composition, the plurality of variant binary complexes exhibit increased ternary complex formation at the target loci of the target nucleic acid and/or increased stability over a range of temperatures, e.g., 20°C to 65°C.

In another example of the composition, the plurality of variant binary complexes exhibit increased ternary complex formation at the target loci of the target nucleic acid and/or increased stability over a range of incubation times.

In another example of the composition, the plurality of variant binary complexes exhibit increased ternary complex formation at the target loci the target nucleic acid and/or increased stability in a buffer having a pH in a range of about 7.3 to about 8.6.

In another example of the composition, the plurality of variant binary complexes exhibit increased ternary complex formation at the target loci of the target nucleic acid and/or increased stability when a T_m value of the binary complex is at least 8°C greater than the T_m value of the parent binary complex.

In another example of the composition, the plurality of parent binary complexes comprise a parent polypeptide that comprises the amino acid sequence of SEQ ID NO: 2.

In another example of the composition, the variant Cas12i2 polypeptide exhibits equivalent or greater enzymatic activity than the parent polypeptide.

In another example of the composition, the equivalent or greater enzymatic activity occurs at a temperature range from about 20°C to about 90°C.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased stability and/or protein-RNA interactions.

In another example of the composition, the plurality of variant binary complexes exhibit increased stability and/or protein-DNA interactions.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased binary complex formation, RNA guide binding activity and/or RNA guide binding specificity.

The disclosure yet further provides a method of complexing a variant binary complex as described herein with a target nucleic acid, e.g., DNA, as described herein.

The disclosure yet further provides a method of complexing a plurality of variant binary complexes described herein with a target nucleic acid, e.g., DNA, as described herein.

In one example of a composition or Cas12i2 polypeptide described herein, the variant Cas12i2 polypeptide comprises at least one of a D581, G624, F626, D835, L836, P868, S879, D911, I926, V1020, V1030, E1035, and S1046 substitution of amino acid sequence of SEQ ID NO: 2.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises at least one of a D581G, D581R, G624R, F626G, F626R, D835G, D835R, L836G, L836R, P868G, P868R, P868T, S879G, S879R, D911G, D911R, I926G, I926R, V1020

G, V1020R, V1030G, V1030R, E1035G, E1035R, S1046G, and S1046R substitution of amino acid sequence of SEQ ID NO: 2.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises at least one of a D581R, G624R, F626R, P868T, D911R, I926R, V1030G, E1035R, and S1046G substitution of amino acid sequence of SEQ ID NO: 2.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises at least one substitution listed in Table 1.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises any one amino acid sequence of SEQ ID NO: 3 to 146 or any one amino acid sequence of SEQ ID NOs: 495 to 512.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide comprises at least one of an epitope peptide, nuclear localization signal, and nuclear export signal.

In another example of the composition, the RNA guide comprises a DNA targeting sequence.

In another example of the composition, the DNA targeting sequence is an RNA guide.

In another example of the composition, the DNA targeting sequence is between 13 to 30 nucleotides.

In another example of the composition, the RNA guide comprises a direct repeat sequence linked to a DNA targeting sequence.

In another example of the composition, the composition further comprises a target nucleic acid.

In another example of the composition, the target nucleic acid is present in a cell.

In another example of the composition, the variant Cas12i2 polypeptide and RNA guide are encoded in a vector, e.g., expression vector.

The disclosure yet further provides a cell comprising a composition as described herein.

The disclosure yet further provides a method of expressing a vector as described herein.

The disclosure yet further provides a method of producing a composition as described herein.

The disclosure yet further provides a method of delivering a composition as described herein.

The disclosure yet further provides a kit or system comprising a composition as described herein or one or more component thereof.

In one example of a composition described herein, the RNA guide comprises or consists of, or about, 43 nucleotides.

In another example of the composition, the RNA guide is a tracr-less RNA guide.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide further exhibits about 40x greater enzymatic activity than parent polypeptide.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide exhibits increased on-target specificity as compared to the parent polypeptide.

In another example of the composition or Cas12i2 polypeptide, the variant Cas12i2 polypeptide exhibits decreased off-target specificity as compared to the parent polypeptide.

In another example of the composition, the variant Cas12i2 polypeptide selectively induces a deletion adjacent to a 5'-NTTN-3' sequence, wherein N is any nucleotide.

In another example of the composition, the deletion is downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the parent polypeptide does not induce the deletion.

5 In another example of the composition, the length of the deletion is greater than the length of a Cas9 polypeptide-induced deletion.

In another example of the composition, the deletion is in a gene of a cell.

In another example of the composition, the deletion is up to about 40 nucleotides in length.

10 In another example of the composition, the deletion is from about 4 nucleotides to 40 nucleotides in length.

In another example of the composition, the deletion is from about 4 nucleotides to 25 nucleotides in length.

In another example of the composition, the deletion is from about 10 nucleotides to 25 nucleotides in length.

15 In another example of the composition, the deletion is from about 10 nucleotides to 15 nucleotides in length.

In another example of the composition, the deletion starts within about 5 nucleotides to about 15 nucleotides of the 5'-NTTN-3' sequence.

20 In another example of the composition, the deletion starts within about 5 nucleotides to about 10 nucleotides of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 10 nucleotides to about 15 nucleotides of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 5 nucleotides to about 15 nucleotides downstream of the 5'-NTTN-3' sequence.

25 In another example of the composition, the deletion starts within about 5 nucleotides to about 10 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 10 nucleotides to about 15 nucleotides downstream of the 5'-NTTN-3' sequence.

30 In another example of the composition, the deletion ends within about 20 nucleotides to about 30 nucleotides of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion ends within about 20 nucleotides to about 25 nucleotides of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion ends within about 25 nucleotides to about 30 nucleotides of the 5'-NTTN-3' sequence.

35 In another example of the composition, the deletion ends within about 20 nucleotides to about 30 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion ends within about 20 nucleotides to about 25 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion ends within about 25 nucleotides to about 30 nucleotides downstream of the 5'-NTTN-3' sequence.

5 In another example of the composition, the deletion starts within about 5 nucleotides to about 15 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 20 nucleotides to about 30 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 5 nucleotides to about 15 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 20 nucleotides to about 25
10 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 5 nucleotides to about 15 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 25 nucleotides to about 30 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 5 nucleotides to about 10
15 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 20 nucleotides to about 30 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 5 nucleotides to about 10 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 20 nucleotides to about 25 nucleotides downstream of the 5'-NTTN-3' sequence.

20 In another example of the composition, the deletion starts within about 5 nucleotides to about 10 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 25 nucleotides to about 30 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 10 nucleotides to about 15 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 20 nucleotides to about 30
25 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 10 nucleotides to about 15 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 20 nucleotides to about 25 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the deletion starts within about 10 nucleotides to about 15
30 nucleotides downstream of the 5'-NTTN-3' sequence and ends within about 25 nucleotides to about 30 nucleotides downstream of the 5'-NTTN-3' sequence.

In another example of the composition, the 5'-NTTN-3' sequence is 5'-NTTY-3', 5'-NTTC-3',
5'-NTTT-3', 5'-NTTA-3', 5'-NTTB-3', 5'-NTTG-3', 5'-CTTY-3', 5'-DTTR-3', 5'-CTTR-3', 5'-DTTT-
3', 5'-ATTN-3', or 5'-GTTN-3', wherein Y is C or T, B is any nucleotide except for A, D is any
35 nucleotide except for C, and R is A or G.

In another example of the composition, the 5'-NTTN-3' sequence is 5'-CTTT-3', 5'-CTTC-3',
5'-GTTT-3', 5'-GTTC-3', 5'-TTTC-3', 5'-GTTA-3', or 5'-GTTG-3'.

In another example of the composition, the deletion is in an exon of the gene, e.g., B2M, TRAC, PDCD1.

In another example of the composition, the deletion overlaps with a mutation in the gene.

In another example of the composition, the deletion overlaps with an insertion in the gene.

5 In another example of the composition, the deletion removes a repeat expansion of the gene.

In another example of the composition, the deletion disrupts one or both alleles of the gene.

In another example of the composition, the deletion is induced in a eukaryotic cell or a prokaryotic cell.

10 In another example of the composition, the deletion is induced in an animal cell, a plant cell, or a fungal cell or the cell is derived from an animal cell, a plant cell, or a fungal cell.

In another example of the composition, the deletion is induced in a mammalian cell or derived from a mammalian cell.

In another example of the composition, the deletion is induced in a human cell or derived from a human cell.

15 In another example of the composition, the deletion is induced in a primary cell.

In another example of the composition, the deletion is induced in a cell line.

In another example of the composition, the deletion is induced in a T cell.

20 In another example of the composition, the deletion is induced in a stem cell (e.g., a totipotent/omnipotent stem cell, a pluripotent stem cell, a multipotent stem cell, an oligopotent stem cell, or an unipotent stem cell), a differentiated cell, or a terminally differentiated cell.

In another example of the composition, 2 or more (e.g., multiplexed targeted deletions) deletions are induced.

The disclosure yet further provides a method of obtaining a deletion in a cell, wherein the method comprises contacting a variant Cas12i2 polypeptide or complex as described herein with DNA in the cell.

25 The disclosure yet further provides a composition or formulation comprising a variant Cas12i2 polypeptide as described herein, an RNA guide, and a cell.

The disclosure yet further provides a method of producing a composition as described herein.

The disclosure yet further provides a method of complexing a variant Cas12i2 polypeptide as described herein with an RNA guide, such as an RNA guide described herein.

30 The disclosure yet further provides a method of complexing a variant binary complex as described herein with a target nucleic acid.

The disclosure yet further provides a method of delivering a composition as described herein.

35 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, wherein the variant Cas12i2 polypeptide comprises a substitution that increases interactions between the variant Cas12i2 polypeptide and a nucleic acid, as compared to a parent polypeptide.

In one example of the composition, the variant Cas12i2 polypeptide comprises the sequence set forth in SEQ ID NO: 4.

In another example of the composition, the variant Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

5 In another example of the composition, the interactions are electrostatic interactions.

In another example of the composition, the interactions are non-specific interactions.

In another example of the composition, the interactions are aromatic, hydrophobic, Van der Waals, and/or cation-pi interactions.

10 In another example of the composition, the substitution is in or adjacent to a nucleic acid interface.

In another example of the composition, the nucleic acid is an RNA guide comprising a direct repeat sequence and a spacer sequence.

In another example of the composition, the direct repeat sequence comprises a nucleotide sequence with at least 95% sequence identity to any one of SEQ ID NO: 492-494.

15 In another example of the composition, the direct repeat sequence comprises a nucleotide sequence set forth in any one of SEQ ID NO: 492-494.

In another example of the composition, the substitution increases interactions between the variant Cas12i2 polypeptide and the direct repeat sequence.

20 In another example of the composition, the substitution increases binary complex formation, as compared to a parent polypeptide.

In another example of the composition, a binary complex comprising the variant Cas12i2 polypeptide exhibits increased stability, as compared to a parent binary complex.

In another example of the composition, the substitution is an arginine, lysine, glutamine, asparagine, histidine, tyrosine, or serine substitution.

25 In another example of the composition, the substitution is in an RNA binding interface.

In another example of the composition, the substitution is a substitution in the Wedge domain or Rec2 domain.

In another example of the composition, the substitution is a substitution listed in Table 4.

30 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495-512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495 to 512 and further having a substitution listed in Table 4.

35 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495 to 512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495 to 512 and further has a substitution listed in Table 4.

5 In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises a sequence set forth in SEQ ID NO: 4.

In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

10 In another example of the composition, the nucleic acid is a target nucleic acid.

In another example of the composition, the nucleic acid is double-stranded DNA.

In another example of the composition, the substitution increases interactions between the variant Cas12i2 polypeptide and double-stranded DNA.

In another example of the composition, the double-stranded DNA comprises a PAM sequence.

15 In another example of the composition, the substitution increases ternary complex formation, as compared to a parent polypeptide.

In another example of the composition, a ternary complex comprising the variant Cas12i2 polypeptide exhibits increased stability, as compared to a parent ternary complex.

In another example of the composition, the substitution is an arginine, lysine, glutamine, asparagine, histidine, or serine substitution.

20 In another example of the composition, the substitution is in a double-stranded DNA binding interface.

In another example of the composition, the substitution is a substitution in the Rec1 domain, PI domain, or Wedge domain.

25 In another example of the composition, the substitution is a substitution listed in Table 5.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

30 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further having a substitution listed in Table 5.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

35 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further has a substitution listed in Table 5.

In another example of the composition, the nucleic acid is single-stranded DNA.

In another example of the composition, the single-stranded DNA comprises a non-target strand.

In another example of the composition, the single-stranded DNA comprises a target strand.

In another example of the composition, the substitution increases ternary complex formation, as compared to a parent polypeptide.

In another example of the composition, a ternary complex comprising the variant Cas12i2 polypeptide exhibits increased stability, as compared to a parent ternary complex.

In another example of the composition, the substitution is an arginine, lysine, glutamine, asparagine, histidine, or alanine substitution.

In another example of the composition, the substitution is in a single-stranded DNA binding interface.

In another example of the composition, the substitution is a substitution in the PI domain, Rec1 domain, Wedge domain, RuvC domain, Rec2 domain, or Nuc domain.

In another example of the composition, the substitution is a substitution listed in Table 6.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495 to 512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495 to 512 and further having a substitution listed in Table 6.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495 to 512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3 to 146 or in any one of SEQ ID NOs: 495 to 512 and further has a substitution listed in Table 6.

In another example of the composition, the substitution increases interactions between the variant Cas12i2 polypeptide and a DNA/RNA hybrid molecule.

In another example of the composition, the DNA/RNA hybrid molecule is a heteroduplex comprising a spacer sequence of an RNA guide and a target strand.

In another example of the composition, the substitution stabilizes the heteroduplex.

In another example of the composition, the substitution increases ternary complex formation, as compared to a parent polypeptide.

In another example of the composition, a ternary complex comprising the variant Cas12i2 polypeptide exhibits increased stability, as compared to a parent ternary complex.

In another example of the composition, the substitution is an arginine, lysine, glutamine, asparagine, histidine, or serine substitution.

In another example of the composition, the substitution is a substitution in the Rec1 domain, PI domain, Rec2 domain, or RuvC2 motif.

In another example of the composition, the substitution is a substitution listed in Table 7.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

5 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further having a substitution listed in Table 7.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

10 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further has a substitution listed in Table 7.

In another example of the composition, the substitution increases interactions between the variant Cas12i2 polypeptide and bases of (a) a double-stranded DNA duplex and/or (b) a heteroduplex comprising a spacer sequence of an RNA guide and a target strand.

15 In another example of the composition, the double-stranded DNA duplex comprises a PAM sequence.

In another example of the composition, the interactions are aromatic, hydrophobic, Van der Waals, and/or cation-pi interactions.

In another example of the composition, the substitution stabilizes the R-loop.

20 In another example of the composition, the substitution increases ternary complex formation, as compared to a parent polypeptide.

In another example of the composition, a ternary complex comprising the variant Cas12i2 polypeptide exhibits increased stability, as compared to a parent ternary complex.

25 In another example of the composition, the substitution is an arginine, lysine, tryptophan, phenylalanine, tyrosine, methionine, histidine, glutamine, threonine, or valine substitution.

In another example of the composition, the substitution is a substitution in the Wedge domain, Rec1 domain, or RuvC domain.

In another example of the composition, the substitution is a substitution listed in Table 8.

30 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further having a substitution listed in Table 8.

35 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further has a substitution listed in Table 8.

5 In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

In another example of the composition, the variant Cas12i2 polypeptide exhibits increased enzymatic activity, as compared to a parent polypeptide.

In another example of the composition, the composition further comprises an RNA guide comprising a direct repeat sequence and a spacer sequence.

10 In another example of the composition, the direct repeat sequence comprises a nucleotide sequence with at least 95% sequence identity to any one of SEQ ID NO: 492-494.

In another example of the composition, the direct repeat sequence comprises a nucleotide sequence set forth in any one of SEQ ID NO: 492-494.

15 In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises a sequence set forth in SEQ ID NO: 4. 239.

In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

20 The disclosure yet further provides a cell comprising a composition described herein.

In one example of the cell, the composition does not substantially affect viability of the cell.

The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, wherein the variant Cas12i2 polypeptide comprises a substitution that increases flexibility of the variant Cas12i2 polypeptide during DNA binding, as compared to a parent polypeptide.

25 In one example of the composition, the variant Cas12i2 polypeptide comprises the sequence set forth in SEQ ID NO: 4.

In another example of the composition, the Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

30 In another example of the composition, the substitution increases binding of the variant Cas12i2 polypeptide to DNA.

In another example of the composition, the substitution increases binding of the variant Cas12i2 polypeptide to double-stranded DNA.

In another example of the composition, the substitution increases binding of the variant Cas12i2 polypeptide to single-stranded DNA.

35 In another example of the composition, the substitution increases ternary complex formation, as compared to a parent polypeptide.

In another example of the composition, a ternary complex comprising the variant Cas12i2 polypeptide exhibits increased stability, as compared to a parent ternary complex.

In another example of the composition, the substitution is a substitution of a bulky amino acid to an amino acid with a smaller side chain.

5 In another example of the composition, the substitution is an alanine, valine, glycine, or serine substitution.

In another example of the composition, the substitution is in the Helical II domain of the variant Cas12i2 polypeptide.

In another example of the composition, the substitution is a substitution listed in Table 9.

10 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of
15 SEQ ID NOs: 495-512 and further having a substitution listed in Table 9.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further has a
20 substitution listed in Table 9.

The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, wherein the variant Cas12i2 polypeptide comprises a substitution that stabilizes a domain-domain interface that forms during ternary complex formation, as compared to a parent polypeptide.

In one example of the composition, the variant Cas12i2 polypeptide comprises the sequence set
25 forth in SEQ ID NO: 4.

In another example of the composition, the variant Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

In another example of the composition, the domain-domain interface forms when single-stranded DNA contacts an active site of the variant Cas12i2 polypeptide.

30 In another example of the composition, the domain-domain interface is a Helical II domain-Nuc domain interface.

In another example of the composition, the substitution increases ternary complex formation, as compared to a parent polypeptide.

In another example of the composition, a ternary complex comprising the variant Cas12i2
35 polypeptide exhibits increased stability, as compared to a parent ternary complex.

In another example of the composition, the substitution is an aspartic acid, glutamic acid, arginine, or lysine substitution.

In another example of the composition, the substitution is a substitution listed in Table 10.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

5 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further having a substitution listed in Table 10.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

10 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further has a substitution listed in Table 10.

In another example of the composition, the parent polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

15 In another example of the composition, the variant Cas12i2 polypeptide exhibits increased enzymatic activity, as compared to a parent polypeptide.

In another example of the composition, the composition further comprises an RNA guide comprising a direct repeat sequence and a spacer sequence.

20 In another example of the composition, the direct repeat sequence comprises a nucleotide sequence with at least 95% sequence identity to any one of SEQ ID NO: 492-494.

In another example of the composition, the direct repeat sequence comprises a nucleotide sequence set forth in any one of SEQ ID NO: 492-494.

In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises a sequence set forth in SEQ ID NO: 4.

25 In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

The disclosure yet further provides a cell comprising the composition as described herein.

In one example of the cell, the composition does not substantially affect viability of the cell.

30 The disclosure yet further provides a composition comprising a variant Cas12i2 polypeptide, wherein the variant Cas12i2 polypeptide comprises a substitution that increases on-target specificity of the variant Cas12i2 polypeptide, as compared to a parent polypeptide.

In one example of the composition, the variant Cas12i2 polypeptide comprises the sequence set forth in SEQ ID NO: 4.

35 In another example of the composition, the variant Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

In another example of the composition, the substitution increases on-target DNA binding.

- In another example of the composition, the substitution decreases off-target DNA binding.
- In another example of the composition, the substitution increases on-target ternary complex formation, as compared to a parent polypeptide.
- In another example of the composition, an on-target ternary complex comprising the variant
- 5 Cas12i2 polypeptide exhibits increased stability, as compared to a parent ternary complex.
- In another example of the composition, the substitution is a substitution of an amino acid contacting the spacer sequence of an RNA guide.
- In another example of the composition, the substitution is a substitution of a bulky amino acid to an amino acid with a smaller side chain.
- 10 In another example of the composition, the substitution is an alanine, serine, valine, glutamine, or asparagine substitution.
- In another example of the composition, the substitution is a substitution in the Wedge domain, Rec1 domain, Rec2 domain, or RuvC domain.
- In another example of the composition, the substitution is a substitution in the Helical II domain.
- 15 In another example of the composition, the substitution is a substitution listed in Table 11.
- In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.
- 20 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further having a substitution listed in Table 11.
- In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.
- 25 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further has a substitution listed in Table 11.
- In another example of the composition, the substitution decreases a catalysis rate (K_{cat}) of the variant Cas12i2 polypeptide.
- 30 In another example of the composition, the substitution is an alanine, serine, threonine, valine, leucine, methionine, asparagine, or isoleucine substitution.
- In another example of the composition, the substitution is a substitution in the Wedge domain, Rec1 domain, Rec2 domain, or RuvC domain.
- In another example of the composition, the substitution is a substitution in the RuvC domain.
- In another example of the composition, the substitution is a substitution listed in Table 12.
- 35 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further having a substitution listed in Table 12.

5 In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512.

In another example of the composition, the variant Cas12i2 polypeptide comprises a sequence set forth in any one of SEQ ID NOs: 3-146 or in any one of SEQ ID NOs: 495-512 and further has a substitution listed in Table 12.

10 In another example of the composition, the variant Cas12i2 polypeptide exhibits increased on-target enzymatic activity, as compared to a parent polypeptide.

In another example of the composition, the variant Cas12i2 polypeptide exhibits decreased off-target enzymatic activity, as compared to a parent polypeptide.

In another example of the composition, the variant Cas12i2 polypeptide exhibits off-target editing that is no more than 10% of the on-target editing.

15 In another example of the composition, the variant Cas12i2 polypeptide exhibits off-target editing that is no more than 5% of the on-target editing.

In another example of the composition, the composition further comprises an RNA guide comprising a direct repeat sequence and a spacer sequence.

20 In another example of the composition, the direct repeat sequence comprises a nucleotide sequence with at least 95% sequence identity to any one of SEQ ID NO: 492-494.

In another example of the composition, the direct repeat sequence comprises a nucleotide sequence set forth in any one of SEQ ID NO: 492-494.

In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises a sequence set forth in SEQ ID NO: 4.

25 In another example of the composition, the direct repeat sequence is set forth in SEQ ID NO: 492 and the variant Cas12i2 polypeptide comprises the sequence set forth in any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 495, and SEQ ID NO: 496.

The disclosure yet further provides a cell comprising the composition as described herein.

In one example of the cell, the composition does not substantially affect viability of the cell.

30 In another example of the composition, the variant Cas12i2 polypeptide exhibits a higher ratio of on-target binding to off-target binding, as compared to a Cas9 polypeptide.

In another example of the composition, the variant Cas12i2 polypeptide exhibits a higher ratio of on-target activity to off-target activity, as compared to a Cas9 polypeptide.

35 In another example of the composition, the variant Cas12i2 polypeptide exhibits a higher ratio of on-target editing to off-target editing, as compared to a Cas9 polypeptide.

In another example of the composition, the variant Cas12i2 polypeptide exhibits less off-target binding, as compared to a Cas9 polypeptide.

In another example of the composition, the variant Cas12i2 polypeptide exhibits less off-target activity, as compared to a Cas9 polypeptide.

In another example of the composition, the variant Cas12i2 polypeptide exhibits less off-target editing, as compared to a Cas9 polypeptide.

5 In another example of the composition, off-target binding, off-target activity, and/or off-target editing by the variant Cas12i2 polypeptide is at least 10% less than off-target binding, off-target activity, and/or off-target editing by a Cas9 polypeptide.

In another example of the composition, off-target binding, off-target activity, and/or off-target editing by the variant Cas12i2 polypeptide is at least 20% less than off-target binding, off-target activity, and/or off-target editing by a Cas9 polypeptide.

10 In another example of the composition, off-target binding, off-target activity, and/or off-target editing by the variant Cas12i2 polypeptide is at least 30% less than off-target binding, off-target activity, and/or off-target editing by a Cas9 polypeptide.

In another example of the composition, off-target binding, off-target activity, and/or off-target editing by the variant Cas12i2 polypeptide is at least 40% less than off-target binding, off-target activity, and/or off-target editing by a Cas9 polypeptide.

15 In another example of the composition, off-target binding, off-target activity, and/or off-target editing by the variant Cas12i2 polypeptide is at least 50% less than off-target binding, off-target activity, and/or off-target editing by a Cas9 polypeptide.

20 In yet another example, the disclosure provides a cell comprising a composition described herein. In one example of the cell, the composition does not substantially affect viability of the cell.

Definitions

25 The present disclosure will be described with respect to particular examples and with reference to certain Figures, but the disclosure is not limited thereto but only by the claims. Terms as set forth hereinafter are generally to be understood in their common sense unless indicated otherwise.

As used herein, the term “activity” refers to a biological activity. In some examples, the activity refers to effector activity. In some examples, activity includes enzymatic activity, e.g., catalytic ability of an effector. For example, activity can include nuclease activity. In some examples, activity includes binding activity, e.g., binding activity of an effector to an RNA guide and/or target nucleic acid.

30 As used herein, the term “adjacent to” refers to a nucleotide or amino acid sequence in close proximity to another nucleotide or amino acid sequence. In some examples, a nucleotide sequence is adjacent to another nucleotide sequence if no nucleotides separate the two sequences. In some examples, a nucleotide sequence is adjacent to another nucleotide sequence if a small number of nucleotides separate the two sequences (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides). In some examples, a first sequence is adjacent to a second sequence if the two sequences are separated by about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides. In some examples,

the term “adjacent to” is used to refer a protein residue that interacts with another protein residue. In some examples, the term “adjacent to” is used to refer a protein residue that interacts with a nucleotide or nucleic acid. In some examples, the term “adjacent to” is used to refer to a protein domain or motif that interacts with another protein domain or motif. In some examples, the term “adjacent to” is used to refer to a protein domain or motif that interacts with a nucleotide or nucleic acid sequence. As used herein, the term “adjacent to” is used to refer to the positioning of an indel (insertion/deletion) in a modified cell of the disclosure.

As used herein, the term “complex” refers to a grouping of two or more molecules. In some examples, the complex comprises a polypeptide and a nucleic acid molecule interacting with (e.g. binding to, coming into contact with, adhering to) one another.

As used herein, the term “binary complex” refers to a grouping of two molecules (e.g., a polypeptide and a nucleic acid molecule). In some examples, a binary complex refers to a grouping of a polypeptide and a targeting moiety (e.g., an RNA guide). In some examples, a binary complex refers to a ribonucleoprotein (RNP). As used herein, the term “variant binary complex” refers to the grouping of a variant Cas12i2 polypeptide and RNA guide. As used herein, the term “parent binary complex” refers to the grouping of a parent polypeptide and RNA guide or a reference polypeptide and RNA guide.

As used herein, the term “ternary complex” refers to a grouping of three molecules (e.g., a polypeptide and two nucleic acid molecules). In some examples, a “ternary complex” refers to a grouping of a polypeptide, an RNA molecule, and a DNA molecule. In some examples, a ternary complex refers to a grouping of a polypeptide, a targeting moiety (e.g., an RNA guide), and a target nucleic acid (e.g., a target DNA molecule). In some examples, a “ternary complex” refers to a grouping of a binary complex (e.g., a ribonucleoprotein) and a third molecule (e.g., a target nucleic acid). As used herein, the terms “variant ternary complex,” “variant Cas12i2 ternary complex,” and “Cas12i2 variant ternary complex” refer to the grouping of a variant Cas12i2 polypeptide, RNA guide, and target nucleic acid (e.g., a variant Cas12i2 ribonucleoprotein and target nucleic acid). As used herein, the terms “parent ternary complex,” “parent Cas12i2 ternary complex,” and “Cas12i2 parent ternary complex” refer to the grouping of a parent polypeptide, RNA guide, and target nucleic acid (e.g., a parent ribonucleoprotein and target nucleic acid) or a reference polypeptide, RNA guide, and target nucleic acid (e.g., a reference ribonucleoprotein and target nucleic acid).

As used herein, the term “deletion” refers to a loss or removal of nucleotides in a nucleic acid sequence. The deletion can be in a genome of an organism. The deletion can be in a cell. The deletion can be a DNA sequence. The deletion can be an RNA sequence. The deletion can be a frameshift mutation or a non-frameshift mutation. A Cas12i2-induced deletion described herein can refer to a deletion of up to about 100 nucleotides, such as from about 4 nucleotides and 100 nucleotides, from about 4 nucleotides and 50 nucleotides, from about 4 nucleotides and 40 nucleotides, from about 4 nucleotides and 25 nucleotides, from about 10 nucleotides and 25 nucleotides, from about 10 nucleotides and 15 nucleotides, from a nucleic acid molecule. In some examples, a Cas12i2-induced deletion described herein occurs downstream of

a 5'-NTTN-3' sequence. In some examples, the term "Cas12i2-induced" refers to a deletion that results from a DNA break induced by a Cas12i2 polypeptide. In some examples, the term "Cas12i2-induced" refers to a deletion that results from a DNA break induced by a Cas12i2 polypeptide and repaired by a cell's DNA repair machinery.

5 As used herein, the term "domain" refers to a distinct functional and/or structural unit of a polypeptide. In some examples, a domain may comprise a conserved amino acid sequence.

As used herein, the terms "editing efficiency" and "indel activity" refer to the ability of an enzyme (e.g., a variant Cas12i2 polypeptide) to introduce an indel (insertion/deletion) into a sequence. For example, in some examples, an enzyme that introduces an indel into each of ten target loci exhibits an editing efficiency of 100%. An enzyme that introduces an indel into five out of ten target loci exhibits an editing efficiency of 50%. In another example, an enzyme that introduces an indel at a target locus in 50% of a plurality of cells exhibits an editing efficiency of 50%. As used herein, the terms "editing efficiency," "indel activity," and "on-target editing" refer to the ability of an enzyme (e.g., a variant Cas12i2 polypeptide) to selectively introduce an indel at a target locus. In some examples, editing efficiency at a target locus is compared to editing efficiency at a non-target locus. In some examples, editing at a target locus is compared to editing at a non-target locus.

As used herein, the term "effector activity" refers to a biological activity. In some examples, effector activity includes enzymatic activity, e.g., catalytic ability of an effector. For example, effector activity can include nuclease activity.

20 As used herein, the term "interface" refers to one or more residues of a variant Cas12i2 polypeptide (e.g., a domain/motif or a portion of a domain/motif) in contact with (e.g., that interact with or are adjacent to) a nucleic acid molecule or a distinct domain/motif or a portion of a distinct domain/motif of the variant Cas12i2 polypeptide. In some examples, an interface is a buried surface area between adjacent domains or motifs. In some examples, an interface is a surface area between the a polypeptide and a ligand (e.g., DNA or RNA) where the polypeptide and ligand make contact. As used herein, the term "nucleic acid interface" refers to residues of the variant Cas12i2 polypeptide that are in close proximity to (e.g., are adjacent to) or interact with a nucleic acid sequence (e.g., a DNA sequence or an RNA sequence). As used herein, the term "RNA binding interface" refers to the residues of the variant Cas12i2 polypeptide that are in close proximity to (e.g., are adjacent to) or interact with an RNA guide (e.g., the direct repeat of the RNA guide). As used herein, the term "double-stranded DNA binding interface" refers to the residues of the variant Cas12i2 polypeptide that are in close proximity to (e.g., are adjacent to) and/or interact with double-stranded DNA. As used herein, the term "single-stranded DNA binding interface" refers to the residues of the variant Cas12i2 polypeptide that are in close proximity to (e.g., are adjacent to) and/or interact with single-stranded DNA. As used herein, the term "domain-domain interface" refers to a domain in close-proximity to (e.g., adjacent to) a separate domain. In some examples, a domain-domain interface (e.g., a Helical II domain-Nuc domain interface) forms upon complex formation (e.g., ternary complex formation).

As used herein, the terms “parent,” “parent polypeptide,” and “parent sequence” refer to an original polypeptide (e.g., starting polypeptide) to which an alteration is made to produce a variant Cas12i2 polypeptide of the present disclosure. In some examples, the parent is a polypeptide having an identical amino acid sequence of the variant with one or more variations at one or more specified positions. In
5 exemplary examples, variations refer to amino acid changes within the polypeptide sequence. The parent may be a naturally occurring (wild-type) polypeptide. In a particular example, the parent is a polypeptide with at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 70%, at least 72%, at least 73%, at least 74%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least
10 96%, at least 97%, at least 98%, at least 99% or 100% identity to a polypeptide of SEQ ID NO: 2. In some examples, variations may include structural changes such as linkages, fusions, or other changes that do not alter the original amino acid sequence of the parent. In some examples, the parent polypeptide sequence includes amino acid variations and structural changes.

As used herein, a “plurality of variant binary complexes” refers to a plurality of binary complexes
15 comprising a plurality of variant Cas12i2 polypeptides. A plurality of binary complexes comprising a plurality of parent polypeptides (e.g., wild-type Cas12i2 polypeptides parent of SEQ ID NO: 2) are referred to herein as a “plurality of parent binary complexes.” A plurality of Cas12i2 polypeptides, e.g., a plurality of variant Cas12i2 polypeptides, and two or more distinct targeting moieties, e.g., two or more distinct RNA guides, individually form two or more binary complexes (e.g., a plurality of binary complexes). In such
20 examples, complex formation may be accomplished simultaneously in a single composition or independently in separate compositions.

As used herein, the term “protospacer adjacent motif” or “PAM” refers to a DNA sequence adjacent to a target sequence to which a binary complex comprising a polypeptide (e.g., an enzyme such as Cas12i2 or a variant thereof) and a targeting moiety (e.g., an RNA guide) binds. In some examples, a PAM is
25 required for enzyme activity. In the case of a double-stranded target, the targeting moiety (e.g., the RNA guide) binds to a first strand of the target, and a PAM sequence as described herein is present in the second, complementary strand. For example, in some examples, the RNA guide binds to the target strand (e.g., the spacer-complementary strand), and the PAM sequence as described herein is present in the non-target strand (i.e., the non-spacer-complementary strand). In some examples, the target strand (i.e., the spacer-complementary strand) comprises a 5’-NAAN-3’ sequence.
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As used herein, the terms “reference composition,” “reference molecule,” “reference sequence,” “reference,” and “reference complex” refer to a control, such as a negative control or a parent (e.g., a parent sequence, a parent protein, a wild-type protein, or a complex comprising a parent sequence). For example, a reference molecule refers to a Cas12i2 polypeptide to which a variant Cas12i2 polypeptide is compared.
35 Likewise, a reference RNA guide refers to a targeting moiety to which a modified RNA guide is compared. The variant or modified molecule may be compared to the reference molecule on the basis of sequence (e.g., the variant or modified molecule may have X% sequence identity or homology with the reference

molecule), thermostability, or activity (e.g., the variant or modified molecule may have X% of the activity of the reference molecule). For example, where the reference molecule is a Cas12i2 polypeptide, a variant or modified molecule may be characterized as having no more than 10% of an activity of the reference Cas12i2 polypeptide or may be characterized as having at least 10% greater of an activity of the reference Cas12i2 polypeptide. Examples of reference Cas12i2 polypeptides include naturally occurring unmodified Cas12i2 polypeptides, e.g., naturally occurring Cas12i2 polypeptide from archaea or other bacterial species. In certain examples, the reference Cas12i2 polypeptide is a naturally occurring Cas12i2 polypeptide having the closest sequence identity or homology with the variant Cas12i2 polypeptide to which it is being compared. In certain examples, the reference Cas12i2 polypeptide is a parental molecule having a naturally occurring or known sequence on which a mutation has been made to arrive at the variant Cas12i2 polypeptide.

As used herein, the terms “RNA guide” or “RNA guide sequence” refer to any RNA molecule that facilitates the targeting of a polypeptide described herein to a target nucleic acid. For example, an RNA guide can be a molecule that recognizes (e.g., binds to) a target nucleic acid. An RNA guide may be designed to be complementary to a specific nucleic acid sequence. An RNA guide comprises a DNA targeting sequence and a direct repeat (DR) sequence. The terms CRISPR RNA (crRNA), pre-crRNA and mature crRNA are also used herein to refer to an RNA guide.

As used herein, the term “substantially identical” refers to a sequence, polynucleotide, or polypeptide, that has a certain degree of identity to a reference sequence.

As used herein, the term “targeting moiety” refers to a molecule or component (e.g., nucleic acid and/or RNA guide) that facilitates the targeting of another molecule or component to a target nucleic acid. In some examples, the targeting moiety specifically interacts or associates with the target nucleic acid.

As used herein, the terms “target nucleic acid,” “target sequence,” “target substrate,” and “on-target locus” refer to a nucleic acid sequence to which a targeting moiety (e.g., RNA guide) specifically binds. In some examples, the DNA targeting sequence of an RNA guide binds to a target nucleic acid. Binding of a binary complex to a target locus is referred to herein as “on-target binding.”

As used herein, the terms “non-target” and “off-target” refer to a nucleic acid sequence other than the sequence to which a targeting moiety specifically binds or is intended to specifically bind. A non-target locus is an unintended target of a targeting moiety (e.g., an RNA guide). Binding of a binary complex to a non-target locus is referred to herein as “off-target binding.” In some examples, a non-target locus is a locus on a target nucleic acid. In some examples, a non-target locus is a locus on a nucleic acid other than the target nucleic acid (e.g., a non-target nucleic acid).

As used herein, the terms “upstream” and “downstream” refer to relative positions within a single nucleic acid (e.g., DNA) sequence in a nucleic acid molecule. As used herein, the terms “upstream” and “downstream” refer to the positioning of two sequences relative to one another. “Upstream” and “downstream” relate to the 5’ to 3’ direction, respectively, in which RNA transcription occurs. A first sequence is upstream of a second sequence when the 3’ end of the first sequence occurs before the 5’ end

CLAIMS

WHAT IS CLAIMED IS:

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1. A variant Cas12i2 polypeptide comprising a sequence having at least 95% identity to a sequence set forth in any one of SEQ ID NOs: 3 to 146 or any one of SEQ ID NOs: 495 to 512.
2. The variant Cas12i2 polypeptide of claim 1, comprising the sequence set forth in SEQ ID NO: 4.
- 10 3. The variant Cas12i2 polypeptide of claim 1 or 2, comprising the sequence set forth in SEQ ID NO: 5.
4. The variant Cas12i2 polypeptide of claim 1, comprising the sequence set forth in SEQ ID NO: 495.
5. The variant Cas12i2 polypeptide of claim 1, comprising the sequence set forth in SEQ ID NO: 496.
6. The variant Cas12i2 polypeptide of any one of claims 1 to 5, further comprising a substitution of
15 Table 2.
7. The variant Cas12i2 polypeptide of any one of claims 1 to 6, wherein the variant Cas12i2 polypeptide is a variant of a Cas12i2 polypeptide comprising the sequence set forth in SEQ ID NO: 2.
8. A composition comprising a variant Cas12i2 polypeptide of any one of claims 1 to 7, wherein the
20 composition further comprises an RNA guide or a nucleic acid encoding the RNA guide, wherein the RNA guide comprises a direct repeat sequence and a spacer sequence.
9. The composition of claim 8, wherein the direct repeat sequence comprises a nucleotide sequence with at least 95% sequence identity to any one of SEQ ID NO: 492-494.
10. The composition of claim 9, wherein the direct repeat sequence comprises a nucleotide sequence
25 set forth in any one of SEQ ID NO: 492-494.
11. The composition of any one of claims 8 to 10, wherein the spacer sequence comprises between about 11 and about 50 nucleotides.
12. The composition of claim 11, wherein the spacer sequence comprises between about 15 and about 35 nucleotides.
- 30 13. The composition of any one of claims 8 to 12, wherein the spacer sequence binds to a target nucleic acid sequence, and wherein the target nucleic acid sequence is adjacent to a 5'-NTTN-3' sequence.
14. The variant Cas12i2 polypeptide of any one of claims 1 to 7, or the composition of any one of any one of claims 8 to 13, wherein the variant Cas12i2 polypeptide further comprises at least one

nuclear localization signal (NLS), at least one nuclear export signal (NES), or at least one NLS and at least one NES.

15. The variant Cas12i2 polypeptide of any one of claims 1 to 7 or 14, or the composition of any one of claims 8 to 14, wherein the variant Cas12i2 polypeptide further comprises a peptide tag, a fluorescent protein, a base-editing domain, a DNA methylation domain, a histone residue modification domain, a localization factor, a transcription modification factor, a light-gated control factor, a chemically inducible factor, or a chromatin visualization factor.
16. The composition of any one of claims 8 to 15, wherein the composition is present in a delivery system comprising a nanoparticle, a liposome, an exosome, a microvesicle, or a gene-gun.
17. A nucleic acid molecule encoding a variant Cas12i2 polypeptide of any one of claims 1 to 7, 14, or 15.
18. A cell comprising the composition or variant Cas12i2 polypeptide of any previous claim.
19. The cell of claim 18, wherein the cell is a eukaryotic cell or a prokaryotic cell.
20. The cell of claim 18 or 19, wherein the cell is a mammalian cell or a plant cell.
21. The cell of any one of claims 18 to 20, wherein the cell is a human cell.
22. A composition or formulation comprising the variant Cas12i2 polypeptide of any one of claims 1 to 7, 14, or 15 and optionally an RNA guide and/or a cell.
23. A composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a complex, and wherein the variant Cas12i2 polypeptide exhibits increased complex formation with the RNA guide as compared to a parent polypeptide.
24. A composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a complex, and wherein the Cas12i2 variant polypeptide exhibits increased binding affinity to the RNA guide, as compared to a parent polypeptide.
25. A composition comprising a variant Cas12i2 polypeptide and an RNA guide, wherein the Cas12i2 variant polypeptide and the RNA guide form a complex, and the Cas12i2 variant polypeptide and the RNA guide exhibit increased protein-RNA interactions, as compared to a parent polypeptide and the RNA guide.

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26. The composition of any one of claims 23 to 25, wherein the variant Cas12i2 polypeptide comprises at least one of a D581G, D581R, G624R, F626G, F626R, D835G, D835R, L836G, L836R, P868G, P868R, P868T, S879G, S879R, D911G, D911R, I926G, I926R, V1020G, V1020R, V1030G, V1030R, E1035G, E1035R, S1046G, and S1046R substitution of amino acid sequence of SEQ ID NO: 2.
27. The composition of any one of claims 23 to 26, wherein the variant Cas12i2 polypeptide comprises at least one of a D581R, G624R, F626R, P868T, D911R, I926R, V1030G, E1035R, and S1046G substitution of amino acid sequence of SEQ ID NO: 2.