

SYNTHETIC GUIDE RNA, COMPOSITIONS, METHODS, AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of, and priority to, U.S. 62/943,158 filed on December 3, 2019, and U.S. 63/031,262 filed on May 28, 2020, the contents of each of which are incorporated
5 herein.

BACKGROUND

Guide RNA molecules (gRNA) in association with Cas endonucleases, and related enzymes—including base editors—are used for applications in gene editing. A common form of gRNA used for therapeutic applications are single, non-natural RNAs of approximately 100
10 nucleotides that form ribonucleoproteins with Cas9. Plasmid DNA and solid-phase synthesis using phosphoramidite chemistry are typical approaches to obtain therapeutic sgRNAs. Use of synthetic RNA is advantageous as it allows for incorporation of modifications that can both increase the chemical stability of sgRNA and reduce editing of genomic DNA at undesired locations (off-targets).

Problems remain in the manufacture of gRNAs, for example: i) the length of sgRNA molecules, typically 100 nucleotides in length, pushes the limit of phosphoramidite chemistry. Phosphoramidite chemistry has a coupling efficiency for RNA of ~0.985X (where X is the number of nucleotides). For example, synthesis of a gRNA of 100 nts long yields approximately 20% full length product before isolation. These lengths are significantly greater than those used in
20 oligonucleotide-based therapeutics currently on the market (siRNA and antisense oligonucleotides (ASOs) are typically 20-50 nucleotides in length and thus more amenable to purification); ii) complete removal of side products formed from incomplete coupling (truncation products), incomplete deprotection, and random insertions of nucleotides (addition products) is not currently achievable for RNA at these length scales by standard purification methods (e.g.,
25 chromatography, electrophoresis); and iii) side-products isolated with the full-length product, which have similar sequence homology to the full-length product, will reduce the activity of the ribonucleoprotein complex and may lead to off-target editing.

SUMMARY ~~OF THE INVENTION~~

Described herein is a process to synthesize gRNA using a chemical and/or enzymatic
30 strategy that overcomes challenges that limit the purity, integrity and final (post-purified) yield of synthetic RNAs. The ~~invention disclosure~~ provides, in some ~~aspect examples~~, a ligation-based approach

where two or more synthetic RNAs are ligated using an enzyme. It was surprisingly found that a ligation-based approach increases the purity, yield and the integrity of the gRNA produced. The resultant purity, yield and integrity of the gRNA allows for increased editing efficiencies and a reduction of off-target editing as compared to previous methods of gRNA synthesis. The ligation-based method for the synthesis of gRNA includes methods comprising using two or more partially complementary synthetic RNAs that are subsequently ligated (“template approach”), and methods that do not require complementarity between two or more synthetic RNAs (“non-templated approach”).

In ~~some one~~ aspects, ~~a method~~ a method is provided comprising, contacting a first RNA with a second RNA, wherein the first RNA and the second RNA comprise at least five RNA nucleotides that are complementary, and wherein the contacting forms a stem structure or a stem loop structure, and ligating the first RNA and the second RNA with a ligating enzyme (i) within the stem structure, or (ii) at an end of the stem structure, thereby forming a loop at the end of the stem structure.

In some ~~embodiment examples~~, the contacting forms a stem structure and the ligating enzyme ligates the first RNA and the second RNA at an end of the stem structure, thereby forming a loop at the end of the stem structure.

In some ~~embodiment examples~~, the contacting forms a stem loop structure and the ligating enzyme ligates the first RNA and the second RNA within a stem of the stem loop structure.

In some ~~embodiment examples~~, the ligating enzyme is selected from the group consisting of T4 RNA ligase 1, T4 RNA Ligase 2, RtcB Ligase, Thermo-stable 5' App DNA/RNA Ligase, ElectroLigase, T4 DNA Ligase, T3 DNA Ligase, T7 DNA Ligase, Taq DNA Ligase, SplintR Ligase, *E. coli* DNA Ligase, 9°N DNA Ligase, CircLigase, CircLigase II, DNA Ligase I, DNA Ligase III, and DNA Ligase IV. Accordingly, in some ~~embodiment examples~~, the ligating enzyme is T4 RNA ligase 1. In some ~~embodiment examples~~, the ligating enzyme is T4 RNA ligase 2. In some ~~embodiment examples~~, the ligating enzyme is RtcB Ligase. In some ~~embodiment examples~~, the ligating enzyme is Thermo-stable 5' App DNA/RNA Ligase. In some ~~embodiment examples~~, the ligating enzyme is ElectroLigase. In some ~~embodiment examples~~, the ligating enzyme is T4 DNA Ligase. In some ~~embodiment examples~~, the ligating enzyme is T3 DNA Ligase. In some ~~embodiment example~~, the ligating enzyme is T7 DNA Ligase. In some ~~embodiment examples~~, the ligating enzyme is Taq DNA Ligase. In some ~~embodiment examples~~, the ligating enzyme is SplintR Ligase, *E. coli* DNA Ligase. In some ~~embodiment examples~~, the ligating enzyme is 9°N DNA Ligase. In some

embodimentexamples, the ligating enzyme is CircLigase. In some embodimentexamples, the ligating enzyme is CircLigase II. In some embodimentexamples, the ligating enzyme is DNA Ligase I. In some embodimentexamples, the ligating enzyme is DNA Ligase III. In some embodimentexamples, the ligating enzyme is DNA Ligase IV.

5 In some embodimentexamples, the first and/or second RNA is chemically synthesized.

In some embodimentexamples, the first RNA is a clustered regularly interspersed short palindromic repeats (CRISPR) RNA (crRNA) and the second RNA is a trans-activating RNA (tracrRNA).

10 In some embodimentexamples, a guide RNA (gRNA) is produced according to the methods herein.

In some embodimentexamples, the first RNA and/or the second RNA is chemically synthesized.

15 In some embodimentexamples, the first and/or the second RNA is enzymatically synthesized.

In some embodimentexamples, the first RNA and/or the second RNA comprises a modified base. Various modified RNA bases are known in the art and include for example, 2'-O-methoxy-ethyl bases (2'-MOE) such as 2-MethoxyEthoxy A, 2-MethoxyEthoxy MeC, 2-MethoxyEthoxy G, 2-MethoxyEthoxy T. Other modified bases include for example, 2'-O-Methyl RNA bases, and fluoro bases. Various fluoro bases are known, and include for example, Fluoro C, Fluoro U, Fluoro A, Fluoro G bases. Various 2'OMethyl modifications can also be used with the methods described herein. For example, the following RNA comprising one or more of the following 2'OMethyl modifications can be used with the methods described: 2'-OMe-5-Methyl-rC, 2'-OMe-rT, 2'-OMe-rI, 2'-OMe-2-Amino-rA, Aminolinker-C6-rC, Aminolinker-C6-rU, 2'-OMe-5-Br-rU, 2'-OMe-5-I-rU, 2'-OMe-7-Deaza-rG.

In some embodimentexamples, the first RNA and/or second RNA comprises one or more of the following modifications: phosphorothioates, 2'-O-methyls, 2' fluoro (2'F), DNA.

In some embodimentexamples, the first RNA and/or the second RNA comprises 2'OMe modifications at the 3' and 5'-ends.

In some embodimentexamples, the first RNA and/or second RNA comprises one or more of the following modifications: 2' -O-2-Methoxyethyl (MOE), locked nucleic acids, bridged nucleic acids, unlocked nucleic acids, peptide nucleic acids, morpholino nucleic acids.

5 In some embodimentexamples, the first RNA and/or second RNA comprises one or more of the following base modifications: 2,6-diaminopurine, 2-aminopurine, pseudouracil, N1-methyl-psuedouracil, 5' methyl cytosine, 2'pyrimidinone (zebularine), thymine.

Other modified bases include for example, 2-Aminopurine, 5-Bromo dU, deoxyUridine, 2,6-Diaminopurine (2-Amino-dA), Dideoxy-C, deoxyInosine, Hydroxymethyl
10 dC, Inverted dT, Iso-dG, Iso-dC, Inverted Dideoxy-T, 5-Methyl dC, 5-Methyl dC, 5-Nitroindole, Super T®, 2'-F-r(C,U), 2'-NH₂-r(C,U), 2,2'-Anhydro-U, 3'-Desoxy-r(A,C,G,U), 3'-O-Methyl-r(A,C,G,U), rT, rI, 5-Methyl-rC, 2-Amino-rA, rSpacer (Abasic), 7-Deaza-rG, 7-Deaza-rA, 8-Oxo-rG, 5-Halogenated-rU, N-Alkylated-rN.

Other chemically modified RNA can be used herein. For example, the first RNA and/or
15 second RNA can comprise a modified base such as, for example, 5', Int, 3' Azide (NHS Ester); 5' Hexynyl; 5', Int, 3' 5-Octadiynyl dU; 5', Int Biotin (Azide); 5', Int 6-FAM (Azide); and 5', Int 5-TAMRA (Azide). Other examples of RNA nucleotide modifications that can be used with the methods described herein include for example phosphorylation modifications, such as 5'-phosphorylation and 3'-phosphorylation. The RNA can also have one or more of the following
20 modifications: an amino modification, biotinylation, thiol modification, alkyne modifier, adenylation, Azide (NHS Ester), Cholesterol-TEG, and Digoxigenin (NHS Ester).

In some embodimentexamples, ligating the first RNA and the second RNA with a ligating enzyme creates phosphodiester linkages between the first and the second RNA.

25 In some embodimentexamples, the first RNA and/or second RNA nucleotide is engineered to allow for non-covalent assembly.

In some embodimentexamples, the stem loop has a length of between about 2-50 nucleotides.

In some embodimentexamples, the first RNA and the second RNA comprise at least two RNA nucleotides that have perfect complementarity.

30 In some embodimentexamples, the first RNA and the second RNA comprise at least three, four, fix, six or seven consecutive RNA nucleotides that have perfect complementarity.

In some [embodimentexamples](#), the RNA nucleotides that have perfect complementarity are present in a top stem and/or in a bottom stem.

In some [embodimentexamples](#), the first RNA and the second RNA comprise at least five, six, or seven consecutive RNA nucleotides that are complementary at a lower stem formed by the first RNA and the second RNA.

In some [embodimentexamples](#), the first RNA and the second RNA comprise at least four to fourteen consecutive RNA nucleotides that are complementary at an upper stem.

In some [embodimentexamples](#), the first RNA and the second RNA comprise four consecutive RNA nucleotides that are complementary at an upper stem.

In some [embodimentexamples](#), the first and the second RNA comprise five consecutive RNA nucleotides that are complementary at an upper stem.

In some [embodimentexamples](#), the first and the second RNA comprise seven consecutive RNA nucleotides that are complementary at an upper stem.

In some [embodimentexamples](#), the first and the second RNA comprises 14 consecutive RNA nucleotides that are complementary at an upper stem.

In some [embodimentexamples](#), the first and the second RNA comprise 7 consecutive RNA nucleotides that are complementary at a lower stem.

In some [embodimentexamples](#), the first RNA and/or the second RNA is engineered to create a ligation site for a ligation enzyme.

In some [embodimentexamples](#), the stem loop comprises a loop of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides. Accordingly, in some [embodimentexamples](#), the stem loop comprises a loop of 4 nucleotides, also referred to herein as a tetraloop. In some [embodimentexamples](#), the stem loop comprises a loop of 5 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 6 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 7 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 8 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 9 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 10 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 11 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of

12 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 13 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 14 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 15 nucleotides. In some [embodimentexamples](#), the stem loop comprises a loop of 15 nucleotides.

5 In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base pairs from the loop. Accordingly, in some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 1 base pair from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 2 base pairs from the loop. In some [embodimentexample](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 3 base pairs from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 4 base pairs from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 5 base pairs from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 6 base pairs from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 7 base pairs from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 8 base pairs from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 9 base pairs from the loop. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 10 base pairs from the loop.

In some [embodimentexamples](#), the ligation site is 2 or 3 base pairs from the loop.

25 In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 base pairs from a bulge. Accordingly, in some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 3 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 4 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 5 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 6 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 7 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a

ligation site that is at least 8 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 9 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 10 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 11 base pairs from a bulge. In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is at least 12 base pairs from a bulge.

In some [embodimentexamples](#), ligating the first RNA and the second RNA occurs at a ligation site that is 3, 4, 5, or 11 base pairs from the bulge.

In some [embodimentexamples](#), the first RNA and/or second RNA is enzymatically produced.

In some [embodimentexamples](#), the first RNA comprises a 3' sequence that is capable of base pairing with a portion of the second RNA.

In some [embodimentexamples](#), the first RNA comprises a phosphate at the 5' terminus.

In some [embodimentexamples](#), the first RNA is a donor RNA.

In some [embodimentexamples](#), the second RNA comprises a variable protospacer region.

In some [embodimentexamples](#), the second RNA is an acceptor RNA.

In some [embodimentexamples](#), the first RNA comprises an adenosine triphosphate at the 5' terminus.

In some [embodimentexamples](#), about 8-50 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-40 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-30 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-20 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-10 nucleotides are complementary and allow for base pairing between the first and the second RNA.

In some [embodimentexamples](#), the 8-50 nucleotides are partially complementary. In some [embodimentexamples](#), about 8-40 nucleotides are partially complementary and allow for base pairing

between the first and the second RNA. In some [embodimentexamples](#), about 8-30 nucleotides are partially complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-20 nucleotides are partially complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-10 nucleotides are partially complementary and allow for base pairing between the first and the second RNA.

In some [embodimentexamples](#), the 8-50 nucleotides are from about 50% to 99% complementary.

In some [embodimentexamples](#), the 8-50 nucleotides are perfectly complementary. In some [embodimentexamples](#), about 8-40 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-30 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-20 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA. In some [embodimentexamples](#), about 8-10 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA

In some [embodimentexamples](#), the first and the second RNA have different nucleotide lengths.

In some [embodimentexamples](#), the first RNA has from about 20-100 nucleotides. In some [embodimentexamples](#), the first RNA has about 20-90 nucleotides. In some [embodimentexamples](#), the first RNA has about 20-80 nucleotides. In some [embodimentexamples](#), the first RNA has about 20-70 nucleotides. In some [embodimentexamples](#), the first RNA has about 20-60 nucleotides. In some [embodimentexamples](#), the first RNA has about 20-50 nucleotides. In some [embodimentexamples](#), the first RNA has about 20-40 nucleotides. In some [embodimentexamples](#), the first RNA has about 20-30 nucleotides.

In some [embodimentexamples](#), the second RNA has from about 20-70 nucleotides. In some [embodimentexamples](#), the second RNA has about 20-60 nucleotides. In some [embodimentexamples](#), the second RNA has about 20-50 nucleotides. In some [embodimentexamples](#), the second RNA has about 20-40 nucleotides. In some [embodimentexamples](#), the second RNA has about 20-30 nucleotides.

In some [embodimentexamples](#), base pairing occurs in a lower stem.

In some [embodimentexamples](#), 7 nucleotides are complementary in the lower stem and allow for base pairing between the first RNA and the second RNA.

In some [embodimentexamples](#), the base pairing occurs in an upper stem.

5 In some [embodimentexamples](#), 2 nucleotides are complementary in the upper stem and allow for base pairing between the first RNA and the second RNA.

10 In some [embodimentexamples](#), the gRNA has a length of about 100 nucleotides, about 125 nucleotides, about 150 nucleotides, about 175 nucleotides, about 200 nucleotides, or greater than about 200 nucleotides. Accordingly, in some [embodimentexamples](#), the gRNA has a length of about 100 nucleotides. In some [embodimentexample](#), the gRNA has a length of about 125 nucleotides. In some [embodimentexamples](#), the gRNA has a length of about 150 nucleotides. In some [embodimentexample](#), the gRNA has a length of about 175 nucleotides. In some [embodimentexamples](#), the gRNA has a length of about 200 nucleotides. In some [embodimentexamples](#), the gRNA has a length of greater than 200 nucleotides.

15 In some [embodimentexamples](#), the gRNA is an extended guide RNA, prime editor guide RNA (pegRNA), or a Cas12 guide RNA such as Cas12a guide RNA, Cas12b guide RNA, Cas12c guide RNA, Cas12d, guide RNA, Cas12e guide RNA, Cas12f guide RNA, Cas12g guide RNA, Cas12h guide RNA, Cas12i guide RNA, Cas12j guide RNA, or Cas12k guide RNA..

20 Accordingly, in some [embodimentexamples](#), the gRNA is an extended guide RNA. In some [embodimentexamples](#), the gRNA is a prime editor guide RNA (pegRNA). In some [embodimentexamples](#), the gRNA is a Cas12 guide RNA. Various Cas12 are known and the art, and include for example Cas12 from Class 2 CRISPR-Cas systems. Exemplary Cas12 include for example, any Cas12 from Class 2 CRISPR-Cas systems. In some [embodimentexamples](#), the methods described herein are suitable to synthesize gRNA for Cas12a, Cas12b, Cas12c, Cas12d, Cas12e, Cas12f, Cas12g, Cas12h, Cas12i, Cas12j, and/or Cas12k. Accordingly, in some
25 [embodimentexamples](#), the gRNA is a Cas12a guide RNA. In some [embodimentexamples](#), the gRNA is a Cas12b guide RNA. In some [embodimentexamples](#), the gRNA is a Cas12c guide RNA. In some [embodimentexamples](#), the gRNA is a Cas12d guide RNA. In some [embodimentexamples](#), the gRNA is a Cas12e guide RNA. In some [embodimentexamples](#), the gRNA is a Cas12f guide RNA. In some [embodimentexamples](#), the gRNA is a Cas12g guide
30 RNA. In some [embodimentexamples](#), the gRNA is a Cas12h guide RNA. In some [embodimentexamples](#), the gRNA is a Cas12i guide RNA. In some

embodimentexamples, the gRNA is a Cas12j guide RNA. In some embodimentexamples, the gRNA is a Cas12k guide RNA.

In some embodimentexamples, the gRNA comprises one or more of the following: a spacer, a lower stem, a bulge, an upper stem, a nexus and a hairpin.

5 In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 0.5:1, 0.6:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1:0.9, 1:0.8, 1:0.7, 1:0.6, or 1:0.5. Accordingly, in some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 0.5:1. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 0.6:1. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 0.7:1. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 0.8:1. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 0.9:1. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 1:1. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 1:0.9. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 1:0.8. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 1:0.7. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 1:0.6. In some embodimentexamples, the first RNA and the second RNA are present at a ratio of about 1:0.5.

20 In some embodimentexamples, the gRNA is produced at a yield of about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more. Accordingly, in some embodimentexamples, the gRNA is produced at a yield of about 50%. In some embodimentexamples, the gRNA is produced at a yield of about 55%. In some embodimentexamples, the gRNA is produced at a yield of about 60%. In some embodimentexamples, the gRNA is produced at a yield of about 65%. In some embodimentexamples, the gRNA is produced at a yield of about 70%. In some embodimentexamples, the gRNA is produced at a yield of about 75%. In some embodimentexamples, the gRNA is produced at a yield of about 80%. In some embodimentexamples, the gRNA is produced at a yield of about 85%. In some embodimentexamples, the gRNA is produced at a yield of about 90%. In some embodimentexamples, the gRNA is produced at a yield of about 95%. In some embodimentexamples, the gRNA is produced at a yield of more than 99%.

30 In some embodimentexamples, the gRNA is produced at 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more improvement in yield as compared to conventional

synthetic methods. Accordingly, in some embodimentexamples, the gRNA is produced at 50% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 55% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 60% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 55% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 60% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 65% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 70% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 75% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 80% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 85% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 90% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at 99% improvement in yield as compared to conventional synthetic methods. In some embodimentexamples, the gRNA is produced at more than 99% improvement in yield as compared to conventional synthetic methods.

In another aspect, the present disclosure provides a method of producing a synthetic guide RNA (gRNA) comprising: providing a first RNA comprising a 5' -monophosphate; providing a second RNA'; providing an oligonucleotide that has partial complementarity to the first RNA and the second RNA, wherein the complementarity of the oligonucleotide allows for base pairing with the first and the second RNA and optionally wherein the oligonucleotide is about 100 nucleotides long; and providing a ligase to catalyze ligation between the first and the second RNA, thus producing the synthetic gRNA.

In some aspectexamples, a method of producing a synthetic guide RNA (gRNA) is provided comprising: providing a first RNA comprising a 5' -monophosphate; providing a second RNA; providing an oligonucleotide that has partial complementarity to the first RNA and the second RNA, wherein the complementarity of the oligonucleotide allows for base pairing with the first and the second RNA; and providing a ligase to catalyze ligation between the first and the second RNA, thus producing the synthetic gRNA.

In ~~some~~another aspects, a method of producing a synthetic guide RNA (gRNA) is provided comprising: providing a first RNA comprising a 5' –monophosphate; providing a second RNA comprising a blocked 3' end; and providing a ligase to catalyze ligation between the first and the second RNA, thus producing the synthetic gRNA.

5 In some ~~embodiment~~examples, the first RNA is a trans-activating RNA (tracrRNA), and the second RNA is a clustered regularly interspersed short palindromic repeats (CRISPR) RNA (crRNA).

In some embodimentexamples, the oligonucleotide is about 100 nucleotides long. In some embodimentexamples, the oligonucleotide is about 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 200 nucleotides long.

In another aspect, the present disclosure provides a method of producing a synthetic guide RNA (gRNA) comprising: providing two or more RNA fragments; providing an oligonucleotide that has partial complementarity to the two or more RNA fragments, wherein the complementarity of the oligonucleotide allows for base pairing with the two or more RNA fragments; and providing a ligase to catalyze ligation between the two or more RNA fragments, thus producing the synthetic guide RNA, optionally wherein the two or more RNA fragments are ligated at an overhang, blunt end, or at a bulge.

In some aspectexamples, a method of producing a synthetic guide RNA (gRNA) is provided comprising:

providing two or more RNA fragments; providing an oligonucleotide that has partial complementarity to the two or more RNA fragments, wherein the complementarity of the oligonucleotide allows for base pairing with the two or more RNA fragments; and providing a ligase to catalyze ligation between the two or more RNA fragments, thus producing the synthetic guide RNA.

In some embodimentexamples, the two or more RNA fragments are ligated at an overhang, blunt end, or at a bulge.

In some-embodimentsanother aspect, a guide RNA (gRNA), or prime editing guide RNA (pegRNA) is synthesized by a method a method described herein. In some embodimentexamples, the guide RNA is a Cas9 guide RNA or a Cas12 guide RNA.

The methods described herein can be used to synthesize Cas12 guide RNA, such as a Cas12b guide RNA. For example, Cas12b RNA harpin loop structures can be targeted as positions to split the sgRNA. Various hairpin loop structures can be targeted as positions to split the sgRNA, for example, such as those hairpin loop structures as shown in FIG. 16. Accordingly, in some embodimentexamples, the Cas12 guide RNA can be synthesized in accordance with the methods described herein by targeting one or more hairpin loop structures. In some embodimentexamples, one or more tetraloops within Cas12 RNA is targeted for ligation. For example, in some embodimentexamples, one or more tetraloops within Cas12b RNA is target for ligation. In some embodimentexamples, the tetraloop that is

targeted is located at the 5' end of the Cas12 RNA. In some embodimentexamples, the tetraloop that is targeted is located at the 3' end of the Cas12 RNA. In some embodimentexamples, the targeted tetraloop is located within about 5-30 nucleotides from the 3' end of the Cas12 RNA. In some embodimentexamples, the targeted tetraloop is about 5-30 nucleotides from the 5' end of the Cas12 RNA.

In another aspect, the present disclosure provides an *in vitro* method for targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification, the method comprising introducing into a eukaryotic cell: (a) a synthetic guide RNA (gRNA) as defined herein; (b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein; wherein interactions between (a) and (b) and a target sequence in chromosomal DNA leads to targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification.

In some aspectexamples, a method for targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification is provided, the method comprising introducing into a eukaryotic cell: (a) a synthetic guide RNA (gRNA)

as defined in any one of the preceding claims; (b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein; wherein interactions between (a) and (b) and a target sequence in chromosomal DNA leads to targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification.

In another aspect, the present disclosure provides an *in vitro* method for targeted RNA modification, the method comprising introducing into a eukaryotic cell: (a) a synthetic guide RNA (gRNA) as defined herein; (b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein; wherein interactions between (a) and (b) and an RNA expressed by chromosomal DNA leads to a modification of the RNA expressed by the chromosomal DNA.

In some ~~aspect~~examples, a method for targeted RNA modification is provided, the method comprising introducing into a eukaryotic cell: (a) a synthetic guide RNA (gRNA) as defined in any one of the preceding claims; (b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein; wherein interactions between (a) and (b) and an RNA expressed by chromosomal DNA leads to a modification of the RNA expressed by the chromosomal DNA.

In some ~~embodiment~~examples, the RNA expressed by the chromosomal DNA is a messenger RNA (mRNA).

In some ~~embodiment~~examples, the CRISPR/Cas protein is selected from Cas9, Cpf1, SaCas, Cas12, Cas13, or modified versions thereof.

In ~~some embodiments~~another aspect, a method for producing synthetic guide RNA (gRNA) is provided according to methods described herein.

In some ~~embodiment~~examples, the second RNA comprises a 3' sequence that is capable of base pairing with a portion of the first RNA.

In some ~~embodiment~~examples, the second RNA comprises a variable protospacer region.

In some ~~embodiment~~examples, the first RNA comprises a phosphate at the 5' terminus.

In some embodiment examples, the contacting forms a stem loop structure and the ligating enzyme ligates the first RNA and the second RNA within a stem of the stem loop structure.

In some embodiment examples, the ligating enzyme is T4 RNA ligase 2.

5 In some embodiment examples, the stem loop comprises GC base pairs in the upper stem.

In some embodiment examples, the upper stem comprises a nucleotide sequence at least about 80% identical to CGAUACGACAGAAC. In some embodiment examples, the upper stem comprises

a nucleotide sequence at least about 85% identical to CGAUACGACAGAAC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 90% identical to CGAUACGACAGAAC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 95% identical to CGAUACGACAGAAC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 99% identical to CGAUACGACAGAAC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence is identical to CGAUACGACAGAAC.

In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 80% identical to CGCCG. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 85% identical to CGCCG. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 90% identical to CGCCG. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 80% identical to CGCCG. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 95% identical to CGCCG. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 99% identical to CGCCG. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence is identical to CGCCG.

In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 80% identical to CGGCCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 85% identical to CGGCCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 90% identical to CGGCCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 95% identical to CGGCCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 99% identical to CGGCCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence is identical to CGGCCGC.

In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 80% identical to CGCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 85% identical to CGCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 90% identical to CGCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 95% identical to CGCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence at least about 99% identical to CGCGC. In some [embodimentexamples](#), the upper stem comprises a nucleotide sequence is identical to CGCGC.

In some embodimentexamples, the upper stem comprises a nucleotide sequence at least about 80% identical to CGAU. In some embodimentexamples, the upper stem comprises a nucleotide sequence at least about 85% identical to CGAU. In some embodimentexamples, the upper stem comprises a nucleotide sequence at least about 90% identical to CGAU. In some

5 embodimentexamples, the upper stem comprises a nucleotide sequence at least about 95% identical to CGAU. In some embodimentexamples, the upper stem comprises a nucleotide sequence at least about 99% identical to CGAU. In some embodimentexamples, the upper stem comprises a nucleotide sequence is identical to CGAU.

In some embodimentexamples, the stem loop comprises GC base pairs in the lower stem.

10 In some embodimentexamples, the lower stem does not comprise GC base pairs.

In some embodimentexamples, the upper stem does not comprise a GC base pair.

In some embodimentexamples, the upper the stem comprises at least 1, 2, 3, 4, 5, or 6, 7, 8, 9, 10, 11, or 12 GC base pairs. In some embodimentexamples, the stem comprises at least 1 GC base pair. In some embodimentexamples, the stem comprises at least 2 GC base pairs. In some

15 embodimentexamples, the stem comprises at least 3 GC base pairs. In some embodimentexamples, the stem comprises at least 4 GC base pairs. In some embodimentexamples, the stem comprises at least 2 GC base pairs. In some embodimentexamples, the stem comprises at least 5 GC base pairs. In some embodimentexamples, the stem comprises at least 6 GC base pairs. In some embodimentexamples, the stem comprises at least 7 GC base pairs. In some embodimentexamples, the stem comprises at least 8 GC base pairs. In some embodimentexamples, the stem comprises at least 9 GC base pairs. In some embodimentexamples, the stem comprises at least 10 GC base pairs. In some embodimentexamples, the stem comprises at least 11 GC base pairs. In some embodimentexamples, the stem comprises at least 12 GC base pairs.

25 In some embodimentexamples, ligating the first and the second RNA results in a yield of at least 60%, 70%, 80%, 90%, or more than 95% of full length product. In some embodimentexamples, the first and the second RNA results in a yield of at least 60% of full length product. In some embodimentexamples, the first and the second RNA results in a yield of at least 70% of full length product. In some embodimentexamples, the first and the second RNA results in a yield of at least 80% of full length product. In some embodimentexamples, the first and the second RNA results in a yield of at least 90% of full length product. In some embodimentexamples, the first and the second

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RNA results in a yield of at least 95% of full length product. In some embodimentexamples, the first and the second RNA results in a yield more than 95% of full length product.

In some embodimentexamples, the gRNA is produced at a quantity of at least 1 gram.

In some embodimentexamples, gRNA is produced at a quantity of at least 5 grams, 10 grams, 20 grams, 30 grams, 40 grams, 50 grams, 60 grams, 70 grams, 80 grams, 90 grams, or 100 grams.

Accordingly, in some embodimentexamples, gRNA is produced at a quantity of at least 5 grams. In some embodimentexamples, gRNA is produced at a quantity of at least 10 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 20 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 30 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 40 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 50 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 60 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 70 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 80 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 90 grams. In some

embodimentexamples, gRNA is produced at a quantity of at least 100 grams.

In some embodimentexamples, the gRNA is produced at a quantity of less than 1 gram.

In some embodimentexamples, the gRNA is produced at a quantity of about 0.05 grams, 0.1 grams, 0.2 grams, 0.3 grams, 0.4 grams, 0.5 grams, 0.6 grams, 0.7 grams, 0.8 grams, or 0.9g. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.05 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.1 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.2 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.3 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.4 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.5 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.6 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.7 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.8 grams. In some

embodimentexamples, the gRNA is produced at a quantity of about 0.9 grams.

In some embodimentexamples, the method produces gRNA at a purity of about 50%, 60%, 70%, 80%, 90%, or more than 90%. In some embodimentexamples, the method produces gRNA at a purity of about 50%. In some embodimentexamples, the method produces gRNA at a purity of about 60%. In some embodimentexamples, the method produces gRNA at a purity of about 70%. In some

embodimentexamples, the method produces gRNA at a purity of about 80%. In some embodimentexamples, the method produces gRNA at a purity of about 90%. In some embodimentexamples, the method produces gRNA at a purity of more than 90%.

In some embodimentexamples, the first RNA is synthesized in a 3' to 5' direction.

5 In some embodimentexamples, the second RNA is synthesized in a 3' to 5' direction.

In some embodimentexamples, the gRNA has a length of about 100 nucleotides, about 125 nucleotides, about 150 nucleotides, about 175 nucleotides, about 200 nucleotides, or greater than about 200 nucleotides. In some embodimentexamples, the gRNA has a length of about 100 nucleotides. In some embodimentexamples, the gRNA has a length of about 125 nucleotides. In some embodimentexamples, the gRNA has a length of about 150 nucleotides. In some embodimentexamples, the gRNA has a length of about 175 nucleotides. In some embodimentexamples, the gRNA has a length of about 200 nucleotides. In some embodimentexamples, the gRNA has a length of greater than 200 nucleotides.

15 In some embodimentexamples, the loop comprises 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides. In some embodimentexamples, the loop comprises 4 nucleotides, also referred to herein as a tetraloop. In some embodimentexamples, the loop comprises 5 nucleotides. In some embodimentexamples, the loop comprises 6 nucleotides. In some embodimentexamples, the loop comprises 7 nucleotides. In some embodimentexamples, the loop comprises 8 nucleotides. In some embodimentexamples, the loop comprises 9 nucleotides. In some embodimentexamples, the loop comprises 10 nucleotides. In some embodimentexamples, the loop comprises 11 nucleotides. In some embodimentexamples, the loop comprises 12 nucleotides. In some embodimentexamples, the loop comprises 13 nucleotides. In some embodimentexamples, the loop comprises 14 nucleotides. In some embodimentexamples, the loop comprises 15 nucleotides. In some embodimentexamples, the loop comprises 16 nucleotides.

25 In some embodimentexamples, ligating the first RNA and the second RNA occurs at a ligation site that is at least about 3 base pairs from the loop.

30 In some embodimentexamples, the ligation site is 1, 2, 3, 4, 5, 6, or 10 base pairs from the loop. In some embodimentexamples, the ligation site is 1 base pair from the loop. In some embodimentexamples, the ligation site is 2 base pairs from the ligation loop. In some embodimentexamples, the ligation site is 3 base pairs from the ligation loop. In some embodimentexamples, the ligation site is 4 base pairs from the ligation loop. In some embodimentexamples, the ligation site is 5 base pairs

from the ligation loop. In some [embodimentexamples](#), the ligation site is 6 base pairs from the ligation loop. In some [embodimentexamples](#), the ligation site is 7 base pairs from the ligation loop. In some [embodimentexamples](#), the ligation site is 8 base pairs from the ligation loop. In some [embodimentexamples](#), the ligation site is 9 base pairs from the ligation loop.

5 In some [embodimentexamples](#), the ligation site is 10 base pairs from the ligation loop.

In some [embodimentexamples](#), the first and/or second RNA comprises one or more backbone modifications.

10 In some [embodimentexamples](#), the one or more backbone modifications comprises a 2' O-methyl or a phosphorothioate modification. Accordingly, in some [embodimentexamples](#), the one or more backbone modifications comprises a 2' O-methyl modification. In some [embodimentexamples](#), the one or more backbone modifications comprises a phosphorothioate modification.

15 In some [embodimentexamples](#), the one or more backbone modifications is selected from 2'-O-methyl 3'-phosphorothioate, 2'-O-methyl, 2'-ribo 3'-phosphorothioate, deoxy, or 5' phosphate modification. Accordingly, in some [embodimentexamples](#), the one or more backbone modifications comprises a 2'-O-methyl 3'-phosphorothioate modification. In some [embodimentexamples](#), the one or more modifications comprises a 2'-ribo 3'-phosphorothioate modification. In some [embodimentexamples](#), the one or more modifications comprises a deoxy modification. In some [embodimentexamples](#), the one or more modifications comprises a 5'

20 phosphate modification.

In some [embodimentexamples](#), the one or more modifications are present at the site of ligation.

25 In some [embodimentexamples](#), the one or more modifications are present in the donor RNA and/or the acceptor RNA. Accordingly, in some [embodimentexamples](#), the one or more modifications are present in the donor RNA. In some [embodimentexamples](#), the one or more modifications are present in the acceptor RNA. In some [embodimentexamples](#), the one or more modifications are present in both the donor and the acceptor RNA.

30 In some [embodimentexamples](#), the 3' and/or the 5' end of the donor RNA has one or more backbone modifications. Accordingly, in some [embodimentexamples](#), the 3' end of the donor RNA has one or more backbone modifications. In some [embodimentexamples](#), the 5' end of the donor RNA has one or more backbone modifications

In some embodimentexamples, the 3' and/or the 5' end of the acceptor RNA has one or more backbone modifications. Accordingly, in some embodimentexamples, the 3' end of the acceptor RNA has one or more backbone modifications. In some embodimentexamples, the 5' end of the acceptor RNA has one or more backbone modifications.

5 In some embodimentexamples, the concentration of the first and/or second RNA is between about 1 g/L and 5 g/L.

In some embodimentexamples, the concentration of the first and/or second RNA is about 1 g/L. In some embodimentexamples, the concentration of the first and/or second RNA is about 2 g/L. In some embodimentexamples, the concentration of the first and/or second
10 RNA is about 3 g/L. In some embodimentexamples, the concentration of the first and/or second RNA is about 4 g/L. In some embodimentexamples, the concentration of the first and/or second RNA is about 5 g/L.

In a further aspect, the present disclosure provides a composition produced by the method as described herein comprising a first RNA comprising a phosphate at a 5' terminus and a second RNA comprising a variable protospacer region, wherein the first and the second
15 RNA are (a) non-covalently bound, or (b) are bound to a ligase, optionally wherein the ligase is a T4 RNA ligase 2.

In some embodimentexamples, a composition produced by a method described herein is provided comprising a first RNA comprising a phosphate at a 5' terminus and a second
20 RNA comprising a variable protospacer region, wherein the first and the second RNA are non-covalently bound.

In some embodimentexamples, a composition produced by a method described herein is provided comprising a first RNA comprising a phosphate at a 5' terminus and a second
25 RNA comprising a variable protospacer region, and wherein the first and the second RNA are bound to a ligase.

In some embodimentexamples, the ligase is a T4 RNA ligase 2.

In a further aspect, the present disclosure provides a composition comprising an RNA comprising: (a) a nucleotide sequence at least about 80% identical to GAUACGACAGAAC,

optionally wherein the nucleotide sequence is identical to CGAUACGACAGAAC; (b) a nucleotide sequence at least about 80% identical to CGCCG, optionally wherein the nucleotide sequence is identical to CGCCG; (c) a nucleotide sequence at least about 80% identical to CGGCCGC, optionally wherein the nucleotide sequence is identical to CGGCCGC; or (d) a nucleotide sequence at least about 80% identical to CGCGC, optionally wherein the nucleotide sequence is identical to CGCGC. In some aspectexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGAUACGACAGAAC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 85% identical to CGAUACGACAGAAC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 90% identical to CGAUACGACAGAAC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 95% identical to CGAUACGACAGAAC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence identical to CGAUACGACAGAAC.

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In some aspectexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGCCG. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 85% identical to CGCCG. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 90% identical to CGCCG. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 95% identical to CGCCG. In some embodimentexamples, the nucleotide sequence is identical to CGCCG.

In some aspectexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGGCCGC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 85% identical to CGGCCGC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 90% identical to CGGCCGC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 95% identical to CGGCCGC. In some embodimentexamples, the nucleotide sequence is identical to CGGCCGC.

In some aspectexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGCGC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 85% identical to CGCGC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 90% identical to CGCGC. In some embodimentexamples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 95% identical to CGCGC. In some embodimentexamples, the nucleotide sequence is identical to CGCGC.

In ~~some-embodiments~~another aspect, a kit is provided comprising a composition described herein.

In yet another aspect, the present disclosure provides a kit comprising a first RNA comprising trans-activating RNA (tracrRNA) sequence, a second RNA comprising a variable protospacer region, and a ligase, optionally wherein the ligase is a T4 RNA Ligase 2. In some

~~aspect~~examples, a kit is provided comprising a first RNA comprising trans-activating RNA (tracrRNA) sequence, a second RNA comprising a variable protospacer region, and a ligase.

In some ~~embodiment~~examples, the kit comprises a T4 RNA Ligase 2.

CLAIMS

1. A method comprising, contacting a first RNA with a second RNA,

wherein the first RNA and the second RNA comprise at least five RNA nucleotides that are complementary, and wherein the contacting forms a stem structure or a stem loop structure, and

ligating the first RNA and the second RNA with a ligating enzyme

- (i) within the stem structure, or
- (ii) at an end of the stem structure, thereby forming a loop at the end of the stem structure.

2. The method of claim 1, wherein (a) the contacting forms a stem structure and the ligating enzyme ligates the first RNA and the second RNA at an end of the stem structure, thereby forming a loop at the end of the stem structure; or (b)

~~3. The method of claim 1, wherein~~ the contacting forms a stem loop structure and the ligating enzyme ligates the first RNA and the second RNA within a stem of the stem loop structure.

~~34.~~ The method of ~~any one of the preceding~~ claims 1 or 2, wherein the ligating enzyme is selected from the group consisting of T4 RNA ligase 1, T4 RNA Ligase 2, RtcB Ligase, Thermo-stable 5' App DNA/RNA Ligase, ElectroLigase, T4 DNA Ligase, T3 DNA Ligase, T7 DNA Ligase, Taq DNA Ligase, SplintR Ligase E. coli DNA Ligase, 9°N DNA Ligase, CircLigase, CircLigase II, DNA Ligase I, DNA Ligase III, and DNA Ligase IV.

~~5. The method of claim 2, wherein the ligating enzyme is T4 RNA ligase 1.~~

~~6. The method of claim 3, wherein the ligating enzyme is T4 RNA ligase 2.~~

~~47.~~ The method of any one of the preceding claims, wherein the first RNA and/or the second RNA is (a) chemically synthesized; or (b) enzymatically synthesized.

~~58.~~ The method of any one of the preceding claims, wherein the first RNA is a clustered regularly interspersed short palindromic repeats (CRISPR) RNA (crRNA) and the second RNA is a trans-activating RNA (tracrRNA).

69. The method of any one of the preceding claims, wherein a guide RNA (gRNA) is produced, optionally wherein the gRNA:

(a) has a length of about 100 nucleotides, about 125 nucleotides, about 150 nucleotides, about 175 nucleotides, about 200 nucleotides, or greater than about 200 nucleotides; and/or

(b) is an extended guide RNA, prime editor guide RNA (pegRNA), or a Cas12 guide RNA such as Cas12a guide RNA, Cas12b guide RNA, Cas12c guide RNA, Cas12d, guide RNA, Cas12e guide RNA, Cas12f guide RNA, Cas12g guide RNA, Cas12h guide RNA, Cas12i guide RNA, Cas12j guide RNA, or Cas12k guide RNA; and/or

(c) comprises one or more of the following: a spacer, a lower stem, a bulge, an upper stem, a nexus and a hairpin; and/or

(d) is produced at a yield of about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more; and/or

(e) is produced at 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more improvement in yield as compared to conventional synthetic methods.

~~10. The method of any one of the preceding claims, wherein the first RNA and/or the second RNA is chemically synthesized.~~

~~11. The method of any one of claims 1-9, wherein the first and/or the second RNA is enzymatically synthesized.~~

742. The method of any one of the preceding claims, wherein ligating the first RNA and the second RNA with a ligating enzyme creates phosphodiester linkages between the first and the second RNA.

813. The method of any one of the preceding claims, wherein the first RNA and/or second RNA nucleotide is engineered to allow for non-covalent assembly.

914. The method of any one of the preceding claims, wherein the stem loop has a length of between about 2-50 nucleotides.

~~1015.~~ The method of any one of the preceding claims, wherein the first RNA and the second RNA comprise:

(a) at least two RNA nucleotides that have perfect complementarity.

~~16. The method of claim 14, optionally~~ wherein the first RNA and the second RNA comprise at least three, four, five, six or seven consecutive RNA nucleotides that have perfect complementarity.

~~17. The method of claim 15 or 16,~~

optionally wherein the RNA nucleotides that have perfect complementarity are present in a top stem and/or in a bottom stem; and/or:

~~18. The method of any one of the preceding claims, wherein the first RNA and the second RNA comprise (b) at least five, six, or seven consecutive RNA nucleotides that are complementary at a lower stem formed by the first RNA and the second RNA; and/or:~~

~~19. The method of any one of the preceding claims, wherein the first RNA and the second RNA comprise (c) at least four to fourteen consecutive RNA nucleotides that are complementary at an upper stem.~~

~~20. The method of claim 19, optionally~~ wherein the first RNA and the second RNA comprise four, five, seven or 14 consecutive RNA nucleotides that are complementary at an upper stem; and/or

~~21. The method of claim 19, wherein the first and the second RNA comprise five consecutive RNA nucleotides that are complementary at an upper stem.~~

~~22. The method of claim 19, wherein the first and the second RNA comprise seven consecutive RNA nucleotides that are complementary at an upper stem.~~

~~23. The method of claim 19, wherein the first and the second RNA comprises 14 consecutive RNA nucleotides that are complementary at an upper stem.~~

~~24. The method of any one of the preceding claims, wherein the first and the second RNA comprise (d) 7seven consecutive RNA nucleotides that are complementary at a lower stem.~~

~~1125.~~ The method of any one of the preceding claims, wherein the first RNA and/or the second RNA is engineered to create a ligation site for a ligation enzyme.

~~1226.~~ The method of any of the preceding claims, wherein the stem loop comprises a loop of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides.

~~27. The method of any one of the preceding claims,~~ optionally wherein the stem loop comprises a tetraloop.

~~28. The method of claim 16, wherein the loop comprises~~ or 7 nucleotides.

~~29. The method of any one of claims 26-28,~~ optionally wherein ligating the first RNA and the second RNA occurs at a ligation site that is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base pairs from the loop.

~~30. The method of claim 29,~~ optionally wherein the ligation site is 2 or 3 base pairs from the loop.

~~1331.~~ The method of any one of the preceding claims, wherein ligating the first RNA and the second RNA occurs at a ligation site that is at least 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 base pairs from a bulge.

~~32. The method of claim 31,~~ optionally wherein ligating the first RNA and the second RNA occurs at a ligation site that is 3, 4, 5, or 11 base pairs from the bulge.

~~33. The method of any one of the preceding claims, wherein the first RNA and/or second RNA is enzymatically produced.~~

~~1434.~~ The method of any one the preceding claims, wherein the first RNA comprises

(a) _____ a 3' sequence that is capable of base pairing with a portion of the second RNA; and/or

~~35. The method of any one of the preceding claims, wherein the first RNA comprises (b) a~~ phosphate at the 5' terminus.

~~36. The method of claim 35,~~ optionally wherein the first RNA is a donor RNA and/or comprises an adenosine triphosphate at the 5' terminus.

~~1537.~~ The method of any one the preceding claims, wherein the second RNA comprises a variable protospacer region.

~~38.~~ ~~The method of claim 37,~~ optionally wherein the second RNA is an acceptor RNA.

~~39.~~ ~~The method of claim 35, wherein the first RNA comprises an adenosine triphosphate at the 5' terminus.~~

~~1640.~~ The method of any one of the preceding claims, wherein about 8-50 nucleotides are complementary and allow for base pairing between the first and the second RNA.

~~41.~~ ~~The method of claim 40,~~ optionally wherein the 8-50 nucleotides are partially complementary.

~~42.~~ ~~The method of claim 41,~~ optionally wherein the 8-50 nucleotides are from about 50% to 99% complementary.

~~43.~~ ~~The method of claim 41, wherein the 8-50 nucleotides~~ or are perfectly complementary.

~~1744.~~ The method of any one of the preceding claims, wherein:

(a) the first and the second RNA have different nucleotide lengths.

~~45.~~ ~~The method of claim 44,~~ optionally wherein the first RNA has from about 20-100 nucleotides; and/or:

~~46.~~ ~~The method of claim any one of the preceding claims, wherein (b)~~ the second RNA has from about 20-70 nucleotides.

~~1847.~~ The method of any one of the preceding claims, wherein base pairing occurs in a lower stem.

~~48.~~ ~~The method of claim 47,~~ optionally wherein 7 nucleotides are complementary in the lower stem and allow for base pairing between the first RNA and the second RNA.

~~49.~~ ~~The method of any claim 47 or 48,~~ optionally wherein the base pairing occurs in an upper stem.

~~50. The method of claim 49, optionally~~ wherein 2 nucleotides are complementary in the upper stem and allow for base pairing between the first RNA and the second RNA.

~~51. The method of any one of claims 9-50, wherein the gRNA has a length of about 100 nucleotides, about 125 nucleotides, about 150 nucleotides, about 175 nucleotides, about 200 nucleotides, or greater than about 200 nucleotides.~~

~~52. The method of any one of the preceding claims, wherein the gRNA is an extended guide RNA, prime editor guide RNA (pegRNA), or a Cas12 guide RNA such as Cas12a guide RNA, Cas12b guide RNA, Cas12c guide RNA, Cas12d, guide RNA, Cas12e guide RNA, Cas12f guide RNA, Cas12g guide RNA, Cas12h guide RNA, Cas12i guide RNA, Cas12j guide RNA, or Cas12k guide RNA.~~

~~53. The method of any one of the preceding claims, wherein the gRNA comprises one or more of the following: a spacer, a lower stem, a bulge, an upper stem, a nexus and a hairpin.~~

1954. The method of any one of the preceding claims, wherein the first RNA and the second RNA are present at a ratio of about 0.5:1, 0.6:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1:0.9, 1:0.8, 1:0.7, 1:0.6, or 1:0.5.

~~55. The method of any one of the preceding claims, wherein the gRNA is produced at a yield of about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more.~~

~~56. The method of any one of the preceding claims, wherein the gRNA is produced at 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more improvement in yield as compared to conventional synthetic methods.~~

2057. A method of producing a synthetic guide RNA (gRNA) comprising:

providing a first RNA comprising a 5' -monophosphate;

providing a second RNA';

providing an oligonucleotide that has partial complementarity to the first RNA and the second RNA, wherein the complementarity of the oligonucleotide allows for base pairing with the first and the second RNA and optionally wherein the oligonucleotide is about 100 nucleotides long; and

providing a ligase to catalyze ligation between the first and the second RNA, thus producing the synthetic gRNA.

2158. A method of producing a synthetic guide RNA (gRNA) comprising:
providing a first RNA comprising a 5' –monophosphate;
providing a second RNA comprising a blocked 3' end; and
providing a ligase to catalyze ligation between the first and the second RNA, thus
producing the synthetic gRNA.

2259. The method of ~~any one of claims 20 or 2157–58~~, wherein the first RNA is a trans-activating RNA (tracrRNA), and the second RNA is a clustered regularly interspersed short palindromic repeats (CRISPR) RNA (crRNA); optionally -

~~60. The method of claim 57,~~ wherein the oligonucleotide is about 100 nucleotides long.

2361. A method of producing a synthetic guide RNA (gRNA) comprising:
providing two or more RNA fragments;
providing an oligonucleotide that has partial complementarity to the two or more RNA fragments, wherein the complementarity of the oligonucleotide allows for base pairing with the two or more RNA fragments; and
providing a ligase to catalyze ligation between the two or more RNA fragments, thus
producing the synthetic guide RNA.

~~62. The method of claim 61,~~ optionally wherein the two or more RNA fragments are ligated at an overhang, blunt end, or at a bulge.

2463. A guide RNA (gRNA) or prime editing guide RNA (pegRNA) synthesized by the method of any one of claim 1-2362.

2564. An *in vitro* method for targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification, the method comprising introducing into a eukaryotic cell:

- (a) a synthetic guide RNA (gRNA) as defined in ~~any one of the preceding claims~~ 24;
- (b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein;

wherein interactions between (a) and (b) and a target sequence in chromosomal DNA leads to targeted transcription activation, targeted transcription repression, targeted epigenome modification, or targeted genome modification.

2665. An *in vitro* method for targeted RNA modification, the method comprising introducing into a eukaryotic cell:

(a) a synthetic guide RNA (gRNA) as defined in ~~any one of the preceding claims~~ 24;

(b) at least one CRISPR/Cas protein or a nucleic acid encoding the at least one CRISPR/Cas protein;

wherein interactions between (a) and (b) and an RNA expressed by chromosomal DNA leads to a modification of the RNA expressed by the chromosomal DNA.

2766. The method of claim 2665, wherein the RNA expressed by the chromosomal DNA is a messenger RNA (mRNA).

~~67. The method of any one of claims 64-66, wherein and/or~~ the CRISPR/Cas protein is selected from Cas9, Cpf1, SaCas, Cas12, Cas13, or modified versions thereof.

2868. A method for producing synthetic guide RNA (gRNA) according to any one of claims 1-23 and 25-2767.

2969. The method of claim 2868, wherein:

(a) _____ the second RNA comprises a 3' sequence that is capable of base pairing with a portion of the first RNA; ~~and/or~~

~~70. The method of claim 68 or 69, wherein (b) _____~~ the second RNA comprises a variable protospacer region; ~~and/or~~

~~71. The method of any one of claims 68-70, wherein (c) _____~~ the first RNA comprises a phosphate at the 5' terminus; ~~and/or~~

~~72. The method of any one of claims 68-71, wherein (d) _____~~ the contacting forms a stem loop structure and the ligating enzyme ligates the first RNA and the second RNA within a stem of the stem loop structure.

~~73. The method of claim 72, optionally~~ wherein the ligating enzyme is T4 RNA ligase 2.

~~74. The method of claim 73, wherein the stem loop comprises GC base pairs in the upper stem.~~

~~75. The method of claim 74, optionally~~ wherein the upper stem comprises a nucleotide sequence:

~~(I) _____ at least about 80% identical to CGAUACGACAGAAC;~~

~~76. The method of claim 74, wherein the upper stem comprises a nucleotide sequence~~

~~(II) _____ at least about 80% identical to CGCCG;~~

~~77. The method of claim 74, wherein the upper stem comprises a nucleotide sequence~~

~~(III) _____ at least about 80% identical to CGGCCGC;~~

~~78. The method of claim 74, wherein the upper stem comprises a nucleotide sequence~~

~~(IV) _____ at least about 80% identical to CGCGC; or~~

~~79. The method of claim 74, wherein the upper stem comprises a nucleotide sequence~~

~~(V) _____ at least about 80% identical to CGAU;~~

~~further 80. The method of claim 72, optionally~~ wherein:

~~A. _____ the stem loop comprises GC base pairs in the lower stem.~~

~~81. The method of any one of claims 68-79; wherein or~~

~~B. _____ the lower stem does not comprise GC base pairs; and/or~~

~~82. The method of any one of claims 68-81, wherein C. _____ the stem loop comprises GC base pairs in the upper stem, optionally wherein the upper the stem comprises at least 1, 2, 3, 4, 5, or 6, 7, 8, 9, 10, 11, or 12 GC base pairs, optionally wherein the upper portion of the stem comprises 2 GC nucleotides; or~~

~~D. _____ the upper stem does not comprise a GC base pair.~~

~~83. The method of any one of claims 68-81, wherein the upper the stem comprises at least 1, 2, 3, 4, 5, or 6, 7, 8, 9, 10, 11, or 12 GC base pairs.~~

~~84. The method of claim 83, wherein the upper portion of the stem comprises 2 GC nucleotides.~~

~~3085. The method of any one of claims 28 or 2968-84, wherein ligating the first and the second RNA results in a yield of at least 60%, 70%, 80%, 90%, or more than 95% of full-length product.~~

~~86. The method of claim 85, optionally wherein ligating the first and the second RNA results in a yield of at least about 60%.~~

~~3187. The method of any one of the claims 28-301-63 or 68-86, wherein the gRNA is produced:~~

~~(a) _____ at a quantity of at least 1 gram.~~

~~88. The method of claim 87, optionally wherein large scale comprises at least 5 grams, 10 grams, 20 grams, 30 grams, 40 grams, 50 grams, 60 grams, 70 grams, 80 grams, 90 grams, or 100 grams; or~~

~~89. The method of any one of claims 1-63 or 68-88, wherein the gRNA is produced (b) _____ at a quantity of less than 1 gram.~~

~~90. The method of claim 89, optionally wherein the gRNA is produced at a quantity of about 0.05 grams, 0.1 grams, 0.2 grams, 0.3 grams, 0.4 grams, 0.5 grams, 0.6 grams, 0.7 grams, 0.8 grams, or 0.9g; and/or.~~

~~91. The method of any one of claims 68-90, wherein the method produces gRNA (c) _____ at a purity of about 50%, 60%, 70%, 80%, 90%, or more than 90%.~~

~~3292. The method of any one of claims 28-3168-91, wherein:~~

~~(a) _____ the first RNA is synthesized in a 3' to 5' direction; and/or.~~

~~93. The method of any one of claims 68-92, wherein (b) _____ the second RNA is synthesized in a 3' to 5' direction; and/or.~~

~~94. The method of any one of claims 68-93, wherein (c) _____ the gRNA has a length of about 100 nucleotides, about 125 nucleotides, about 150 nucleotides, about 175 nucleotides, about 200 nucleotides, or greater than about 200 nucleotides; and/or.~~

~~95. The method of any one of claims 68-94, wherein (d) _____ the loop comprises 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides.~~

~~96. The method of claim 95, optionally wherein the loop is a tetraloop.~~

~~97. The method of claim 95, wherein the loop or comprises 7 nucleotides; and/or.~~

~~98. The method of any one of claims 68-97, wherein (e) _____ ligating the first RNA and the second RNA occurs at a ligation site that is at least about 3 base pairs from the loop.~~

~~99. The method of claim 98, optionally wherein the ligation site is 1, 2, 3, 4, 5, 6, or 10 base pairs from the loop.~~

~~334100. The method of any one of claims 1-2363 or 25-3268-99, wherein the first and/or second RNA comprises one or more backbone modifications, optionally wherein:~~

~~101. The method of claim 100, wherein (a) _____ the one or more backbone modifications comprises a 2' O-methyl or a phosphorothioate modification; or~~

~~102. The method of claim 100, wherein (b) _____ the one or more backbone modifications is selected from 2'-O-methyl 3'-phosphorothioate, 2'O-methyl, 2'-ribo 3'-phosphorothioate, deoxy, or 5' phosphate modification;~~

~~103. The method of claim 101 or 102, optionally wherein the one or more modifications are present at the site of ligation.~~

~~104. The method of claim 103, optionally wherein the one or more modifications are present in the donor RNA and/or the acceptor RNA, optionally wherein:~~

~~105. The method of claim 104, wherein (I) _____ the 3' and/or the 5' end of the donor RNA has one or more backbone modifications; or~~

~~106. The method of claim 104, wherein (II) _____ the 3' and/or the 5' end of the acceptor RNA has one or more backbone modifications.~~

~~34107. The method of any one of claims 1-2363 or 25-3368-106, wherein the concentration of the first and/or second RNA is between about 1g/L and 5 g/L.~~

~~108. The method of claim 107,~~ optionally wherein the concentration of the first and/or second RNA is about 1 g/L.

~~109. The method of claim 107, wherein the concentration of the first and/or second RNA is or~~
about 3 g/L.

~~35110.~~ A composition produced by the method of any one of ~~the preceding~~ claims 1-23 or 25-34 comprising a first RNA comprising a phosphate at a 5' terminus and a second RNA comprising a variable protospacer region, wherein the first and the second RNA are (a) non-covalently bound.

~~111. A composition produced by the method of any one of claims 1-103 comprising a first RNA comprising a phosphate at a 5' terminus and a second RNA comprising a variable protospacer region, and wherein the first and the second RNA, or (b) are bound to a ligase.~~

~~112. The composition of claim 111,~~ optionally wherein the ligase is a T4 RNA ligase 2.

~~36113.~~ A composition comprising an RNA comprising:

(a) a nucleotide sequence at least about 80% identical to CGAUACGACAGAAC.

~~114. The composition of claim 113,~~ optionally wherein the nucleotide sequence is identical to CGAUACGACAGAAC;

~~115. A composition comprising an RNA comprising (b) a nucleotide sequence at least about 80% identical to CGCCG.~~

~~116. The composition of claim 115,~~ optionally wherein the nucleotide sequence is identical to CGCCG;

~~117. A composition comprising an RNA comprising (c) a nucleotide sequence at least about 80% identical to CGGCCGC.~~

~~118. The composition of claim 117,~~ optionally wherein the nucleotide sequence is identical to CGGCCGC; or

~~119. A composition comprising an RNA comprising (d) a nucleotide sequence at least about 80% identical to CGCGC.~~

~~120. The composition of claim 119, optionally~~ wherein the nucleotide sequence is identical to CGCGC.

~~37121.~~ A kit comprising the composition of ~~any one of claims 35 or 36110-120.~~

~~38122.~~ A kit comprising a first RNA comprising trans-activating RNA (tracrRNA) sequence, a second RNA comprising a variable protospacer region, and a ligase.

~~123. The kit of claim 122, optionally~~ wherein the ligase is a T4 RNA Ligase 2.