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 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 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 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., chromatography, electrophorese); 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

Described herein is a process to synthesize gRNA using a chemical and/or enzymatic strategy that overcomes challenges that limit the purity, integrity and final (post-purified) yield of synthetic RNAs. The disclosure provides, in some 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 one aspect, 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 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 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 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 examples, the ligating enzyme is T4 RNA ligase 1. In some examples, the ligating enzyme is T4 RNA ligase 2. In some examples, the ligating enzyme is RtcB Ligase. In some examples, the ligating enzyme is Thermo-stable 5′ App DNA/RNA Ligase. In some examples, the ligating enzyme is ElectroLigase. In some examples, the ligating enzyme is T4 DNA Ligase. In some examples, the ligating enzyme is T3 DNA Ligase. In some examples, the ligating enzyme is T7 DNA Ligase. In some examples, the ligating enzyme is Taq DNA Ligase. In some examples, the ligating enzyme is SplintR Ligase *E. coli* DNA Ligase. In some examples, the ligating enzyme is 9°N DNA Ligase. In some

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

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

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

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

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

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

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

In some examples, 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.

In some examples, 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 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 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 modifications: an amino modification, biotinylation, thiol modification, alkyne modifier, adenylation, Azide (NHS Ester), Cholesterol-TEG, and Digoxigenin (NHS Ester).

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

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

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

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

In some examples, 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 examples, the RNA nucleotides that have perfect complementarity are present in a top stem and/or in a bottom stem.

In some examples, 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 examples, 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 examples, the first RNA and the second RNA comprise four consecutive RNA nucleotides that are complementary at an upper stem.

In some examples, the first and the second RNA comprise five consecutive RNA nucleotides that are complementary at an upper stem.

In some examples, the first and the second RNA comprise seven consecutive RNA nucleotides that are complementary at an upper stem.

In some examples, the first and the second RNA comprises 14 consecutive RNA nucleotides that are complementary at an upper stem.

In some examples, the first and the second RNA comprise 7 consecutive RNA nucleotides that are complementary at a lower stem.

In some examples, the first RNA and/or the second RNA is engineered to create a ligation site for a ligation enzyme.

In some examples, 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 examples, the stem loop comprises a loop of 4 nucleotides, also referred to herein as a tetraloop. In some examples, the stem loop comprises a loop of 5 nucleotides. In some examples, the stem loop comprises a loop of 6 nucleotides. In some examples, the stem loop comprises a loop of 7 nucleotides. In some examples, the stem loop comprises a loop of 8 nucleotides. In some examples, the stem loop comprises a loop of 9 nucleotides. In some examples, the stem loop comprises a loop of 10 nucleotides. In some examples, the stem loop comprises a loop of 11 nucleotides. In some examples, the stem loop comprises a loop of

12 nucleotides. In some examples, the stem loop comprises a loop of 13 nucleotides. In some examples, the stem loop comprises a loop of 14 nucleotides. In some examples, the stem loop comprises a loop of 15 nucleotides. In some examples, the stem loop comprises a loop of 15 nucleotides.

In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, the ligation site is 2 or 3 base pairs from the loop.

In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, the first RNA and/or second RNA is enzymatically produced.

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

In some examples, the first RNA comprises a phosphate at the 5′ terminus.

In some examples, the first RNA is a donor RNA.

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

In some examples, the second RNA is an acceptor RNA.

In some examples, the first RNA comprises an adenosine triphosphate at the 5′ terminus.

In some examples, about 8-50 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-40 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-30 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-20 nucleotides are complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-10 nucleotides are complementary and allow for base pairing between the first and the second RNA

In some examples, the 8-50 nucleotides are partially complementary. In some examples, about 8-40 nucleotides are partially complementary and allow for base pairing

between the first and the second RNA. In some examples, about 8-30 nucleotides are partially complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-20 nucleotides are partially complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-10 nucleotides are partially complementary and allow for base pairing between the first and the second RNA.

In some examples, the 8-50 nucleotides are from about 50% to 99% complementary.

In some examples, the 8-50 nucleotides are perfectly complementary. In some examples, about 8-40 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-30 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-20 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA. In some examples, about 8-10 nucleotides are perfectly complementary and allow for base pairing between the first and the second RNA

In some examples, the first and the second RNA have different nucleotide lengths.

In some examples, the first RNA has from about 20-100 nucleotides. In some examples, the first RNA has about 20-90 nucleotides. In some examples, the first RNA has about 20-80 nucleotides. In some examples, the first RNA has about 20-70 nucleotides. In some examples, the first RNA has about 20-60 nucleotides. In some examples, the first RNA has about 20-50 nucleotides. In some examples, the first RNA has about 20-40 nucleotides. In some examples, the first RNA has about 20-30 nucleotides.

In some examples, the second RNA has from about 20-70 nucleotides. In some examples, the second RNA has about 20-60 nucleotides. In some examples, the second RNA has about 20-50 nucleotides. In some examples, the second RNA has about 20-40 nucleotides. In some examples, the second RNA has about 20-30 nucleotides.

In some examples, base pairing occurs in a lower stem.

In some examples, 7 nucleotides are complementary in the lower stem and allow for base pairing between the first RNA and the second RNA.

In some examples, the base pairing occurs in an upper stem.

In some examples, 2 nucleotides are complementary in the upper stem and allow for base pairing between the first RNA and the second RNA.

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

In some examples, 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.. Accordingly, in some examples, the gRNA is an extended guide RNA. In some examples, the gRNA is a prime editor guide RNA (pegRNA). In some examples, 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 examples, 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 examples, the gRNA is a Cas12a guide RNA. In some examples, the gRNA is a Cas12b guide RNA. In some examples, the gRNA is a Cas12c guide RNA. In some examples, the gRNA is a Cas12d guide RNA. In some examples, the gRNA is a Cas12e guide RNA. In some examples, the gRNA is a Cas12f guide RNA. In some examples, the gRNA is a Cas12g guide RNA. In some examples, the gRNA is a Cas12h guide RNA. In some examples, the gRNA is a Cas12i guide RNA. In some

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

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

In some examples, 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 examples, the first RNA and the second RNA are present at a ratio of about 0.5:1. In some examples, the first RNA and the second RNA are present at a ratio of about 0.6:1. In some examples, the first RNA and the second RNA are present at a ratio of about 0.7:1. In some examples, the first RNA and the second RNA are present at a ratio of about 0.8:1. In some examples, the first RNA and the second RNA are present at a ratio of about 0.9:1. In some examples, the first RNA and the second RNA are present at a ratio of about 1:1. In some examples, the first RNA and the second RNA are present at a ratio of about 1:0.9. In some examples, the first RNA and the second RNA are present at a ratio of about 1:0.8. In some examples, the first RNA and the second RNA are present at a ratio of about 1:0.7. In some examples, the first RNA and the second RNA are present at a ratio of about 1:0.6. In some examples, the first RNA and the second RNA are present at a ratio of about 1:0.5.

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

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

In some 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 examples, the oligonucleotide is about 100 nucleotides long. In some examples, 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 examples, 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 examples, the two or more RNA fragments are ligated at an overhang, blunt end, or at a bulge.

In another aspect, a guide RNA (gRNA), or prime editing guide RNA (pegRNA) is synthesized by a method a method described herein. In some examples, 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 examples, the Cas12 guide RNA can be synthesized in accordance with the methods described herein by targeting one or more hairpin loop structures. In some examples, one or more tetraloops within Cas12 RNA is targeted for ligation. For example, in some examples, one or more tetraloops within Cas12b RNA is target for ligation. In some examples, the tetraloop that is targeted is located at the 5' end of the Cas12 RNA. In some examples, the tetraloop that is targeted is located at the 3’ end of the Cas12 RNA. In some

examples, the targeted tetraloop is located within about 5-30 nucleotides from the 3' end of the Cas12 RNA. In some examples, 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 examples, 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 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 examples, the RNA expressed by the chromosomal DNA is a messenger RNA (mRNA).

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

Inanother aspect, a method for producing synthetic guide RNA (gRNA) is provided according to methods described herein.

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

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

In some examples, the first RNA comprises a phosphate at the 5′ terminus.

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

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

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

a nucleotide sequence at least about 85% identical to CGAUACGACAGAAC. In some examples, the upper stem comprises a nucleotide sequence at least about 90% identical to CGAUACGACAGAAC. In some examples, the upper stem comprises a nucleotide sequence at least about 95% identical to CGAUACGACAGAAC. In some examples, the upper stem comprises a nucleotide sequence at least about 99% identical to CGAUACGACAGAAC. In some examples, the upper stem comprises a nucleotide sequence is identical to CGAUACGACAGAAC.

In some examples, the upper stem comprises a nucleotide sequence at least about 80% identical to CGCCG. In some examples, the upper stem comprises a nucleotide sequence at least about 85% identical to CGCCG. In some examples, the upper stem comprises a nucleotide sequence at least about 90% identical to CGCCG. In some examples, the upper stem comprises a nucleotide sequence at least about 80% identical to CGCCG. In some examples, the upper stem comprises a nucleotide sequence at least about 95% identical to CGCCG. In some examples, the upper stem comprises a nucleotide sequence at least about 99% identical to CGCCG. In some examples, the upper stem comprises a nucleotide sequence is identical to CGCCG.

In some examples, the upper stem comprises a nucleotide sequence at least about 80% identical to CGGCCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 85% identical to CGGCCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 90% identical to CGGCCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 95% identical to CGGCCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 99% identical to CGGCCGC. In some examples, the upper stem comprises a nucleotide sequence is identical to CGGCCGC.

In some examples, the upper stem comprises a nucleotide sequence at least about 80% identical to CGCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 85% identical to CGCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 90% identical to CGCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 95% identical to CGCGC. In some examples, the upper stem comprises a nucleotide sequence at least about 99% identical to CGCGC. In some examples, the upper stem comprises a nucleotide sequence is identical to CGCGC.

In some examples, the upper stem comprises a nucleotide sequence at least about 80% identical to CGAU. In some examples, the upper stem comprises a nucleotide sequence at least about 85% identical to CGAU. In some examples, the upper stem comprises a nucleotide sequence at least about 90% identical to CGAU. In some examples, the upper stem comprises a nucleotide sequence at least about 95% identical to CGAU. In some examples, the upper stem comprises a nucleotide sequence at least about 99% identical to CGAU. In some examples, the upper stem comprises a nucleotide sequence is identical to CGAU.

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

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

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

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

In some examples, 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 examples, the first and the second RNA results in a yield of at least 60% of full length product. In some examples, the first and the second RNA results in a yield of at least 70% of full length product. In some examples, the first and the second RNA results in a yield of at least 80% of full length product. In some examples, the first and the second RNA results in a yield of at least 90% of full length product. In some examples, the first and the second

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

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

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

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

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

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

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

In some examples, the first RNA is synthesized in a 3′ to 5′ direction.

In some examples, the second RNA is synthesized in a 3′ to 5′ direction.

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

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

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

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

from the ligation loop. In some examples, the ligation site is 6 base pairs from the ligation loop. In some examples, the ligation site is 7 base pairs from the ligation loop. In some examples, the ligation site is 8 base pairs from the ligation loop. In some examples, the ligation site is 9 base pairs from the ligation loop. In some examples, the ligation site is 10 base pairs from the ligation loop.

In some examples, the first and/or second RNA comprises one or more backbone modifications.

In some examples, the one or more backbone modifications comprises a 2′ O-methyl or a phosphorothioate modification. Accordingly, in some examples, the one or more backbone modifications comprises a 2′ O-methyl modification. In some examples, the one or more backbone modifications comprises a phosphorothioate modification.

In some examples, 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 examples, the one or more backbone modifications comprises a 2′-O-methyl 3′-phosphorothioate modification. In some examples, the one or more modifications comprises a 2′-ribo 3′-phosphorothioate modification. In some examples, the one or more modifications comprises a deoxy modification. In some examples, the one or more modifications comprises a 5′ phosphate modification.

In some examples, the one or more modifications are present at the site of ligation.

In some examples, the one or more modifications are present in the donor RNA and/or the acceptor RNA. Accordingly, in some examples, the one or more modifications are present in the donor RNA. In some examples, the one or more modifications are present in the acceptor RNA. In some examples, the one or more modifications are present in both the donor and the acceptor RNA.

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

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

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

In some examples, the concentration of the fist and/or second RNA is about 1 g/L. In some examples, the concentration of the fist and/or second RNA is about 2 g/L. In some examples, the concentration of the first and/or second RNA is about 3 g/L. In some examples, the concentration of the fist and/or second RNA is about 4 g/L. In some examples, the concentration of the fist 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 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 examples, a composition produced by a method described herein is provided 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 non-covalently bound.

In some examples, a composition produced by a method described herein is provided 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 are bound to a ligase.

In some examples, 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 examples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 80% identical to CGAUACGACAGAAC. In some examples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 85% identical to CGAUACGACAGAAC. In some examples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 90% identical to CGAUACGACAGAAC. In some examples, a composition is provided comprising an RNA comprising a nucleotide sequence at least about 95% identical to CGAUACGACAGAAC. In some examples, a composition is provided comprising an RNA comprising a nucleotide sequence identical to CGAUACGACAGAAC.

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

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

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

In 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 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 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) 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.

3. The method of claim 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.

4. 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.

5. 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).

6. 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.

7. 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.

8. 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.

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

10. 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, optionally

wherein the first RNA and the second RNA comprise at least three, four, fix, six or seven consecutive RNA nucleotides that have perfect complementarity,

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

(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

(c) at least four to fourteen consecutive RNA nucleotides that are complementary at an upper stem, 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

(d) seven consecutive RNA nucleotides that are complementary at a lower stem.

11. 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.

12. 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, optionally wherein the stem loop comprises a tetraloop or 7 nucleotides, 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, optionally wherein the ligation site is 2 or 3 base pairs from the loop.

13. 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, 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.

14. 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

(b) a phosphate at the 5′ terminus, optionally wherein the first RNA is a donor RNA and/or comprises an adenosine triphosphate at the 5′ terminus.

15. The method of any one the preceding claims, wherein the second RNA comprises a variable protospacer region, optionally wherein the second RNA is an acceptor RNA.

16. 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, optionally

wherein the 8-50 nucleotides are partially complementary, optionally wherein the 8-50 nucleotides are from about 50% to 99% complementary or are perfectly complementary.

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

(a) the first and the second RNA have different nucleotide lengths, optionally wherein the first RNA has from about 20-100 nucleotides; and/or

(b) the second RNA has from about 20-70 nucleotides.

18. The method of any one of the preceding claims, wherein base pairing occurs in a lower stem, optionally wherein 7 nucleotides are complementary in the lower stem and allow for base pairing between the first RNA and the second RNA, optionally wherein the base pairing occurs in an upper stem, optionally wherein 2 nucleotides are complementary in the upper stem and allow for base pairing between the first RNA and the second RNA.

19. 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.

20. 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.

21. 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.

22. The method of claim 20 or 21, 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

wherein the oligonucleotide is about 100 nucleotides long.

23. 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.

24. A guide RNA (gRNA) or prime editing guide RNA (pegRNA) synthesized by the method of any one of claim 1-23.

25. 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 claim 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.

26. 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 claim 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.

27. The method of claim 26, wherein the RNA expressed by the chromosomal DNA is a messenger RNA (mRNA) and/or the CRISPR/Cas protein is selected from Cas9, Cpf1, SaCas, Cas12, Cas13, or modified versions thereof.

28. A method for producing synthetic guide RNA (gRNA) according to any one of claims 1-23 and 25-27.

29. The method of claim 28, wherein:

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

(b) the second RNA comprises a variable protospacer region; and/or

(c) the first RNA comprises a phosphate at the 5′ terminus; and/or

(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, optionally wherein the ligating enzyme is T4 RNA ligase 2, optionally wherein the upper stem comprises a nucleotide sequence:

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

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

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

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

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

further optionally wherein:

A. the stem loop comprises GC base pairs in the lower stem; or

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

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.

30. The method of claim 28 or 29, 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, optionally wherein ligating the first and the second RNA results in a yield of at least about 60%.

31. The method of any one of the claims 28-30, wherein the gRNA is produced:

(a) at a quantity of at least 1 gram, 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

(b) at a quantity of less than 1 gram, 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

(c) at a purity of about 50%, 60%, 70%, 80%, 90%, or more than 90%.

32. The method of any one of claims 28-31, wherein:

(a) the first RNA is synthesized in a 3′ to 5′ direction; and/or

(b) the second RNA is synthesized in a 3′ to 5′ direction; and/or

(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

(d) the loop comprises 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides, optionally wherein the loop is a tetraloop or comprises 7 nucleotides; and/or

(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, optionally wherein the ligation site is 1, 2, 3, 4, 5, 6, or 10 base pairs from the loop.

33. The method of any one of claims 1-23 or 25-32, wherein the first and/or second RNA comprises one or more backbone modifications, optionally wherein:

(a) the one or more backbone modifications comprises a 2′ O-methyl or a phosphorothioate modification; or

(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;

optionally wherein the one or more modifications are present at the site of ligation, optionally wherein the one or more modifications are present in the donor RNA and/or the acceptor RNA, optionally wherein:

(I) the 3' and/or the 5' end of the donor RNA has one or more backbone modifications; or

(II) the 3' and/or the 5' end of the acceptor RNA has one or more backbone modifications.

34. The method of any one of claims 1-23 or 25-33, wherein the concentration of the first and/or second RNA is between about 1g/L and 5 g/L, optionally wherein the concentration of the fist and/or second RNA is about 1 g/L or about 3 g/L.

35. A composition produced by the method of any one of 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, or (b) are bound to a ligase, optionally wherein the ligase is a T4 RNA ligase 2.

36. A composition comprising an RNA comprising:

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

37. A kit comprising the composition of claim 35 or 36.

38. 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.