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(54) Title: CIRCULAR POLYRIBONUCLEOTIDES ENCODING GLUCAGON-LIKE PEPTIDE 2 (GLP-2) AND USES THEREOF

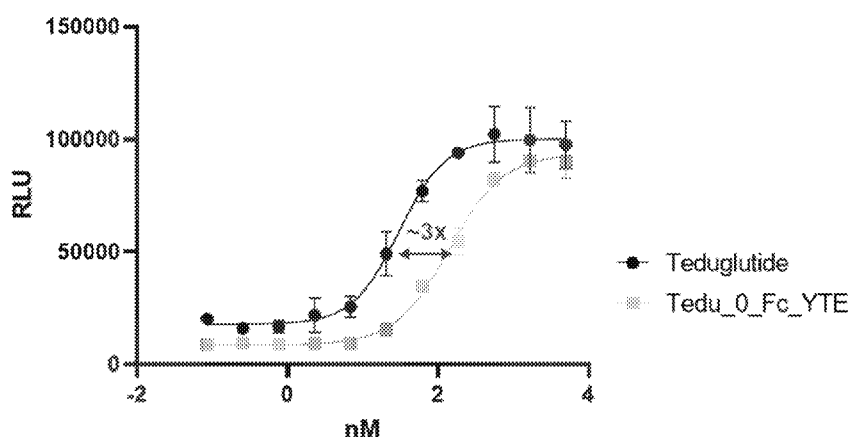


FIG. 1

(57) Abstract: The present disclosure provides circular polyribonucleotides including a sequence encoding a polypeptide including (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, (ii) a half-life extension moiety, and (iii) a secretion signal sequence, and kits and compositions thereof. The present disclosure also provides methods of treating GLP-2 related diseases with compositions described herein.

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CIRCULAR POLYRIBONUCLEOTIDES ENCODING GLUCAGON-LIKE PEPTIDE 2 (GLP-2) AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application No.
5 63/600,527, filed on November 17, 2023, and U.S. Provisional Patent Application No.
63/614,264, filed on December 22, 2023, the content of each of which is hereby incorporated by
reference in its entirety.

SEQUENCE LISTING

10 This application contains a Sequence Listing that has been submitted electronically as an
XML file named 56929-0005WO1_SL_ST26.xml. The XML file, created on November 12,
2024, is 228,547 bytes in size. The material in the XML file is hereby incorporated by reference
in its entirety.

TECHNICAL FIELD

15 The present disclosure generally relates to compositions including circular
polyribonucleotides and modified RNAs, and their use in treatment and/or prevention of GLP-2-
related diseases, such as short bowel syndrome.

BACKGROUND

20 Glucagon-like peptide (GLP)-2 is a peptide hormone derived from tissue-specific
posttranslational processing of the pro-glucagon peptide. GLP-2 and GLP-2 analogs have been
used to treat intestinal diseases, such as short bowel syndrome.

SUMMARY

25 The present disclosure is based, at least in part, on the development of circular
polyribonucleotides and modified RNAs encoding a GLP-2 polypeptide or analog thereof.
These circular polyribonucleotides and modified RNAs provide for prolonged and stable
expression of the GLP-2 polypeptide or analog thereof in a subject (e.g., subjects having a GLP-
30 2-related disease, e.g., intestinal diseases such as short bowel syndrome).

Thus, provided herein are circular polyribonucleotides including a sequence encoding a polypeptide including (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, (ii) a half-life extension moiety, and (iii) a secretion signal sequence.

In some embodiments, the GLP-2 polypeptide or analog thereof includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13. In some embodiments, the GLP-2 polypeptide or analog thereof includes an amino acid sequence of any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

In some embodiments, the sequence encoding the polypeptide includes a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14. In some embodiments, the sequence encoding the polypeptide includes a nucleic acid sequence of any one of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14.

In some embodiments, the GLP-2 analog includes teduglutide, glepaglutide, dapiglutide, apraglutide, HM15912, and SHP-681.

In some embodiments, the half-life extension moiety includes a peptide moiety. In some embodiments, the peptide moiety includes an Fc domain, an albumin polypeptide, a nanobody, or a post-translational modification site. In some embodiments, the Fc domain includes an IgG1 Fc domain, an IgG2 Fc domain, an IgG3 Fc domain, or an IgG4 Fc domain. In some embodiments, the Fc domain includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identical to SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain). In some embodiments, the Fc domain includes an amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain). In some embodiments, the IgG4 Fc domain includes an amino acid sequence of SEQ ID NO: 21.

In some embodiments, the sequence encoding the polypeptide includes a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 16 (IgG1 Fc domain), SEQ ID NO: 18 (IgG2 Fc domain), SEQ ID NO: 20 (IgG3 Fc

domain), or SEQ ID NO: 22 (IgG4 Fc domain). In some embodiments, the sequence encoding the polypeptide includes a nucleic acid sequence of SEQ ID NO: 16 (IgG1 Fc domain), SEQ ID NO: 18 (IgG2 Fc domain), SEQ ID NO: 20 (IgG3 Fc domain), or SEQ ID NO: 22 (IgG4 Fc domain).

5 In some embodiments, the Fc domain includes a mutation. In some embodiments, the Fc domain includes the amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain), except the amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain) includes an amino acid substitution selected from one or more of: M252Y/S254T/T256E (YTE); L284A/L285A; M428L/N434S (LS); T307A/E380A/N434A (AAA); T250Q/M428L (QL), and V308P.

15 In some embodiments, the albumin polypeptide includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 23. In some embodiments, the peptide moiety includes a nanobody that binds to albumin.

In some embodiments, the circular polyribonucleotide includes one or more modifications. In some embodiments, the one or more modifications include one or more modifications to a portion of the sequence encoding the GLP-2 polypeptide or analog thereof. In some embodiments, the one or more modifications include a modification to a sugar, a nucleobase, an internucleoside linkage, or a combination thereof.

20 In some embodiments, the circular polyribonucleotide includes a translation initiation sequence operably linked to the sequence encoding the polypeptide.

In some embodiments, the circular polyribonucleotide includes an internal ribosome entry site (IRES) operatively linked to the sequence encoding the polypeptide.

25 In some embodiments, the circular polyribonucleotide includes a translation termination sequence. In some embodiments, the circular polyribonucleotide lacks a translation termination sequence.

In some embodiments, the circular polyribonucleotide includes a stagger element at a 3' end of the sequence encoding the polypeptide. In some embodiments, the stagger element is configured to stall a ribosome during rolling circle translation.

30 In some embodiments, the circular polyribonucleotide includes a replication element.

In some embodiments, the circular polyribonucleotide includes a 5' untranslated region operably linked to the sequence encoding the polypeptide. In some embodiments, the circular polyribonucleotide does not include a poly(A) sequence operably linked to the sequence encoding the polypeptide. In some embodiments, the circular polyribonucleotide does not include a 5' untranslated region operably linked to the sequence encoding the polypeptide.

In some embodiments, the secretion signal sequence is a liver-specific secretion signal sequence, where the liver-specific secretion signal includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to any one of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, or SEQ ID NO: 139.

In some embodiments, the secretion signal sequence includes an IL-2 secretion signal sequence, a proglucagon secretion signal sequence, a Gaussia luciferase secretion signal sequence, or a erythropoietin (EPO) secretion signal sequence. In some embodiments, the IL-2 secretion signal sequence includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 29. In some embodiments, the proglucagon secretion signal sequence includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 31. In some embodiments, the Gaussia luciferase secretion signal sequence includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 33. In some embodiments, the EPO secretion signal sequence includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 35.

In some embodiments, the sequence encoding the polypeptide includes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to one of SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, or SEQ ID NO: 39.

5 In some embodiments, the circular polyribonucleotide includes at least one spacer sequence.

In some embodiments, the polypeptide includes a protease cleavage site and the at least one spacer sequence encodes the protease cleavage site. In some embodiments, the protease cleavage site is positioned between (a) the secretion signal and (b) the GLP-2 polypeptide or analog thereof and the half-life extension moiety.

10 In some embodiments, the polypeptide includes at least one linker sequence. In some embodiments, the at least one linker sequence encodes an amino acid sequence selected from (GS)_x, (GGS)_x, (GGGS)_x (SEQ ID NO: 141), (GGSG)_x (SEQ ID NO: 142), and (SGGG)_x (SEQ ID NO: 143), where x is an integer from 1 to 50. In some embodiments, the at least one linker sequence is disposed between the GLP-2 polypeptide or analog thereof and the half-life
15 extension moiety.

In some embodiments, the circular polyribonucleotide encodes an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1. In some embodiments, the circular polyribonucleotide includes an amino acid sequence of SEQ ID NO: 1. In some embodiments, the circular polyribonucleotide includes a nucleic acid sequence
20 that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 2. In some embodiments, the circular polyribonucleotide includes a nucleic acid sequence of SEQ ID NO: 2.

Also provided herein are pharmaceutical compositions including any of the circular polyribonucleotides described herein and a pharmaceutically acceptable excipient.

25 In some embodiments, the circular polyribonucleotide is formulated as a lipid nanoparticle. In some embodiments, the circular polyribonucleotide is formulated for unencapsulated delivery or natural particle delivery.

In some embodiments, the pharmaceutical composition is formulated for intravenous, subcutaneous, intrahepatic, or intramuscular administration. In some embodiments, the
30 pharmaceutical composition is formulated for local administration. In some embodiments, the pharmaceutical composition is formulated for systemic administration.

In some embodiments, the pharmaceutical composition includes one or more of the following features: (i) a concentration of the circular polyribonucleotide of between 0.1 ng/mL and 500 mg/mL; (ii) a concentration of deoxyribonucleotide of no more than 100 ng/mL; (iii) a concentration of protein of less than 100 ng of protein per milligram (mg) of the circular polyribonucleotide; (iv) an A260/A280 absorbance ratio of from about 1.6 to 2.3 as measured by a spectrophotometer; (v) substantially free of a process-related impurity selected from a cell protein, a cell deoxyribonucleic acid, an enzyme, a reagent component, a gel component, or a chromatographic material; and (vi) a reduced level of one or more markers of an immune or inflammatory response after purification compared to prior to purification.

Also provided herein are methods of treating a glucagon-like peptide-2 (GLP-2)-related disease in a subject in need thereof, the method including administering to the subject an effective amount of any of the circular polyribonucleotides described herein or any of the pharmaceutical compositions described herein.

In some embodiments, the GLP-2-related disease is selected from the group consisting of: short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn's disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury.

Also provided herein are methods of increasing intestinal growth in a subject in need thereof, the method including administering to the subject an effective amount of any of the circular polyribonucleotides described herein or any of the pharmaceutical compositions described herein.

In some embodiments, increasing intestinal growth includes increasing crypt cell growth.

Also provided herein are methods of increasing intestinal absorption of fluids and nutrients in a subject in need thereof including administering to the subject an effective amount of any of the circular polyribonucleotides described herein or any of the pharmaceutical compositions described herein.

In some embodiments, the subject has short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large

intestinal inflammation, Crohn's disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury.

In some embodiments, any of the circular polyribonucleotides described herein or any of the pharmaceutical compositions described herein is administered one or more times to the
5 subject.

In some embodiments, any of the circular polyribonucleotides described herein or any of the pharmaceutical compositions described herein is administered locally. In some embodiments, any of the circular polyribonucleotides described herein or any of the pharmaceutical compositions described herein is administered systemically.

10 In some embodiments, the method includes administering one or more additional therapeutic agents to the subject. In some embodiments, the subject is a human subject.

Also provided herein are kits including: (a) (i) a composition including any of the circular polyribonucleotides described herein or (ii) any of the pharmaceutical compositions described herein; and (b) instructions for administering the composition or the pharmaceutical composition
15 to a subject in need thereof.

Also provided herein are circular polyribonucleotides including a sequence encoding a polypeptide including (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a half-life extension moiety.

Also provided herein are circular polyribonucleotides including a sequence encoding a
20 polypeptide including (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a half-life extension moiety, where the half-life extension moiety is an Fc domain including a M252Y/S254T/T256E (YTE) mutation.

Also provided herein are modified linear RNAs including a sequence encoding a polypeptide including a glucagon-like peptide-2 (GLP-2) or analog thereof.

25 Also provided herein are modified linear RNAs including a sequence encoding a polypeptide including (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a secretion signal sequence.

In some embodiments, the modified linear RNA further includes a half-life extension moiety (e.g., any of the half-life extension moieties described herein).

30 All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent,

patent application, or item of information was specifically and individually indicated to be incorporated by reference. To the extent publications, patents, patent applications, and items of information incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

Where values are described in terms of ranges, it should be understood that the description includes the disclosure of all possible sub-ranges within such ranges, as well as specific numerical values that fall within such ranges irrespective of whether a specific numerical value or specific sub-range is expressly stated.

The term “each,” when used in reference to a collection of items, is intended to identify an individual item in the collection but does not necessarily refer to every item in the collection, unless expressly stated otherwise, or unless the context of the usage clearly indicates otherwise.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings illustrate certain embodiments of the features and advantages of this disclosure. These embodiments are not intended to limit the scope of the appended claims in any manner. Like reference symbols in the drawings indicate like elements.

FIG. 1 includes a graph showing activity of teduglutide in cells treated with circular RNA encoding teduglutide-Fc or recombinant teduglutide.

FIGs. 2A-2B include graphs showing expression (**FIG. 2A**) and activity (**FIG. 2B**) of teduglutide-Fc in mammalian cells transfected with circular RNA constructs encoding teduglutide-Fc.

FIG. 3 includes a graph showing levels of teduglutide-Fc in mice injected with circular RNA encoding teduglutide-Fc and reported levels of glepaglutide, teduglutide, apraglutide, and HM15912 in rats injected with recombinant glepaglutide, teduglutide, apraglutide, or HM15912.

FIGs. 4A-4F include data from characterization of various LNP formulations including expression levels of teduglutide-Fc (**FIG. 4A**), complete blood cell count (CBC) (**FIGs. 4B-4C**), ALT levels (**FIG. 4D**), IFN α levels (**FIG. 4E**), and cytokine levels (**FIG. 4F**).

FIGs. 5A-5G include dose response data in mice injected with LNP formulations of circular RNA encoding teduglutide-Fc. The data include expression of GLP2-Fc (**FIGs. 5A-B**), cytokine levels (**FIG. 5C-F**), and anti-target antibody(ATA) response (**FIG. 5G**)

FIGs. 6A-F include data from characterization of GLP-2 Fc expressed from circular RNA in non-human primates including Teduglutide-Fc expression (**FIG. 6A**), antibody production (**FIG. 6B**), complete blood count, including lymphocyte count (**FIG. 6C**), cytokine responses (**FIG. 6D**), CRP levels (**FIG. 6E**), and activate partial thromboplastin (**FIG. 6F**).

DETAILED DESCRIPTION

Provided herein are circular polyribonucleotides and modified RNAs encoding a GLP-2 polypeptide or analog thereof and related pharmaceutical compositions and kits. Circular polyribonucleotides and modified RNAs described herein comprise a sequence encoding a polypeptide comprising (i) a GLP-2 polypeptide or analog thereof, (ii) a half-life extension moiety, and optionally, (iii) a secretion signal sequence. In some embodiments, the circular polyribonucleotides and modified RNAs comprise a sequence encoding a polypeptide comprising (i) a GLP-2 polypeptide or analog thereof and (ii) a half-life extension moiety. In some embodiments, the circular polynucleotides and modified RNAs comprise a sequence encoding a polypeptide comprising (i) a GLP-2 polypeptide or analog thereof, (ii) a half-life extension moiety, and (iii) a secretion signal sequence. The circular polyribonucleotides and modified RNAs described herein are useful in the treatment or prevention of a GLP-2-related disease (e.g., short bowel syndrome).

I. Circular Polyribonucleotides/Modified RNAs

Circular polyribonucleotides and modified RNAs described herein comprise a sequence encoding a polypeptide comprising: (i) a GLP-2 polypeptide or analog thereof, (ii) a half-life extension moiety, and optionally, (iii) a secretion signal sequence, each of which is described herein. In some embodiments, a circular polyribonucleotide or a modified RNA comprises a sequence encoding a polypeptide comprising (i) a GLP-2 polypeptide or analog thereof and (ii) a half-life extension moiety. In some embodiments, a circular polyribonucleotide or a modified RNA comprise a sequence encoding a polypeptide comprising a sequence encoding a (i) a GLP-2 polypeptide or analog thereof, (ii) a half-life extension moiety, and (iii) a secretion signal sequence. In some embodiments, a circular polyribonucleotide or modified RNA can include two or more copies of the sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein). Non-limiting additional sequences that can be included in a circular polyribonucleotide and a modified RNA are described herein.

As used herein a “modified RNA” or a “linear modified RNA” refers to an RNA molecule that includes one or more (e.g., 2, 3, 4, 5, 10 or more) modified ribonucleotides described herein or modifications any of the exemplary modifications known in the art. Non-limiting examples of modified ribonucleotides include 5-methylcytidine, N1-methylpseudouridine, 5-methoxyuridine, and pseudouridine.

A non-limiting example of a sequence encoding a polypeptide that can be present in any of the circular polyribonucleotides or modified RNAs described herein are provided in Table 1 (SEQ ID NO: 2). A non-limiting example of a polypeptide comprising (i) a GLP-2 polypeptide or analog thereof, (ii) a half-life extension moiety, and (iii) a secretion signal sequence is provided in Table 1 (SEQ ID NO: 1).

Table 1. Non-limiting example of a polypeptide and a sequence encoding a polypeptide

SEQ ID NO:	Description	Sequence
1	GLP2-Fc (IL2 Secretion Signal – Teduglutide-Fc)	MYRMQLLSICIALSLALVTNSHGDGSFSDENNTILDNLAARDFINWLIQTKITDGGGG GGGSGGGSGGGGSAESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKGLPSSIEKTIKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYP SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMEHA LHNHYTQKSLSLGLG

2	GLP2- Fc (IL2 Secretion Signal – Teduglutide-Fc)	ATGTACCGGATGCAGCTGCTCAGCTGCATCGCTCTGAGTCTGGCCCTGGTGACCAAC AGCCACGGCGACGGAAGCTTTAGCGACGAGATGAACACCATCCTGGACAACCTGGCC GCTCGGGACTTCATCAACTGGCTGATCCAGACCAAGATCACCGACGGAGGAGGAGGC GGAGGAGGAAGCGGAGGCGGGGCGAGCGGCGGAGGCGGCAGCGCCGAGAGCAAGTAC GGCCCCCTTGTCTCCTTGCCCCGCCCCGAGGCCGCCGGCGGGGCCAGCGTGTTC CTGTTCCCCCAAAGCCCAAGGACACCCTGATGATCAGCCGGACCCCCGAGGTGACC TGCGTGGTTCGTGGACGTGAGTCAGGAAGACCCCGAGGTGCAGTTCAACTGGTACGTG GACGGCGTGGAGGTGCACAACGCCAAGACCAAACCCGCGAGGAACAGTTCAACAGC ACCTACCGGGTGGTTAGCGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAAG GAGTACAAGTGCAAGGTGAGCAACAAGGGCCTGCCAGCTCCATCGAGAAGACCATC AGCAAGGCCAAGGGCCAGCCCCGGGAGCCCCAGGTGTACACCCTGCCCCCAGCCAG GAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTGAAGGGCTTCTACCCC AGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCGAGAACAATTACAAGACC ACACCCCCCGTGCTGGACAGCGACGGCAGCTTCTTTCTGTACAGCCGGCTGACCGTG GACAAGAGCCGGTGGCAGGAGGGCAACGTGTTGAGCTGCAGCGTGATGCACGAGGCC CTGCACAACCACTACACCCAGAAAAGTCTCAGTCTCAGCCTGGGC
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In some embodiments, the polypeptide comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1. In some embodiments, the polypeptide comprises an amino acid sequence of SEQ ID NO: 1.

5 In some embodiments, the sequence (encoding the polypeptide) comprises a sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 2. In some embodiments, the sequence (encoding the polypeptide) comprises a sequence of SEQ ID NO: 2.

10 (i) *GLP-2 and Analogs Thereof*

Pro-glucagon is cleaved to glucagon by prohormone convertase 2 (PC2) in pancreatic α -cells (and other organs), and it is cleaved to glucagon-like peptide-2 (GLP-2) by prohormone convertase 1 (PC1).

15 GLP-2 is a 33 amino acid peptide-encoded carboxyterminal to the sequence of GLP-1 in the proglucagon gene. Importantly, GLP-2 also significantly enhances the surface area of the mucosal epithelium via stimulation of crypt cell proliferation and inhibition of apoptosis in the enterocyte and crypt compartments. Both GLP-1 and GLP-2 are secreted from gut endocrine cells and promote nutrient absorption through distinct mechanisms of action.

20 GLP-2 is also known to regulate gastric motility, gastric acid secretion, intestinal hexose transport, and increases the barrier function of the gut epithelium. GLP-2 has been shown to reduce mortality and decrease mucosal injury, cytokine expression, and bacterial septicemia in

the setting of small and large bowel inflammation. GLP-2 also enhances nutrient absorption and gut adaptation in rodents or humans with short bowel syndrome.

Activation of GLP-2 receptor signaling in heterologous cells promotes resistance to apoptotic injury *in vitro* via a G protein-coupled receptor expressed in gut endocrine cells of the stomach, small bowel, and colon. The cytoprotective, reparative, and energy-retentive properties of GLP-2 suggests that GLP-2 may potentially be useful for the treatment of human disorders characterized by injury and/or dysfunction of the intestinal mucosal epithelium.

A “GLP-2 analog” refers to a polypeptide having one or more alterations (e.g., substitutions, deletions, and/or insertions) in an amino acid sequence of wild-type GLP-2 and having at least partial GLP-2 activity as compared to a wild-type GLP-2 polypeptide. Non-limiting examples of GLP-2 analogs include teduglutide, glepaglutide, dapiglutide, apraglutide, HM15912, and SHP-681. Further non-limiting examples of GLP-2 analogs are described in WO 2020/020904, which is incorporated herein by reference in its entirety.

Non-limiting examples of amino acid sequences of pro-glucagon, GLP-2, and GLP-2 analogs are shown in Table 2 below. Non-limiting examples of nucleic acid sequence encoding pro-glucagon, GLP-2, and GLP-2 analogs are also shown in Table 2 below.

Table 2. Non-limiting examples of sequences of pro-glucagon, GLP-2, and GLP-2 analogs

SEQ ID NO:	Description	Sequence
3	Pro-glucagon	MKSIYFVAGLFVMLVQGSWQSRSLQDTEEKSRFSASQADPLSDPDQMNEDKRHSQ GTFTSDYSKYLDSRRAQDFVQWLMNTKRNRRNNIAKRHDEFERHAEFTTSDVSSY LEGQAAKEFIAWLVKGRGRDRDFPEEVAIVEELGRRHADGSFSDENMTILDNLAAAR DFINWLIQTKITDRK
4	Pro-glucagon	ATGAAAAGCATTTACTTTGTGGCTGGGTTATTTGTAATGCTGGTACAAGGCAGCT GGCAACGTTCCCTTCAAGACACAGAGGAGAAATCCAGATCATTCTCAGCTTCCCA GGCAGACCCACTCAGTGATCCTGATCAGATGAACGAGGACAAGCGCCATTCACAG GGCACATTCAACAGTGACTACAGCAAGTATCTGGACTCCAGGCGTGCCCAAGATT TTGTGCAGTGGTTGATGAATACCAAGAGGAACAGGAATAACATTGCCAAACGTCA CGATGAATTTGAGAGACATGCTGAAGGGACCTTTACCAGTGATGTAAGTTCTTAT TTGGAAGGCCAAGCTGCCAAGGAATTCATTGCTTGGCTGGTGAAAGGCCGAGGAA GGCGAGATTTCCAGAAGAGGTGCGCCATTGTTGAAGAACTTGGCCGCAGACATGC TGATGGTTCTTTCTCTGATGAGATGAACACCATTCTTGATAATCTTGCCGCCAGG GACTTTATAAACTGGTTGATTTCAGACCAAAATCACTGACAGGAAATAA
5	GLP-2 sequence	HADGSFSDENMTILDNLAAARDFINWLIQTKITD
6	GLP-2 sequence	catgcggatggcagcttttagcgatgaaatgaacaccattctggataacctggcgg cgcgcgattttattaactggctgattcagaccaaattaccgat

7	Teduglutide	HGDGSFSDEMNTILDNLAARDFINWLIQTKITD
8	Teduglutide	catggcgatggcagcttttagcgatgaaatgaacaccattctggataacctggcgg cgcgcgattttattaactggctgattcagaccaaattaccgat
9	Glepaglutide	HGEGTFSSSELATILDALAARDFIAWLIATKITDKKKKKK-NH ₂
10	Glepaglutide	catggcgaaggcaccttttagcagcgaactggcgaccattctggatgcgctggcgg cgcgcgattttattgctggctgattgcgaccaaattaccgataaaaaaaaaa aaaaaaa
11	Dapiglutide	HAEGSFTSELATILDKQAARDFIAWLIQHKITD
12	Dapiglutide	catgcggaaggcagctttaccagcgaactggcgaccattctggataaacaggcgg cgcgcgattttattgctggctgattcagcataaaattaccgat
13	Apraglutide	HGDGSFSDEX ₁ X ₂ TILDLAARDFINWLIQTKITD-NH ₂
14	Apraglutide	catggcgatggcagcttttagcgatgaannntttaccattctggatctgctggcgg cgcgcgattttattaactggctgattcagaccaaattaccgat
167	GLP-2 consensus analog #1	X ₁ X ₂ X ₃ X ₄ SFSDEMNTILDNLAARDFINWLIQTKITD

In some embodiments, the GLP-2 polypeptide or analog thereof comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

In some embodiments, the GLP-2 polypeptide or analog thereof comprises an amino acid sequence of any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

In some embodiments, the sequence (encoding the GLP-2 polypeptide or analog thereof) comprises a sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14.

In some embodiments, the sequence (encoding the GLP-2 polypeptide or analog thereof) comprises a sequence of any one of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14.

In some embodiments, the GLP-2 polypeptide or analog thereof comprises an amino acid sequence that is a functional fragment of any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

In some embodiments, the GLP-2 polypeptide or analog thereof comprises a functional fragment that has a sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

5 In some embodiments, the GLP-2 polypeptide or analog thereof comprises an amino acid sequence of SEQ ID NO: 13. In some embodiments, the amino acid at position X₁ is norleucine. In some embodiments, the amino acid at position X₂ is D-phenylalanine.

In some embodiments, GLP-2 polypeptide or analog thereof comprises an amino acid sequence of SEQ ID NO: 167. In some embodiments, the amino acid at positions X₁, X₂, X₃, and X₄ of SEQ ID NO: 167 (e.g., positions 1, 2, 3, and 4 of SEQ ID NO: 5) can be any amino acid. For example, each of X₁, X₂, X₃, and X₄ can independently be any of amino acids alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, or valine, with the proviso that the combination of X₁ of histidine, X₂ of alanine, X₃ of aspartic acid, and X₄ of glycine is excluded.

As used herein a “functional fragment” is a fragment that retains at least 50% (e.g., at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%) of the activity of the wildtype polypeptide from which it is derived. In some embodiments, a functional fragment can have an activity greater than the wildtype polypeptide from which it is derived.

In some embodiments, the GLP-2 polypeptide or analog thereof comprises an amino acid sequence having at least one mutation. The term “substitution” refers to a substitution of a residue within a sequence (e.g., a nucleic acid or an amino acid sequence) with another residue, or a deletion or insertion of one or more residues within a sequence (e.g., a nucleic acid or an amino acid sequence). Substitutions are typically described herein by identifying the original residue followed by the position of the residue within the sequence and by the identity of the newly substituted residue. Various methods for making the amino acid substitutions provided herein are well known in the art, and are provided by, e.g., Green and Sambrook, Molecular Cloning: A Laboratory Manual (4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2012)). The GLP-2 polypeptide or analog thereof can comprise any number and/or any type of substitutions.

In some embodiments, the sequence encoding the GLP-2 polypeptide or analog thereof comprises one or more mutations as compared to the wild type sequence. The one or more mutations can be silent, *i.e.*, they do not modify the amino acid sequence of any encoded protein (or otherwise result in a substituted amino acid sequence). Alternatively, the one or more mutations in a nucleic acid sequence can result in modifications to the encoded amino acid sequence of GLP-2 or analog thereof, resulting in GLP-2 or analog thereof having one or more amino acid substitutions (*e.g.*, having 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more amino acid substitutions) relative to the wild-type protein sequence.

In some embodiments, the polypeptide comprises (i) a GLP-2 polypeptide or analog thereof (*e.g.*, any of the GLP-2 polypeptides or analogs thereof described herein or known in the art) and (ii) a half-life extension moiety (*e.g.*, any of the exemplary half-life extension moieties described herein or known in the art). In some embodiments, the polypeptide comprises (i) a secretion signal sequence (*e.g.*, any of the exemplary secretion signal sequences described herein or known in the art), (ii) a GLP-2 polypeptide or analog thereof (*e.g.*, any of the exemplary GLP-2 polypeptides or analogs thereof described herein or known in the art), and (iii) a half-life extension moiety (*e.g.*, any of the exemplary half-life extension moieties described herein or known in the art). Non-limiting examples of half-life extension moieties are described herein.

In some embodiments, the polypeptide may further comprise an epitope tag for detection or purification (*e.g.*, a HiBiT-tag) and/or a fluorescent tag for visualization (*e.g.*, a GFP-tag).

Also provided herein are modified linear RNAs including a sequence encoding a polypeptide including a glucagon-like peptide-2 (GLP-2) or analog thereof.

Also provided herein are modified linear RNAs including a sequence encoding a polypeptide including (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a secretion signal sequence.

In some embodiments, the modified linear RNA includes a half-life extension moiety (*e.g.*, any of the half-life extension moieties described herein).

(ii) Half-Life Extension Moieties

In some embodiments, the polypeptide (encoded by a sequence in a circular polyribonucleotide or a modified RNA described herein) comprises a half-life extension moiety. The term “half-life extension moiety” refers to any moiety that extends the circulating serum

half-life of a polypeptide comprising a GLP-2 polypeptide or analog thereof *in vivo*. Circulating serum half-life refers to the amount of time it takes for the concentration or amount of a polypeptide comprising a GLP-2 polypeptide or analog thereof in the serum to be reduced by half (i.e., 50%).

- 5 In some embodiments, the half-life extension moiety is a peptide moiety. Non-limiting examples of a half-life extension moiety include an antibody or fragment thereof (e.g., an Fc domain, *e.g.*, an IgG1 Fc domain, an IgG2 Fc domain, an IgG3 Fc domain, or an IgG4 Fc domain), a serum protein or fragment thereof (e.g., albumin or fragment thereof (e.g., human serum albumin or a fragment thereof), fibronectin or fragment thereof (e.g., human fibronectin or
- 10 a fragment thereof), or transferrin or fragment thereof (e.g., human transferrin or a fragment thereof), and antibodies or an antigen-binding fragments thereof or peptides that bind to a serum protein (e.g., an antibody or antigen-binding fragment thereof that binds to human albumin, human fibronectin, or human transferrin). Antigen-binding fragments of antibodies include, e.g., single-chain antibodies (scFv), single-domain antibodies (also called nanobodies), Fab
- 15 fragments, Fab' fragments, and F(ab')₂ Fab₂ fragments, diabodies, triabodies, and tetrabodies.

Non-limiting examples of amino acid sequences (and corresponding nucleic acid sequences) of half-life extension moieties are shown in Table 3 below.

Table 3. Non-limiting examples of sequences of half-life extension moieties

SEQ ID NO:	Description	Sequence
15	IgG1 Fc Domain (P238-K447)	PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSV MHEALHNHYTQKSLSLSPGK
16	IgG1 Fc Domain (P238-K447)	CCGTCAGTCTTCTCTTCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCTGAGGTCACATGCGTGGTGGTGGAGCTGAGCCACGAAGACCCTGAGGTCAAGTTC AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAG CAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGG CTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATC GAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTG CCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTCAAA GGCTTCTATCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAAC AACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGC AAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTG ATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGT AAA
17	IgG2 Fc Domain (P238-K447)	PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTL

		PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPMLDSDGSFFLYS KLTVDKSRWQQGNV FSCSV MHEALHNHYTQKSLSLSPGK
18	IgG2 Fc Domain (P238-K447)	CCGTCAAGTCTTCTCTTCCCCCAAAACCAAGGACACCTCATGATCTCCCGGACC CCTGAGGTACAGTGCCTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTC AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCACGGGAGGAG CAGTTCAACAGCACGTTCCTGTGGTCAAGCTCCTCACCCTGTGTGCACCAAGGACTGG CTGAACGGCAAGGAGTACAAGTGAAGGTCTCCAACAAAGGCCTCCAGCCCCCATC GAGAAAACCATCTCCAAACCAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTG CCCCCATCCCGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTCAAA GGCTTCTACCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAAC AACTACAAGACCACACCTCCCATGCTGGACTCCGACGGCTCCTTCTTCTCTACAGC AAGCTCACCCTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTG ATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGT AAA
19	IgG3 Fc Domain (P238-K447)	PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFKWYVDGVEVHNAKTKPREE QFNSTFRVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKTKGQPREPQVYTL PPSREEMTKNQVSLTCLVKGFYPSDIAVEWESSGQPENNYNTTPMLDSDGSFFLYS KLTVDKSRWQQGNIFSCSV MHEALHNRTQKSLSLSPGK
20	IgG3 Fc Domain (P238-K447)	CCGTCAAGTCTTCTCTTCCCCCAAAACCAAGGATACCTTATGATTTCCCGGACC CCTGAGGTACAGTGCCTGGTGGTGGACGTGAGCCACGAAGACCCCGAGGTCCAGTTC AAGTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGAGGAG CAGTTCAACAGCACGTTCCTGTGGTCAAGCTCCTCACCCTGCTGCACCAAGGACTGG CTGAACGGCAAGGAGTACAAGTGAAGGTCTCCAACAAAGGCCTCCAGCCCCCATC GAGAAAACCATCTCCAAACCAAGGACAGCCCCGAGAACCACAGGTGTACACCCTG CCCCCATCCCGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTCAAA GGCTTCTACCCAGCGACATCGCCGTGGAGTGGGAGAGCAGCGGGCAGCCGGAGAAC AACTACAACACCACGCCTCCCATGCTGGACTCCGACGGCTCCTTCTTCTCTACAGC AAGCTCACCCTGGACAAGAGCAGGTGGCAGCAGGGGAACATCTTCTCATGCTCCGTG ATGCATGAGGCTCTGCACAACCGCTTACAGCAGAAGAGCCTCTCCCTGTCTCCGGGT AAA
21	IgG4 Fc Domain (P238-K447)	PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTI SKAKGQPREPQVYTL PPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYS RLTVDKSRWQEGNV FSCSV MHEALHNHYTQKSLSLSLGK
22	IgG4 Fc Domain (P238-K447)	CCATCAAGTCTTCTGTTCCTCCCCCAAAACCAAGGACACTCTCATGATCTCCCGGACC CCTGAGGTACAGTGCCTGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTC AACTGGTACGTGGATGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGAGGAG CAGTTCAACAGCACGTACCGTGTGGTCAAGCTCCTCACCCTGCTGCACCAAGGACTGG CTGAACGGCAAGGAGTACAAGTGAAGGTCTCCAACAAAGGCCTCCCGTCTCCATC GAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAGCCACAGGTGTACACCCTG CCCCCATCCAGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTCAAA GGCTTCTACCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAAC AACTACAAGACCACGCCTCCCGTGTGGACTCCGACGGCTCCTTCTTCTCTACAGC AGGCTAACCGTGGACAAGAGCAGGTGGCAGGAGGGGAATGTCTTCTCATGCTCCGTG ATGCATGAGGCTCTGCACAACCACTACACACAGAAGAGCCTCTCCCTGTCTCTGGGT AAA
23	Human albumin	MKWVTFISLLFLFSSAYS RGVFRDAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQ CPFEDHVKL VNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADC CAKQEPERNECFLQHKDDNP NLPRLVRPEVDVMCTAFHDNEETFLKKYLYE IARRHP YFYAPELLFFAKRYKAAFT ECCCQAADKAACLLPKLDEL RDEGKASSAKQRLKCASLQ KFGERAFAWAVARLSQRFPAEFAEVSKLVTDLT KVHTECCHGDLLECADDRADLA KYI CENQDSISSKLKECCEKPLEKSHCIAEVENDEMPADLP SLAADFVESKDVCKN YAEAKDVFLGMFLY EYARRHPDYSV LLLRLAKTYETTLEKCCAAADPHECYAKVFD EFKPLVEEPQNLIKQNCLEFELGEYKFQNALLVRYTKVPQVSTPTLVEVSRNLGK VGSCKKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCTESLVNRRPCFS

		ALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKA VMDDFAAFVEKCKKADDKETCFEEGKKLVAASQAALGL
24	Human albumin	AGCTTTTCTCTTCTGTCAACCCACACGCCTTTGGCACAATGAAGTGGGTAACCTTT ATTTCCCTTCTTTTCTCTTTAGCTCGGCTTATTCAGGGGTGTGTTTCGTGAGAT GCACACAAGAGTGAGGTTGCTCATCGGTTTAAAGATTTGGGAGAAGAAAATTTCAA GCCTTGGTGTGATTGCCTTTGCTCAGTATCTTCAGCAGTGTCCATTTGAAGATCAT GTAAAATTAGTGAATGAAGTAACTGAATTTGCAAAAACATGTGTTGCTGATGAGTCA GCTGAAAATTGTGACAAATCACTTCATACCCTTTTGGAGACAAATTATGCACAGTT GCAACTCTTCGTGAAACCTATGGTGAAATGGCTGACTGCTGTGCAAAACAAGAACCT GGGAGAAATGAATGCTTCTTGCAACACAAAGATGACAACCCAAACCTCCCCGATTG GTGAGACCAGAGGTTGATGTGATGTGCACTGCTTTTCATGACAATGAAGAGACATTT TTGAAAAATACTTATATGAAATTGCCAGAAGACATCCTTACTTTTATGCCCCGGAA CTCCTTTTCTTTGCTAAAAGGTATAAAGCTGCTTTTACAGAATGTTGCCAAGCTGCT GATAAAGCTGCCTGCCTGTTGCCAAAGCTCGATGAACTTCGGGATGAAGGGAAGGCT TCGTCTGCCAAACAGAGACTCAAGTGTGCCAGTCTCCAAAATTTGGAGAAAGAGCT TTCAAAGCATGGGCAGTAGCTCGCTGAGCCAGAGATTTCCCAAAGCTGAGTTTGCA GAAGTTTCCAAGTTAGTGACAGATCTTACCAAAGTCCACACGGAATGCTGCCATGGA GATCTGCTTGAATGTGCTGATGACAGGGCGGACCTTGCCAAGTATATCTGTGAAAT CAAGATTCGATCTCCAGTAACTGAAGGAATGCTGTGAAAAACCTCTGTTGGAAAAA TCCCCTGCATTGCCGAAGTGGAAAAATGATGAGATGCCTGCTGACTTGCCCTTCATTA GCTGCTGATTTTGTGAAAGTAAGGATGTTTGCAAAAATATGCTGAGGCAAAGGAT GTCTTCTTGGGCATGTTTTGTATGAATATGCAAGAAGGCATCCTGATTACTCTGTC GTGCTGCTGCTGAGACTTGCCAAGACATATGAAACCACTCTAGAGAAGTGTGTGCC GCTGCAGATCCTCATGAATGCTATGCCAAAGTGTTCGATGAATTTAAACCTCTTG GAAGAGCCTCAGAATTTAATCAAACAAAATTTGTGAGCTTTTTGAGCAGCTTGAGAG TACAAATTCAGAATGCGCTGTTAGTTTCGTTACACCAAGAAAGTACCCGAAGTGTCA ACTCCAACCTCTGTAGAGGTCTCAAGAAACCTAGGAAAAGTGGGCAGCAAATGTTGT AAACATCCTGAAGCAAAAAGAATGCCCTGTGCAGAAAGACTATCTATCCGTGGTCCTG AACCAGTTATGTGTGTTGCATGAGAAAACGCCAGTAAGTGACAGAGTCACCAAATGC TGCACAGAATCCTTGGTGAACAGGCGACCATGCTTTTCAGCTCTGGAAGTCGATGAA ACATACGTTCCCAAAGAGTTAATGCTGAAACATTCACCTTCCATGCAGATATATGC ACACTTTCTGAGAAGGAGAGACAAATCAAGAAAACAACTGCACTTGTTGAGCTCGTG AAACACAAGCCCAAGGCAACAAAAGAGCAACTGAAAGCTGTTATGGATGATTTGCT GCTTTTGTAGAGAAGTGTGCAAGGCTGACGATAAGGAGACCTGCTTTGCCGAGGAG GGTAAAAAATTTGTGCTGCAAGTCAAGCTGCCTTAGGCTTATAACATCACATTTAA AAGCATCTCAGCCTACCATGAGAATAAGAGAAAAGAAAATGAAGATCAAAGCTTATT CATCTGTTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCTAAAAACATAAAT TTCTTTAATCATTTTGCCTCTTTTCTCTGTGCTTCAATTAATAAAAAATGGAAAGAA TCT
25	Human Fibronectin	MLRGPGLLLLLAVQCLGTAVPSTGASKSKRQAQQMVQPQSPVAVSQSKPGCYDNGK HYQINQQWERTYLGNALVCTCYGSSRGFNCEKPEAEETCFDKYTGNTYRVGDTYER PKDSMIWDCTCIGAGRGRISCTIANRCHEGGQSYKIGDTWRRPHETGGYMLECVCLG NGKGEWTCKPIAEKCFDHAAGTSYVGETWEKPYQGWMVDCTCLGEGSGRITCTSR NRCNDQDTRTSYRIGDTWSKKDNRGNLLQCICTGNRGGEWKERHTSVQTTSSGSGP FTDVRAAVYQPQPHPQPPYGHCVTDSGVVYSVGMQWLKTQGNKQMLCTCLNGVSC QETAVTQTYGGNSNGEPCVLPFTYNDRTDSTTSNYEQDQKYSFCTDHTVLVQTRGGN SNGALCHFPLYNHNYTDCTSEGRDNMKWCGTTQNYDADQKFGFCPMAAHEEICT TNEGVMYRIGDQWDKQHDMGHMMRCTCVGNRGGEWTCIAYSQLRDQCIVDITYNVN DTFHKRHEEGHMLNCTCFGQGRGRWKCDPVDQCQDSETGTFYQIGDSWEKYVHGVRY QCYCYGRGIGEWHCQPLQTYPSSSGPVEVFITETPSQPNSHPIQWNAPQPSHISKYI LRWRPVSIPPRNLGY
26	Human Fibronectin	ATGCTTAGGGGTCCGGGGCCCGGGCTGCTGCTGCTGGCCGTCCAGTGCCTGGGGACA GCGGTGCCCTCCACGGGAGCCTCGAAGAGCAAGAGGCAGGCTCAGCAAATGGTTTCAG CCCCAGTCCCCGGTGGCTGTGAGTCAAAGCAAGCCCGGTTGTTATGACAATGAAAA CACTATCAGATAAATCAACAGTGGGAGCGGACCTACCTAGGCAATGCGTTGGTTTGT ACTTGTATGAGGAAGCCGAGGTTTAACTGCGAGAGTAAACCTGAAGCTGAAGAG

		ACTTGCTTTGACAAGTACACTGGGAACACTTACCGAGTGGGTGACACTTATGAGCGT CCTAAAGACTCCATGATCTGGGACTGTACCTGCATCGGGGCTGGGCGAGGGAGAATA AGCTGTACCATCGCAAACCGCTGCCATGAAGGGGGTCAGTCTACAAGATTGGTGAC ACCTGGAGGAGACCACATGAGACTGGTGGTTACATGTTAGAGTGTGTGTCTTGGT AATGGAAAAGGAGAATGGACCTGCAAGCCCATAGCTGAGAAGTGTTTTGATCATGCT GCTGGGACTTCCTATGTGGTCCGAGAAAACGTGGGAGAAGCCCTACCAAGGCTGGATG ATGGTAGATTGTACTTGCCCTGGGAGAAGGCAGCGGACGCATCACTTGCACCTCTAGA AATAGATGCAACGATCAGGACACAAGGACATCCTATAGAATTGGAGACACCTGGAGC AAGAAGGATAATCGAGGAAACCTGCTCCAGTGCATCTGCACAGGCAACGGCCGAGGA GAGTGGAAGTGTGAGAGGCACACCTCTGTGCAGACCACATCGAGCGGATCTGGCCCC TTCACCGATGTTCTGTCAGCTGTTTACCAACCGCAGCCTCACCCCCAGCCTCCTCCC TATGGCCACTGTGTACAGACAGTGGTGTGGTCTACTCTGTGGGGATGCAGTGGCTG AAGACACAAGGAAATAAGCAAATGCTTTGCACGTGCCTGGGCAACGGAGTCAGCTGC CAAGAGACAGCTGTAACCCAGACTTACGGTGGCAACTCAAATGGAGAGCCATGTGTC TTACCATTACCTACAACGACAGGACGGACAGCACAACCTTCGAATTATGAGCAGGAC CAGAAATACTCTTTCTGCACAGACCACACTGTTTTGGTTTCACTCGAGGAGGAAAT TCCAATGGTGCCTTGTGCCACTTCCCCTTCTATACAACAACCACAATTACACTGAT TGCACCTCTGAGGGCAGAAGAGACAACATGAAGTGGTGTGGGACCACACAGAACTAT GATGCCGACCAGAAGTTTGGGTTCTGCCCCATGGCTGCCACGAGGAAATCTGCACA ACCAATGAAGGGGTGATGTACCGCATTGAGATCAGTGGGATAAGCAGCATGACATG GGTCACATGATGAGGTGCACGTGTGTTGGGAATGGTCTGTTGGGAATGGACATGCATT GCCTACTCGCAGCTTCGAGATCAGTGCATTGTTGATGACATCACTTACAATGTGAAC GACACATTCCACAAGCGTCATGAAGAGGGGCACATGCTGAACTGTACATGCTTCGGT CAGGGTCGGGGCAGGTGGAAGTGTGATCCCGTCGACCAATGCCAGGATTCAAGAGCT GGGACGTTTTATCAAATTGGAGATTCATGGGAGAAAGTATGTGCATGGTGTGAGATAC CAGTGTCTACTGCTATGGCCGTGGCATTGGGGAGTGGCATTGCCAACCTTTACAGACC TATCCAAGCTCAAGTGGTCTGTGCGAAGTATTTATCACTGAGACTCCGAGTCAGCCC AACTCCCACCCCATCCAGTGGAAATGCACCACAGCCATCTCACATTTCCAAGTACATT CTCAGGTGGAGACCTGTGAGTATCCCACCCAGAAACCTTGATACTGA
27	Human Transferrin	MRLAVGALLVCAVLGLCLAVPDKTVRWCAVSEHEATKCQSFRDHMSVIPS DGPSVA CVKKASYLDCIRAIANEADAVTL DAGLVYDAY LAPNNLKPVVAEFYGSKEDEPQTFY YAVAVVKKDSGFQMNQLRGKKSCHTGLGRSAGWNIPIGLLYCDLPEPRKPLEKAVAN FFSGSCAPCADGTD FPQLCQLCPGCGCSTLNQYFGYSGAFKCLKDGAGDVA FVKHST IFENLANKADRDQYELLCLDNTRKPVDEYKDCHLAQVPSHTVVARSMGKEDLIWEL LNQAQEHFQKDKSKEFQLFSSPHGKDLLFKDSAHGFLKVP PRMDAKMYLG EYVTAI RNLREGTCPEAPTDECKPVKWCALSHHERLKCDEWSVNSVGKIECVSAETTEDCIAK IMNGEADAMSLDGGFVYIAGKCGLPVLAENYNKSDNCE TPEAGYFAVAVVKK SAS DLTWDNLKGKKSCHTAVGRTAGWNI PMGLLYNKINHCRFDEFFSEGCAPGSKKDSSL CKLCMGSGNLNCEPNNKEGYGYTGAFRC LVEKGDVA FVKHQTV PONTGGKNPD PWA KNLNEKDYE LLLCLDGT RKPVEEYANCHLARA PNHAVVTRKDKEACVHKILRQQOHLF GSNVTDCSGNFCLFRSETKDLLFRDDTVCLAKLHDRNTYEKYLGE EYVKAVGNLRKC STSSLLEACTFRRP
28	Human Transferrin	ATGAGGCTCGCCGTGGGAGCCCTGCTGGTCTGCGCCGTCTCTGGGGCTGTGTCTGGCT GTCCCTGATAAACTGTGAGATGGTGTGCACTGTGCGGAGCATGAGGCCACTAAGTGC CAGAGTTTCCGCGACCATATGAAAAGCGTCATTCCATCCGATGGTCCCAGTGTGCT TGTGTGAAGAAAGCCTCCTACCTTGATTGCATCAGGGCCATTGCGGCAAACGAAGCG GATGCTGTGACACTGGATGCAGGTTTGGTGTATGATGCTTACTTGGCTCCCAATAAC CTGAAGCCTGTGGTGGCAGAGTTCTATGGGTCAAAAAGAGGATCCACAGACTTTCTAT TATGCTGTTGCTGTGGTGAAGAAGGATAGTGGCTTCCAGATGAACCAGCTTCGAGGC AAGAAGTCCTGCCACACGGGTCTAGGCAGGTCCGCTGGGTGGAACATCCCCATAGGC TTACTTTACTGTGACTTACCTGAGCCACGTAAACCTCTTGAGAAAGCAGTGGCCAAT TTCTTCTCGGGCAGCTGTGCCCTTGTGCGGATGGGACGGACTTCCCCCAGCTGTGT CAACTGTGTCCAGGTGTGGCTGCTCCACCCTTAACCAATACTTCGGCTACTCGGGA GCCTTCAAGTGTCTGAAGGATGGTGTGGGGATGTGGCCTTTGTCAAGCACTCGACT ATATTTGAGAACTTGCAACAAGGCTGACAGGGACCAAGTATGAGCTGCTTTGCCTA GACAACACCCGGAAGCCGGTAGATGAATACAAGGACTGCCACTTGGCCAGGTCCCT

		TCTCATACCGTCGTTGGCCCCGAAGTATGGGCGGCAAGGAGGACTTGATCTGGGAGCTT CTCAACCAGGCCCCAGGAACATTTTGGCAAAGACAAATCAAAAGAATTCCAACATATTC AGCTCTCCTCATGGGAAGGACCTGCTGTTTAAGGACTCTGCCACGGGTTTTTAAAA GTCCCCCAAGGATGGATGCCAAGATGTACCTGGGCTATGAGTATGTCACTGCCATC CGGAATCTACGGGAAGGCACATGCCAGAAAGCCCCAACAGATGAATGCAAGCCTGTG AAGTGGTGTGCGCTGAGCCACCACGAGAGGCTCAAGTGTGATGAGTGGAGTGTTAAC AGTGTAGGGAAAATAGAGTGTGTATCAGCAGAGACCACCGAAGACTGCATCGCCAAG ATCATGAATGGAGAAGCTGATGCCATGAGCTTGGATGGAGGGTTTGTCTACATAGCG GGCAAGTGTGGTCTGGTGCCTGTCTTGGCAGAAAACCTACAATAAGAGCGATAATTGT GAGGATACACCAGAGGCAGGGTATTTTGTCTGATGAGTGGTGAAGAAATCAGCTTCT GACCTCACCTGGGACAATCTGAAAGGCAAGAGTCCTGCCATACGGCAGTTGGCAGA ACCGCTGGCTGGAACATCCCCATGGGCCTGCTCTACAATAAGATCAACCACTGCAGA TTTGATGAATTTTTTCAAGTGAAGTTGTGCCCTGGGTCTAAGAAAGACTCCAGTCTC TGTAAGCTGTGTATGGGCTCAGGCCATAACCTGTGTGAACCAACAACAAAGAGGGA TACTACGGCTACACAGGCGCTTTCAGGTGTCTGGTTGAGAAGGGAGATGTGGCCTTT GTGAAACACCAGACTGTCCACAGAACTGGGGGAAAAAACCTGATCCATGGGCT AAGAATCTGAATGAAAAAGACTATGAGTTGCTGTGCCTTGATGGTACCAGGAAACCT GTGGAGGAGTATGCGAAGTCCACCTGGCCAGAGCCCCGAATCACGCTGTGGTCACA CGGAAAGATAAGGAAGCTTGCCTCCACAAGATATTACGTCAACAGCAGCACCTATTT GGAAGCAACGTAACCTGACTGCTCGGGCAACTTTTGTGTGTTCCGGTCGGAAACCAAG GACCTTCTGTTCAGAGATGACACAGTATGTTTGGCCAACTTCATGACAGAAACACA TATGAAAAATACTTAGGAGAAGAATATGTCAAGGCTGTTGGTAACCTGAGAAAATGC TCCACCTCATCACTCCTGGAAGCCTGCACTTTCCGTAGACCTTAA
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In some embodiments, the half-life extension moiety comprises an antibody or fragment thereof. Non-limiting examples of antibody fragments include a Fab, a Fab', a F(ab')₂, a Fc, a Fv, an scFv, a diabody, a triabody, a tetrabody, and a nanobody.

5 In some embodiments, the half-life extension moiety comprises an Fc domain (e.g., an IgG1 Fc domain (e.g., a human IgG1 Fc domain), an IgG2 Fc domain (e.g., a human IgG2 Fc domain), an IgG3 Fc domain (e.g., a human IgG3 Fc domain, or an IgG4 Fc domain (e.g., a human IgG4 Fc domain).

10 In some embodiments, the half-life extension moiety comprises an Fc domain comprising an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 15 (human IgG1 Fc domain), SEQ ID NO: 17 (human IgG2 Fc domain), SEQ ID NO: 19 (human IgG3 Fc domain), or SEQ ID NO: 21 (human IgG4 Fc domain).

15 In some embodiments, the half-life extension moiety comprises an Fc domain comprising an amino acid sequence of SEQ ID NO: 15 (human IgG1 Fc domain), SEQ ID NO: 17 (human IgG2 Fc domain), SEQ ID NO: 19 (human IgG3 Fc domain), or SEQ ID NO: 21 (human IgG4 Fc domain).

In some embodiments, the sequence encoding the Fc domain comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID

NO: 16 (human IgG1 Fc domain), SEQ ID NO: 18 (human IgG2 Fc domain), SEQ ID NO: 20 (human IgG3 Fc domain), or SEQ ID NO: 22 (human IgG4 Fc domain).

In some embodiments, the sequence encoding the Fc domain comprises a nucleic acid sequence of SEQ ID NO: 16 (human IgG1 Fc domain), SEQ ID NO: 18 (human IgG2 Fc domain), SEQ ID NO: 20 (human IgG3 Fc domain), or SEQ ID NO: 22 (human IgG4 Fc domain).

In some embodiments, the Fc domain comprises an amino acid sequence having at least one substitution. The Fc domain can comprise any number and/or any type of substitution. The Fc domain can comprise any substitution known in the art or described herein. Non-limiting examples of substitutions that can be included in an Fc domain include M252Y/S254T/T256E (YTE); L234A/L235A (LALA); M428L/N434S (LS); T307A/E380A/N434A (AAA); T250Q/M428L (QL); and V308P.

In some embodiments, the Fc domain can comprise the amino acid sequence of SEQ ID NO: 15 (human IgG1 Fc domain), except that it comprises an amino acid substitution(s) selected from one or more of: M252Y/S254T/T256E (YTE); L234A/L235A (LALA); M428L/N434S (LS); T307A/E380A/N434A (AAA); T250Q/M428L (QL); and V308P.

In some embodiments, the Fc domain can comprise the amino acid sequence of SEQ ID NO: 17 (human IgG2 Fc domain), except that it comprises an amino acid substitution(s) selected from one or more of: M252Y/S254T/T256E (YTE); L234A/L235A (LALA); M428L/N434S (LS); T307A/E380A/N434A (AAA); T250Q/M428L (QL); and V308P.

In some embodiments, the Fc domain can comprise the amino acid sequence of SEQ ID NO: 19 (human IgG3 Fc domain), except that it comprises an amino acid substitution(s) selected from one or more of: M252Y/S254T/T256E (YTE); L234A/L235A (LALA); M428L/N434S (LS); T307A/E380A/N434A (AAA); T250Q/M428L (QL); and V308P.

In some embodiments, the Fc domain can comprise the amino acid sequence of SEQ ID NO: 21 (human IgG4 Fc domain), except that it comprises an amino acid substitution(s) comprising an amino acid substitution selected from one or more of: M252Y/S254T/T256E (YTE); L234A/L235A (LALA); M428L/N434S (LS); T307A/E380A/N434A (AAA); T250Q/M428L (QL); and V308P.

In some embodiments, the half-life extension moiety comprises an albumin polypeptide or a functional fragment thereof (e.g., a human albumin polypeptide or fragment thereof), a

fibronectin polypeptide or fragment thereof (e.g., a human fibronectin polypeptide or fragment thereof), or a transferrin polypeptide or fragment thereof (e.g., a human transferrin polypeptide or fragment thereof).

In some embodiments, the albumin polypeptide or a functional fragment thereof
5 comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 23. In some embodiments, the albumin polypeptide comprises an amino acid sequence of SEQ ID NO: 23. In some embodiments, the albumin polypeptide comprises an amino acid sequence having at least one substitution (e.g., one, two, three, four, five, six, seven, eight, nine, or ten amino acid substitutions and/or deletions) as compared to the
10 corresponding wildtype albumin polypeptide (e.g., wildtype human albumin polypeptide, e.g., SEQ ID NO: 23). The albumin polypeptide can comprise one, two, three, four, five, six, seven, eight, nine, or ten of any type of substitution.

In some embodiments, the albumin polypeptide or a functional fragment thereof is encoded by a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or
15 99% identical to SEQ ID NO: 24. In some embodiments, the albumin polypeptide is encoded by a nucleic acid sequence of SEQ ID NO: 24.

In some embodiments, the fibronectin polypeptide or a functional fragment thereof comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 25. In some embodiments, the fibronectin polypeptide comprises
20 an amino acid sequence of SEQ ID NO: 25. In some embodiments, the fibronectin polypeptide comprises an amino acid sequence having at least one substitution (e.g., one, two, three, four, five, six, seven, eight, nine, or ten amino acid substitutions and/or deletions) as compared to the corresponding wildtype fibronectin polypeptide (e.g., wildtype human fibronectin polypeptide, e.g., SEQ ID NO: 25). The fibronectin polypeptide can comprise one, two, three, four, five, six,
25 seven, eight, nine, or ten of any type of substitution.

In some embodiments, the fibronectin polypeptide or a functional fragment thereof is encoded by a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 26. In some embodiments, the fibronectin polypeptide is encoded
by a nucleic acid sequence of SEQ ID NO: 26.

30 In some embodiments, the transferrin polypeptide or a functional fragment thereof comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or

99% identical to SEQ ID NO: 27. In some embodiments, the transferrin polypeptide comprises an amino acid sequence of SEQ ID NO: 27. In some embodiments, the transferrin polypeptide comprises an amino acid sequence having at least one substitution (e.g., one, two, three, four, five, six, seven, eight, nine, or ten amino acid substitutions and/or deletions) as compared to the corresponding wildtype transferrin polypeptide (e.g., wildtype human transferrin polypeptide, e.g., SEQ ID NO: 27). The transferrin polypeptide can comprise one, two, three, four, five, six, seven, eight, nine, or ten of any type of substitution.

In some embodiments, the transferrin polypeptide or a functional fragment thereof is encoded by a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 28. In some embodiments, the transferrin polypeptide is encoded by a nucleic acid sequence of SEQ ID NO: 28.

In some embodiments, the half-life extension moiety comprises an antibody or antigen-binding fragment thereof (e.g., any of the exemplary types of antigen-binding antibody fragments described herein or known in the art) that binds to a serum protein (e.g., albumin (e.g., human albumin), fibronectin (e.g., human fibronectin), or transferrin (e.g., human transferrin)). For example, the half-life extension moiety comprises antigen-binding antibody fragments, e.g., single-chain antibodies (scFv), single-domain antibodies (also called nanobodies), Fab fragments, Fab' fragments, and F(ab')₂ Fab₂ fragments, diabodies, triabodies, and tetrabodies, that bind to albumin (e.g., human albumin), fibronectin (e.g., human fibronectin), or transferrin (e.g., human transferrin). In some embodiments, the half-life extension moiety comprises a nanobody that binds specifically to human albumin, human fibronectin, or human transferrin.

Non-limiting examples of antibodies, antigen-binding fragments, and peptides that bind to a serum protein that can be used as half-life extension moieties are provided in WO 2013/128027; US 2015/0037334; US 9,376,495; US 8,349,569; US 10,407,508; and Michot, N., et al, Albumin binding Nanofitins, a new scaffold to extend half-life of biologics – a case study with exenatide peptide, *Peptides*, 152 (2022), the contents of each of which is incorporated herein by reference in its entirety.

In some embodiments, the half-life extension moiety can comprise one or more post-translational modification site(s). A post-translational modification site includes amino acid(s) in the polypeptide where a cellular enzyme can modify the amino acid to add a post-translational modification (e.g., phosphorylation, glycosylation, hydroxylation, methylation, acetylation,

deamidation, and lipidation (e.g., N-myristoylation, palmitoylation, glycosylphosphatidylinositol (GPI)-anchor addition) that results in an increase in the half-life of the polypeptide.

(iii) Secretion Signal Sequences

5 In some embodiments, the polypeptide (encoded by the circular polyribonucleotides and modified RNAs described herein) can further comprise a secretion signal sequence. The term “secretion signal sequence” refers to a polypeptide sequence (e.g., between about 10 and about 30 amino acids in length) that is present at the N-terminus of a protein and that targets at least a portion of the protein to the cellular secretory pathway.

10 In some embodiments, a sequence encoding a secretion signal sequence (e.g., any of the exemplary secretion signal sequences described herein or known in the art) is operably linked to a sequence encoding a GLP-2 polypeptide or analog thereof (and a sequence encoding the half-life extension moiety). For example, the sequence encoding the secretion signal sequence can be operably linked at a 5' end of a sequence encoding a GLP-2 polypeptide or analog thereof.

15 In some embodiments, the secretion signal sequence is a liver-specific secretion signal sequence. Non-limiting examples of a secretion signal sequence include an IL-2 secretion signal sequence, a proglucagon secretion signal sequence, a Gaussia lucifersase secretion signal sequence, and an erythropoietin (EPO) secretion signal sequence.

20 Non-limiting examples of amino acid sequences (and corresponding nucleic acid sequences) of an IL-2 secretion signal sequence, a proglucagon secretion signal sequence, a Gaussia lucifersase secretion signal sequence, and an EPO secretion signal sequence are shown in Table 4 below.

Table 4. Non-limiting examples of signal secretion sequences

SEQ ID NO:	Description	Sequence
29	IL2 secretion signal sequence	MYRMQLLSICIALSLALVTNS
30	IL2 secretion signal sequence	ATGTACCGGATGCAGTTGTTGTCCTGTATAGCTCTTCCCTTGCATTGGTC ACTAATTCT
31	proglucagon secretion signal sequence	MKSIYFVAGLFVMLVQGSWQ

32	proglucagon secretion signal sequence	ATGAAAAGCATTTACTTTGTGGCTGGGTTATTTGTAATGCTGGTACAAGG CAGCTGGCAA
33	Gaussia luciferase secretion signal sequence	MGVKVLFALICIAVAEAK
34	Gaussia luciferase secretion signal sequence	ATGGGAGTCAAAGTTCTGTTTGGCCTGATCTGCATCGCTGTGGCCGAGGC CAAG
35	EPO secretion signal sequence	MGVHECPAWLWLLLSLLSLPLGLPVLGA
36	EPO secretion signal sequence	AUGGGAGUGCACGAGUGUCCCGCGUGGUUGUGGUUGCUGCUGUCGCUC UUGAGCCUCCACUGGGACUGCCUGUGCUGGGGGCA
37	IL2co2CC-2 secretion signal sequence	MRMQLLLLIALLSLALVTNS
38	IL2co2CC-2 secretion signal sequence	ATGAGGATGCAGCTGCTGCTCCTGATCGCCCTGAGCCTGGCCCTGGTGAC CAACAGC
39	A1AT secretion signal sequence	MPSSVSWGILLLAGLCCLVPVSLA
40	A1AT secretion signal sequence	ATGCCAGCAGCGTTTCCTGGGGCATCCTGCTGCTGGCCGGCCTGTGCTG CCTGGTGCCCGTGAGCCTGGCC
41	AACT secretion signal sequence	MERMLPLLALGLLAAGFCPAVLC
42	AACT secretion signal sequence	ATGGAGCGCATGCTGCCCCTGCTGGCCCTGGGACTGCTGGCCGCCGGCTT CTGCCCCGCCGTCTGTGC
43	A1AG1 secretion signal sequence	MALSWVLTVLSLLPLLEA
44	A1AG1 secretion signal sequence	ATGGCCCTGTCTGGGTGCTGACCGTCCTGAGCCTGCTGCCCCTGCTGGA AGCC
45	ALBU secretion signal sequence	MKWVTFISLLFLFSSAYS
46	ALBU secretion signal sequence	ATGAAGTGGGTGACCTTCATTTCCCTCCTGTTCTCTCCAGCGCCTAC TCC
47	HPT secretion signal sequence	MSALGAVIALLLWGQLFA
48	HPT secretion signal sequence	ATGAGCGCCCTGGGGGCCGTGATCGCCCTGCTGCTCTGGGGCCAACTTTT CGCC
49	EST1 secretion signal sequence	MWLRAFILATLSASA AW
50	EST1 secretion signal sequence	ATGTGGCTCCGGGCCTTTATCCTGGCCACCCTGAGCGCCTCCGCCGCCTG G
51	PAI1 secretion signal sequence	MQMSPALTCLVLGLALVFGESGA
52	PAI1 secretion signal sequence	ATGCAGATGTCCCCGCCCTGACCTGCCTGGTGCTGGGCCTGGCCCTGGT GTTCGGCGAGGGCTCCGCC
53	SAA1 secretion signal sequence	MKLLTGLVFCSLVLGVSS
54	SAA1 secretion signal sequence	ATGAAGCTGCTGACCGGCCTGGTCTTCTGCTCCCTGGTCCTGGGCGTGAG CTCC

55	B2MG secretion signal sequence	MSRSVALAVLALLSLSGLEA
56	B2MG secretion signal sequence	ATGAGCAGGAGCGTGGCCCTGGCCGTCCTGGCCCTCCTGAGCCTGAGCGG CCTGGAGGCC
57	PPIB secretion signal sequence	MLRLSERNMKVLLAAALIAGSVFLLLPGPSAA
58	PPIB secretion signal sequence	ATGCTGCGCCTTTCCGAGAGGAACATGAAGGTGCTGCTGGCCGCCGCCCT GATCGCCGGCTCCGTTTTCTTCCTGCTGCTGCCCCGGCCCCCTCCGCTGCA
59	RET4 secretion signal sequence	MKWVWALLLLAALGSGRA
60	RET4 secretion signal sequence	ATGAAGTGGGTCTGGGCCCTGCTGCTGCTGGCCGCCCTGGGCTCCGGCAG AGCC
61	IC1 secretion signal sequence	MASRLTLLTLLLLLAGDRASS
62	IC1 secretion signal sequence	ATGGCCTCCAGACTGACCCTGCTGACCTTGCTGCTGCTCCTGCTGGCCGGC GACAGGGCCTCCTCC
63	FETUA secretion signal sequence	MKSLVLLCLAQWLGHS
64	FETUA secretion signal sequence	ATGAAGTCCCTGGTGCTGCTGCTGTGCCTGGCCAGCTCTGGGGCTGCCA CAGC
65	CO3 secretion signal sequence	MGPTSGPSLLLLLLTHLPLALG
66	CO3 secretion signal sequence	ATGGGCCCCACCTCCGGCCCCCTCCCTGCTGCTCCTGCTGCTGACCCACCTG CCCCTGGCCCTGGGC
67	APOA1 secretion signal sequence	MKAAVLTAVLFLTGSQA
68	APOA1 secretion signal sequence	ATGAAGGCCGCCGTGCTGACCCTGGCCGTCCTGTTCTGACCGGCTCCCA GGCC
69	PDIA1 secretion signal sequence	MLRRALLCLAVAALVRA
70	PDIA1 secretion signal sequence	ATGCTGCGGAGAGCCCTCCTGTGCCTGGCCGTGGCCGCCCTGGTGCGCGC C
71	CALR secretion signal sequence	MLLSVPLLLGLLGLAVA
72	CALR secretion signal sequence	ATGCTGCTGTCCGTGCCCTGCTCCTGGGCCTGCTGGGCCTGGCCGTGGCC
73	PDIA3 secretion signal sequence	MRLRRLALFPGVALLLAAARLAAA
74	PDIA3 secretion signal sequence	ATGCGCCTGAGGAGGCTGGCCCTTTTCCCCGGAGTGGCCCTGCTGCTGGC TGCCGCCCCGCTGGCCGCCGCC
75	CFAB secretion signal sequence	MGSNLSPQLCLMPFILGLLSGGVTT
76	CFAB secretion signal sequence	ATGGGCAGCAACCTGAGCCCCCAACTTTCCTGATGCCCTTTATCCTGGG CCTGCTGAGCGGCGGCGTGACCACC
77	CRP secretion signal sequence	MEKLLCFLVLTSLSHAFG
78	CRP secretion signal sequence	ATGGAGAAGCTGCTCTGCTTCCTGGTGCTGACCTCCCTGAGCCACGCCTTC GGG
79	H1 secretion signal sequence	MELGLSWIFLLAILKGVQC

80	H1 secretion signal sequence	ATGGAGCTGGGCCTGTCCTGGATCTTCCTGCTGGCCATCCTGAAGGGCGTGCAGTGC
81	H2 secretion signal sequence	MELGLRWVFLVAILEGVQC
82	H2 secretion signal sequence	ATGGAACCTGGGCCTGCGCTGGGTTTTCTGCTGGCCATCCTCGAGGGCGTGCAGTGC
83	H3 secretion signal sequence	MKHLWFFLLVAAPRWLS
84	H3 secretion signal sequence	ATGAAGCACCTTTGGTTTTCTGCTCCTGGTGGCCGCCCCACGCTGGGTGCTTTCC
85	H4 secretion signal sequence	MDWTWRILFLVAAATGAHS
86	H4 secretion signal sequence	ATGGACTGGACCTGGAGGATCCTGTTCTGCTGGCCGCCGCCACCGGCGCCACTCC
87	H5 secretion signal sequence	MDWTWRFLFVAAATGVQS
88	H5 secretion signal sequence	ATGGACTGGACCTGGCGCTTCCTGTTCTGCTGGTGGCCGCCGCCACCGGCGTGCAGAGC
89	H6 secretion signal sequence	MEFGLSWLFLVAILKGVQC
90	H6 secretion signal sequence	ATGGAGTTCGGCCTGTCCTGGCTGTTCTGCTGGCCATCCTGAAGGGCGTGCAGTGC
91	H7 secretion signal sequence	MEFGLSWVFLVALFRGVQC
92	H7 secretion signal sequence	ATGGAATTCGGCCTGAGCTGGGTGTTCTGCTGGCCCTGTTCCGCGGGGTGCAGTGC
93	H8 secretion signal sequence	MDLLHKNMKHLWFFLLVAAPRWLS
94	H8 secretion signal sequence	ATGGACCTGCTGCACAAGAACATGAAGCACCTGTGGTTTTCTGCTCCTGGTGGCCGCCCCCTCGCTGGGTCTTGAGC
95	L1 secretion signal sequence	MDMRVPAQLLGLLLWLSGARC
96	L1 secretion signal sequence	ATGGACATGCGGGTGCCCGCCAGCTCCTGGGCCTGCTGCTCCTGTGGCTTTCCGGCGCCCGCTGC
97	L2 secretion signal sequence	MKYLLPTAAAGLLLLAAQPAMA
98	L2 secretion signal sequence	ATGAAGTACCTGCTGCCCCACCGCCGCCGCTGGCCTGCTGCTGCTGGCCGCCAGCCCCGCGCATGGCC
99	Ab-IgKVIII secretion signal sequence	MDMRVPAQLLGLLLWLRGARC
100	Ab-IgKVIII secretion signal sequence	ATGGACATGAGAGTGCCCGCCAGCTCCTGGGCCTGCTGCTGCTCTGGCTGCGGGGCGCCCGCTGC
101	IL20 secretion signal sequence	MKASSLAFSLLSAFYLLWTPSTG
102	IL20 secretion signal sequence	ATGAAGGCCTCCTCCCTGGCCTTTTCCCTGCTGAGCGCCGCCTTTTACCTGCTCTGGACCCCTTCCACCGGG
103	IL10 secretion signal sequence	MHSSALLCCLVLLTGVR
104	IL10 secretion signal sequence	ATGCACTCCTCCGCCCTGCTCTGCTGCCTGGTGTGCTGCTGACCGGCGTGCGGCC
105	IL23A secretion signal sequence	MLGSRAVMLLLLLPWTAGQ

106	IL23A secretion signal sequence	ATGCTGGGCTCCCGCGCCGTGATGCTGCTCCTGCTGCTGCCCTGGACCGC CCAGGGC
107	IL23R secretion signal sequence	MNQVTIQWDAVIALYILFSWCHG
108	IL23R secretion signal sequence	ATGAACCAGGTGACCATCCAGTGGGACGCCGTGATCGCCCTGTACATCCT GTTCTCCTGGTGCCACGGC
109	IL2RA secretion signal sequence	MDSYLLMWGLLTFIMVPGCQA
110	IL2RA secretion signal sequence	ATGGACAGCTACCTGCTGATGTGGGGCCTGCTGACTTTCATCATGGTGCC CGGCTGCCAGGCC
111	IL6 secretion signal sequence	MNSFSTSAFGPVAFSLGLLLVLPAAFPAP
112	IL6 secretion signal sequence	ATGAACTCCTTTTCCACCTCCGCCTTCGGCCCCGTGGCCTTTTCCCTGGGC CTGCTCCTGGTGCTGCCCCGCCGCCTTCCCCGCCCCC
113	IL9 secretion signal sequence	MLLAMVLTSALLCSVAG
114	IL9 secretion signal sequence	ATGCTGCTGGCCATGGTGCTGACCTCCGCCCTGCTGCTGTGCAGCGTGGC CGGC
115	IL12RB1 secretion signal sequence	MEPLVTWVPLFLFLLSRQGAA
116	IL12RB1 secretion signal sequence	ATGGAGCCCCTGGTGACCTGGGTCGTGCCCCCTCCTGTTCTTTTCTGCTT TCCCGCCAGGGCGCCGCC
117	IL12RB2 secretion signal sequence	MAHTFRGCSLAFMFIITWLLIKA
118	IL12RB2 secretion signal sequence	ATGGCCACACCTCCGAGGCTGCTCCCTGGCCTTCATGTTTCATTACC TGGCTGCTGATCAAGGCT
119	IFN γ secretion signal sequence	MKYTSYILAFQLCIVLGS LGCYC
120	IFN γ secretion signal sequence	ATGAAGTACACCTCCTACATCCTGGCCTTCCAGCTGTGCATCGTGCTGGG CTCCCTGGGCTGCTACTGC
121	TCR-mVa2 secretion signal sequence	MDKILTASFLLLGLHLAGVNGQQKEKHDQQQVRQSP
122	TCR-mVa2 secretion signal sequence	ATGGACAAGATCCTGACCGCCAGCTTCCTGCTCCTGGGCCTGCACCTGGC CGGCGTGAACGGACAGCAGAAGGAGAAGCACGACCAGCAGCAAGTGCG GCAGAGCCCA
123	TCR-hVa17 secretion signal sequence	MDKILGASFLVLWLQLCWVSGQQKEKSDQQQVKQSP
124	TCR-hVa17 secretion signal sequence	ATGGACAAGATCCTGGGCGCTTCCTTCCTGGTCCTGTGGCTGCAGCTCTGC TGGGTTTCCGGGCAGCAGAAGGAGAAGTCCGACCAGCAGCAGGTGAAAC AGAGCCCC
125	TCR-hVa21 secretion signal sequence	MAMLLGASVLILWLQPDWVNSQQKNNDQQVKQNS
126	TCR-hVa21 secretion signal sequence	ATGGCCATGCTCCTGGGCGCCAGCGTCCTGATCCTTTGGCTCCAGCCCGA CTGGGTGAACTCCCAGCAGAAGAACAACGACCAGCAGGTGAAGCAGAAC AGC
127	TCR-CTL-L17 secretion signal sequence	MAMLLGASVLILWLQPDWVNSQQKNDD

128	TCR-CTL-L17 secretion signal sequence	ATGGCCATGCTGCTGGGCGCCTCCGTCCTGATCCTTTGGCTGCAACCCGA CTGGGTGAACTCCCAGCAGAAGAACGACGAC
129	TCR-HPB-MLT-TAV1 secretion signal sequence	MIFASLLRAVIASICVVSSMA
130	TCR-HPB-MLT-TAV1 secretion signal sequence	ATTTTCGCCAGCCTGCTGCGCGCCGTGATCGCCTCCATTGCGTGGTGTCC TCCATGGCC
131	TCR-PY14 secretion signal sequence	MLLLLVPVLEVIFTLGGTRA
132	TCR-PY14 secretion signal sequence	ATGCTGCTGCTCCTGGTGCCCGTGCTGGAGGTGATCTTTACCCTGGGCGGC ACCAGAGCC
133	Gluc secretion signal sequence	MGVKVLFALICIAVAEA
134	Gluc secretion signal sequence	ATGGGCGTGAAGGTCCTGTTCGCCCTGATCTGCATCGCCGTGGCCGAGGC C
135	VSVG secretion signal sequence	MKCLLYLAFLFIGVNC
136	VSVG secretion signal sequence	ATGAAGTGCCTGCTGTACCTGGCCTTCCTGTTCATCGGCGTGAAGTGC
137	INS secretion signal sequence	MALWMRLLPLLALLALWGPDPAAA
138	INS secretion signal sequence	ATGGCCCTTTGGATGCGCCTGCTGCCCCTGCTGGCCCTGCTCGCCCTCTGG GGCCCTGACCCCGCCGCCGCC
139	gE-HSV2 secretion signal sequence	MARGAGLVFFVGVVWVSCLA
140	gE-HSV2 secretion signal sequence	ATGGCCAGGGGCGCCGGCCTGGTCTTCTTCGTGGGCGTTTGGGTAGTGAG CTGCCTGGCC

In some embodiments, an IL-2 secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, or 99% identical to SEQ ID NO: 29. In some embodiments, an IL-2 secretion signal sequence comprises an amino acid sequence of SEQ ID NO: 29.

In some embodiments, a proglucagon secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, or 99% identical to SEQ ID NO: 31. In some embodiments, a proglucagon secretion signal sequence comprises an amino acid sequence of SEQ ID NO: 31.

In some embodiments, a Gaussia luciferase secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, or 99% identical to SEQ ID NO: 33. In

some embodiments, a Gaussia luciferase secretion signal sequence comprises an amino acid sequence of SEQ ID NO: 33.

In some embodiments, an EPO secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, or 99% identical to SEQ ID NO: 35. In some
5 embodiments, an EPO secretion signal sequence comprises an amino acid sequence of SEQ ID NO: 35.

In some embodiments, a signal secretion sequence comprises an amino acid sequence having at least 70%, 80%, 90%, 95%, or 99% identical to one of SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ
10 ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO:
15 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, or SEQ ID NO: 139.

In some embodiments, a signal secretion sequence comprises an amino acid sequence of
20 SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71,
25 SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125,
30 SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, or SEQ ID NO: 139.

In some embodiments, the sequence encoding a secretion signal sequence comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, or 99% identical to one of SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, or SEQ ID NO: 36.

5 In some embodiments, the sequence encoding a secretion signal sequence comprises a nucleic acid sequence of SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, or SEQ ID NO: 36.

In some embodiments, the sequence encoding a secretion signal sequence comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, or 99% identical to one of SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID
10 NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO:
15 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, SEQ ID NO: 128, SEQ ID NO: 130, SEQ ID NO: 132, SEQ ID NO: 134, SEQ ID NO: 136, SEQ ID NO: 138, or SEQ ID NO: 140.

In some embodiments, the sequence encoding a secretion signal sequence comprises a
20 nucleic acid sequence of SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, SEQ ID NO: 68, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80,
25 SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, SEQ ID NO: 128, SEQ ID NO: 130, SEQ ID NO: 132, SEQ ID NO:
30 134, SEQ ID NO: 136, SEQ ID NO: 138, or SEQ ID NO: 140.

(iv) Spacer Sequences

As used herein, a “spacer” or “spacer sequence” or “spacer region” refers to any contiguous nucleotide sequence (e.g., of one or more nucleotides) that provides distance or flexibility between two adjacent polynucleotide regions.

5 In some embodiments, the circular polyribonucleotide or linear modified RNA as disclosed herein includes at least one spacer sequence. In some embodiments, the circular polyribonucleotide or linear modified RNA includes 1, 2, 3, 4, 5, 6, 7, or more spacer sequences (e.g., identical or different spacer sequences). In some embodiments, a first spacer sequence and a second spacer sequence (e.g., a third spacer sequence, a fourth spacer sequence, a fifth spacer
10 sequence, etc.) are about the same length. In some embodiments, a first spacer sequence and a second spacer sequence (e.g., a third spacer sequence, a fourth spacer sequence, a fifth spacer sequence, etc.) are different lengths.

The spacer sequence can be a nucleic acid sequence having low GC content, e.g., less than 65%, 60%, 55%, 50%, 55%, 50%, 45%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%,
15 32%, 31 %, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11 %, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1 %, across the full length of the spacer, or across at least 50%, 60%, 70%, 80%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% contiguous nucleic acid residues of the spacer. In some embodiments, the spacer sequence is substantially free of a secondary structure, such as less than 40 kcal/mol,
20 less than -39, -38, -37, -36, -35, -34, -33, -32, -31, -30, -29, -28, -27, -26, -25, -24, -23, -22, -20, -19, -18, -17, -16, -15, -14, -13, -12, -11, -10, -9, -8, -7, -6, -5, -4, -3, -2 or -1 kcal/mol.

In some embodiments, the spacer sequence comprises: (i) a polyA region comprising 80% to 100% (e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100%) adenosine residues; (ii) a polyAC region
25 comprising between 80% to 100% (e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100%) adenosine or cytosine residues; (iii) a polyAU region comprising between 80% to 100% (e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100%) adenosine or uridine residues; or (iii) a polyAG region comprising between 80% to 100%
30 (e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100%) adenosine or guanosine residues.

The spacer sequence can be a non-coding sequence or a coding sequence. When the spacer is a non-coding sequence, a start codon may be provided in the coding sequence of an adjacent sequence. When the spacer is a coding sequence, a start codon may be provided in the spacer sequence. In some embodiments, the spacer is operably linked to another sequence described herein.

In some embodiments, the circular polyribonucleotide or modified linear RNA includes an IRES operably linked to an expression sequence encoding a polypeptide. In some embodiments, the circular polyribonucleotide further includes a spacer region between the IRES and the 3' exon fragment or the 5' exon fragment. The spacer region may be, e.g., at least 5 (e.g., at least 10, at least 15, at least 20) ribonucleotides in length. The spacer region may be, e.g., from 5 to 500 (e.g., 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, or 500) ribonucleotides. In some embodiments, the spacer region includes a polyA sequence. In some embodiments, the spacer region includes a polyA-C sequence. In some embodiments, the spacer region includes a polyA-G sequence. In some embodiments, the spacer region includes a polyA-T sequence. In some embodiments, the spacer region includes a polyU sequence. In some embodiments, the spacer region includes a random sequence. In some embodiments, the spacer region includes a random sequence.

In some embodiments, the circular polyribonucleotide is from 50 to 20,000, e.g., 100 to 20,000, e.g., 200 to 20,000, e.g., 300 to 20,000 (e.g., 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 11,000, 12,000, 13,000, 14,000, 15,000, 16,000, 17,000, 18,000, 19,000, or 20,000) ribonucleotides in length. In embodiments, the circular polyribonucleotide is, e.g., at least 25, at least 50, at least 100, at least 125, at least 1150, at least 200, at least 300, at least 400, at least 500, at least 1,000, at least 2,000, at least 3,000, at least 4,000, or at least 5,000 ribonucleotides in length.

A spacer can be specifically engineered depending on the IRES. In some embodiments, an RNA folding computer software, such as RNAFold, can be utilized to guide designs of the various elements of the vector, including the spacers.

In some embodiments, the polyribonucleotide includes a 5' spacer sequence (e.g., between the 5' annealing region and the polyribonucleotide cargo). In some embodiments, the 5' spacer sequence is at least 10 nucleotides in length. In another embodiment, the 5' spacer

sequence is at least 15 nucleotides in length. In a further embodiment, the 5' spacer sequence is at least 30 nucleotides in length. In some embodiments, the 5' spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the 5' spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides in length. In some embodiments the 5' spacer sequence is between 20 and 50 nucleotides in length. In certain embodiments, the 5' spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In one embodiment, the 5' spacer sequence is a polyA sequence. In another embodiment, the 5' spacer sequence is a polyA-C sequence. In some embodiments, the 5' spacer sequence includes a polyA-G sequence. In some embodiments, the 5' spacer sequence includes a polyA-T sequence. In some embodiments, the 5' spacer sequence includes a random sequence.

In some embodiments, the polyribonucleotide includes a 3' spacer sequence (e.g., between the 3' annealing region and the polyribonucleotide cargo). In some embodiments, the 3' spacer sequence is at least 10 nucleotides in length. In another embodiment, the 3' spacer sequence is at least 15 nucleotides in length. In a further embodiment, the 3' spacer sequence is at least 30 nucleotides in length. In some embodiments, the 3' spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the 3' spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides in length. In some embodiments the 3' spacer sequence is from 20 to 50 nucleotides in length. In certain embodiments, the 3' spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38,

39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In one embodiment, the 3' spacer sequence is a polyA sequence. In another embodiment, the 3' spacer sequence is a polyA-C sequence. In some embodiments, the 3' spacer sequence includes a polyA-G sequence. In some embodiments, the 3' spacer sequence includes a polyA-T sequence. In some embodiments, the 3' spacer sequence includes a random sequence.

In one embodiment, the polyribonucleotide includes a 5' spacer sequence, but not a 3' spacer sequence. In another embodiment, the polyribonucleotide includes a 3' spacer sequence, but not a 5' spacer sequence. In another embodiment, the polyribonucleotide includes neither a 5' spacer sequence, nor a 3' spacer sequence. In another embodiment, the polyribonucleotide

does not include an IRES sequence. In a further embodiment, the polyribonucleotide does not include an IRES sequence, a 5' spacer sequence or a 3' spacer sequence.

In some embodiments, the spacer sequence includes at least 3 ribonucleotides, at least 4 ribonucleotides, at least 5 ribonucleotides, at least about 8 ribonucleotides, at least about 10 ribonucleotides, at least about 12 ribonucleotides, at least about 15 ribonucleotides, at least about 20 ribonucleotides, at least about 25 ribonucleotides, at least about 30 ribonucleotides, at least about 40 ribonucleotides, at least about 50 ribonucleotides, at least about 60 ribonucleotides, at least about 70 ribonucleotides, at least about 80 ribonucleotides, at least about 90 ribonucleotides, at least about 100 ribonucleotides, at least about 120 ribonucleotides, at least about 150 ribonucleotides, at least about 200 ribonucleotides, at least about 250 ribonucleotides, at least about 300 ribonucleotides, at least about 400 ribonucleotides, at least about 500 ribonucleotides, at least about 600 ribonucleotides, at least about 700 ribonucleotides, at least about 800 ribonucleotides, at least about 900 ribonucleotides, or at least about 1000 ribonucleotides.

Exemplary spacer sequences are shown in Table 5 below.

Table 5. Non-limiting examples of spacer sequences

SEQ ID NO:	Description	Sequence
224	AU120	AAAAAAAAUAAAAAAAAAUAAAAAAAAAUUUAAAAAAAAAUAAAAAAAAUUU AAAAAAAAAUAAAAAAAAAUAAAAAAAAAUAAAAAAAAAUAAAAAAAAUUUAAA AAAAAAUAAAAAAA
225	AC120	AAAAAAACAAAAAAAAACCAAAAAAAAAACCAAAAAAAAAACCAAAAAACCA AAAAAAACCAAAAAAAAAACAAAAACCAAAAAACCAAAAAAAAAACCAAAAA AAAACAAAAAAA
226	AC50	AAAAAAACAAAAAAAAACCAAAAAAAAAACCAAAAAAAAAACCAAAAAAA

Additional spacer sequences are further described in WO 2023/044006, WO 2024/192420, and WO 2019/118919, each of which is incorporated herein by reference in its entirety.

(v) Linkers

In some embodiments, the circular polyribonucleotide or modified RNA further comprises a sequence encoding a linker sequence. For example, the circular polyribonucleotide or modified RNA can comprise a sequence encoding a linker sequence disposed between the sequence encoding the GLP-2 polypeptide or analog thereof and the sequence encoding the half-life extension moiety. In such instances, the polypeptide comprises a linker sequence disposed between the GLP-2 polypeptide or analog thereof and the half-life extension moiety in the polypeptide. Non-limiting examples of linker sequences are shown in Table 6 below.

Additional examples of linker sequences are well known in the art (*See e.g.*, Chen, X., Fusion Protein Linkers: Property, Design and Functionality, *Adv Drug Deliv Rev.*, 65(10): 1357-1369 (2013), the content of which is incorporated herein by reference in its entirety).

Table 6. Non-limiting examples of linker sequences

SEQ ID NO:	Description	Sequence
141	Linker Sequence	(GGGGS) _x wherein x is an integer from 1 to 50
142	Linker Sequence	(GGSG) _x wherein x is an integer from 1 to 50
143	Linker Sequence	(SGGG) _x wherein x is an integer from 1 to 50
210	Linker Sequence	(Gly) ₈
211	Linker Sequence	(Gly) ₆
212	Linker Sequence	(EAAAK) _x wherein x is an integer from 1 to 3
213	Linker Sequence	A(EAAAK) ₄ ALEA(EAAAK) ₄ A
214	Linker Sequence	PAPAP
215	Linker Sequence	AEAAAKEAAKA
216	Linker Sequence	(Ala-Pro) _x (10 – 34 aa) wherein x is an integer between 5 and 17
217	Linker Sequence	(Glu-Pro) _x (10 – 34 aa) wherein x is an integer between 5 and 17
218	Linker Sequence	(Lys-Pro) _x (10 – 34 aa) wherein x is an integer between 5 and 17
219	Linker Sequence	KESGSVSSEQLAQFRSLD
220	Linker Sequence	EGKSSGSGSESKST
221	Linker Sequence	GSAGSAAGSGEF

222	Linker Sequence	A(EAAAK) _x A wherein x is an integer between 2 and 5
223	Linker Sequence	DKTHT

(vi) Modifications

Any of the circular polyribonucleotides or elements thereof described herein can include one or more modifications (e.g., any of the exemplary modifications described herein or known in the art). Any of the modified RNAs described herein can include one or more modifications (e.g., any of the exemplary modifications described herein or known in the art).

The one or more modifications can be included at any position in the circular polyribonucleotide or modified RNA. For example, the sequence encoding a GLP-2 polypeptide or analog thereof can comprise one or more modifications (e.g., the sequence encoding a GLP-2 polypeptide or analog thereof comprises one or more modified bases).

In some embodiments, the circular polyribonucleotide or modified RNA can comprise a post-transcriptional modification (e.g., capping, cleavage, polyadenylation, and splicing).

A circular polyribonucleotide or elements thereof, or a modified RNA or elements thereof can include one or more nucleoside modifications (e.g., any of those described in Rozenski et al., *Nucl. Acids Res.* 27: 196-197, 1999).

In some embodiments, the circular polyribonucleotide or modified RNA comprises one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) modification(s) to a sugar, a nucleobase, an internucleoside linkage, or a combination thereof. In some embodiments, the circular polyribonucleotide or modified RNA can include one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) pyrimidine nucleobases having one or more atoms substituted with an amino, a thiol, an alkyl (e.g., methyl or ethyl), or halo (e.g., chloro or fluoro) group.

In some embodiments, the circular polyribonucleotide or modified RNA can include one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) ribonucleotides that have been modified into deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), and/or locked nucleic acids (LNAs).

In some embodiments, the circular polyribonucleotide or modified RNA includes at least one (e.g., two, three, four, or five) N(6)methyladenosine (m6A) modification to increase translation efficiency. In some embodiments, the N(6)methyladenosine (m6A) modification can

reduce immunogenicity (e.g., reduce the level of one or more marker of an immune or inflammatory response) of the circular polyribonucleotide or modified RNA.

In some embodiments, a modification may include a chemical or cellular induced modification. For example, some non-limiting examples of intracellular RNA modifications are described in Lewis et al., *Nature Reviews Mol. Cell Biol.* 18:202-210, 2017, herein incorporated by reference.

In some embodiments, chemical modifications to the ribonucleotides of a circular polyribonucleotide or modified RNA may enhance immune evasion. The circular polyribonucleotide or modified RNA may be synthesized and/or modified by methods well established in the art, such as those described in “Current protocols in nucleic acid chemistry,” Beaucage, S.L. et al. (Eds.), John Wiley & Sons, Inc., New York, NY, USA, herein incorporated by reference. Modifications include, for example, end modifications, e.g., 5' end modifications (phosphorylation (mono-, di- and tri-), conjugation, inverted linkages), 3' end modifications (conjugation, DNA nucleotides, inverted linkages), base modifications (e.g., replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners), removal of bases (abasic nucleotides), or conjugated bases. The modified ribonucleotide bases may also include 5- methylcytidine, N1-methylpseudouridine, 5-methoxyuridine, and pseudouridine. In some embodiments, base modifications may modulate expression, immune response, stability, subcellular localization, to name a few functional effects, of the circular polyribonucleotide or modified RNA. In some embodiments, the modification includes a bi-orthogonal nucleotide, e.g., an unnatural base. See, e.g., Kimoto et al., Chem. Comm. (Camb) 53:12309, 2017, which is herein incorporated by reference.

In some embodiments, one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) ribonucleotides of the circular polyribonucleotide or modified RNA can include a sugar modification (e.g., at the 2' position or 4' position) and optionally, a backbone modification, including modification or replacement of the phosphodiester linkages. Specific examples of circular polyribonucleotides or modified RNAs include, but are not limited to, circular polyribonucleotides or modified RNAs including modified backbones or no natural internucleoside linkages, such as internucleoside modifications, including modification or replacement of the phosphodiester linkages. Circular polyribonucleotides and modified RNAs having modified backbones include, among others, those that do not have a phosphorus atom in

the backbone. In particular embodiments, the circular polyribonucleotide or modified RNA will include ribonucleotides with a phosphorus atom in its internucleoside backbone.

Modified backbones of circular polyribonucleotides and modified RNAs can include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates, such as 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates, such as 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity, wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts, and free acid forms of the circular polyribonucleotides and modified RNAs are also included. In some embodiments, the circular polyribonucleotides or modified RNAs may be negatively- or positively-charged.

The modified nucleotide(s), which may be incorporated into the circular polyribonucleotides or modified RNAs, can be modified on the internucleoside linkage (e.g., phosphate backbone). Herein, in the context of the polynucleotide backbone, the phrases "phosphate" and "phosphodiester" are used interchangeably. Backbone phosphate groups can be modified by replacing one or more of the oxygen atoms with a different substituent. Further, the modified nucleosides and nucleotides can include the wholesale replacement of an unmodified phosphate moiety with another internucleoside linkage as described herein. Examples of modified phosphate groups include, but are not limited to, phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. The phosphate linker can also be modified by the replacement of a linking oxygen with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates), and carbon (bridged methylene -phosphonates).

A-thio substituted phosphate moieties can be included to confer stability to circular polyribonucleotides and modified RNAs through the unnatural phosphorothioate backbone linkages. Phosphorothioate RNAs have increased nuclease resistance and subsequently a longer half-life in a cellular environment. Phosphorothioate linkages in a circular polyribonucleotide or

modified RNA is expected to reduce the innate immune response through weaker binding/activation of cellular innate immune molecules.

In specific embodiments, a modified nucleoside can include an alpha-thio-nucleoside (e.g., 5'-O-(1-thiophosphate)-adenosine, 5'-O-(1-thiophosphate)-cytidine (a-thio-cytidine), 5'-O-(1-thiophosphate)-guanosine, 5'-O-(1-thiophosphate)-uridine, or 5'-O-(1-thiophosphate)-pseudouridine).

Other internucleoside linkages that may be employed according to the present disclosure, including internucleoside linkages which do not contain a phosphorous atom, are described herein.

A circular polyribonucleotide or modified RNA may or may not be uniformly modified along the entire length of the molecule. For example, one or more or all types of nucleotide (e.g., naturally-occurring nucleotides, purine or pyrimidine, or any one or more or all of A, G, U, C, I, pU) may or may not be uniformly modified in the circular polyribonucleotide or modified RNA, or in a given predetermined subregion thereof. In some embodiments, the circular polyribonucleotide or modified RNA includes a pseudouridine. In some embodiments, the circular polyribonucleotide or modified RNA includes an inosine, which may aid in the immune system characterizing the circular polyribonucleotide or modified RNA as an endogenous RNA versus a viral RNA. The incorporation of inosine may also mediate improved RNA stability/reduced degradation. See for example, Yu, Z. et al., *Cell Res.* 25, 1283–1284, 2015, which is incorporated by reference in its entirety.

In some embodiments, all or substantially all nucleotides in a circular polyribonucleotide or modified RNA (or in a given subregion thereof) are modified. In some embodiments, the modification may include an m6A, which may augment expression; an inosine, which may attenuate an immune response; pseudouridine, which may increase RNA stability, or translational readthrough (stagger element); an m5C, which may increase stability; and a 2,2,7-trimethylguanosine, which aids subcellular translocation (e.g., nuclear localization).

Different sugar modifications, nucleotide modifications, and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in a circular polyribonucleotide or modified RNA. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of the circular polyribonucleotide or modified RNA, such that the function of the circular polyribonucleotide or modified RNA is not

substantially decreased. A modification can be included in a coding sequence or a non-coding sequence.

In some embodiments, the circular polyribonucleotide or modified RNA may include about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, e.g., any one or more of A, G, U, or C) or any intervening percentage (e.g., about 1% to about 10%, about 1% to about 15%, about 1% to about 20%, from about 1% to about 25%, about 1% to about 30%, about 1% to about 40%, about 1% to about 50%, about 1% to about 60%, about 1% to about 70%, about 1% to about 80%, about 1% to about 90%, about 1% to about 95%, about 10% to about 20%, about 10% to about 25%, about 10% to about 30%, about 10% to about 40%, about 10% to about 50%, about 10% to about 60%, about 10% to about 70%, about 10% to about 80%, about 10% to about 90%, about 10% to about 95%, about 10% to about 100%, about 20% to about 25%, about 20% to about 30%, about 20% to about 40%, about 20% to about 50%, about 20% to about 60%, about 20% to about 70%, about 20% to about 80%, about 20% to about 90%, about 20% to about 95%, about 20% to about 100%, about 30% to about 40%, about 30% to about 50%, about 30% to about 60%, about 30% to about 70%, about 30% to about 80%, about 30% to about 90%, about 30% to about 100%, about 40% to about 50%, about 40% to about 60%, about 40% to about 70%, about 40% to about 80%, about 40% to about 90%, about 40% to about 100%, about 50% to about 60%, about 50% to about 70%, about 50% to about 80%, about 50% to about 90%, about 50% to about 95%, about 50% to about 100%, about 60% to about 70%, about 60% to about 80%, about 60% to about 90%, about 60% to about 100%, about 70% to about 80%, about 70% to about 90%, about 70% to about 95%, about 70% to about 100%, about 80% to about 90%, about 80% to about 95%, about 80% to about 100%, about 90% to about 95%, about 90% to about 100%, and about 95% to about 100%).

(vii) Internal Ribosome Entry Site (IRES)

In some embodiments, the circular polyribonucleotides or modified RNAs described herein includes one or more internal ribosome entry site(s) (IRES(s)). In some embodiments, an IRES is operably linked to the sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein). In some embodiments, an IRES is located at or proximal to the

5' end of the sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein).

A suitable IRES includes an RNA sequence capable of engaging a eukaryotic ribosome.

In some embodiments, an IRES is at least about 50 nucleotides, at least about 100 nucleotides, at

5 least about 200 nucleotides, at least about 250 nucleotides, at least about 350 nucleotides, or at

least about 500 nucleotides. In some embodiments, an IRES is about 50 nucleotides to about 500

nucleotides, about 50 nucleotides to about 450 nucleotides, about 50 nucleotides to about 400

nucleotides, about 50 nucleotides to about 350 nucleotides, about 50 nucleotides to about 300

nucleotides, about 50 nucleotides to about 250 nucleotides, about 50 nucleotides to about 200

10 nucleotides, about 50 nucleotides to about 150 nucleotides, about 50 nucleotides to about 100

nucleotides, about 100 nucleotides to about 500 nucleotides, about 100 nucleotides to about 450

nucleotides, about 100 nucleotides to about 400 nucleotides, about 100 nucleotides to about 350

nucleotides, about 100 nucleotides to about 300 nucleotides, about 100 nucleotides to about 250

nucleotides, about 100 nucleotides to about 200 nucleotides, about 100 nucleotides to about 150

15 nucleotides, about 150 nucleotides to about 500 nucleotides, about 150 nucleotides to about 450

nucleotides, about 150 nucleotides to about 400 nucleotides, about 150 nucleotides to about 350

nucleotides, about 150 nucleotides to about 300 nucleotides, about 150 nucleotides to about 250

nucleotides, about 150 nucleotides to about 200 nucleotides, about 200 nucleotides to about 500

nucleotides, about 200 nucleotides to about 450 nucleotides, about 200 nucleotides to about 400

20 nucleotides, about 200 nucleotides to about 350 nucleotides, about 200 nucleotides to about 300

nucleotides, about 200 nucleotides to about 250 nucleotides, about 250 nucleotides to about 500

nucleotides, about 250 nucleotides to about 450 nucleotides, about 250 nucleotides to about 400

nucleotides, about 250 nucleotides to about 350 nucleotides, about 250 nucleotides to about 300

nucleotides, about 300 nucleotides to about 500 nucleotides, about 300 nucleotides to about 450

25 nucleotides, about 300 nucleotides to about 400 nucleotides, about 300 nucleotides to about 350

nucleotides, about 350 nucleotides to about 500 nucleotides, about 350 nucleotides to about 450

nucleotides, about 350 nucleotides to about 400 nucleotides, about 400 nucleotides to about 500

nucleotides, about 400 nucleotides to about 450 nucleotides, or about 450 nucleotides to about

500 nucleotides.

30 In some embodiments, the IRES is derived from the DNA of an organism including, but not limited to, a virus, a mammal, and *Drosophila*. Such viral DNA may be derived from, but

not limited to, picornavirus complementary DNA (cDNA), encephalomyocarditis virus (EMCV) cDNA, and poliovirus cDNA. In some embodiments, *Drosophila* DNA from which an IRES is derived includes, but is not limited to, an Antennapedia gene from *Drosophila melanogaster*.

Herein, the term “derived from” in the context of a nucleic acid, i.e., for a nucleic acid
 5 “derived from” (another) nucleic acid, means that the nucleic acid, which is derived from (another) nucleic acid, shares e.g. at least 60%, 70%, 80%, 81 %, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with the nucleic acid from which it is derived. The skilled person is aware that sequence identity is typically calculated for the same types of nucleic acids, i.e., for DNA sequences or for RNA
 10 sequences. Thus, it is understood, if a DNA is “derived from” an RNA or if an RNA is “derived from” a DNA, in a first step the RNA sequence is converted into the corresponding DNA sequence (in particular by replacing the uracils (U) by thymidines (T) throughout the sequence) or, vice versa, the DNA sequence is converted into the corresponding RNA sequence (in particular by replacing the T by U throughout the sequence). Thereafter, the sequence identity of
 15 the DNA sequences or the sequence identity of the RNA sequences is determined. Preferably, a nucleic acid “derived from” a nucleic acid also refers to nucleic acid, which is modified in comparison to the nucleic acid from which it is derived, e.g., in order to increase RNA stability even further and/or to prolong and/or increase protein production. In the context of amino acid sequences, the term “derived from” means that the amino acid sequence, which is derived from
 20 (another) amino acid sequence, shares e.g. at least 60%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with the amino acid sequence from which it is derived.

In some embodiments, if present, the IRES is an IRES of Taura syndrome virus, Triatoma virus, Theiler's encephalomyelitis virus, simian Virus 40, Solenopsis invicta virus 1,
 25 *Rhopalosiphum padi* virus, Reticuloendotheliosis virus, human poliovirus 1, Plautia stall intestine virus, Kashmir bee virus, Human rhinovirus 2, Homalodisca coagulata virus- 1, Human Immunodeficiency Virus type 1, Homalodisca coagulata virus- 1, Himetobi P virus, Hepatitis C virus, Hepatitis A virus, Hepatitis GB virus, foot and mouth disease virus, Human enterovirus 71, Equine rhinitis virus, Ectropis obliqua picorna-like virus, Encephalomyocarditis virus
 30 (EMCV), *Drosophila* C Virus, Crucifer tobamo virus, Cricket paralysis virus, Bovine viral diarrhea virus 1, Black Queen Cell Virus, Aphid lethal paralysis virus, Avian encephalomyelitis

virus, Acute bee paralysis virus, Hibiscus chlorotic ringspot virus, Classical swine fever virus, Human FGF2, Human SFTPA1, Human AML1/RUNX1, *Drosophila antennapedia*, Human AQP4, Human AT1R, Human BAG-1, Human BCL2, Human BiP, Human c-IAP1, Human c-myc, Human eIF4G, Mouse NDST4L, Human LEF1, Mouse HIF1 alpha, Human n-myc, Mouse
 5 Gtx, Human p27kipl, Human PDGF2/c-sis, Human p53, Human Pim-1, Mouse Rbm3, *Drosophila reaper*, Canine Scamper, *Drosophila Ubx*, Human UNR, Mouse UtrA, Human VEGF-A, Human XIAP, Salivirus, Cosavirus, Parechovirus, *Drosophila hairless*, *S. cerevisiae* TFIID, *S. cerevisiae* YAP1, Human c-src, Human FGF-1, Simian picomavirus, Turnip crinkle virus, Aichivirus, Crohivirus, Echovirus 11, an aptamer to eIF4G, Cocksackievirus B3 (CVB3), or
 10 Cocksackievirus A (CVB1/2). In yet another embodiment, the IRES is an IRES of Cocksackievirus B3 (CVB3). In a further embodiment, the IRES is an IRES of Encephalomyocarditis virus. In some embodiments, the IRES sequence has more than 90% sequence identify with one of the foregoing IRES sequences.

In some embodiments, The IRES sequence may have the sequence of wild-type CVB3
 15 IRES sequence having the nucleic acid sequence of:
 TTAAAACAGCCTGTGGGTTGATCCCACCCACAGGCCCATTTGGGCGCTAGCACTCTGG
 TATCACGGTACCTTTGTGCGCCTGTTTTATACCCCTCCCCCAACTGTAAGTTAGAAG
 TAACACACACCGATCAACAGTCAGCGTGGCACACCAGCCACGTTTTGATCAAGCAC
 TTCTGTTACCCCGGACTGAGTATCAATAGACTGCTCACGCGGTTGAAGGAGAAAGCG
 20 TTCGTTATCCGGCCAACTACTTCGAAAAACCTAGTAACACCGTGGAAGTTGCAGAGT
 GTTTCGCTCAGCACTACCCAGTGTAGATCAGGTCGATGAGTCACCGCATTCCCCAC
 GGGCGACCGTGGCGGTGGCTGCGTTGGCGGCCTGCCATGGGGAAACCCATGGGAC
 GCTCTAATACAGACATGGTGCGAAGAGTCTATTGAGCTAGTTGGTAGTCCTCCGGCC
 CCTGAATGCGGCTAATCCTAACTGCGGAGCACACACCCTCAAGCCAGAGGGCAGTG
 25 TGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCATT
 TTATTCCTATACTGGCTGCTTATGGTGACAATTGAGAGATCGTTACCATATAGCTATT
 GGATTGGCCATCCGGTGACTAATAGAGCTATTATATATCCCTTTGTTGGGTTTATACC
 ACTTAGCTTGAAAGAGGTTAAAACATTACAATTCATTGTTAAGTTGAATACAGCAAA
 (SEQ ID NO: 227).

The IRES sequence may have a modified sequence in comparison to the wildtype IRES sequence. In some embodiments, when the last nucleotide of the wild-type IRES is not a cytosine nucleic acid residue, the last nucleotide of the wild-type IRES sequence may be modified such that it is a cytosine residue. For example, the IRES sequence may be a CVB3

5 IRES sequence wherein the terminal adenosine residue is modified to cytosine residue. In some embodiments, the modified CVB3 IRES may have the nucleic acid sequence of:
 TTAAACAGCCTGTGGGTTGATCCCACCCACAGGCCATTGGGCGCTAGCACTCTGG
 TATCACGGTACCTTTGTGCGCCTGTTTTATACCCCTCCCCCAACTGTAAGTCTAGAAG
 TAACACACACCGATCAACAGTCAGCGTGGCACACCAGCCACGTTTTGATCAAGCAC
 10 TTCTGTTACCCCGGACTGAGTATCAATAGACTGCTCACGCGGTTGAAGGAGAAAGCG
 TTCGTTATCCGGCCAACTACTTCGAAAAACCTAGTAACACCGTGGAAGTTGCAGAGT
 GTTTCGCTCAGCACTACCCAGTGTAGATCAGGTCGATGAGTCACCGCATTCCCCAC
 GGGCGACCGTGGCGGTGGCTGCGTTGGCGGCCTGCCATGGGGAAACCCATGGGAC
 GCTCTAATACAGACATGGTGCGAAGAGTCTATTGAGCTAGTTGGTAGTCCTCCGGCC
 15 CCTGAATGCGGCTAATCCTAACTGCGGAGCACACACCCTCAAGCCAGAGGGCAGTG
 TGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCATT
 TTATTCCTATACTGGCTGCTTATGGTGACAATTGAGAGATCGTTACCATATAGCTATT
 GGATTGGCCATCCGGTGACTAATAGAGCTATTATATATCCCTTTGTTGGGTTTATACC
 ACTTAGCTTGAAAGAGGTTAAACATTACAATTCATTGTTAAGTTGAATACAGCAAC
 20 (SEQ ID NO: 168).

In some embodiments, the modified CVB3 IRES may have the nucleic acid sequence of:
 CCAACTGTAAGTCTAGAAGTAACACACACCGATCAACAGTCAGCGTGGCACACCAGC
 CACGTTTTGATCAAGCACTTCTGTTACCCCGGACTGAGTATCAATAGACTGCTCACG
 CGGTTGAAGGAGAAAGCGTTCGTTATCCGGCCAACTACTTCGAAAAACCTAGTAAC
 25 ACCGTGGAAGTTGCAGAGTGTTTCGCTCAGCACTACCCAGTGTAGATCAGGTCGAT
 GAGTCACCGCATTCCCCACGGGCGACCGTGGCGGTGGCTGCGTTGGCGGCCTGCC
 ATGGGGAAACCCATGGGACGCTCTAATACAGACATGGTGCGAAGAGTCTATTGAGC
 TAGTTGGTAGTCCTCCGGCCCCCTGAATGCGGCTAATCCTAACTGCGGAGCACACACC
 CTCAAGCCAGAGGGCAGTGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACT
 30 TTGGGTGTCCGTGTTTCATTTTATTCCTATACTGGCTGCTTATGGTGACAATTGAGAG
 ATCGTTACCATATAGCTATTGGATTGGCCATCCGGTGACTAATAGAGCTATTATATA

TCCCTTTGTTGGGTTTATAACCACTTAGCTTGAAAGAGGTTAAACATTACAATTCATT
GTTAAGTTGAATACAGCAAC (SEQ ID NO: 228)

In some embodiments, the IRES sequence is an encephalomyocarditis virus (EMCV) IRES. In some embodiments, the ECMV IRES may have the nucleic acid sequence of:

5 ACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTAT
TTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTT
CTTGACGAGCATTCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTT
GAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGT
AGCGACCCCTTTCAGGCAGCGGAACCCCCACCTGGCGACAGGTGCCTCTGCGGCC
10 AAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCAGTGCCACGTT
GTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAG
GGGCTGAAGGATGCCCAGAAGGTACCCCATTTGTATGGGATCTGATCTGGGGCCTCG
GTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAACGTCTAGGCCCCCGAAC
CACGGGGACGTGGTTTTCTTTGAAAAACACGATGATAATA (SEQ ID NO: 229).

15 In some embodiments, the IRES sequence is an Enterovirus 71 (EV71) IRES. In some
embodiments, the IRES sequence is a wild-type EV71 sequence having the nucleic acid
sequence of:

TTAAACAGCTGTGGGTTGTCACCCACCCACAGGGTCCACTGGGCGCTAGTACACTG
GTATCTCGGTACCTTTGTACGCCTGTTTTATACCCCTCCCTGATTTGCAACTTAGAA
20 GCAACGCAAACCAGATCAATAGTAGGTGTGACATACCAGTCGCATCTTGATCAAGC
ACTTCTGTATCCCCGGACCGAGTATCAATAGACTGTGCACACGGTTGAAGGAGAAA
ACGTCCGTTACCCGGCTAACTACTTCGAGAAGCCTAGTAACGCCATTGAAGTTGCAG
AGTGTTTCGCTCAGCACTCCCCCGTGTAGATCAGGTCGATGAGTCACCGCATTCCC
CACGGGCGACCGTGGCGGTGGCTGCGTTGGCGGCCTGCCTATGGGGTAACCCATAG
25 GACGCTCTAATACGGACATGGCGTGAAGAGTCTATTGAGCTAGTTAGTAGTCCTCCG
GCCCCTGAATGCGGCTAATCCTAACTGCGGAGCACATAACCCTTAATCCAAAGGGCA
GTGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTC
TTTTTATTCTTGTATTGGCTGCTTATGGTGACAATTAAAGAATTGTTACCATATAGCT
ATTGGATTGGCCATCCAGTGTCAAACAGAGCTATTGTATATCTCTTTGTTGGATTAC
30 ACCTCTCACTCTTGAAACGTTACACACCCTCAATTACATTATACTGCTGAACACGAA
GCG (SEQ ID NO: 169).

In some embodiments, the terminal guanosine residue of the EV71 IRES sequence is modified to a cytosine residue.

In some embodiments, the polyribonucleotide includes at least one IRES flanking at least one (e.g., 2, 3, 4, 5 or more) expression sequence. In some embodiments, the IRES flanks both sides of at least one (e.g., 2, 3, 4, 5 or more) expression sequence. In some embodiments, the polyribonucleotide includes one or more IRES sequences on one or both sides of each expression sequence, leading to separation of the resulting peptide(s) and or polypeptide(s). For example, a polyribonucleotide described herein may include a first IRES operably linked to a first expression sequence and a second IRES operably linked to a second expression sequence.

For example, an exemplary construct described above includes a construct with two IRES sequences each of which is followed by an ORF sequence. In some embodiments, a spacer sequence is disposed between the first IRES-ORF sequence and the second IRES-ORF sequence. In some embodiments, both the first ORF and the second ORF sequence encode a GLP-2 polypeptide or analog thereof (e.g., GLP-2 Fc). In some embodiments, the first ORF encodes a GLP-2 polypeptide or analog thereof (e.g., GLP-2 Fc) and the second ORF encodes a different polypeptide.

In some embodiments, the circular polyribonucleotides or modified RNAs described herein can include a modified IRES, such as those described in WO 2020/198403, which is incorporated herein by reference in its entirety or an IRES, such as those described in Fan et al. Nature Communications 13(1):3751, 2022 doi: 10.1038/s41467-022-31327-y; Chen et al. Mol. Cell 81:1-19, 2021; Jopling et al. Oncogene 20:2664-2670, 2001; Baranick et al. PNAS 105(12):4733-4738, 2008; Lang et al. Molecular Biology of the Cell 13(5):1792-1801, 2002; Dorokhov et al. PNAS 99(8):5301-5306, 2002; Wang et al. Nucleic Acids Research 33(7):2248-2258, 2005; Petz et al. Nucleic Acids Research 35(8):2473-2482, 2007; and WO 2021/263124 A2, each of, which is hereby incorporated by reference in its entirety.

(viii) Translation initiation sequence

In some embodiments, the circular polyribonucleotides or modified RNAs described herein can include at least one translation initiation sequence. In some embodiments, the circular polyribonucleotide or modified RNA includes a translation initiation sequence operably linked to

the sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein).

In some embodiments, the translation initiation sequence includes, e.g., a start codon. In some embodiments, the translation initiation sequence includes a Kozak or Shine-Dalgarno sequence. In some embodiments, the circular polyribonucleotide or modified RNA includes the translation initiation sequence, e.g., Kozak sequence, adjacent to the sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein). In some embodiments, the translation initiation sequence, e.g., Kozak sequence, is present on one or both sides of each sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein), leading to separation of the expression products. In some embodiments, the circular polyribonucleotide or modified RNA includes at least one translation initiation sequence adjacent to the sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein). In some embodiments, the translation initiation sequence provides conformational flexibility to the circular polyribonucleotide or modified RNA. In some embodiments, the translation initiation sequence is within a substantially single stranded region of the circular polyribonucleotide or modified RNA.

The circular polyribonucleotide may include more than 1 start codon such as, but not limited to, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 50, at least 60, or more than 60 start codons. In such embodiments, translation may initiate on the first start codon or may initiate downstream of the first start codon.

In some embodiments, the circular polyribonucleotide or modified RNA may initiate at a codon that is not the first start codon, e.g., AUG. Translation of the circular polyribonucleotide or modified RNA may initiate at an alternative translation initiation sequence, such as, but not limited to, ACG, AGG, AAG, CTG/CUG, GTG/GUG, ATA/AUA, ATT/AUU, or TTG/UUG. In some embodiments, translation begins at an alternative translation initiation sequence under selective conditions, e.g., stress-induced conditions. As a non-limiting example, the translation of the circular polyribonucleotide or modified RNA may begin at alternative translation initiation sequence, such as ACG. As another non-limiting example, the circular polyribonucleotide or modified RNA translation may begin at alternative translation initiation sequence, CTG/CUG.

As yet another non-limiting example, the circular polyribonucleotide or modified RNA translation may begin at alternative translation initiation sequence, GTG/GUG. As yet another non-limiting example, the circular polyribonucleotide or modified RNA may begin translation at a repeat-associated non-AUG (RAN) sequence, such as an alternative translation initiation sequence that includes short stretches of repetitive RNA, e.g., CGG, GGGGCC, CAG, or CTG. Nucleotides flanking a codon that initiates translation may affect the translation efficiency and the length and/or the structure of the circular polyribonucleotide or modified RNA. Masking any of the nucleotides flanking a codon that initiates translation may be used to alter the position of translation initiation, translation efficiency, and length, and/or structure of the circular polyribonucleotide or modified RNA.

In some embodiments, a masking agent may be used near the start codon or alternative start codon in order to mask or hide the codon to reduce the probability of translation initiation at the masked start codon or alternative start codon. Non-limiting examples of masking agents include antisense locked nucleic acids (LNA) oligonucleotides and exon-junction complexes (EJCs). In some embodiments, a masking agent may be used to mask a start codon of the circular polyribonucleotide or modified RNA in order to increase the likelihood that translation will initiate at an alternative start codon.

In some embodiments, translation is initiated under selective conditions, such as, but not limited to, viral-induced selection in the presence of GRSF-1 and the circular polyribonucleotide or modified RNA includes GRSF-1 binding sites.

In some embodiments, translation is initiated by eukaryotic initiation factor 4A (eIF4A) treatment with Rocaglates. Translation may be repressed by blocking 43S scanning, leading to premature, upstream translation initiation and reduced protein expression from transcripts bearing the RocA-eIF4A target sequence.

(ix) Translation Termination Sequences

In some embodiments, the circular polyribonucleotides or modified RNAs can further include at least one translation termination sequence. In some embodiments, the translation termination sequence is operably linked to the 3' end of the sequence encoding the polypeptide (e.g., any of the exemplary polypeptides described herein).

In some embodiments, the circular polyribonucleotide or modified RNA includes a termination element at the end of a sequence encoding the polypeptide (e.g., any of the polypeptides described herein). In some embodiments, a circular polyribonucleotide or modified RNA can include two or more termination elements in succession. Generally, translation termination sequences include an in-frame nucleotide triplet that signals termination of translation, e.g., UAA, UGA, or UAG. In some embodiments, one or more translation termination sequences in the circular polyribonucleotide or modified RNA are frame-shifted termination elements, such as but not limited to, off-frame or -1 and + 1 shifted reading frames (e.g., hidden stop) that may terminate translation. Frame-shifted termination elements include nucleotide triples, e.g., TAA, TAG, or TGA, that appear in the second and third reading frames of the sequence encoding the polypeptide. Frame-shifted termination elements may be important in preventing misreads of the sequence encoding the polypeptide, which is often detrimental to a cell. In some embodiments, the termination element is a stop codon.

Further examples of translation termination sequences are described in paragraphs [0169] - [0170] of WO2019/118919, which is hereby incorporated by reference in its entirety.

(x) Stagger Elements

A circular polyribonucleotide of the disclosure can include a cleavage domain (e.g., a stagger element or a cleavage sequence).

In some embodiments, the circular polyribonucleotide includes a stagger sequence or a stagger element. As used herein, the term “stagger element” or a “stagger sequence” is a moiety, such as a nucleotide sequence, that induces ribosomal pausing during translation. To avoid production of a continuous expression product, e.g., peptide or polypeptide, while maintaining rolling circle translation, a stagger sequence can be included to induce ribosomal pausing during translation. The stagger sequence may include a 2A-like or CHYSEL (cis-acting hydrolase element) sequence. In some embodiments, the stagger element encodes a sequence with a C-terminal consensus sequence that is $X_1X_2X_3EX_5NP GP$ (SEQ ID NO: 144), where X_1 is absent or G or H, X_2 is absent or D or G, X_3 is D, V, I, S, or M, and X_5 is any amino acid. In some embodiments, the stagger element comprises a non-conserved sequence of amino-acids with a strong alpha-helical propensity followed by the consensus sequence -D(V/I)EXNP GP (SEQ ID

NO: 145), where X is any amino acid. Some non-limiting examples of stagger elements include any of the stagger sequences shown in Table 7.

In some embodiments, a stagger element described herein terminates translation and/or cleaves an expression product between G and P of the consensus sequence described herein. As one non-limiting example, the circular polyribonucleotide or modified RNA includes at least one stagger sequence to terminate translation and/or cleave the encoded polypeptide. In some embodiments, the circular polyribonucleotide or modified RNA includes a stagger element adjacent to at least one sequence encoding a polypeptide (e.g., any of the exemplary polypeptides described herein). In some embodiments, the circular polyribonucleotide or modified RNA includes a stagger element after each sequence encoding a polypeptide (e.g., any of the exemplary polypeptides described herein). In some embodiments, the circular polyribonucleotide or modified RNA includes a stagger element present on one or both sides of each sequence encoding a polypeptide (e.g., any of the exemplary polypeptides described herein), leading to translation of individual peptide(s) and or polypeptide(s) from each expression sequence.

Table 7. Non-limiting examples of stagger elements

SEQ ID NO:	Description	Sequence
146	Stagger Sequence	GDVESNPGP
147	Stagger Sequence	GDIEENPGP
148	Stagger Sequence	VEPNPGP
149	Stagger Sequence	IETNPGP
150	Stagger Sequence	GDIESNPGP
151	Stagger Sequence	GDVELNPGP
152	Stagger Sequence	GDIE'TNPGP
153	Stagger Sequence	GDVENPGP
154	Stagger Sequence	GDVEENPGP
155	Stagger Sequence	GDVEQNPGP
156	Stagger Sequence	IESNPGP

157	Stagger Sequence	GDIELNPGP
158	Stagger Sequence	HDIETNPGP
159	Stagger Sequence	HDVETNPGP
160	Stagger Sequence	HDVEMNPGP
161	Stagger Sequence	GDMESNPGP
162	Stagger Sequence	GDVETNPGP
163	Stagger Sequence	GDIEQNPGP
164	Stagger Sequence	DSEFNPGP

In some embodiments, the stagger element may include a chemical moiety, such as glycerol, a non-nucleic acid linking moiety, a chemical modification, a modified nucleic acid, or any combination thereof.

5

(xi) Replication Elements

Replication of a circular polyribonucleotide can occur by generating a complement circular polyribonucleotide. In some embodiments, the circular polyribonucleotide can include a motif to initiate transcription, where transcription is driven by either endogenous cellular machinery (DNA-dependent RNA polymerase) or an RNA-dependent RNA polymerase encoded by the circular polyribonucleotide. The product of the rolling-circle transcriptional event can be cut by a ribozyme to generate either complementary or propagated circular polyribonucleotide at unit length. The ribozyme can be encoded by the circular polyribonucleotide, its complement, or by an RNA sequence in trans. In some embodiments, the encoded ribozyme can include a sequence or motif that regulates (inhibits or promotes) activity of the ribozyme to control circular polyribonucleotide propagation. In some embodiments, unit-length sequences can be ligated into a circular form by a cellular RNA ligase. In some embodiments, the circular polyribonucleotide includes a replication element that aids in self-amplification. Examples of such replication elements include HDV replication domains and replication competent circular RNA sense and/or antisense ribozymes, such as antigenomic 5'-

15

20

CGGGUCGGCAUGGCAUCUCCACCUCCUCGC

GGUCCGACCUGGGCAUCCGAAGGAGGACGCACGUCCACUCGGAUGGCUAAGGGAG

AGCCA-3' (SEQ ID NO: 165) or genomic 5'-

UGGCCGGCAUGGUCCCAGCCUCCUCGCUGGCGCCGGCUGGGCAACAUAU

CCGAGGGGACCGUCCCCUCGGUAAUGGCGAAUGGGACCCA-3' (SEQ ID NO: 166).

In some embodiments, the circular polyribonucleotide includes at least one cleavage
5 sequence as described herein to aid in replication. A cleavage sequence within the circular
polyribonucleotide can cleave long transcripts replicated from the circular polyribonucleotide to
a specific length that can subsequently circularize to form a complement to the circular
polyribonucleotide.

In another embodiment, the circular polyribonucleotide includes at least one ribozyme
10 sequence to cleave long transcripts replicated from the circular polyribonucleotide to a specific
length, where another encoded ribozyme cuts the transcripts at the ribozyme sequence.
Circularization forms a complement to the circular polyribonucleotide.

In some embodiments, the circular polyribonucleotide is substantially resistant to
degradation, e.g., by exonucleases. A "substantially resistant" circular polyribonucleotide can
15 refer to one that has at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or
99% resistance as compared to a reference polyribonucleotide.

In some embodiments, the circular polyribonucleotide replicates within a cell. In some
embodiments, the circular polyribonucleotide replicates within in a cell at a rate of about 10%-
20%, 20%-30%, 30%-40%, 40%-50%, 50%-60%, 60%-70%, 70%-75%, 75%-80%, 80%-85%,
20 85%-90%, 90%-95%, 95%-99%, or any percentage therebetween. In some embodiments, the
circular polyribonucleotide replicates within a cell and is passed to daughter cells. In some
embodiments, a cell passes at least one circular polyribonucleotide to daughter cells with an
efficiency of at least 25%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, or 99%. In some
embodiments, a cell undergoing meiosis passes the circular polyribonucleotide to daughter cells
25 with an efficiency of at least 25%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, or 99%. In some
embodiments, a cell undergoing mitosis passes the circular polyribonucleotide to daughter cells
with an efficiency of at least 25%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, or 99%.

In some embodiments, the circular polyribonucleotide replicates within the host cell. In some
embodiments, the circular polyribonucleotide is capable of replicating in a mammalian cell, e.g.,
30 a human cell.

While in some embodiments the circular polyribonucleotide replicates in the host cell, the circular polyribonucleotide does not integrate into the genome of the host, e.g., with the host's chromosomes. In some embodiments, the circular polyribonucleotide has a negligible recombination frequency, e.g., with the host's chromosomes. In some embodiments, the circular polyribonucleotide has a recombination frequency, e.g., less than about 1.0 cM/Mb, 0.9 cM/Mb, 0.8 cM/Mb, 0.7 cM/Mb, 0.6 cM/Mb, 0.5 cM/Mb, 0.4 cM/Mb, 0.3 cM/Mb, 0.2 cM/Mb, 0.1 cM/Mb, or less, e.g., with the host's chromosomes.

(xii) Production Methods

The disclosure provides methods for producing circular polyribonucleotides described herein, including, e.g., recombinant technology or chemical synthesis. For example, a DNA molecule used to produce a circular RNA molecule can include a DNA sequence of a naturally occurring nucleic acid sequence, a modified version thereof, or a DNA sequence encoding a synthetic polypeptide not normally found in nature (e.g., chimeric molecules or fusion proteins).

DNA and RNA molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, polymerase chain reaction (PCR) amplification or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof.

The circular polyribonucleotides may be prepared according to any available technique, including, but not limited to chemical synthesis and enzymatic synthesis. In some embodiments, a linear primary construct or linear polyribonucleotide for circularization may be cyclized or concatenated to create a circular RNA described herein. In some embodiments, the linear polyribonucleotide for circularization may be cyclized in vitro prior to formulation and/or delivery. In some embodiments, the circular polyribonucleotide may be in a mixture with linear polyribonucleotides. In some embodiments, the linear polyribonucleotides have the same nucleic acid sequence as the circular polyribonucleotides.

The mechanism of cyclization or concatenation may occur through methods such as, e.g., chemical, enzymatic, splint ligation, or ribozyme-catalyzed methods. The newly formed 5'-3'

linkage may be an intramolecular linkage or an intermolecular linkage. For example, a splint ligase, such as a SplintR® ligase, can be used for splint ligation. According to this method, a single stranded polynucleotide (splint), such as a single-stranded DNA or RNA, can be designed to hybridize with both termini of a linear polyribonucleotide, so that the two termini can be juxtaposed upon hybridization with the single-stranded splint. Splint ligase can thus catalyze the ligation of the juxtaposed two termini of the linear polyribonucleotide, generating a circular polyribonucleotide. In some embodiments, a DNA or RNA ligase may be used in the synthesis of the circular polynucleotides. As a non-limiting example, the ligase may be a circ ligase or circular ligase.

In some embodiments, either the 5' or 3' end of the linear polyribonucleotide can encode a ligase ribozyme sequence such that during in vitro transcription, the resultant linear polyribonucleotide for circularization includes an active ribozyme sequence capable of ligating the 5' end of the linear polyribonucleotide for circularization to the 3' end of the linear polyribonucleotide for circularization. The ligase ribozyme may be derived from the Group I Intron, Hepatitis Delta Virus, Hairpin ribozyme or may be selected by SELEX (systematic evolution of ligands by exponential enrichment).

In another example, a linear polyribonucleotide may be cyclized or concatenated by using at least one non-nucleic acid moiety. In one aspect, the at least one non-nucleic acid moiety may react with regions or features near the 5' terminus or near the 3' terminus of the linear polyribonucleotide for circularization in order to cyclize or concatenate the linear polyribonucleotide for circularization. In another aspect, the at least one non-nucleic acid moiety may be located in or linked to or near the 5' terminus or the 3' terminus of the linear polyribonucleotide for circularization. The non-nucleic acid moieties may be homologous or heterologous. As a non-limiting example, the non-nucleic acid moiety may be a linkage such as a hydrophobic linkage, ionic linkage, a biodegradable linkage, or a cleavable linkage. As another non-limiting example, the non-nucleic acid moiety is a ligation moiety. As yet another non-limiting example, the non-nucleic acid moiety may be an oligonucleotide or a peptide moiety, such as an aptamer or a non-nucleic acid linker as described herein.

In some embodiments, a linear polyribonucleotide for circularization may include a 5' triphosphate of the nucleic acid converted into a 5' monophosphate, e.g., by contacting the 5' triphosphate with RNA 5' pyrophosphohydrolase (RppH) or an ATP diphosphohydrolase

(apyrase). In some embodiments, the 5' end of at least a portion of the linear polyribonucleotides includes a monophosphate moiety. In some embodiments, a population of polyribonucleotides including circular and linear polyribonucleotides is contacted with RppH prior to digesting at least a portion of the linear polyribonucleotides with a 5' exonuclease and/or a 3' exonuclease. Alternately, converting the 5' triphosphate of the linear polyribonucleotide for circularization into a 5' monophosphate may occur by a two-step reaction including: (a) contacting the 5' nucleotide of the linear polyribonucleotide for circularization with a phosphatase (e.g., Antarctic Phosphatase, Shrimp Alkaline Phosphatase, or Calf Intestinal Phosphatase) to remove all three phosphates; and (b) contacting the 5' nucleotide after step (a) with a kinase (e.g., Polynucleotide Kinase) that adds a single phosphate. In another aspect, linear polyribonucleotides for circularization may be cyclized or concatenated by self-splicing. In some embodiments, the linear polyribonucleotide may include a sequence that mediates self-ligation. In some embodiments, the linear polyribonucleotides may include loop E sequence (e.g., in PSTVd) to self-ligate. In some embodiments, the linear polyribonucleotide may include a HDV sequence, e.g., HDV replication domain conserved sequence,

GGCUCAUCUCGACAAGAGGCGGCAGUCCUCAGUACUCUACUCUUUUCUGUAAAG
 AGGAGACUGCUGGACUCGCCGCCCAAGUUCGAGCAUGAGCC (SEQ ID NO: 170) or
 GGCUAGAGGCGGCAGUCCUCAGUACUCUACUCUUUUCUGUAAAGAGGAGACUG
 CUGGACUCGCCGCCCGAGCC (SEQ ID NO: 171), to self-ligate. In another embodiment,

the linear polyribonucleotides may include a self-circularizing intron, e.g., a 5' and 3' splice junction, or a self-circularizing catalytic intron such as a Group I, Group II, or Group III Introns. Nonlimiting examples of group I intron self-splicing sequences may include self-splicing permuted intron-exon sequences derived from T4 bacteriophage gene td, and the intervening sequence (IVS) rRNA of Tetrahymena, cyanobacterium Anabaena pre-tRNA-Leu gene, or a Tetrahymena pre-rRNA.

In some embodiments, the polyribonucleotide includes catalytic intron fragments, such as a 3' half of Group I catalytic intron fragment and a 5' half of Group I catalytic intron fragment. The first and second annealing regions may be positioned within the catalytic intron fragments. Group I catalytic introns are self-splicing ribozymes that catalyze their own excision from mRNA, tRNA, and rRNA precursors via two-metal ion phosphoryl transfer mechanism.

Importantly, the RNA itself self-catalyzes the intron removal without the requirement of an exogenous enzyme, such as a ligase.

In some embodiments, the 3' half of Group I catalytic intron fragment and the 5' half of Group I catalytic intron fragment are from a cyanobacterium *Anabaena* pre-tRNA-Leu gene, or a
5 Tetrahymena pre-rRNA.

In some embodiments, the 3' half of Group I catalytic intron fragment and the 5' half of Group I catalytic intron fragment are from a Cyanobacterium *Anabaena* pre-tRNA-Leu gene, and the 3' exon fragment includes the first annealing region and the 5' exon fragment includes the second annealing region. The first annealing region may include, e.g., from 5 to 50, e.g., from
10 10 to 15 (e.g., 10, 11, 12, 13, 14, or 15) ribonucleotides and the second annealing region may include, e.g., from 5 to 50, e.g., from 10 to 15 (e.g., 10, 11, 12, 13, 14, or 15) ribonucleotides.

In some embodiments, the 3' half of Group I catalytic intron fragment and the 5' half of Group I catalytic intron fragment are from a Tetrahymena pre-rRNA, and the 3' half of Group I catalytic intron fragment includes the first annealing region and the 5' exon fragment includes
15 the second annealing region. In some embodiments, the 3' exon includes the first annealing region and the 5' half of Group I catalytic intron fragment includes the second annealing region. The first annealing region may include, e.g., from 6 to 50, e.g., from 10 to 16 (e.g., 10, 11, 12, 13, 14, 15, or 16) ribonucleotides, and the second annealing region may include, e.g., from 6 to 50, e.g., from 10 to 16 (e.g., 10, 11, 12, 13, 14, 15, or 16) ribonucleotides.

20 In some embodiments, the 3' half of Group I catalytic intron fragment and the 5' half of Group I catalytic intron fragment are from a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a Tetrahymena pre-rRNA, or a T4 phage td gene.

In some embodiments, the 3' half of Group I catalytic intron fragment and the 5' Group I catalytic intron fragment are from a T4 phage td gene. The 3' exon fragment may include the
25 first annealing region and the 5' half of Group I catalytic intron fragment may include the second annealing region. The first annealing region may include, e.g., from 2 to 16 (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16) ribonucleotides, and the second annealing region may include, e.g., from 2 to 16 (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16) ribonucleotides.

In some embodiments, the 3' half of Group I catalytic intron fragment is the 5' terminus
30 of the linear polynucleotide.

In some embodiments, the 5' half of Group I catalytic intron fragment is the 3' terminus of the linear polyribonucleotide.

In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

5 AACAAACAGATAACTTACAGCTAGTCGGAAGGTGCAGAGACTCGACGGGAGCTACCC
TAACGTCAAGACGAGGGTAAAGAGAGAGTCCAATTCTCAAAGCCAATAGGCAGTAG
CGAAAGCTGCGGGAGAATG-3' (SEQ ID NO: 172).

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

10 AAATAATTGAGCCTTAGAGAAGAAATTCTTTAAGTGGATGCTCTCAAACCTCAGGGA
AACCTAAATCTAGCTATAGACAAGGCAATCCTGAGCCAAGCCGAAGTAGTAATTAG
TAAGTT-3' (SEQ ID NO: 173)).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 172 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ
15 ID NO: 173.

In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

CTTCTGTTGATATGGATGCAGTTCACAGACTAAATGTTCGGTCGGGGAAGATGTATTC
TTCTCATAAGATATAGTCGGACCTCTCCTTAATGGGAGCTAGCGGATGAAGTGATGC
20 AACACTGGAGCCGCTGGGAACTAATTTGTATGCGAAAGTATATTGATTAGTTTTTGA
GTACTCG-3' (SEQ ID NO: 174).

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

AAATAGCAATATTTACCTTTGGAGGGAAAAGTTATCAGGCATGCACCTGGTAGCTAG
25 TCTTTAAACCAATAGATTGCATCGGTTTAAAAGGCAAGACCGTCAAATTGCGGGAA
AGGGGTCAACAGCCGTTCAGTACCAAGTCTCAGGGGAACTTTGAGATGGCCTTGC
AAAGGGTATGGTAATAAGCTGACGGACATGGTCCTAACCACGCAGCCAAGTCCTAA
GTCAACAGAT-3' (SEQ ID NO: 175).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 174 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ
30 ID NO: 175.

In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - GGTTCTACATAAATGCCTAACGACTATCCCTTTGGGGAGTAGGGTCAAGTGA CT CGA AACGATAGACA ACTTGCTTTAACAAGTTGGAGATATAGTCTGCTCTGCATGGTGACA

5 TGCAGCTGGATATAATTCCGGGGTAAGATTAACGACCTTATCTGAACATAATG-3' (SEQ ID NO: 176).

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - TAATTGAGGCCTGAGTATAAGGTGACTTATACTTGTAATCTATCTAAACGGGGAACC

10 TCTCTAGTAGACAATCCCGTGCTAAATTGTAGGACT-3' (SEQ ID NO: 177).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 176 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 177.

In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - TAAACA ACTAACAGCTTTAGAAGGTGCAGAGACTAGACGGGAGCTACCCTAACGGA TTCAGCCGAGGGTAAAGGGATAGTCCAATTCTCAACATCGCGATTGTTGATGGCAGC GAAAGTTGCAGAGAGAATGAAAATCCGCTGACTGTAAAGGTCGTGAGGGTTCGAGT CCCTCCGCCCCCA-3' (SEQ ID NO: 178).

15

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - ACGGTAGACGCAGCGGACTTAGAAA ACTGGGCCTCGATCGCGAAAGGGATCGAGTG GCAGCTCTCAA ACTCAGGGAAACCTAAAACTTTAAACATTMAAGTCATGGCAATCC TGAGCCAAGCTAAAGC-3' (SEQ ID NO: 179).

20

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 178 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 179.

25

In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - TTAAACTCAA AATTTAAAATCCCAAATTCAA AATTCCGGGAAGGTGCAGAGACTCG ACGGGAGCTACCCTAACGTAAAGCCGAGGGTAAAGGGAGAGTCCAATTCTCAAAGC

30

CTGAAGTTGCTGAAGCAACAAGGCAGTAGTGAAAGCTGCGAGAGAATGAAAATCCG
TTGACTGTAAAAAGTCGTGGGGGTTCAAGTCCCCCACCACC-3' (SEQ ID NO: 180).

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

5 ATGGTAGACGCTACGGACTTAGAAAAGTGAAGCCTTGATAGAGAAATCTTTTAAGTG
GAAGCTCTCAAATTCAGGGAAACCTAAATCTGAATACAGATATGGCAATCCTGAGC
CAAGCCCAGAAAATTTAGACTTGAGATTTGATTTTGGAG-3' (SEQ ID NO: 181).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 180 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ
10 ID NO: 181.

In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

GGCTTTCAATTTGAAATCAGAAATTCAAATTCAGGGAAGGTGCAGAGACTCGACG
GGAGCTACCCTAACGTAAAGGCGAGGGTAAAGGGAGAGTCCAATTCTTAAAGCCTG
15 AAGTTGTGCAAGCAACAAGGCAACAGTGAAAGCTGTGGAAGAATGAAAATCCGTTG
ACCTTAAACGGTCGTGGGGGTTCAAGTCCCCCACCACC-3' (SEQ ID NO: 182).

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

20 ATGGTAGACGCTACGGACTTAGAAAAGTGAAGCCTTGATAGAGAAATCTTTCAAGTG
GAAGCTCTCAAATTCAGGGAAACCTAAATCTGAATACAGATATGGCAATCCTGAGC
CAAGCCCGGAAATTTTAGAATCAAGATTTTATTTT-3' (SEQ ID NO: 183).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 182 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ
ID NO: 183.

25 In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

AGAAATGGAGAAGGTGTAGAGACTGGAAGGCAGGCACCCTAACGTAAAGGCGAG
GGTGAAGGGACAGTCCAGACCACAAACCAGTAAATCTGGGCAGCGAAAGCTGTAGA
TGGTAAGCATAACCCGAAGGTCAGTGGTTCAAATCCACTTCCCGCCACCAAATTAAA
30 AAAACAATAA-3' (SEQ ID NO: 184).

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - AGAAATGGAGAAGGTGTAGAGACTGGAAGGCAGGCACCCTAACGTTAAAGGCGAG GGTGAAGGGACAGTCCAGACCACAAACCAGTAAATCTGGGCAGCGAAAGCTGTAGA
 5 TGGTAAGCATAACCCGAAGGTCAGTGGTTCAAATCCACTTCCCGCCACCAAATTAAA AAAACAATAA-3' (SEQ ID NO: 185).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 184 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 185.

10 In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - ACAACAGATAACTTACTAATTACAGCTAGTCGGAAGGTGCAGAGACTCGACGGGA GCTACCCTAACGTCAAGACGAGGGTAAAGAGAGAGTCCAATTCTCAAAGCCAATAG GCAGTAGCGAAAGCTGCGGGAGAATGAAAATCCGTAGCGTCTAAACGGTCGTGTGG
 15 GTTCAAGTCCCTCCACCCCCA-3' (SEQ ID NO: 186).

In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - AGACGCTACGGACTTAAATAATTGAGCCTTAGAGAAGAAATTCTTTAAGTGGATGCT CTCAAACCTCAGGGAAACCTAAATCTAGCTATAGACAAGGCAATCCTGAGCCAAGCC
 20 GAAGTAGTAATTAGTAAGTTAGTAAGTT-3' (SEQ ID NO: 187).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 186 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ ID NO: 187.

In some embodiments, the 3' half of Group I catalytic intron fragment has at least 80%
 25 (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' - AACAAACAGATAACTTACTAGTTACTAGTCGGAAGGTGCAGAGACTCGACGGGAGCT ACCCTAACGTCAAGACGAGGGTAAAGAGAGAGTCCAATTCTCAAAGCCAATAGGCA GTAGCGAAAGCTGCGGGAGAATGAAAATCCGTAGCGTCTAAACGGTCGTGTGGGTT CAAGTCCCTCCACCCCCA-3' (SEQ ID NO: 188).

30 In some embodiments, the 5' half of Group I catalytic intron fragment has at least 80% (e.g., at least 85%, 90%, 95%, 97%, 99%, or 100%) sequence identity to the sequence of 5' -

AGACGCTACGGACTTAAATAATTGAGCCTTAGAGAAGAAATTCTTTAAGTGGATGCT
CTCAAACCTCAGGGAAACCTAAATCTAGCTATAGACAAGGCAATCCTGAGCCAAGCC
GAAGTAGTAATTAGTAAGTT-3' (SEQ ID NO: 189).

In some embodiments, the 3' half of Group I catalytic intron fragment has the sequence
5 of SEQ ID NO: 188 and the 5' half of Group I catalytic intron fragment has the sequence of SEQ
ID NO: 189.

In some embodiments, the Group I catalytic intron fragment is from the T4 phage nrdB
gene or nrdD gene. In some embodiments, the 3' half of Group I catalytic intron fragment of
includes a sequence having at least 80% sequence identity to 5'-

10 TTGCAAAACAAGGTTCAACGACTAGTCTTCGGACGTAGGGTCAAGCGACTCGAAAT
GGGGAGAATCCCTCCGGGATTGTGATATAGTCTGGACTGCATGGTAACATGCAGCA
GTTTCATAAGAGAACGGGTTGAGAATTAGCGAGCTCAATCGAACATACG-3' (SEQ ID
NO: 190).

In some embodiments, the 3' half of Group I catalytic intron fragment of (A) includes a
15 sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

TTGCAAAACAAGGTTCAACGACTAGTCTTCGGACGTAGGGTCAAGCGACTCGAAAT
GGGGAGAATCCCTCCGGGATTGTGATATAGTCTGGACTGCATGGTAACATGCAGCA
GTTTCATAAGAGAACGGGTTGAGAATTAGCGAGCTCAATCGAACATACG-3' (SEQ ID
NO: 191).

20 In some embodiments, the 5' half of Group I catalytic intron fragment from the T4 phage
nrdB gene. In some embodiments, the 3' half of Group I catalytic intron fragment is from the T4
phage nrdB gene and the 5' half of Group I catalytic intron fragment is from the T4 phage nrdB
gene.

In some embodiments, the 5' half of Group I catalytic intron includes a sequence having
25 at least 80% sequence identity to 5'-

AAAATGCGCCTTTAAACGGTAACGTTTATCGAAAACCTCTTAATTGCTGGAAAGTC
CTTTATGGAAAACCTAGCAGCCAAGGTTTTGCTT-3' (SEQ ID NO: 192).

In some embodiments, the 5' half of Group I catalytic intron includes a sequence having
at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

30 AAAATGCGCCTTTAAACGGTAACGTTTATCGAAAACCTCTTAATTGCTGGAAAGTC
CTTTATGGAAAACCTAGCAGCCAAGGTTTTGCTT-3' (SEQ ID NO: 193).

In some embodiments, the 3' half of Group I catalytic intron fragment is from the T4 phage nrdD gene.

In some embodiments, the 3' half of Group I catalytic intron fragment includes a sequence having at least 80% sequence identity to 5'-

5 CAGTAGCTGTAAATGCCCAACGACTATCCCTGATGAATGTAAGGGAGTAGGGTCAA
GCGACCCGAAACGGCAGACAACCTCTAAGAGTTGAAGATATAGTCTGAACTGCATGG
TGACATGCAGCTGTTTATCCTCGTATAAATATGAATACGAGGTGAAACGATGAAATG
AATTACATTGTTTCATATAAACGGGTAGAGAAGTAGCGAACTCTACTGAACACATTG
-3' (SEQ ID NO: 194).

10 In some embodiments, the 3' half of Group I catalytic intron fragment includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

CAGTAGCTGTAAATGCCCAACGACTATCCCTGATGAATGTAAGGGAGTAGGGTCAA
GCGACCCGAAACGGCAGACAACCTCTAAGAGTTGAAGATATAGTCTGAACTGCATGG
TGACATGCAGCTGTTTATCCTCGTATAAATATGAATACGAGGTGAAACGATGAAATG
15 AATTACATTGTTTCATATAAACGGGTAGAGAAGTAGCGAACTCTACTGAACACATTG
-3' (SEQ ID NO: 195).

In some embodiments, the 5' half of Group I catalytic intron fragment is from the T4 phage nrdD gene. In some embodiments, the 3' half of Group I catalytic intron fragment is from the T4 phage nrdD gene and the 5' half of Group I catalytic intron fragment is from the T4 phage
20 nrdD gene.

In some embodiments, the 5' half of Group I catalytic intron includes a sequence having at least 80% sequence identity to 5'-

TAACGTAAGTCAAGCTCATGTAAAATCTGCCTAAAACGGGAACTCTCACTGAGAC
AATCCGTTGCTAAATCAG-3' (SEQ ID NO: 196).

25 In some embodiments, the 5' half of Group I catalytic intron includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

TAACGTAAGTCAAGCTCATGTAAAATCTGCCTAAAACGGGAACTCTCACTGAGAC
AATCCGTTGCTAAATCAG-3' (SEQ ID NO: 197).

In some embodiments, the 3' exon fragment includes a sequence having at least 80%
30 sequence identity to 5'-

GTACCTTTAACTTCCATAAGAACATGGAAATCATGGAAGGTAATGCCAAG-3' (SEQ ID NO: 198).

In some embodiments, the 3' exon fragment includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

5 GTACCTTTAACTTCCATAAGAACATGGAAATCATGGAAGGTAATGCCAAG-3' (SEQ ID NO: 199).

In some embodiments, the 3' exon fragment includes a sequence having at least 80% sequence identity to 5'-

10 GTACCTTTAACTTCCAAAAGATACATAAAAATCATGGAAGGTAATGCCAAG-3' (SEQ ID NO: 200).

In some embodiments, the 3' exon fragment includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

GTACCTTTAACTTCCAAAAGATACATAAAAATCATGGAAGGTAATGCCAAG-3' (SEQ ID NO: 201).

15 In some embodiments, the 5' exon fragment includes a sequence having at least 80% sequence identity to 5'-TTTTTATGTATCTTTTGCGT-3' (SEQ ID NO: 202).

In some embodiments, the 5' exon fragment includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-TTTTTATGTATCTTTTGCGT-3' (SEQ ID NO: 203).

20 In some embodiments, the 3' exon fragment Includes a sequence having at least 80% sequence identity to 5'-

ATGAAGTGAACACGTTATTCAGTTCAAACGGACAGACTCCTTTTGTAACA -3' (SEQ ID NO: 204).

25 In some embodiments, the 3' exon fragment includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

ATGAAGTGAACACGTTATTCAGTTCAAACGGACAGACTCCTTTTGTAACA -3' (SEQ ID NO: 205).

In some embodiments, the 3' exon fragment includes a sequence having at least 80% sequence identity to 5'-

30 ATGAAGTGAACACGTTACATAAGCTTGGAATGCAGACTCCTTTTGTAACA -3' (SEQ ID NO: 206).

In some embodiments, the 3' exon fragment includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'-

ATGAAGTGAACACGTTACATAAGCTTGGAATGCAGACTCCTTTTGTAACA -3' (SEQ ID NO: 207).

5 In some embodiments, the 5' exon fragment includes a sequence having at least 80% sequence identity to 5'- TGCATTCCAAGCTTATGAGT -3' (SEQ ID NO: 208).

In some embodiments, the 5' exon fragment includes a sequence having at least 85%, 90%, 95%, 97%, 99%, or 100% sequence identity to 5'- TGCATTCCAAGCTTATGAGT -3' (SEQ ID NO: 209).

10 In another aspect, a linear polyribonucleotide for circularization may be cyclized or concatenated by a non-nucleic acid moiety that causes an attraction between atoms, molecular surfaces at, near, or linked to the 5' and 3' ends of the linear polyribonucleotide for circularization. In one embodiment, one or more linear polyribonucleotides is cyclized or concatenated by intermolecular forces or intramolecular forces. Non-limiting examples of
15 intermolecular forces include dipole-dipole forces, dipole-induced dipole forces, induced dipole-induced dipole forces, Van der Waals forces, and London dispersion forces. Non-limiting examples of intramolecular forces include covalent bonds, metallic bonds, ionic bonds, resonant bonds, agnostic bonds, dipolar bonds, conjugation, hyperconjugation and antibonding.

In some embodiments, a linear polyribonucleotide for circularization may include a
20 ribozyme RNA sequence near the 5' terminus and near the 3' terminus. The ribozyme RNA sequence may covalently link to a peptide when the sequence is exposed to the remainder of the ribozyme. In one aspect, the peptides covalently linked to the ribozyme RNA sequence near the 5' terminus and the 3' terminus may associate with each other causing a linear polyribonucleotide to cyclize or concatenate. In another example, the peptides covalently linked
25 to the ribozyme RNA near the 5' terminus and the 3' terminus may cause the linear primary construct or linear mRNA to cyclize or concatenate after being subjected to ligation using various methods known in the art such as, but not limited to, protein ligation. Non-limiting examples of ribozymes for use in the linear primary constructs or linear polyribonucleotides of the present invention or a non-exhaustive listing of methods to incorporate or covalently link
30 peptides are described in U.S. Patent Publication No. U.S. 2003/0082768, the contents of which is here in incorporated by reference in its entirety.

In another aspect, chemical methods of circularization may be used to generate the circular polyribonucleotide. Such methods may include but are not limited to click chemistry (e.g., alkyne and azide-based methods, or clickable bases), olefin metathesis, phosphoramidate ligation, hemiaminal-imine crosslinking, base modification, and any combination thereof. In some chemical methods, the 5'-end and the 3'-end of a linear polyribonucleotide for circularization includes chemically reactive groups that, when close together, may form a new covalent linkage between the 5'-end and the 3'-end of the molecule. The 5'-end may contain an NHS-ester reactive group and the 3'-end may contain a 3'-amino-terminated nucleotide such that in an organic solvent the 3'-amino-terminated nucleotide on the 3'-end of a linear RNA molecule will undergo a nucleophilic attack on the 5'-NHS-ester moiety forming a new 5'-/3'-amide bond. In another aspect, the circular polyribonucleotide may be produced using a deoxyribonucleotide template transcribed in a cell-free system (e.g., by in vitro transcription) to produce a linear RNA. The linear polyribonucleotide produces a splicing-compatible polyribonucleotide, which may be self-spliced to produce a circular polyribonucleotide.

In some embodiments, the disclosure provides a method of producing a circular polyribonucleotide (e.g., in a cell-free system) by providing a linear polyribonucleotide; and self-splicing linear polyribonucleotide under conditions suitable for splicing of the 3' and 5' splice sites of the linear polyribonucleotide; thereby producing a circular polyribonucleotide.

In some embodiments, the disclosure provides a method of producing a circular polyribonucleotide by providing a deoxyribonucleotide encoding the linear polyribonucleotide; transcribing the deoxyribonucleotide in a cell-free system to produce the linear polyribonucleotide; optionally purifying the splicing-compatible linear polyribonucleotide; and self-splicing the linear polyribonucleotide under conditions suitable for splicing of the 3' and 5' splice sites of the linear polyribonucleotide, thereby producing a circular polyribonucleotide.

In some embodiments, the disclosure provides a method of producing a circular polyribonucleotide by providing a deoxyribonucleotide encoding a linear polyribonucleotide; transcribing the deoxyribonucleotide in a cell-free system to produce the linear polyribonucleotide, wherein the transcribing occurs in a solution under conditions suitable for splicing of the 3' and 5' splice sites of the linear polyribonucleotide, thereby producing a circular polyribonucleotide. In some embodiments, the linear polyribonucleotide comprises a 5' split-intron and a 3' split-intron (e.g., a self-splicing construct for producing a circular

polyribonucleotide). In some embodiments, the linear polyribonucleotide comprises a 5' annealing region and a 3' annealing region.

Suitable conditions for in vitro transcriptions and or self-splicing may include any conditions (e.g., a solution or a buffer, such as an aqueous buffer or solution) that mimic physiological conditions in one or more respects. In some embodiments, suitable conditions include between 0.1-100mM Mg^{2+} ions or a salt thereof (e.g., 1-100mM, 1-50mM, 1-20mM, 5-50mM, 5-20 mM, or 5-15mM). In some embodiments, suitable conditions include between 1-1000mM K^{+} ions or a salt thereof such as KCl (e.g., 1-1000mM, 1-500mM, 1-200mM, 50-500mM, 100-500mM, or 100-300mM). In some embodiments, suitable conditions include between 1-1000mM Cl^{-} ions or a salt thereof such as KCl (e.g., 1-1000mM, 1-500mM, 1-200mM, 50- 500mM, 100-500mM, or 100-300mM). In some embodiments, suitable conditions include between 0.1-100mM Mn^{2+} ions or a salt thereof such as $MnCl_2$ (e.g., 0.1-100mM, 0.1-50mM, 0.1-20mM, 0.1-10mM, 0.1-5mM, 0.1-2mM, 0.5- 50mM, 0.5-20 mM, 0.5-15mM, 0.5-5mM, 0.5-2mM, or 0.1-10mM). In some embodiments, suitable conditions include dithiothreitol (DTT) (e.g., 1-1000 μ M, 1-500 μ M, 1-200 μ M, 50- 500 μ M, 100-500 μ M, 100-300 μ M, 0.1-100mM, 0.1-50mM, 0.1-20mM, 0.1-10mM, 0.1-5mM, 0.1-2mM, 0.5- 50mM, 0.5-20 mM, 0.5-15mM, 0.5-5mM, 0.5-2mM, or 0.1-10mM). In some embodiments, suitable conditions include between 0.1mM and 100mM ribonucleoside triphosphate (NTP) (e.g., 0.1-100 mM, 0.1-50mM, 0.1-10mM, 1- 100mM, 1-50mM, or 1-10mM). In some embodiments, suitable conditions include a pH of 4 to 10 (e.g., pH of 5 to 9, pH of 6 to 9, or pH of 6.5 to 8.5). In some embodiments, suitable conditions include a temperature of 4°C to 50°C (e.g., 10°C to 40°C, 15°C to 40°C, 20°C to 40°C, or 30°C to 40°C),

In some embodiments the linear polyribonucleotide is produced from a deoxyribonucleic acid, e.g., a deoxyribonucleic acid described herein, such as a DNA vector, a linearized DNA vector, or a cDNA. In some embodiments, the linear polyribonucleotide is transcribed from the deoxyribonucleic acid by transcription in a cell-free system (e.g., in vitro transcription).

In another aspect, the circular polyribonucleotide may be produced in a cell, e.g., a prokaryotic cell or a eukaryotic cell. In some embodiments, an exogenous polyribonucleotide is provided to a cell (e.g., a linear polyribonucleotide described herein or a DNA molecule encoding for the transcription of a linear polyribonucleotide described here). The linear polyribonucleotides may be transcribed in the cell from an exogenous DNA molecule provided to the cell. The linear

polyribonucleotide may be transcribed in the cell from an exogenous recombinant DNA molecule transiently provided to the cell. In some embodiments, the exogenous DNA molecule does not integrate into the cell's genome. In some embodiments, the linear polyribonucleotide is transcribed in the cell from a recombinant DNA molecule that is incorporated into the cell's genome.

In some embodiments, the cell is a prokaryotic cell. In some embodiments, the prokaryotic cell including the polyribonucleotides described herein is a bacterial cell or an archaeal cell. For example, the prokaryotic cell including the polyribonucleotides described herein may be *E. coli*, halophilic archaea (e.g., *Haloferax volcanii*), *Sphingomonas*, *cyanobacteria* (e.g., *Synechococcus elongatus*, *Spirulina* (*Arthrospira*) spp., and *Synechocystis* spp.), *Streptomyces*, *actinomycetes* (e.g., *Nonomuraea*, *Kitasatospora*, or *Thermobifida*), *Bacillus* spp. (e.g., *Bacillus subtilis*, *Bacillus anthracis*, *Bacillus cereus*), betaproteobacteria (e.g., *Burkholderia*), alphaproteobacterial (e.g., *Agrobacterium*), *Pseudomonas* (e.g., *Pseudomonas putida*), and enterobacteria. The prokaryotic cells may be grown in a culture medium. The prokaryotic cells may be contained in a bioreactor.

In some embodiments, the cell is a eukaryotic cell. In some embodiments, the eukaryotic cell is a unicellular eukaryotic cell. In some embodiments, the unicellular eukaryotic is a unicellular fungal cell such as a yeast cell (e.g., *Saccharomyces cerevisiae* and other *Saccharomyces* spp., *Brettanomyces* spp., *Schizosaccharomyces* spp., *Torulaspora* spp, and *Pichia* spp.). In some embodiments, the unicellular eukaryotic cell is a unicellular animal cell. A unicellular animal cell may be a cell isolated from a multicellular animal and grown in culture, or the daughter cells thereof. In some embodiments, the unicellular animal cell is dedifferentiated. In some embodiments, the unicellular eukaryotic cell is a unicellular plant cell. A unicellular plant cell may be a cell isolated from a multicellular plant and grown in culture, or the daughter cells thereof. In some embodiments, the unicellular plant cell is dedifferentiated. In some embodiments, the unicellular plant cell is from a plant callus. In embodiments, the unicellular cell is a plant cell protoplast. In some embodiments, the unicellular eukaryotic cell is a unicellular eukaryotic algal cell, such as a unicellular green alga, a diatom, an euglenid, or a dinoflagellate. Non-limiting examples of unicellular eukaryotic algae of interest include *Dunaliella salina*, *Chlorella vulgaris*, *Chlorella zofingiensis*, *Haematococcus pluvialis*, *Neochloris oleoabundans* and other *Neochloris* spp., *Protosiphon botryoides*, *Botryococcus*

braunii, *Cryptococcus* spp., *Chlamydomonas reinhardtii* and other *Chlamydomonas* spp. In some embodiments, the unicellular eukaryotic cell is a protist cell. In some embodiments, the unicellular eukaryotic cell is a protozoan cell.

In some embodiments, the eukaryotic cell is a cell of a multicellular eukaryote. For example, the multicellular eukaryote may be selected from the group consisting of a vertebrate animal, an invertebrate animal, a multicellular fungus, a multicellular alga, and a multicellular plant. In some embodiments, the eukaryotic organism is a human. In some embodiments, the eukaryotic organism is a non-human vertebrate animal. In some embodiments, the eukaryotic organism is an invertebrate animal. In some embodiments, the eukaryotic organism is a multicellular fungus. In some embodiments, the eukaryotic organism is a multicellular plant. In some embodiments, the eukaryotic cell is a cell of a human or a cell of a non-human mammal such as a non-human primate (e.g., monkeys, apes), ungulate (e.g., bovids including cattle, buffalo, bison, sheep, goat, and musk ox; pig; camelids including camel, llama, and alpaca; deer, antelope; and equids including horse and donkey), carnivore (e.g., dog, cat), rodent (e.g., rat, mouse, guinea pig, hamster, squirrel), or lagomorph (e.g., rabbit, hare). In some embodiments, the eukaryotic cell is a cell of a bird, such as a member of the avian taxa Galliformes (e.g., chickens, turkeys, pheasants, quail), Anseriformes (e.g., ducks, geese), Paleognathae (e.g., ostriches, emus), Columbiformes (e.g., pigeons, doves), or Psittaciformes (e.g., parrots). In some embodiments, the eukaryotic cell is a cell of an arthropod (e.g., insects, arachnids, crustaceans), a nematode, an annelid, a helminth, or a mollusc. In some embodiments, the eukaryotic cell is a cell of a multicellular plant, such as an angiosperm plant (which can be a dicot or a monocot) or a gymnosperm plant (e.g., a conifer, a cycad, a gnetophyte, a Ginkgo), a fern, horsetail, clubmoss, or a bryophyte. In some embodiments, the eukaryotic cell is a cell of a eukaryotic multicellular alga.

The eukaryotic cells may be grown in a culture medium. The eukaryotic cells may be contained in a bioreactor.

In some embodiments, any method of producing a circular polyribonucleotide described herein may be performed in a bioreactor. A bioreactor refers to any vessel in which a chemical or biological process is carried out which involves organisms or biochemically active substances derived from such organisms. Bioreactors may be compatible with the cell-free methods for production of circular RNA described herein. A vessel for a bioreactor may include a culture flask, a dish, or a bag that may be single use (disposable), autoclavable, or sterilizable. A

bioreactor may be made of glass, or it may be polymer-based, or it may be made of other materials.

Examples of bioreactors include, without limitation, stirred tank (e.g., well mixed) bioreactors and tubular (e.g., plug flow) bioreactors, airlift bioreactors, membrane stirred tanks, spin filter stirred tanks, vibromixers, fluidized bed reactors, and membrane bioreactors. The mode of operating the bioreactor may be a batch or continuous processes. A bioreactor is continuous when the reagent and product streams are continuously being fed and withdrawn from the system. A batch bioreactor may have a continuous recirculating flow, but no continuous feeding of reagents or product harvest. Some methods of the present disclosure are directed to large-scale production of circular polyribonucleotides. For large-scale production methods, the method may be performed in a volume of 1 liter (L) to 50 L, or more (e.g., 5 L, 10 L, 15 L, 20 L, 25 L, 30 L, 35 L, 40 L, 45 L, 50 L, or more). In some embodiments, the method may be performed in a volume of 5 L to 10 L, 5 L to 15 L, 5 L to 20 L, 5 L to 25 L, 5 L to 30 L, 5 L to 35 L, 5 L to 40 L, 5 L to 45 L, 10 L to 15 L, 10 L to 20 L, 10 L to 25 L, 20 L to 30 L, 10 L to 35 L, 10 L to 40 L, 10 L to 45 L, 10 L to 50 L, 15 L to 20 L, 15 L to 25 L, 15 L to 30 L, 15 L to 35 L, 15 L to 40 L, 15 L to 45 L, or 15 to 50 L. In some embodiments, a bioreactor may produce at least 1g of circular RNA. In some embodiments, a bioreactor may produce 1-200g of circular RNA (e.g., 1-10g, 1-20g, 1-50g, 10-50g, 10-100g, 50-100g, or 50-200g of circular RNA). In some embodiments, the amount produced is measured per liter (e.g., 1-200g per liter), per batch or reaction (e.g., 1-200g per batch or reaction), or per unit time (e.g., 1-200g per hour or per day). In some embodiments, more than one bioreactor may be utilized in series to increase the production capacity (e.g., one, two, three, four, five, six, seven, eight, or nine bioreactors may be used in series).

In some embodiments, circularization efficiency is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or 100%. In some embodiments, circularization efficiency is at least about 40%. In some embodiments, circularization efficiency is between about 10% and about 100%; for example, circularization efficiency is about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%,

and about 99%. In some embodiments, circularization efficiency is between about 20% and about 80%. In some embodiments, circularization efficiency is between about 30% and about 60%. In some embodiments, circularization efficiency is about 40%.

Additional methods of making the circular polyribonucleotides described herein are described in, for example, Khudyakov & Fields, *Artificial DNA: Methods and Applications*, CRC Press (2002); in Zhao, *Synthetic Biology: Tools and Applications*, (First Edition), Academic Press (2013); Muller and Appel, *from RNA Biol*, 2017, 14(8):1018-1027; and Egli & Herdewijn, *Chemistry and Biology of Artificial Nucleic Acids*, (First Edition), Wiley-VCH (2012). Other methods of making circular polyribonucleotides are described, for example, in International Publication No. WO2023/044006, International Publication No. WO2022/247943, U.S. Patent No. 11000547, International Publication No. 2018/191722, International Publication No. WO2019/236673, International Publication No. WO2020/023595, International Publication No. WO2022/204460, International Publication No. WO2022/204464, International Publication No. WO2022/204466, and International Publication No. 2022/261490, the contents of each of which are herein incorporated by reference in their entirety).

Additional methods of synthesizing circular polyribonucleotides are also described elsewhere (see, e.g., U.S. Patent No. 6210931, U.S. Patent No. 5773244, U.S. Patent No. 5766903, U.S. Patent No. 5712128, U.S. Patent No. 5426180, U.S. Patent Publication No. US20100137407, International Publication No. WO1992/001813, International Publication No. WO2010/084371, and Petkovic et al., *Nucleic Acids Res.* 43:2454-65 (2015); the contents of each of which are herein incorporated by reference in their entirety).

(xiii) Purification Methods

One or more purification steps may be included in the methods described herein. For example, in some embodiments, the linear polyribonucleotide is substantively enriched or pure (e.g., purified) prior to self-splicing the linear polyribonucleotide. In other embodiments, the linear polyribonucleotide is not purified prior to self-splicing the linear polyribonucleotide. In some embodiments, the resulting circular polyribonucleotide is purified.

Purification may include separating or enriching the desired reaction product from one or more undesired components, such as any unreacted starting material, byproducts, enzymes, or other reaction components. For example, purification of linear polyribonucleotide following

transcription in a cell-free system (e.g., in vitro transcription) may include separation or enrichment from the DNA template prior to self-splicing the linear polyribonucleotide.

Purification of the circular RNA product following splicing may be used to separate or enrich the circular polyribonucleotide from its corresponding linear polyribonucleotide. Methods of
5 purification of polyribonucleotides are known to those of skill in the art and include enzymatic purification or by chromatography.

In some embodiments, the methods of purification result in a circular polyribonucleotide that has less than 50% (e.g., less than 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1%) linear polyribonucleotides.

10 In some embodiments, the reference criterion for the amount of linear polyribonucleotide molecules present in a preparation (e.g., pharmaceutical preparation) is the presence of no more than 1 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 50 ng/ml, 60 ng/ml, 70 ng/ml, 80 ng/ml, 90 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml, 600 ng/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400
15 µg/ml, 500 µg/ml, 600 µg/ml, 700 µg/ml, 800 µg/ml, 900 µg/ml, 1 mg/ml, 1.5 mg/ml, or 2 mg/ml of linear polyribonucleotide molecules.

In some embodiments, the reference criterion for the amount of circular polyribonucleotide molecules present in a preparation (e.g., pharmaceutical preparation) is at least 30% (w/w), 40% (w/w), 50% (w/w), 60% (w/w), 70% (w/w), 80% (w/w), 85% (w/w), 90%
20 (w/w), 91% (w/w), 92% (w/w), 93% (w/w), 94% (w/w), 95% (w/w), 96% (w/w), 97% (w/w), 98% (w/w), 99% (w/w), 99.1% (w/w), 99.2% (w/w), 99.3% (w/w), 99.4% (w/w), 99.5% (w/w), 99.6% (w/w), 99.7% (w/w), 99.8% (w/w), 99.9% (w/w), or 100% (w/w) molecules of the total ribonucleotide molecules in the preparation.

In some embodiments, the reference criterion for the amount of linear polyribonucleotide
25 molecules present in a preparation (e.g., pharmaceutical preparation) is no more than 0.5% (w/w), 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), 40% (w/w), 50% (w/w) linear polyribonucleotide molecules of the total ribonucleotide molecules in the preparation.

In some embodiments, the reference criterion for the amount of nicked
30 polyribonucleotide molecules present in a preparation (e.g., pharmaceutical preparation) is no

more than 0.5% (w/w), 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), or 15% (w/w) nicked polyribonucleotide molecules of the total ribonucleotide molecules in the preparation.

In some embodiments, the reference criterion for the amount of combined nicked and linear polyribonucleotide molecules present in a preparation (e.g., pharmaceutical preparation) is no more than 0.5% (w/w), 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), 40% (w/w), 50% (w/w) combined nicked and linear polyribonucleotide molecules of the total ribonucleotide molecules in the preparation. In some embodiments, a preparation (e.g., pharmaceutical preparation) is an intermediate preparation of a final circular polyribonucleotide drug product. In some embodiments, a preparation (e.g., pharmaceutical preparation) is a drug substance or active pharmaceutical ingredient (API). In some embodiments, a preparation (e.g., pharmaceutical preparation) is a drug product for administration to a subject.

In some embodiments, a preparation (e.g., pharmaceutical preparation) of circular polyribonucleotides is (before, during or after the reduction of linear polyribonucleotide) further processed to substantially remove DNA, protein contamination (e.g., cell protein such as a host cell protein or protein process impurities), endotoxin, mononucleotide molecules, and/or a process-related impurity.

II. Pharmaceutical Compositions

Provided herein are compositions and pharmaceutical compositions comprising (i) any of the circular polyribonucleotides or modified RNAs described herein and (ii) a pharmaceutically acceptable excipient. Pharmaceutical compositions can comprise one or more additional therapeutic agents, e.g., therapeutically and/or prophylactically active agents. Methods of making pharmaceutical compositions including circular polyribonucleotides or modified RNAs are described in WO 2020/181013, which is incorporated herein by reference in its entirety.

Pharmaceutical compositions provided herein are suitable for administration to humans or any other animal, e.g., to non-human animals, e.g., non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood. Non-limiting examples of subjects that can be administered any of the pharmaceutical compositions described herein include humans, non-human primate, mammals (e.g., commercially-relevant mammals,

such as cattle, pigs, horses, sheep, cats, dogs, mice, and rats), and birds (e.g., including commercially relevant birds such as poultry, chickens, ducks, geese, and/or turkeys).

Pharmaceutical compositions provided herein can be formulated for any suitable mode of administration. Non-limiting examples include formulation of the pharmaceutical composition for intravenous, subcutaneous, intrahepatic, or intramuscular administration. In some embodiments, a pharmaceutical composition is formulated for local administration. In some embodiments, a pharmaceutical composition is formulated for systemic administration.

Pharmaceutical compositions provided herein can include an unencapsulated circular polyribonucleotide of modified RNA, a partially encapsulated circular polyribonucleotide or modified RNA, or a completely encapsulated circular polyribonucleotide or modified RNA.

Any of the circular polyribonucleotides or modified RNAs described herein can be included in a pharmaceutical composition with a carrier or without a carrier.

In some embodiments, pharmaceutical compositions described herein can be formulated with a carrier, e.g., pharmaceutical carrier and/or a polymeric carrier, e.g., a liposome. In such instances, the circular polyribonucleotide or modified RNA can be formulated as a lipid nanoparticle. In some embodiments, the circular polyribonucleotide or modified RNA is formulated for unencapsulated delivery or natural nanoparticle delivery.

In some embodiments, the natural nanoparticle is composed of a natural source selected from a plant, bacteria, animal, insect, archaea, or fungi. In some embodiments the circular polyribonucleotide is formulated within (a) a plurality of lipid nanoparticles (LNP) comprising synthetic structural lipids and an ionizable lipid or within (b) a natural nanoparticle comprising natural lipids and an ionizable lipid.

One or more naturally occurring and/or synthetic lipid compounds may be used in the preparation of a lipid-based carrier (or lipid nanoformulation).

As described herein, a lipid-based carrier (or lipid nanoformulation) may be a lipid particle that has a complexity characterized by comprising a wide variety of lipids, including both synthetic lipids or lipids extracted from one or more natural sources (such as plants or bacteria). Natural lipids can be derived from a variety of arthropod, fungi, plant, archaea, or bacteria, or one or more parts thereof (e.g., segments, organs, eggs, spores, mycelium, tissue, membrane or cell wall). In some embodiments, the lipid-based carrier is a modified natural nanoparticle. As used herein, the term "modified natural particle" refers to a natural lipid

nanoparticles composition including both naturally derived lipids and one or more synthetically derived lipids. In some embodiments, modified natural nanoparticles may include ionizable lipids, non-cationic lipids, sterols, and PEGylated lipids. More embodiments of natural lipids, complex lipid particles, and methods of producing and delivering natural nanoparticles may be found in PCT Application No. PCT/US2023/021094, filed on May 5, 2023, PCT Application No. PCT/US24/25134, filed on April 18, 2024, and PCT Application No. PCT/US24/25270, filed on April 18, 2024, the contents of which are incorporated herein by reference in their entirety. In some embodiments, the modified natural nanoparticle is plant-based, e.g. as described in International Patent Publication Nos. WO2011/097480, WO2013/070324, WO2017/004526, WO2020/041784, WO2021/041301, WO2023/069498, WO2023/122080, or WO2024/102434. In some embodiments, the modified natural nanoparticle is bacteria-derived, e.g. as described in International Patent Publication Nos. WO2023/096858.

In the lipid nanoparticle or natural nanoparticle composition, more than one ionizable lipid can be used for the ionizable lipid component.

More embodiments of ionizable lipids may be found in PCT Application No. PCT/US22/50725, filed on November 22, 2022, PCT Application No. PCT/US23/16300, filed on March 24, 2023, PCT Application No. PCT/US22/50111, filed on November 16, 2022, and PCT Application No. PCT/US23/31669, filed on August 31, 2023, the contents of which are incorporated herein by reference in their entirety. Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in International Patent Publication WO2016/118725, WO2016/118724, WO2013/063468, WO2016/205691, WO2015/184256, WO2016/004202, WO2015/199952, WO2017/004143, WO2017/075531, WO2017/117528, WO2017/049245, WO2017/173054, WO2015/095340, WO2016/118724, WO2016/118725, WO2016/187531, WO2017/176974, WO2018/078053, WO2019/027999, WO2019/036030, WO2019/089828, WO2019/099501, WO2020/072605, WO2020/081938, WO2020/118041, WO2020/146805, or WO2020/219876 each of which is incorporated herein by reference in its entirety. Other suitable lipids for use in the RNA composition and methods for making and using thereof include the lipids as described in J. McClellan, M. C. King, Cell 2010, 141, 210-217 and in Whitehead et al., Nature Communications (2014) 5:4277, which is incorporated herein by reference in its entirety.

In some embodiments, the ionizable lipid may be 1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl) (2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), MD1 (cKK-E12), OF2, EPC, ZA3-Ep10, TT3, LP01, 5A2-SC8, Lipid 5, SM-102 (Lipid H), or ALC-315. In one embodiment, the ionizable lipid included is LP01.

The natural nanoparticle may include one or more exogenous lipids, e.g., lipids that are exogenous to the natural source (e.g., originating from a source that is not the source or source part from which the particle is produced). The lipid composition may include 0%, less than 1%, or at least 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more than 95% exogenous lipid. In some examples, the exogenous lipid (e.g., ionizable lipid) is added to amount to 25% or 40% (w/w) of total lipids in the preparation. In some examples, the exogenous lipid is added to the preparation prior to step (b), e.g., mixed with extracted natural lipids prior to step (b).

As used herein, the term "exogenous lipid" refers to a lipid that is exogenous to the natural source (e.g., plant, bacteria), i.e., a lipid originates from a source that is not the natural source from which the lipids are extracted (e.g., a lipid that is added to the lipid particle formulation using method described herein). The term "exogenous lipid" does not exclude a natural-derived lipid (such as a plant-derived sterol). That is to say, an exogenous lipid can be a natural-derived lipid (such as a plant-derived sterol that is exogenous to the plant source from which the lipids are extracted, e.g., an exogenous lipid can be a plant derived sterol that is added to the lipid particle formulation). As another example, an exogenous lipid can be a natural-derived lipid that is exogenous to the particular natural source from which the lipids are extracted (e.g., a bacteria-derived lipid that is exogenous to the plant source from which the lipids are extracted, or vice versa). An exogenous lipid may be a cell-penetrating agent, may be capable of increasing delivery of one or more polynucleotides by the lipid formulation (e.g. lipid nanoparticle or natural nanoparticle) to a cell, and/or may be capable of increasing loading (e.g., loading efficiency or loading capacity) of a polynucleotide. In some embodiments, the exogenous lipid may be a stabilizing lipid. In some embodiments, the exogenous lipid may be a structural lipid (e.g., a synthetic structural lipid). Exemplary exogenous lipids include ionizable lipids, synthetic structural lipids, sterols, and PEGylated lipids. Exogenous lipids may also include cationic lipids. As used herein, the term "cationic lipid" refers to an amphiphilic

molecule (e.g., a lipid or a lipidoid) that is positively charged, containing a cationic group (e.g., a cationic head group). As used herein, the term “ionizable lipid” refers to an amphiphilic molecule (e.g., a lipid or a lipidoid, e.g., a synthetic lipid or lipidoid) containing a group (e.g., a head group) that can be ionized, e.g., dissociated to produce one or more electrically charged species, under a given condition (e.g., pH).

In some embodiments, the natural nanoparticle may contain 3-1000 lipids extracted from one or more natural (e.g., plant, bacteria) sources. In some embodiments, the natural nanoparticle contains at least 10 natural lipids belonging to one or more of the classes selected from the group consisting of fatty acyls (FA), fatty acyl conjugates, phospholipids, glycerolipids, glycolipids, glycerophospholipids, sphingolipids, waxes, and sterol.

In some embodiments, the lipid nanoparticle or natural nanoparticle composition comprises one or more ionizable lipids, one or more structural lipids (synthetic or natural), a sterol, and one or more PEG-modified lipids.

In some embodiments, the natural nanoparticle may further comprise a neutral lipid as a helper lipid. In some embodiments, the natural lipids may be used in combination with a neutral lipid as a structural lipid component.

In some embodiments, the particle has a size of less than about 200 nm. In some embodiments, the RNA and particle composition has an N:P ratio of at least 3, for instance, an N:P ratio of 3 to 100, 3 to 50, 3 to 30, 3 to 20, 3 to 15, 3 to 12, 6 to 30, 6 to 20, 6 to 15, or 6 to 12.

In some embodiments, pharmaceutical compositions described herein can be formulated without a carrier, e.g., in a naked delivery formulation. A naked delivery formulation refers to a formulation that is free from a carrier.

In some embodiments, a naked delivery formulation can be free of any or all of: transfection reagents, cationic carriers, carbohydrate carriers, nanoparticle carriers, or protein carriers. For example, a naked delivery formulation can be free from phytoglycogen octenyl succinate, phytoglycogen beta-dextrin, anhydride-modified phytoglycogen beta-dextrin, lipofectamine, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-

Propane (DOTAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B—[N—(N,N'-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HC1), diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin.

In some embodiments, the naked delivery formulation can comprise a non-carrier excipient. In some embodiments, a non-carrier excipient may comprise an inactive ingredient that does not exhibit an active cell-penetrating effect. In some embodiments, a non-carrier excipient may comprise a buffer, for example PBS. In some embodiments, a non-carrier excipient may be a solvent, a non-aqueous solvent, a diluent, a suspension aid, a surface active agent, an isotonic agent, a thickening agent, an emulsifying agent, a preservative, a polymer, a peptide, a protein, a cell, a hyaluronidase, a dispersing agent, a granulating agent, a disintegrating agent, a binding agent, a buffering agent, a lubricating agent, or an oil.

In some embodiments, the naked delivery formulation can comprise a diluent (*e.g.*, a parenterally acceptable diluent). A diluent can be a liquid diluent or a solid diluent. In some embodiments, a diluent can be an RNA solubilizing agent, a buffer, or an isotonic agent. Non-limiting examples of an RNA solubilizing agent include water, ethanol, methanol, acetone, formamide, and 2-propanol. Examples of a buffer include 2-(N-morpholino)ethanesulfonic acid (MES), Bis-Tris, 2-[(2-amino-2-oxoethyl)-(carboxymethyl)amino]acetic acid (ADA), N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES), 3-(N-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris, Tricine, Gly-Gly, Bicine, or phosphate. Non-limiting examples of an isotonic agent include glycerin, mannitol, polyethylene glycol, propylene glycol, trehalose, or sucrose.

Purity of the pharmaceutical composition comprising the circular polyribonucleotide or modified RNA can be measured using any method known in the art, *e.g.*, chromatography,

electrophoresis, mass spectrometry, fluorescence, light scattering, refractive index, microscopy, circular dichroism (CD) spectroscopy, spectrophotometry, or surface plasmon resonance (SPR).

In some embodiments, the pharmaceutical composition comprising the circular polyribonucleotide or modified RNA is at least 30% (w/w), 40% (w/w), 50% (w/w), 60% (w/w), 70% (w/w), 80% (w/w), 85% (w/w), 90% (w/w), 91% (w/w), 92% (w/w), 93% (w/w), 94% (w/w), 95% (w/w), 96% (w/w), 97% (w/w), 98% (w/w), 99% (w/w), or 100% (w/w) pure on a mass basis.

In some embodiments, a concentration of the circular polyribonucleotide or modified RNA in the pharmaceutical composition is at least 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL, 70 µg/mL, 80 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 500 µg/mL, 1 mg/mL, 2 mg/mL, 3 mg/mL, 5 mg/mL, 10 mg/mL, 100 mg/mL, or 500 mg/mL.

In some embodiments, the pharmaceutical composition is substantially free of mononucleotide or has a mononucleotide content of no more than 1 pg/ml, 10 pg/ml, 0.1 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 50 ng/ml, 60 ng/ml, 70 ng/ml, 80 ng/ml, 90 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml, 1000 µg/mL, 5000 µg/mL, 10,000 µg/mL, or 100,000 µg/mL.

In some embodiments, the pharmaceutical composition has a mononucleotide content of no more than 0.1% (w/w), 0.2% (w/w), 0.3% (w/w), 0.4% (w/w), 0.5% (w/w), 0.6% (w/w), 0.7% (w/w), 0.8% (w/w), 0.9% (w/w), 1% (w/w), 2% (w/w), 3% (w/w), 4% (w/w), 5% (w/w), 6% (w/w), 7% (w/w), 8% (w/w), 9% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), or any percentage therebetween of total nucleotides on a mass basis, wherein total nucleotide content is the total mass of deoxyribonucleotide molecules and ribonucleotide molecules.

In some embodiments, the pharmaceutical composition has a linear RNA content, *e.g.*, linear RNA counterpart or RNA fragments, of no more than 1 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 50 ng/ml, 60 ng/ml, 70 ng/ml, 80 ng/ml, 90 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml, 600 ng/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 g/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml, 600 µg/ml, 700 µg/ml, 800 µg/ml, 900 µg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 5 mg/mL, 10 mg/mL, 50 mg/mL,

100 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL, 500 mg/mL, 600 mg/mL, 650 mg/mL, 700 mg/mL, or 750 mg/mL.

In some embodiments, the pharmaceutical composition has a nicked RNA content of no more than 10% (w/w), 9.9% (w/w), 9.8% (w/w), 9.7% (w/w), 9.6% (w/w), 9.5% (w/w), 9.4%
5 (w/w), 9.3% (w/w), 9.2% (w/w), 9.1% (w/w), 9% (w/w), 8% (w/w), 7% (w/w), 6% (w/w), 5% (w/w), 4% (w/w), 3% (w/w), 2% (w/w), 1% (w/w), 0.5% (w/w), or 0.1% (w/w), or percentage therebetween.

In some embodiments, the pharmaceutical composition is substantially free of DNA content, *e.g.*, template DNA or cell DNA (*e.g.*, host cell DNA), has a DNA content, as low as
10 zero, or has a DNA content of no more than 1 pg/ml, 10 pg/ml, 0.1 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 50 ng/ml, 60 ng/ml, 70 ng/ml, 80 ng/ml, 90 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml, 1000 µg/mL, 5000 µg/mL, 10,000 µg/mL, or 100,000 µg/mL.

In some embodiments, the pharmaceutical composition is substantially free of an
15 impurity (*e.g.*, a cell protein, a cell nucleic acid, an enzyme, a reagent component, a gel component, or a chromatographic material, protein contamination, or endotoxin contamination).

In some embodiments, the pharmaceutical composition comprises a concentration of protein that is less than 0.1 ng, 1 ng, 5 ng, 10 ng, 15 ng, 20 ng, 25 ng, 30 ng, 35 ng, 40 ng, 50 ng,
20 60 ng, 70 ng, 80 ng, 90 ng, 100 ng, 200 ng, 300 ng, 400 ng, or 500 ng of protein per milligram (mg) of the circular polyribonucleotide.

In some embodiments, the pharmaceutical composition has an A260/A280 absorbance ratio from about 1.6 to about 2.3, *e.g.*, as measured by spectrophotometer. In some embodiments, the A260/A280 absorbance ratio is about 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, or any number therebetween.

25 In some embodiments, the pharmaceutical composition comprises an amount of endotoxin that is less than 20 EU/kg (weight), 10 EU/kg, 5 EU/kg, 1 EU/kg, or is below a predetermined threshold, *e.g.*, the pharmaceutical composition comprises a level of endotoxin below a limit of detection by a specified method.

In some embodiments, the pharmaceutical composition is a sterile drug product or
30 substantially free of microorganisms (*e.g.*, supports growth of fewer than 100 viable microorganisms as tested under aseptic conditions).

In some embodiments, the pharmaceutical composition comprises a bioburden of less than 100 CFU/100 mL, 50 CFU/100 mL, 40 CFU/100 mL, 30 CFU/100 mL, 200 CFU/100 mL, 10 CFU/100 mL, or 10 CFU/100 mL before sterilization.

In some embodiments, the pharmaceutical composition comprising the circular polyribonucleotide that has undergone a purification step produces a reduced level of one or more markers of an immune or inflammatory response after administration to a subject compared to a pharmaceutical composition comprising an unpurified circular polyribonucleotide. In some embodiments, the one or more markers of an immune or inflammatory response is a cytokine or immune response related gene. In some embodiments, the one or more markers of an immune or inflammatory response is expression of a gene, such as RIG-I, MDA5, PKR, IFN-beta, OAS, and OASL.

III. Uses of Circular Polyribonucleotides and Modified RNAs

Also provided herein are methods for treating or reducing the risk of developing a GLP-2-related disease in a subject that include administering to the subject a therapeutically effective amount of any of the circular polyribonucleotides or modified RNAs described herein, or any of the pharmaceutical compositions described herein. The term “GLP-2 related disease” refers to any disease in which cells engage in aberrant GLP-2 signaling, e.g., aberrant insulin signaling.

Non-limiting examples of GLP-2-related diseases include short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn’s disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury.

To practice a method disclosed herein, an effective amount of any of the circular polyribonucleotides, modified RNAs, or pharmaceutical compositions described herein is administered to a subject (e.g., a mammalian subject, e.g., a human) having or at risk for developing a GLP-2 related disease (e.g., short bowel syndrome) via a suitable route (e.g., local administration and/or systemic administration). In some embodiments of any of the methods described herein, the subject has been previously identified or diagnosed as having a GLP-2-related disease. In some embodiments of any of the methods described herein, the subject has been previously identified as having an elevated risk of developing a GLP-2-related disease.

The term “subject” refers to a subject who needs treatment as described herein. In some embodiments, the subject is a human or a non-human mammal (e.g., cat, dog, horse, cow, goat, or sheep). A human subject who needs treatment can be a human patient having, suspected of having, or at risk for having a GLP-2-related disease, *e.g.*, short bowel syndrome (SBS), short
5 bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn’s disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury

An “effective amount” or a “therapeutically effective amount” as used herein refers to the
10 amount of each active agent required to confer therapeutic effect on the subject, either alone or in combination with one or more other active agents. Effective amounts vary, as recognized by those skilled in the art, depending on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size, weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of
15 administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known in the medical field. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons,
20 psychological reasons, or virtually any other reason.

Empirical considerations such as the half-life of an agent will generally contribute to the determination of the dosage. Frequency of administration can be determined and adjusted over the course of therapy, and is generally, but not necessarily, based on treatment and/or suppression and/or amelioration and/or delay of a GLP-2-related disease (*e.g.*, short bowel
25 syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn’s disease, chemotherapy-induced mucositis, intestinal ischemia–reperfusion injury, or a combination thereof).

In some embodiments, dosages of any of the circular polyribonucleotides, modified
30 RNAs, or pharmaceutical compositions described herein can be determined empirically in individuals who have been given one or more administration(s) of the circular polyribonucleotide

or modified RNA. For example, individuals are given incremental dosages of any of the circular polyribonucleotides, modified RNAs, and pharmaceutical compositions described herein and an indicator and/or a symptom of a GLP-2-related disease can be followed to assess efficacy.

Any suitable dosing regimen can be used in methods described herein. In some
5 embodiments, the dosage regimen depends on the pattern of pharmacokinetic decay that the practitioner wishes to achieve. In some embodiments, dosing frequency is once every day, once every other day, once every week, or longer. In some embodiments, dosing frequency is multiple times per day. For repeated administrations over several days or longer, depending on the condition, the treatment can be sustained until a desired suppression of symptoms occurs or
10 until sufficient therapeutic levels are achieved to alleviate a GLP-2-related disease, or a symptom thereof. In some embodiments, dosing regimens (including inhibitor used) can vary over time.

A circular or linear polyribonucleotide described herein may be administered in a single dose or in multiple doses to a cell, tissue or subject. A circular or linear polydeoxyribonucleotide described herein may be administered in a single dose or in multiple doses to a cell, tissue or
15 subject.

A method of administering multiple doses of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition) comprises providing two or more compositions, over a period of time, to a cell,
20 tissue or subject (e.g. a mammal). According to certain embodiments, multiple doses of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition) may be administered to a subject over a defined time course. The methods according to this aspect of the invention comprise sequentially
25 administering to a subject multiple doses of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition). As used herein, “sequentially administering” means that each dose of composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear
30 polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition) is administered to the subject at a different point in time, e.g.,

on different days separated by a predetermined interval (e.g., hours, days, weeks or months). In some embodiments, the present invention provides methods which comprise sequentially administering to the subject a single initial dose of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition), followed by one or more secondary doses of the composition, and optionally followed by one or more tertiary doses of the composition.

The terms “initial dose,” “secondary doses,” and “tertiary doses,” refer to the temporal sequence of administration of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition). Thus, the “initial dose” is the dose which is administered at the beginning of the treatment regimen (also referred to as the “baseline dose”); the “secondary doses” are the doses which are administered after the initial dose; and the “tertiary doses” are the doses which are administered after the secondary doses.

The initial, secondary, and tertiary doses may all contain the same amount of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition), and in certain embodiments, may differ from one another in terms of frequency of administration. In certain embodiments, the amount of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition) contained in the initial, secondary and/or tertiary doses varies from one another (e.g., adjusted up or down as appropriate) during the course of treatment. In certain embodiments, one or more (e.g., 2, 3, 4, or 5) doses are administered at the beginning of the treatment regimen as “loading doses” followed by subsequent doses that are administered on a less frequent basis (e.g., “maintenance doses”).

In certain embodiments, each secondary and/or tertiary dose is administered after the immediately preceding dose. The phrase “the immediately preceding dose,” as used herein, means, in a sequence of multiple administrations, the dose of the composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical

composition) which is administered to a subject prior to the administration of the very next dose in the sequence with no intervening doses. In certain embodiments, each secondary and/or tertiary dose is administered every day, every 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days after the immediately preceding dose. In certain embodiments, each secondary and/or tertiary dose is administered every 0.5 weeks, 1 week, 2 weeks, 3 weeks, or 4 weeks after the immediately preceding dose.

The methods according to this aspect of the invention may comprise administering to a subject any number of secondary and/or tertiary doses of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical composition). For example, in certain embodiments, only a single secondary dose is administered to the subject. In other embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) secondary doses are administered to the subject. Likewise, in certain embodiments, only a single tertiary dose is administered to the subject. In other embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) tertiary doses are administered to the subject.

In certain embodiments, the frequency at which the secondary and/or tertiary doses are administered to a subject can vary over the course of the treatment regimen. The frequency of administration may also be adjusted during the course of treatment.

In some embodiments, multiple doses are provided to produce a level of the composition or express a level of the GLP-2 or GLP-2 analog in a cell, tissue or subject. In some embodiments, multiple doses are provided to produce or maintain a level of the composition, or to produce or maintain a level of the GLP-2 or GLP-2 analog, in a cell, tissue or subject for a period of time, for instance, for at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150 days, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, 21, or 24 months, or at least 1, 2, 3, 4, or 5 years.

In some embodiments, the method comprises providing (e.g., administering) at least a first composition and a second composition to the cells, tissue, or subject (e.g., a mammal, e.g., a human). In some embodiments, the method further comprises providing (e.g., administering) a third composition, fourth composition, fifth composition, sixth composition, seventh composition, eighth composition, ninth composition, tenth composition, or more. In some embodiments, additional compositions are provided for the duration of the life of the cell. In some embodiments, additional compositions are provided (e.g., administered) while the cell,

tissue or subject obtains a benefit from the composition, e.g., stimulated insulin secretion, altered metabolism, improved glucose homeostasis, reduced body weight, improved liver function, and/or amelioration of fibrosis.

In some embodiments, a first composition in a multiple dosing regimen comprises a first
5 amount of the nucleic acid molecule (e.g., circular polyribonucleotide) disclosed herein. In some
embodiments, a second composition in a multiple dosing regimen comprises a second amount of
the nucleic acid molecule (e.g., circular polyribonucleotide) disclosed herein. In some
embodiments, a third composition, a fourth composition, a fifth composition, a sixth
composition, a seventh composition, an eighth composition, a ninth composition, a tenth
10 composition, or more in a multiple dosing regimen comprises a third, fourth, fifth, sixth, seventh,
eighth, ninth, tenth or more amount of the nucleic acid molecule (e.g., circular
polyribonucleotide) disclosed herein. In some embodiments, the second amount of the nucleic
acid molecule (e.g., circular polyribonucleotide) is the same as the first amount of the nucleic
acid molecule (e.g., circular polyribonucleotide). In some embodiments, the third amount of the
15 nucleic acid molecule (e.g., circular polyribonucleotide) is the same as the first amount of the
nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the fourth, fifth,
sixth, seventh, eighth, ninth, tenth, or more amount of the nucleic acid molecule (e.g., circular
polyribonucleotide) is the same as the first amount of the nucleic acid molecule (e.g., circular
polyribonucleotide). In some embodiments, the second amount of the nucleic acid molecule
20 (e.g., circular polyribonucleotide) is less than the first amount of the nucleic acid molecule (e.g.,
circular polyribonucleotide). In some embodiments, the third amount of the nucleic acid
molecule (e.g., circular polyribonucleotide) is less than the first amount of the nucleic acid
molecule (e.g., circular polyribonucleotide). In some embodiments, the fourth, fifth, sixth,
seventh, eighth, ninth, tenth, or more amount of the nucleic acid molecule (e.g., circular
25 polyribonucleotide) is less than the first amount of the nucleic acid molecule (e.g., circular
polyribonucleotide). In some embodiments, the second amount of the nucleic acid molecule
(e.g., circular polyribonucleotide) is greater than the first amount of the nucleic acid molecule
(e.g., circular polyribonucleotide). In some embodiments, the third amount of the nucleic acid
molecule (e.g., circular polyribonucleotide) is greater than the first amount of the nucleic acid
30 molecule (e.g., circular polyribonucleotide). In some embodiments, the fourth, fifth, sixth,
seventh, eighth, ninth, tenth, or more amount of the nucleic acid molecule (e.g., circular

polyribonucleotide) is greater than the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the second composition varies by no more than 1%, 5%, 10%, 15%, 20%, or 25% of an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the second composition is no more than 1%, 5%, 10%, 15%, 20%, or 25% less than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is from 0.1-fold to 1000-fold higher than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold higher than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a subsequent composition (e.g., a composition administered after a first composition) is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold higher than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is from 0.1-fold to 1000-fold lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a subsequent composition (e.g., a composition administered after a first composition) is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a subsequent composition (e.g., after a first composition of an amount of nucleic acid molecule (e.g., circular polyribonucleotide)) is from 0.1-fold to 1000-fold higher or lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular

polyribonucleotide) of a subsequent composition (e.g., after a first composition of an amount of nucleic acid molecule (e.g., circular polyribonucleotide)) is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold higher or lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. For example, a first composition comprises 1-fold nucleic acid molecule (e.g., circular polyribonucleotide), a second composition comprises 5-fold nucleic acid molecule (e.g., circular polyribonucleotide) compared to the first composition, and a third composition comprises 0.2-fold nucleic acid molecule (e.g., circular polyribonucleotide) compared to the first composition. In some embodiments, the second composition comprises at least 5-fold nucleic acid molecule (e.g., circular polyribonucleotide) compared to an amount of nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition.

In some embodiments, the first composition comprises a higher amount of the nucleic acid molecule (e.g., circular polyribonucleotide) than the second composition. In some embodiments, the first composition comprises a higher amount of the nucleic acid molecules (e.g., circular polyribonucleotides) than the third, fourth, fifth, sixth, seventh, eighth, ninth, or tenth composition.

In some embodiments, the plurality (e.g., two or more) of compositions of a nucleic acid molecule (e.g., circular polyribonucleotide) encoding a GLP-2 or GLP-2 analog, which are administered in a multiple dosing regimen as described herein, are the same compositions. In some embodiments, the plurality (e.g., two or more) of compositions of a nucleic acid molecule (e.g., circular polyribonucleotide) encoding a GLP-2 or GLP-2 analog, which are administered in a multiple dosing regimen as described herein, are different compositions. In some embodiments, the same compositions comprise the nucleic acid molecules (e.g., circular polyribonucleotides) encoding the same GLP-2 or GLP-2 analog. In some embodiments, the different compositions comprise the nucleic acid molecules (e.g., circular polyribonucleotides) encoding different a GLP-2 or GLP-2 analog, or a combination thereof.

A composition of the nucleic acid molecule (e.g., a circular polyribonucleotide) disclosed herein can induce a response in a subject. In some embodiments, the response may be stimulated insulin secretion, altered metabolism, improved glucose homeostasis, reduced body weight, improved liver function, and/or amelioration of fibrosis.

In some embodiments, in a multiple dosing regimen, the method of administering the nucleic acid molecule (e.g., circular polyribonucleotide) provided herein includes administering

to a subject in need thereof the nucleic acid molecule for multiple times (multiple doses), e.g., at least 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 30, 40, 50, 60, 100, 150, 200, or 500 times, with an interval of from 1 day to 56 days, such as about 49 days, 42 days, 35 days, 28 days, 21 days, 14 days, or 7 days. In some embodiments, in a multiple dosing regimen, the method provided herein

5 includes administering to a subject in need thereof the nucleic acid molecule for at least 3 times, with an interval of about 7 days. In some embodiments, in a subject that receives administration of multiple doses of the nucleic acid molecule (e.g., at least 3, 4, 5, 6, 7, 8, or 9 doses) provided herein, a level of the GLP-2 or GLP-2 analog (e.g., a plasma GLP-2 or GLP-2 level) is maintained at a level with variation of less than 50%, 40%, 30%, 20%, or 10% for a period of
10 longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, or 20 weeks after the last dose. In some embodiments, in a subject that receives administration of multiple doses of the nucleic acid molecule (e.g., at least 3, 4, 5, 6, 7, 8, or 9 doses) provided herein, a level of the GLP-2 or GLP-2 analog (e.g., a plasma GLP-2 or GLP-2 level) is maintained at a first level for a period of longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, or 20 weeks after the second,
15 third, fourth, fifth, sixth, seventh, eighth, or the last dose, wherein the first level is higher than a level of the GLP-2 or GLP-2 analog measured shortly after the first dose (e.g., measured about 12, 24, 36, or 48 hours after the first dose). In some embodiments, in a subject that receives administration of multiple doses of the nucleic acid molecule (e.g., at least 3 doses) provided herein with an interval of about 7 days, a level of the GLP-2 or GLP-2 analog (e.g., a plasma
20 GLP-2 or GLP-2 level) is maintained at a first level for a period of longer than 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 weeks after the second, third, fourth, fifth, sixth, seventh, eighth, or the last dose, wherein the first level is higher than a level of the GLP-2 or GLP-2 analog measured shortly after the first dose (e.g., measured about 12, 24, 36, or 48 hours after the first dose).

25 As used herein, the term “treating” refers to the application or administration of a composition including one or more active agents to a subject who has a GLP-2-related disease (e.g., short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn’s disease, chemotherapy-
30 induced mucositis, intestinal ischemia–reperfusion injury, or a combination thereof), a symptom of a GLP-2-related disease, and/or an increased risk of developing a GLP-2-related disease, with

the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the GLP-2-related disease, the symptom of the GLP-2 related disease, and/or the risk of developing a GLP-2-related disease.

Alleviating a GLP-2-related disease includes delaying the development or progression of the disease, and/or reducing disease severity. Alleviating the disease does not necessarily require curative results.

As used herein, “delaying” the development of a GLP-2-related disease (*e.g.*, short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn’s disease, chemotherapy-induced mucositis, intestinal ischemia–reperfusion injury, or a combination thereof) means to defer, hinder, slow, retard, stabilize, and/or postpone progression of the GLP-2 related disease. This delay can be of varying lengths of time, depending on the history of the GLP-2-related disease and/or individuals being treated. A method that “delays” or alleviates the development of a GLP-2-related disease and/or delays the onset of the GLP-2-related disease is a method that reduces probability of developing one or more symptoms of the GLP-2-related disease in a given time frame and/or reduces extent of the symptoms in a given time frame, when compared to not using the method. Such comparisons are typically based on clinical studies, using a number of subjects sufficient to give a statistically significant result.

“Development” or “progression” of a disease means initial manifestations and/or ensuing progression of the GLP-2-related disease. Development of the GLP-2-related disease can be detectable and assessed using standard clinical techniques known in the art. However, development also refers to progression that may be undetectable. For purposes of this disclosure, development or progression refers to the biological course of the symptoms. “Development” includes occurrence, recurrence, and onset. As used herein, “onset” or “occurrence” of a GLP-2-related disease includes initial onset and/or recurrence.

In some embodiments, any of the circular polyribonucleotides, modified RNAs, or pharmaceutical compositions described herein is administered to a subject in an amount sufficient to increase levels of GLP-2-mediated signaling by at least 5% (*e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or more).

In some embodiments, any of the circular polyribonucleotides, modified RNAs, or pharmaceutical compositions described herein is administered to a subject in an amount sufficient to increase intestinal growth in a subject by at least 5% (*e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or more). In some embodiments, increasing intestinal growth includes increasing intestinal tissue and/or cells. In some embodiments, the cells are crypt cells.

In some embodiments, any of the circular polyribonucleotides, modified RNAs, or pharmaceutical compositions described herein is administered to a subject in an amount sufficient to increase intestinal absorption of fluids and nutrients in a subject by at least 5% (*e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or more).

In some embodiments, methods for treating a GLP-2-related disease involve restoring, at least in part, expression and/or activity of GLP-2 using any of the circular polyribonucleotides, modified RNAs, or pharmaceutical compositions described herein.

Any of the circular polyribonucleotides, modified RNAs, or pharmaceutical compositions described herein can be administered using any suitable method for achieving delivery of the circular polyribonucleotide or modified RNA to the subject in need thereof. The route of administration can depend on various factors such as the type of GLP-2-related disease to be treated and the site of the disease. In some embodiments, any of the circular polyribonucleotides, modified RNAs, or pharmaceutical composition described herein can be administered topically, locally, nasally, parenterally, buccally, or by inhalation. Parenteral administration includes, but is not limited to, subcutaneous, intracutaneous, intravenous, intramuscular, or intrasynovial injection, or infusion techniques. In some embodiments, any of the circular polyribonucleotides, modified RNAs, or pharmaceutical composition described herein is administered locally (*e.g.*, intramuscular injection). In some embodiments, any of the circular polyribonucleotides, the modified RNAs, or the pharmaceutical composition described herein is administered systemically (*e.g.*, intravenous infusion).

In some embodiments, any of the circular polyribonucleotides, the modified RNAs, or the pharmaceutical compositions described herein is administered one or more times to the subject.

Alternatively, or in addition, any of the circular polyribonucleotides, the modified RNAs, or the

pharmaceutical compositions described herein can be administered as part of a combination therapy with an additional therapeutic agent.

Any therapeutic agent suitable for treating a GLP-2-related disease can be used as an additional therapeutic agent in methods and/or pharmaceutical compositions described herein.

5 Non-limiting examples of additional therapeutic agents include anti-inflammatory agents (e.g., steroids, such as corticosteroids)), immunosuppressants (e.g., methotrexate, cyclosporine), or insulin. Alternatively, in some embodiments, no other agents are administered.

IV. Kits

10 The present disclosure also provides kits that can be used, e.g., in any of the methods described herein.

Such kits include (a) (i) a composition comprising any of the circular polyribonucleotides described herein or any of the modified RNAs described herein or (ii) any of the pharmaceutical compositions described herein; and optionally, (b) instructions for administering the composition
15 or the pharmaceutical composition to a subject in need thereof.

V. Nucleic Acids

The present disclosure further provides nucleic acids (e.g., DNA or RNA) encoding any of the polypeptides described herein. In some embodiments, the nucleic acid encodes a
20 polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof (e.g., any of the GLP-2 polypeptides or analogs described herein), (ii) a half-life extension moiety (e.g., any of the exemplary half-life extension moieties described herein), and (iii) a secretion signal sequence (e.g., any of the exemplary secretion signal sequences described herein). In some embodiments, the nucleic acid encodes a polypeptide comprising (i) a
25 glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof (e.g., any of the exemplary GLP-2 polypeptides or analogs described herein) and (ii) a half-life extension moiety (e.g., any of the exemplary half-life extension moieties described herein). In some embodiments, the nucleic acid encodes a polypeptide comprising a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof (e.g., any of exemplary the GLP-2 polypeptides or analogs described herein).

30

VI. Polypeptides

The present disclosure also provides polypeptides generated from any of the circular polyribonucleotides, any of the modified RNAs, and any of the nucleic acids described herein. In some embodiments, the polypeptide comprises (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof (e.g., any of the exemplary GLP-2 polypeptides or analogs described herein), (ii) a half-life extension moiety (e.g., any of the exemplary half-life extension moieties described herein), and (iii) a secretion signal sequence (e.g., any of the exemplary secretion signal sequences described herein). In some embodiments, the polypeptide comprises (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof (e.g., any of the exemplary GLP-2 polypeptides or analogs described herein) and (ii) a half-life extension moiety (e.g., any of the exemplary half-life extension moieties described herein). In some embodiments, the polypeptide comprises a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof (e.g., any of the exemplary GLP-2 polypeptides or analogs described herein).

In some embodiments, the polypeptides can include an IgG constant domain (e.g., IgG1, IgG2, IgG3, IgG4), a serum protein or fragment thereof (e.g., albumin or fragment thereof (e.g., human serum albumin or a fragment thereof), fibronectin or fragment thereof (e.g., human fibronectin or a fragment thereof), or transferrin or fragment thereof (e.g., human transferrin or a fragment thereof)), and an antibody or an antigen-binding fragment thereof that binds to a serum protein (e.g., an antibody or antigen-binding fragment thereof that binds to human albumin, human fibronectin, or human transferrin). In some embodiments, the IgG constant domain includes a half-life extension substitution (e.g., any of the half-life extension substitutions described herein).

EXAMPLES

In order that the disclosure described may be more fully understood, the following examples are set forth. The examples described in this application are offered to illustrate the methods provided herein and are not to be construed in any way as limiting their scope.

Example 1: GLP-2 Analog Expressed from Circular RNA is Less Active Than Recombinant GLP-2 Analog

The activity of the GLP-2 analog teduglutide expressed from a circular RNA construct was tested in mammalian cells. The circular RNA construct included teduglutide linked to IgG4

YTE via a (GGGS)₃ (SEQ ID NO: 141) linker (also referred to as teduglutide-Fc).

Recombinant teduglutide was used as a control. The circular RNA construct was transfected into HeLa cells using Lipofectamine™ MessengerMAX™ (Invitrogen LMRNA001) according to the manufacturer's instructions. Activity was measured using a G protein-coupled receptor (GPCR) assay in which cyclic adenosine monophosphate (cAMP) production were measured with a fluorescently labelled cAMP probe.

As shown in **FIG. 1**, teduglutide-Fc expressed from circular RNA showed approximately 3-fold reduced activity level as compared to recombinant teduglutide in a GPCR assay. These results demonstrate that a functionally active GLP-2 analog can be expressed from circular RNA.

Example 2: Expression of Functionally Active GLP-2 Analog from Circular RNA in Mammalian Cells

The expression of the GLP-2 analog teduglutide from various circular RNA constructs (**Table 8**) was tested in mammalian cells (HEK293T cells). Circular RNA constructs were transfected into HeLa cells using Lipofectamine™ MessengerMAX™ (Invitrogen LMRNA001) according to the manufacturer's instructions. Expression was measured using RT-qPCR at 3 days post-transfection using the Luna® Universal One-Step RT-qPCR kit following manufacturer's instructions. Activity was measured using a GPCR assay in which cAMP production was measured with a fluorescently labelled cAMP probe.

Table 8: Circular RNA constructs

Construct Name	Description
2860 0	IL2 K274(Shire GLP2 Fc) G4S HiBit
3539 0	IL2 K274(Shire GLP2 Fc) G4S HiBit
3538 0	IL2 K274(Shire GLP2 Fc) G4S HiBit
2862 0	IL2 K274(Shire GLP2 Fc) G4S HiBit
4158 0	IL2 K274(Shire GLP2 Fc) G4S HiBit Bicistronic
4139 0	IL2 K274(Shire GLP2 Fc) G4S HiBit Bicistronic
4157 0	IL2 K274(Shire GLP2 Fc) G4S HiBit Bicistronic
4166 0	IL2 K274(Shire GLP2 Fc) G4S HiBit Bicistronic
4165 0	IL2 K274(Shire GLP2 Fc) G4S HiBit Bicistronic
4133 0	IL2 Tedu IgG4 G4S HiBit
4159 0	IL2 Tedu IgG4 G4S HiBit
4134 0	IL2 Tedu IgG4 G4S HiBit
4135 0	IL2 Tedu IgG4 G4S HiBit
4160 0	IL2 Tedu IgG4 G4S HiBit

4137_0	IL2_Tedu_IgG4_G4S_HiBit_Bicistronic
4161_0	IL2_Tedu_IgG4_G4S_HiBit_Bicistronic
4163_0	IL2_Tedu_IgG4_G4S_HiBit_Bicistronic
4162_0	IL2_Tedu_IgG4_G4S_HiBit_Bicistronic
4225_0	IL2_Teduglutide_IG4YTE
4229_0	IL2_Teduglutide_IG4YTE_Bicistronic
mRNA	Trilink modified mRNA
4106_0	IL2_Teduglutide_IG4YTE_G4S_HiBit
4106_1	IL2_Teduglutide_IG4YTE_G4S_HiBit
4245_0	IL2_Teduglutide_IG4YTE_G4S_HiBit
4244_0	IL2_Teduglutide_IG4YTE_G4S_HiBit
4243_0	IL2_Teduglutide_IG4YTE_G4S_HiBit
4138_0	IL2_Teduglutide_IG4YTE_G4S_HiBit_Bicistronic
4246_0	IL2_Teduglutide_IG4YTE_G4S_HiBit_Bicistronic
4248_0	IL2_Teduglutide_IG4YTE_G4S_HiBit_Bicistronic
4247_0	IL2_Teduglutide_IG4YTE_G4S_HiBit_Bicistronic
3075_0	None_GLP2_IgG4_None

IL2: IL2 secretion signal; K274(Shire_GLP2_Fc): Teduglutide linked to IgG1 via G4S linker; G4S: linker sequence with four glycine and one serine; HiBiT: HiBiT epitope tag; IG4YTE: IgG4 with YTE mutation

As shown in **FIG. 2A**, expression levels of teduglutide from circular RNA constructs was similar to the expression level of teduglutide from mRNA. As shown in **FIG. 2B**, teduglutide expressed from the circular RNA constructs described in **Table 9** showed robust activity in a GPCR assay.

Table 9: Circular RNA constructs

Construct	Polypeptide	IRES	C-Terminal	CE	5' Spacer	3' Spacer	
3538_0	K274(Shire_GLP2_Fc)	Modified CVB3	G4S_HiBit	Anabaena2.3	AU120	AU120	
3539_0	K274(Shire_GLP2_Fc)	Modified CVB3	G4S_HiBit	Anabaena2.3	AU120	None	
2862_0	K274(Shire_GLP2_Fc)	Modified CVB3	G4S_HiBit	Anabaena2	AU50	None	

K274(Shire_GLP2_Fc): Teduglutide linked to IgG1 via G4S linker; G4S: linker sequence with four glycine and one serine; HiBiT: HiBiT epitope tag; IG4YTE: IgG4 with YTE mutation; CVB3: IRES from Cocksackievirus B3 (CVB3); AU50: poly(adenosine-uridine) spacer sequence having a length of 50 nucleotides; AU120: poly(adenosine-uridine) spacer sequence having a length of 120 nucleotides; CE: circularization element; Anabaena2.3: A specific intron based circularization element; Anabaena2: A specific intron based circularization element.

Taken together, these results demonstrate that functionally active teduglutide can be expressed from various circular RNA constructs.

Example 3: Pharmacokinetic (PK) Profiles of GLP-2 Fc Expressed from Circular RNA Compared to Recombinant GLP-2 Analogs

The PK profile of teduglutide-Fc expressed from circular RNA encoding teduglutide-Fc was evaluated in mice. Circular RNA encoding teduglutide-Fc (1.5 mg/kg) was injected into mice via subcutaneous injection in the interscapular area. Blood was collected from the tail-vein of each mouse. Serum was collected from blood and the concentration and pharmacokinetic (PK) parameters for teduglutide-Fc were determined.

The PK profile of teduglutide-Fc expressed from circular RNA in mice was then compared to the reported PK profiles of various recombinant GLP-2 analogs. Reported PK profiles were obtained from Hargrove et al., “Pharmacological Characterization of Apraglutide, a Novel Long-Acting Peptidic Glucagon-Like Peptide-2 Agonist, for the Treatment of Short Bowel Syndrome,” J Pharmacol Exp Ther; 373(2):193-203 (2020) and Choi et al., “HM15912, a Novel Long-acting Glucagon-like Peptide-2 Analog, Improves Intestinal Growth and Absorption Capacity in a Male Rat Model of Short Bowel Syndrome,” J Pharmacol Exp Ther; JPET-AR-2022-001381 (2022).

As shown in **FIG. 3** and **Table 10**, higher levels of teduglutide-Fc were detected in mice injected with circular RNA encoding teduglutide-Fc compared to reported levels of teduglutide and HM15912 in rats. The C_{max} (μg/mL) of teduglutide-Fc was approximately 10-fold higher in mice injected with circular RNA encoding teduglutide-Fc compared to rats injected with recombinant teduglutide.

Table 10: PK parameters for mice injected with circular RNA encoding GLP-2 Fc or recombinant GLP-2 analog

GLP-2 Fc Analog	Dose (mg/kg)	Species*	C _{max} (g/mL)	T _{max} (hr)	Terminal t _{1/2} (hr)
3539	1.5	CD1 mouse	30.5	24	60
Teduglutide	0.5**	SD rat	2.5	1	0.5
HM15912	1.5	SD rat	9.1	24	42.3

* Clearance rate of GLP-2 is similar between mouse and rat

** Approved dose of teduglutide in humans is 0.05 mg/kg.

Thus, these results demonstrate that expression of teduglutide-Fc from circular RNA can achieve a preferred PK profile compared to recombinant teduglutide.

Example 4: Characterization of Lipid Nanoparticle (LNP) Formulations

5 Various LNP formulations were tested for tolerability and expression of teduglutide-Fc. Circular RNA encoding teduglutide-Fc (4133_3) was formulated in different LNPs. LNP formulations (0.75 mg/kg) were injected into mice. Negative control mice were injected with PBS. Blood was collected from the tail-vein at 4 hours, 6 hours, and 24 hours after injection. Expression of teduglutide-Fc at 6 and 24 hours (**FIG. 4A**), complete blood cell count (CBC) at 10 24 hours (**FIGs. 4B-4C**), ALT levels at 24 hours (**FIG. 4D**), IFN α levels at 4 hours (**FIG. 4E**), and cytokine levels at 24 hours (**FIG. 4F**) were determined.

Example 5: Dose Response in Mice

Circular RNA (7297) and mRNA containing the modified nucleoside N1-methylpseudouridine (m¹ψ) (7377) encoding teduglutide-Fc was formulated into LNPs (LRN28). 15 A dose response of the LNP formulations of teduglutide-Fc was evaluated in either WT or NSG (NOD-*scid* IL2Rg^{null}) mice. Circular RNA in LNPs encoding teduglutide-Fc (1, 0.3, 0.1, or 0.03 mg/kg) in WT and NSG mice and m¹ψ mRNA in LNP encoding teduglutide-Fc (0.3 mg/kg) in NSG mice only were injected via subcutaneous injection in the interscapular area. Blood was 20 collected from the tail-vein of each mouse at baseline and 6h, D1, D2, D10, D17, and D28 after subcutaneous administration. Expression of GLP2-Fc (**FIGs. 5A-B**), cytokine levels (**FIGs. 5C-F**), and anti-target antibody(ATA) response (**FIG. 5G**) were measured at various timepoints.

Example 6: Characterization of GLP-2 Fc Expressed from Circular RNA in Non-Human Primates

25 Circular RNA (7297) and mRNA containing the modified nucleoside N1-methylpseudouridine (m¹ψ) (7377) encoding teduglutide-Fc was formulated into LNPs (LRN28). The circular RNA formulations had 2 purification levels: pre-Reverse Phase (pre-RP) or reverse phase (RP). The LNP formulations of teduglutide-Fc were intravenously dosed in non-human 30 primates (NHPs) at 0.5 mg/kg with or without steroids, as shown in **Table 11**. The steroid, if given, was dexamethasone intramuscularly administered at 1mpk 24h and 1h pre-dose and 24h

post-dose. Blood was collected at baseline (D -14), post-infusion, 6hr, 24h, 48h, 72h, D5, D7, D14, D21, and D28. Teduglutide-Fc expression (**FIG. 6A**), antibody production (**FIG. 6B**), complete blood count, including lymphocyte count (**FIG. 6C**), cytokine responses (**FIG. 6D**), CRP levels (**FIG. 6E**), and activate partial thromboplastin (**FIG. 6F**) were measured. Blood counts and safety levels measured throughout were comparable.

Table 11: Experimental Design

Arm	Group	Carrier	Steroid	ROA	Dose (mg/kg)	N
1	Ctrl	PBS	-	IV infusion	/	3
2	7297 (Pre-RP)	LNP-LRN28	+		0.5	
3	7297 (RP)		-		0.5	
4			+		0.5	
5	7377 m1ψ mRNA		-		0.5	
6			+		0.5	

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

Embodiment 1 is a circular polyribonucleotide comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, (ii) a half-life extension moiety, and (iii) a secretion signal sequence.

Embodiment 2 is the circular polyribonucleotide of embodiment 1, wherein the GLP-2 polypeptide or analog thereof comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

Embodiment 3 is the circular polyribonucleotide of embodiment 1 or 2, wherein the GLP-2 polypeptide or analog thereof comprises an amino acid sequence of any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

Embodiment 4 is the circular polyribonucleotide of any one of embodiments 1-3, wherein
5 the sequence encoding the polypeptide comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14.

Embodiment 5 is the circular polyribonucleotide of any one of embodiments 1-4, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence of any one of SEQ ID
10 NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14.

Embodiment 6 is the circular polyribonucleotide of any one of embodiments 1-5, wherein the GLP-2 analog comprises teduglutide, glepaglutide, dapiglutide, apraglutide, HM15912, and SHP-681.

Embodiment 7 is the circular polyribonucleotide of any one of embodiments 1-6, wherein
15 the half-life extension moiety comprises a peptide moiety.

Embodiment 8 is the circular polyribonucleotide of embodiment 7, wherein the peptide moiety comprises an Fc domain, an albumin polypeptide, a nanobody, or a post-translational modification site.

Embodiment 9 is the circular polyribonucleotide of embodiment 8, wherein the Fc
20 domain comprises an IgG1 Fc domain, an IgG2 Fc domain, an IgG3 Fc domain, or an IgG4 Fc domain.

Embodiment 10 is the circular polyribonucleotide of embodiment 8 or 9, wherein the Fc domain comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identical to SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc
25 domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain).

Embodiment 11 is the circular polyribonucleotide of any one of embodiments 8-10, wherein the Fc domain comprises an amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain).

Embodiment 12 the circular polyribonucleotide of embodiment 11, wherein the IgG4 Fc
30 domain comprises an amino acid sequence of SEQ ID NO: 21.

Embodiment 13 is the circular polyribonucleotide of any one of embodiments 8-12, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 16 (IgG1 Fc domain), SEQ ID NO: 18 (IgG2 Fc domain), SEQ ID NO: 20 (IgG3 Fc domain), or SEQ ID NO: 22 (IgG4 Fc domain).

Embodiment 14 is the circular polyribonucleotide of embodiment 13, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence of SEQ ID NO: 16 (IgG1 Fc domain), SEQ ID NO: 18 (IgG2 Fc domain), SEQ ID NO: 20 (IgG3 Fc domain), or SEQ ID NO: 22 (IgG4 Fc domain).

Embodiment 15 is the circular polyribonucleotide of any one of embodiments 8-14, wherein the Fc domain comprises a mutation.

Embodiment 16 is the circular polyribonucleotide of any one of embodiments 8-10, 13, and 15, wherein the Fc domain comprises the amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain), except the amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain) comprises an amino acid substitution selected from one or more of: M252Y/S254T/T256E (YTE); L284A/L285A; M428L/N434S (LS); T307A/E380A/N434A (AAA); T250Q/M428L (QL), and V308P.

Embodiment 17 is the circular polyribonucleotide of embodiment 8, wherein the albumin polypeptide comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 23.

Embodiment 18 is the circular polyribonucleotide of embodiment 8, wherein the peptide moiety comprises a nanobody that binds to albumin.

Embodiment 19 is the circular polyribonucleotide of any one of embodiments 1-18, wherein the circular polyribonucleotide comprises one or more modifications.

Embodiment 20 is the circular polyribonucleotide of embodiment 19, wherein the one or more modifications comprise one or more modifications to a portion of the sequence encoding the GLP-2 polypeptide or analog thereof.

Embodiment 21 is the circular polyribonucleotide of embodiment 19 or embodiment 20, wherein the one or more modifications comprise a modification to a sugar, a nucleobase, an internucleoside linkage, or a combination thereof.

Embodiment 22 is the circular polyribonucleotide of any one of embodiments 1-21, wherein the circular polyribonucleotide further comprises a translation initiation sequence operably linked to the sequence encoding the polypeptide.

Embodiment 23 is the circular polyribonucleotide of any one of embodiments 1-22, wherein the circular polyribonucleotide further comprises an internal ribosome entry site (IRES) operatively linked to the sequence encoding the polypeptide.

Embodiment 24 is the circular polyribonucleotide of any one of embodiments 1-23, wherein the circular polyribonucleotide further comprises a translation termination sequence.

Embodiment 2 is the circular polyribonucleotide of any one of embodiments 1-24, wherein the circular polyribonucleotide lacks a translation termination sequence.

Embodiment 26 is the circular polyribonucleotide of any one of embodiments 1-25, wherein the circular polyribonucleotide further comprises a stagger element at a 3' end of the sequence encoding the polypeptide.

Embodiment 27 is the circular polyribonucleotide of embodiment 26, wherein the stagger element is configured to stall a ribosome during rolling circle translation.

Embodiment 28 is the circular polyribonucleotide of any one of embodiments 1-27, wherein the circular polyribonucleotide further comprises a replication element.

Embodiment 29 is the circular polyribonucleotide of any one of embodiments 1-28, wherein the circular polyribonucleotide further comprises a 5' untranslated region operably linked to the sequence encoding the polypeptide.

Embodiment 30 is the circular polyribonucleotide of any one of embodiments 1-29, wherein the circular polyribonucleotide does not comprise a poly(A) sequence operably linked to the sequence encoding the polypeptide.

Embodiment 31 is the circular polyribonucleotide of any one of embodiments 1-30, wherein the circular polyribonucleotide does not comprise a 5' untranslated region operably linked to the sequence encoding the polypeptide.

Embodiment 32 is the circular polyribonucleotide of any one of embodiments 1-31, wherein the secretion signal sequence is a liver-specific secretion signal sequence, wherein the

liver-specific secretion signal comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to any one of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, or SEQ ID NO: 139.

Embodiment 33 is the circular polyribonucleotide of any one of embodiments 1-31, wherein the secretion signal sequence comprises an IL-2 secretion signal sequence, a proglucagon secretion signal sequence, a Gaussia luciferase secretion signal sequence, or an erythropoietin (EPO) secretion signal sequence.

Embodiment 34 is the circular polyribonucleotide of embodiment 33, wherein the IL-2 secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 29.

Embodiment 35 is the circular polyribonucleotide of embodiment 33, wherein the proglucagon secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 31.

Embodiment 36 is the circular polyribonucleotide of embodiment 33, wherein the Gaussia luciferase secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 33.

Embodiment 37 is the circular polyribonucleotide of embodiment 33, wherein the EPO secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 35.

Embodiment 38 is the circular polyribonucleotide of embodiment 33, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence that is at least 70%, 80%,

90%, 95%, 99%, or 100% identical to one of SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, or SEQ ID NO: 39.

Embodiment 39 is the circular polyribonucleotide of any one of embodiments 1-38, wherein the circular polyribonucleotide further comprises at least one spacer sequence.

5 Embodiment 40 is the circular polyribonucleotide of embodiment 39, wherein the polypeptide comprises a protease cleavage site and the at least one spacer sequence encodes the protease cleavage site.

Embodiment 41 is the circular polyribonucleotide of embodiment 40, wherein the protease cleavage site is positioned between (a) the secretion signal and (b) the GLP-2
10 polypeptide or analog thereof and the half-life extension moiety.

Embodiment 42 is the circular polyribonucleotide of any one of embodiments 1-41, wherein the polypeptide further comprises at least one linker sequence.

Embodiment 43 is the circular polyribonucleotide of embodiment 42, wherein the at least one linker sequence encodes an amino acid sequence selected from (GS)_x, (GGS)_x, (GGGS)_x
15 (SEQ ID NO: 141), (GGSG)_x (SEQ ID NO: 142), and (SGGG)_x (SEQ ID NO: 143), wherein x is an integer from 1 to 50.

Embodiment 44 is the circular polyribonucleotide of embodiment 42 or 43, wherein the at least one linker sequence is disposed between the GLP-2 polypeptide or analog thereof and the half-life extension moiety.

20 Embodiment 45 is the circular polyribonucleotide of any one of embodiments 1-44, wherein the circular polyribonucleotide comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1.

Embodiment 46 is the circular polyribonucleotide of any one of embodiments 1-45, wherein the circular polyribonucleotide comprises an amino acid sequence of SEQ ID NO: 1.

25 Embodiment 47 is the circular polyribonucleotide of any one of embodiments 1-46, wherein the circular polyribonucleotide comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 2.

Embodiment 48 is the circular polyribonucleotide of any one of embodiments 1-47, wherein the circular polyribonucleotide comprises a nucleic acid sequence of SEQ ID NO: 2.

Embodiment 49 is a pharmaceutical composition comprising the circular polyribonucleotide of any one of embodiments 1-48, and a pharmaceutically acceptable excipient.

Embodiment 50 is the pharmaceutical composition of embodiment 49, wherein the circular polyribonucleotide is formulated as a lipid nanoparticle.

Embodiment 51 is the pharmaceutical composition of embodiment 49, wherein the circular polyribonucleotide is formulated for unencapsulated delivery or natural particle delivery.

Embodiment 52 is the pharmaceutical composition of any one of embodiments 49-51, wherein the pharmaceutical composition is formulated for intravenous, subcutaneous, intrahepatic, or intramuscular administration.

Embodiment 53 is the pharmaceutical composition of any one of embodiments 49-51, wherein the pharmaceutical composition is formulated for local administration.

Embodiment 54 is the pharmaceutical composition of any one of embodiments 49-51, wherein the pharmaceutical composition is formulated for systemic administration.

Embodiment 55 is the pharmaceutical composition of any one of embodiments 49-54, wherein the pharmaceutical composition comprises one or more of the following features: (i) a concentration of the circular polyribonucleotide of between 0.1 ng/mL and 500 mg/mL; (ii) a concentration of deoxyribonucleotide of no more than 100 ng/mL; (iii) a concentration of protein of less than 100 ng of protein per milligram (mg) of the circular polyribonucleotide; (iv) an A260/A280 absorbance ratio of from about 1.6 to 2.3 as measured by a spectrophotometer; (v) substantially free of a process-related impurity selected from a cell protein, a cell deoxyribonucleic acid, an enzyme, a reagent component, a gel component, or a chromatographic material; and (vi) a reduced level of one or more markers of an immune or inflammatory response after purification compared to prior to purification.

Embodiment 56 is a method of treating a glucagon-like peptide-2 (GLP-2)-related disease in a subject in need thereof, the method comprising administering to the subject an effective amount of a circular polyribonucleotide of any one of embodiments 1-48 or the pharmaceutical composition of any one of embodiments 49-55.

Embodiment 57 is the method of embodiment 56, wherein the GLP-2-related disease is selected from the group consisting of: short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total

parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn's disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury.

Embodiment 58 is a method of increasing intestinal growth in a subject in need thereof, the method comprising administering to the subject an effective amount of a circular

5 polyribonucleotide of any one of embodiments 1-48 or the pharmaceutical composition of any one of embodiments 49-55.

Embodiment 59 is the method of embodiment 59, wherein increasing intestinal growth comprises increasing crypt cell growth.

10 Embodiment 60 is a method of increasing intestinal absorption of fluids and nutrients in a subject in need thereof comprising administering to the subject an effective amount of a circular polyribonucleotide of any one of embodiments 1-48 or the pharmaceutical composition of any one of embodiments 49-55.

Embodiment 61 is the method of any one of embodiments 58-60, wherein the subject has short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric
15 SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn's disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury.

Embodiment 62 is the method of any one of embodiments 56-61, wherein the circular polyribonucleotide of embodiments 1-48 or the pharmaceutical composition of any one of
20 embodiments 49-55 is administered one or more times to the subject.

Embodiment 63 is the method of any one of embodiments 56-62, wherein the circular polyribonucleotide of embodiments 1-48 or the pharmaceutical composition of any one of embodiments 49-55 is administered locally.

Embodiment 64 is the method of any one of embodiments 56-62, wherein the circular
25 polyribonucleotide of embodiments 1-48 or the pharmaceutical composition of any one of embodiments 49-55 is administered systemically.

Embodiment 65 is the method of any one of embodiments 56-64, further comprising administering one or more additional therapeutic agents to the subject.

Embodiment 66 is the method of any one of embodiments 56-65, wherein the subject is a
30 human subject.

Embodiment 67 is a kit comprising: (a) (i) a composition comprising the circular polyribonucleotide of any one of embodiments 1-48 or (ii) the pharmaceutical composition of any one of embodiments 49-55; and (b) instructions for administering the composition or the pharmaceutical composition to a subject in need thereof.

5 Embodiment 68 is a circular polyribonucleotide comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a half-life extension moiety.

 Embodiment 69 is a circular polyribonucleotide comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and
10 (ii) a half-life extension moiety, wherein the half-life extension moiety is an Fc domain comprising a M252Y/S254T/T256E (YTE) mutation.

Embodiment 70 is a modified linear RNA comprising a sequence encoding a polypeptide comprising a glucagon-like peptide-2 (GLP-2) or analog thereof.

 Embodiment 71 is a modified linear RNA comprising a sequence encoding a polypeptide
15 comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a secretion signal sequence.

Embodiment 72 is the modified linear RNA of embodiment 70 or 71, further comprising a half-life extension moiety.

WHAT IS CLAIMED IS:

1. A circular polyribonucleotide comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, (ii) a half-life extension moiety, and (iii) a secretion signal sequence.

2. The circular polyribonucleotide of claim 1, wherein the GLP-2 polypeptide or analog thereof comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

3. The circular polyribonucleotide of claim 1 or 2, wherein the GLP-2 polypeptide or analog thereof comprises an amino acid sequence of any one of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, or SEQ ID NO: 13.

4. The circular polyribonucleotide of any one of claims 1-3, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14.

5. The circular polyribonucleotide of any one of claims 1-4, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence of any one of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO: 14.

6. The circular polyribonucleotide of any one of claims 1-5, wherein the GLP-2 analog comprises teduglutide, glepaglutide, dapiglutide, apraglutide, HM15912, and SHP-681.

7. The circular polyribonucleotide of any one of claims 1-6, wherein the half-life extension moiety comprises a peptide moiety.

8. The circular polyribonucleotide of claim 7, wherein the peptide moiety comprises an Fc domain, an albumin polypeptide, a nanobody, or a post-translational modification site.

9. The circular polyribonucleotide of claim 8, wherein the Fc domain comprises an IgG1 Fc domain, an IgG2 Fc domain, an IgG3 Fc domain, or an IgG4 Fc domain.

10. The circular polyribonucleotide of claim 8 or 9, wherein the Fc domain comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identical to SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain).

11. The circular polyribonucleotide of any one of claims 8-10, wherein the Fc domain comprises an amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain).

12. The circular polyribonucleotide of claim 11, wherein the IgG4 Fc domain comprises an amino acid sequence of SEQ ID NO: 21.

13. The circular polyribonucleotide of any one of claims 8-12, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 16 (IgG1 Fc domain), SEQ ID NO: 18 (IgG2 Fc domain), SEQ ID NO: 20 (IgG3 Fc domain), or SEQ ID NO: 22 (IgG4 Fc domain).

14. The circular polyribonucleotide of claim 13, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence of SEQ ID NO: 16 (IgG1 Fc domain), SEQ ID NO: 18 (IgG2 Fc domain), SEQ ID NO: 20 (IgG3 Fc domain), or SEQ ID NO: 22 (IgG4 Fc domain).

15. The circular polyribonucleotide of any one of claims 8-14, wherein the Fc domain comprises a mutation.

16. The circular polyribonucleotide of any one of claims 8-10, 13, and 15, wherein the Fc domain comprises the amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain), except the amino acid sequence of SEQ ID NO: 15 (IgG1 Fc domain), SEQ ID NO: 17 (IgG2 Fc domain), SEQ ID NO: 19 (IgG3 Fc domain), or SEQ ID NO: 21 (IgG4 Fc domain) comprises an amino acid substitution selected from one or more of:

M252Y/S254T/T256E (YTE);

L284A/L285A;

M428L/N434S (LS);

T307A/E380A/N434A (AAA);

T250Q/M428L (QL); and

V308P.

17. The circular polyribonucleotide of claim 8, wherein the albumin polypeptide comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 23.

18. The circular polyribonucleotide of claim 8, wherein the peptide moiety comprises a nanobody that binds to albumin.

19. The circular polyribonucleotide of any one of claims 1-18, wherein the circular polyribonucleotide comprises one or more modifications.

20. The circular polyribonucleotide of claim 19, wherein the one or more modifications comprise one or more modifications to a portion of the sequence encoding the GLP-2 polypeptide or analog thereof.

21. The circular polyribonucleotide of claim 19 or claim 20, wherein the one or more modifications comprise a modification to a sugar, a nucleobase, an internucleoside linkage, or a combination thereof.

22. The circular polyribonucleotide of any one of claims 1-21, wherein the circular polyribonucleotide further comprises a translation initiation sequence operably linked to the sequence encoding the polypeptide.

23. The circular polyribonucleotide of any one of claims 1-22, wherein the circular polyribonucleotide further comprises an internal ribosome entry site (IRES) operatively linked to the sequence encoding the polypeptide.

24. The circular polyribonucleotide of any one of claims 1-23, wherein the circular polyribonucleotide further comprises a translation termination sequence.

25. The circular polyribonucleotide of any one of claims 1-24, wherein the circular polyribonucleotide lacks a translation termination sequence.

26. The circular polyribonucleotide of any one of claims 1-25, wherein the circular polyribonucleotide further comprises a stagger element at a 3' end of the sequence encoding the polypeptide.

27. The circular polyribonucleotide of claim 26, wherein the stagger element is configured to stall a ribosome during rolling circle translation.

28. The circular polyribonucleotide of any one of claims 1-27, wherein the circular polyribonucleotide further comprises a replication element.

29. The circular polyribonucleotide of any one of claims 1-28, wherein the circular polyribonucleotide further comprises a 5' untranslated region operably linked to the sequence encoding the polypeptide.

30. The circular polyribonucleotide of any one of claims 1-29, wherein the circular polyribonucleotide does not comprise a poly(A) sequence operably linked to the sequence encoding the polypeptide.

31. The circular polyribonucleotide of any one of claims 1-30, wherein the circular polyribonucleotide does not comprise a 5' untranslated region operably linked to the sequence encoding the polypeptide.

32. The circular polyribonucleotide of any one of claims 1-31, wherein the secretion signal sequence is a liver-specific secretion signal sequence, wherein the liver-specific secretion signal comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to any one of SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO: 79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, or SEQ ID NO: 139.

33. The circular polyribonucleotide of any one of claims 1-31, wherein the secretion signal sequence comprises an IL-2 secretion signal sequence, a proglucagon secretion signal sequence, a Gaussia luciferase secretion signal sequence, or an erythropoietin (EPO) secretion signal sequence.

34. The circular polyribonucleotide of claim 33, wherein the IL-2 secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 29.

35. The circular polyribonucleotide of claim 33, wherein the proglucagon secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 31.

36. The circular polyribonucleotide of claim 33, wherein the Gaussia luciferase secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 33.

37. The circular polyribonucleotide of claim 33, wherein the EPO secretion signal sequence comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 35.

38. The circular polyribonucleotide of claim 33, wherein the sequence encoding the polypeptide comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 99%, or 100% identical to one of SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, or SEQ ID NO: 39.

39. The circular polyribonucleotide of any one of claims 1-38, wherein the circular polyribonucleotide further comprises at least one spacer sequence.

40. The circular polyribonucleotide of claim 39, wherein the polypeptide comprises a protease cleavage site and the at least one spacer sequence encodes the protease cleavage site.

41. The circular polyribonucleotide of claim 40, wherein the protease cleavage site is positioned between (a) the secretion signal and (b) the GLP-2 polypeptide or analog thereof and the half-life extension moiety.

42. The circular polyribonucleotide of any one of claims 1-41, wherein the polypeptide further comprises at least one linker sequence.

43. The circular polyribonucleotide of claim 42, wherein the at least one linker sequence encodes an amino acid sequence selected from (GS)_x, (GGS)_x, (GGGS)_x (SEQ ID NO: 141), (GGSG)_x (SEQ ID NO: 142), and (SGGG)_x (SEQ ID NO: 143), wherein x is an integer from 1 to 50.

44. The circular polyribonucleotide of claim 42 or 43, wherein the at least one linker sequence is disposed between the GLP-2 polypeptide or analog thereof and the half-life extension moiety.

45. The circular polyribonucleotide of any one of claims 1-44, wherein the circular polyribonucleotide comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1.

46. The circular polyribonucleotide of any one of claims 1-45, wherein the circular polyribonucleotide comprises an amino acid sequence of SEQ ID NO: 1.

47. The circular polyribonucleotide of any one of claims 1-46, wherein the circular polyribonucleotide comprises a nucleic acid sequence that is at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 2.

48. The circular polyribonucleotide of any one of claims 1-47, wherein the circular polyribonucleotide comprises a nucleic acid sequence of SEQ ID NO: 2.

49. A pharmaceutical composition comprising the circular polyribonucleotide of any one of claims 1-48, and a pharmaceutically acceptable excipient.

50. The pharmaceutical composition of claim 49, wherein the circular polyribonucleotide is formulated as a lipid nanoparticle.

51. The pharmaceutical composition of claim 49, wherein the circular polyribonucleotide is formulated for unencapsulated delivery or natural particle delivery.

52. The pharmaceutical composition of any one of claims 49-51, wherein the pharmaceutical composition is formulated for intravenous, subcutaneous, intrahepatic, or intramuscular administration.

53. The pharmaceutical composition of any one of claims 49-51, wherein the pharmaceutical composition is formulated for local administration.

54. The pharmaceutical composition of any one of claims 49-51, wherein the pharmaceutical composition is formulated for systemic administration.

55. The pharmaceutical composition of any one of claims 49-54, wherein the pharmaceutical composition comprises one or more of the following features:

- (i) a concentration of the circular polyribonucleotide of between 0.1 ng/mL and 500 mg/mL;
- (ii) a concentration of deoxyribonucleotide of no more than 100 ng/mL;
- (iii) a concentration of protein of less than 100 ng of protein per milligram (mg) of the circular polyribonucleotide;
- (iv) an A260/A280 absorbance ratio of from about 1.6 to 2.3 as measured by a spectrophotometer;
- (v) substantially free of a process-related impurity selected from a cell protein, a cell deoxyribonucleic acid, an enzyme, a reagent component, a gel component, or a chromatographic material; and
- (vi) a reduced level of one or more markers of an immune or inflammatory response after purification compared to prior to purification.

56. A method of treating a glucagon-like peptide-2 (GLP-2)-related disease in a subject in need thereof, the method comprising administering to the subject an effective amount of a circular polyribonucleotide of any one of claims 1-48 or the pharmaceutical composition of any one of claims 49-55.

57. The method of claim 56, wherein the GLP-2-related disease is selected from the group consisting of: short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn's disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury.

58. A method of increasing intestinal growth in a subject in need thereof, the method comprising administering to the subject an effective amount of a circular polyribonucleotide of any one of claims 1-48 or the pharmaceutical composition of any one of claims 49-55.

59. The method of claim 58, wherein increasing intestinal growth comprises increasing crypt cell growth.

60. A method of increasing intestinal absorption of fluids and nutrients in a subject in need thereof comprising administering to the subject an effective amount of a circular polyribonucleotide of any one of claims 1-48 or the pharmaceutical composition of any one of claims 49-55.

61. The method of any one of claims 58-60, wherein the subject has short bowel syndrome (SBS), short bowel syndrome with intestinal failure (SBS-IF), pediatric SBS, adult SBS, major small bowel resection, total parenteral nutrition (TPN)-induced intestinal hypoplasia, small and large intestinal inflammation, Crohn's disease, chemotherapy-induced mucositis, and intestinal ischemia–reperfusion injury.

62. The method of any one of claims 56-61, wherein the circular polyribonucleotide of claims 1-48 or the pharmaceutical composition of any one of claims 49-55 is administered one or more times to the subject.

63. The method of any one of claims 56-62, wherein the circular polyribonucleotide of claims 1-48 or the pharmaceutical composition of any one of claims 49-55 is administered locally.

64. The method of any one of claims 56-62, wherein the circular polyribonucleotide of claims 1-48 or the pharmaceutical composition of any one of claims 49-55 is administered systemically.

65. The method of any one of claims 56-64, further comprising administering one or more additional therapeutic agents to the subject.

66. The method of any one of claims 56-65, wherein the subject is a human subject.

67. A kit comprising:

(a) (i) a composition comprising the circular polyribonucleotide of any one of claims 1-48 or (ii) the pharmaceutical composition of any one of claims 49-55; and

(b) instructions for administering the composition or the pharmaceutical composition to a subject in need thereof.

68. A circular polyribonucleotide comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a half-life extension moiety.

69. A circular polyribonucleotide comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a half-life extension moiety, wherein the half-life extension moiety is an Fc domain comprising a M252Y/S254T/T256E (YTE) mutation.

70. A modified linear RNA comprising a sequence encoding a polypeptide comprising a glucagon-like peptide-2 (GLP-2) or analog thereof.

71. A modified linear RNA comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, and (ii) a secretion signal sequence.

72. The modified linear RNA of claim 70 or 71, further comprising a half-life extension moiety.

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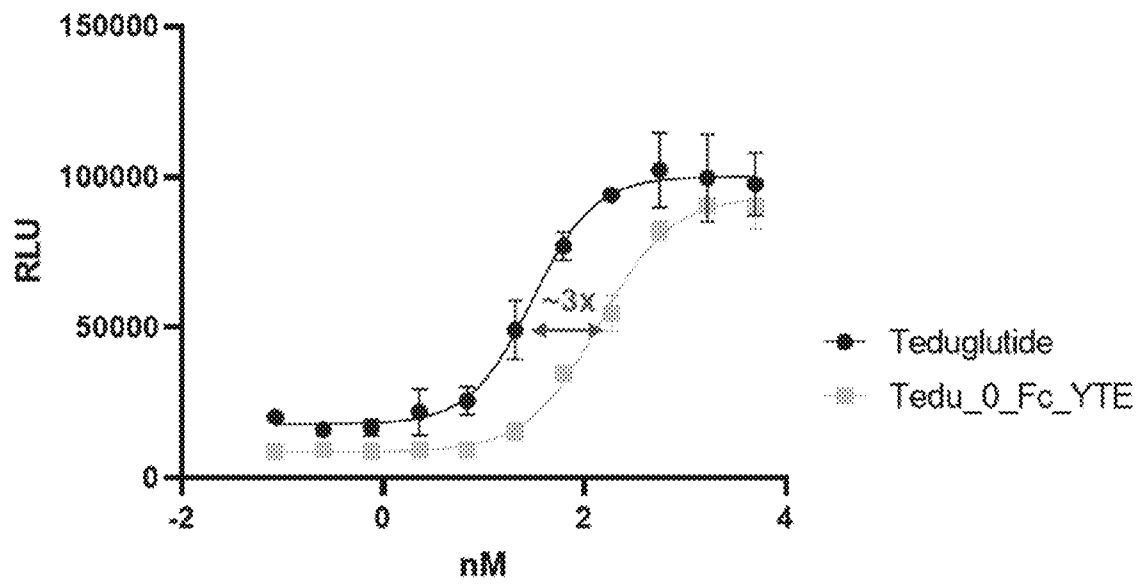
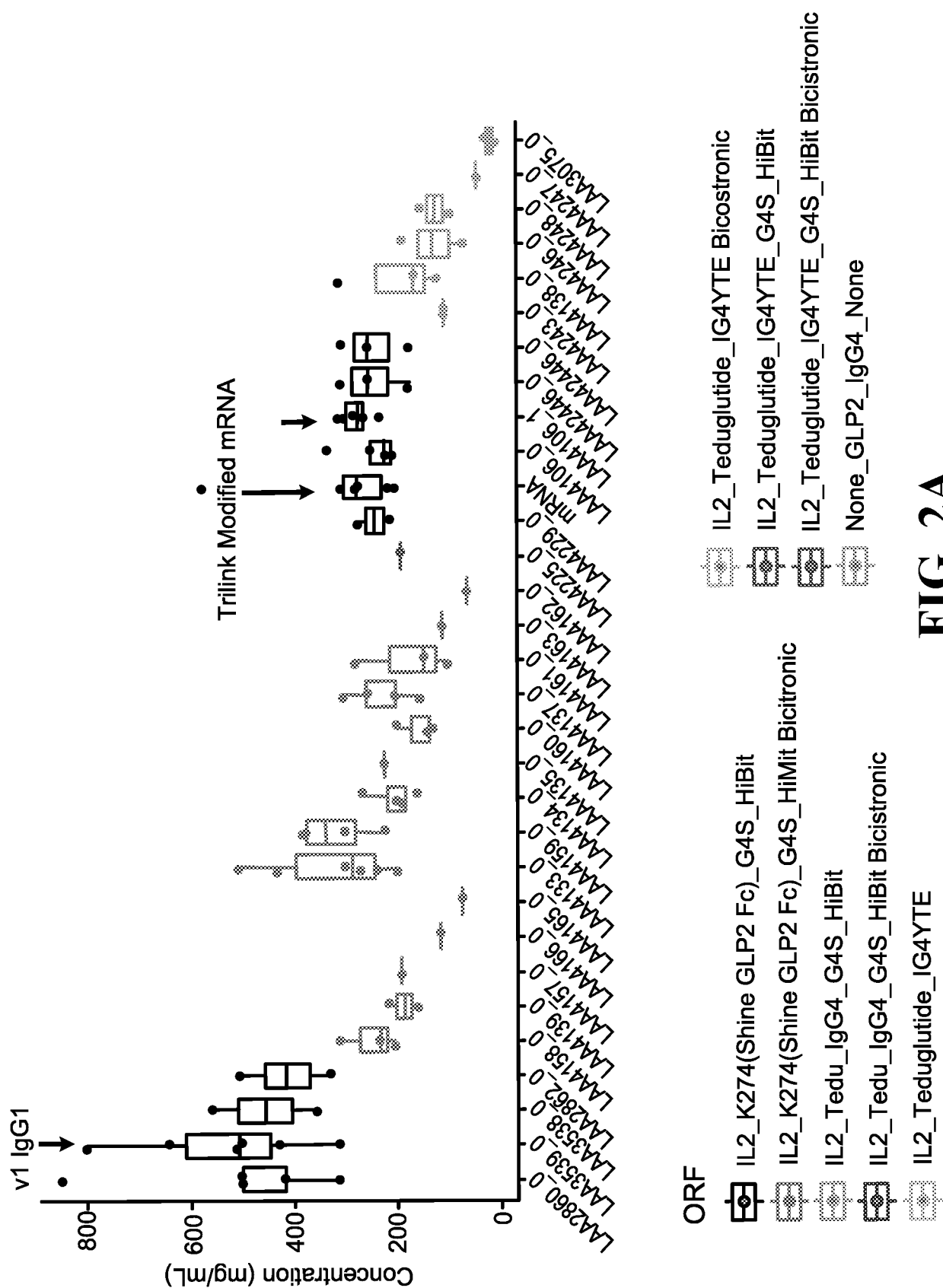


FIG. 1



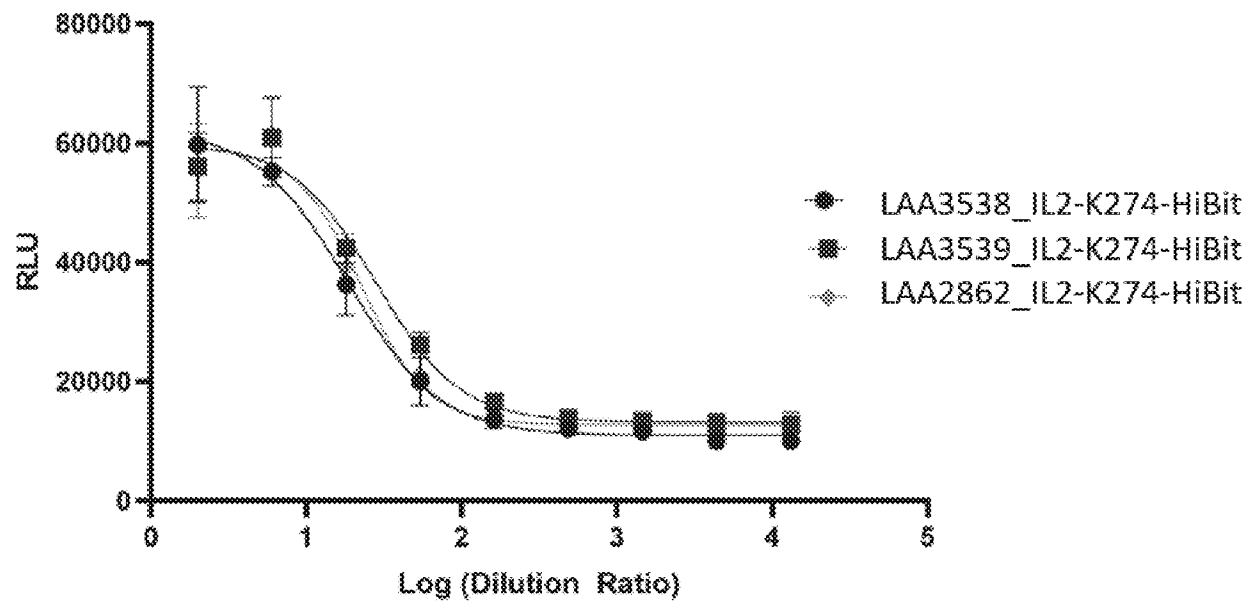


FIG. 2B

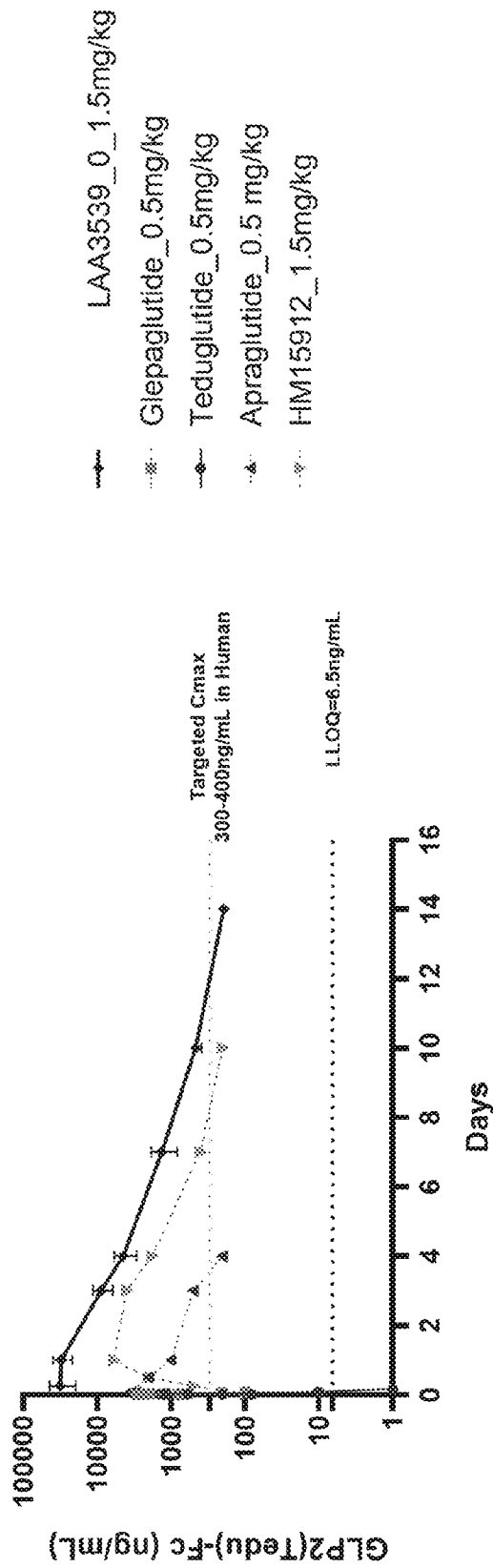


FIG. 3

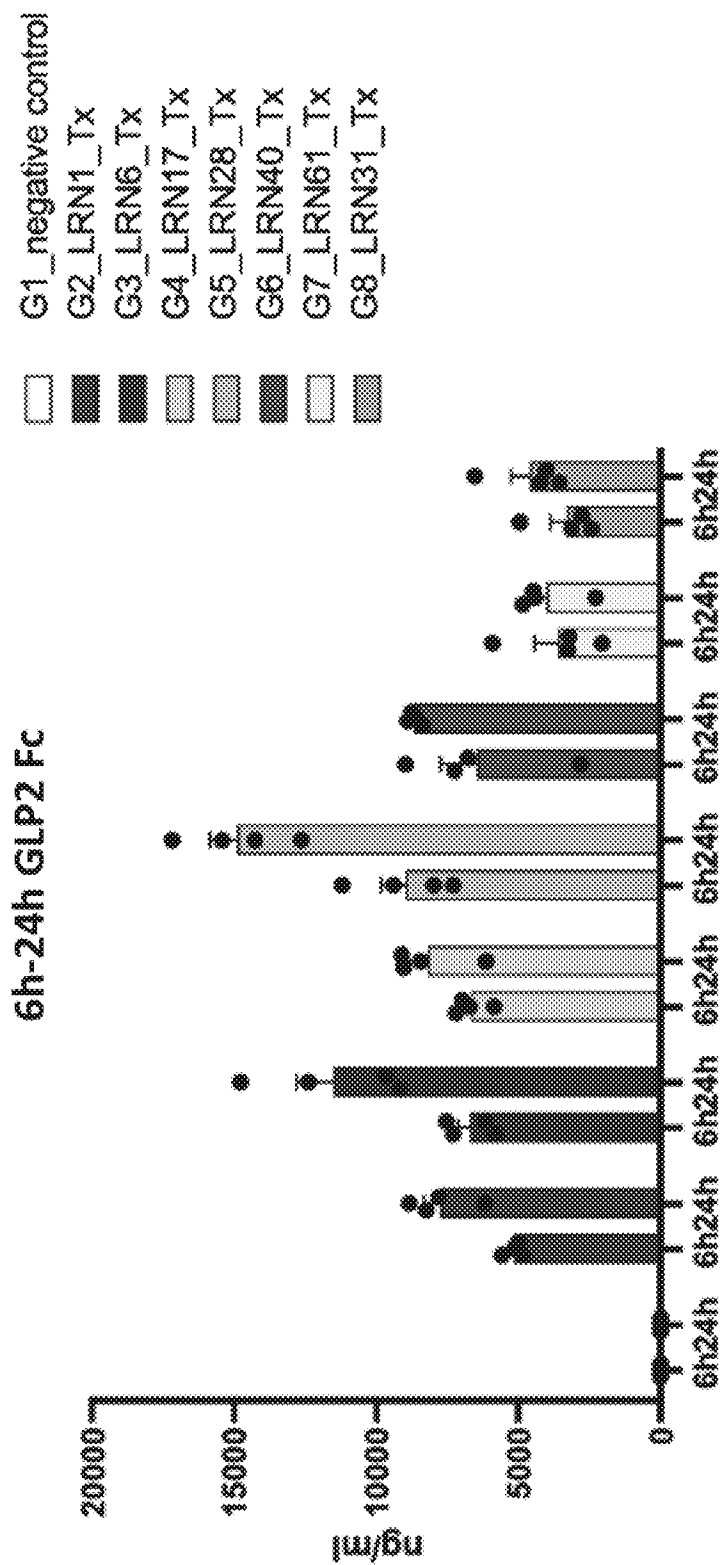


FIG. 4A

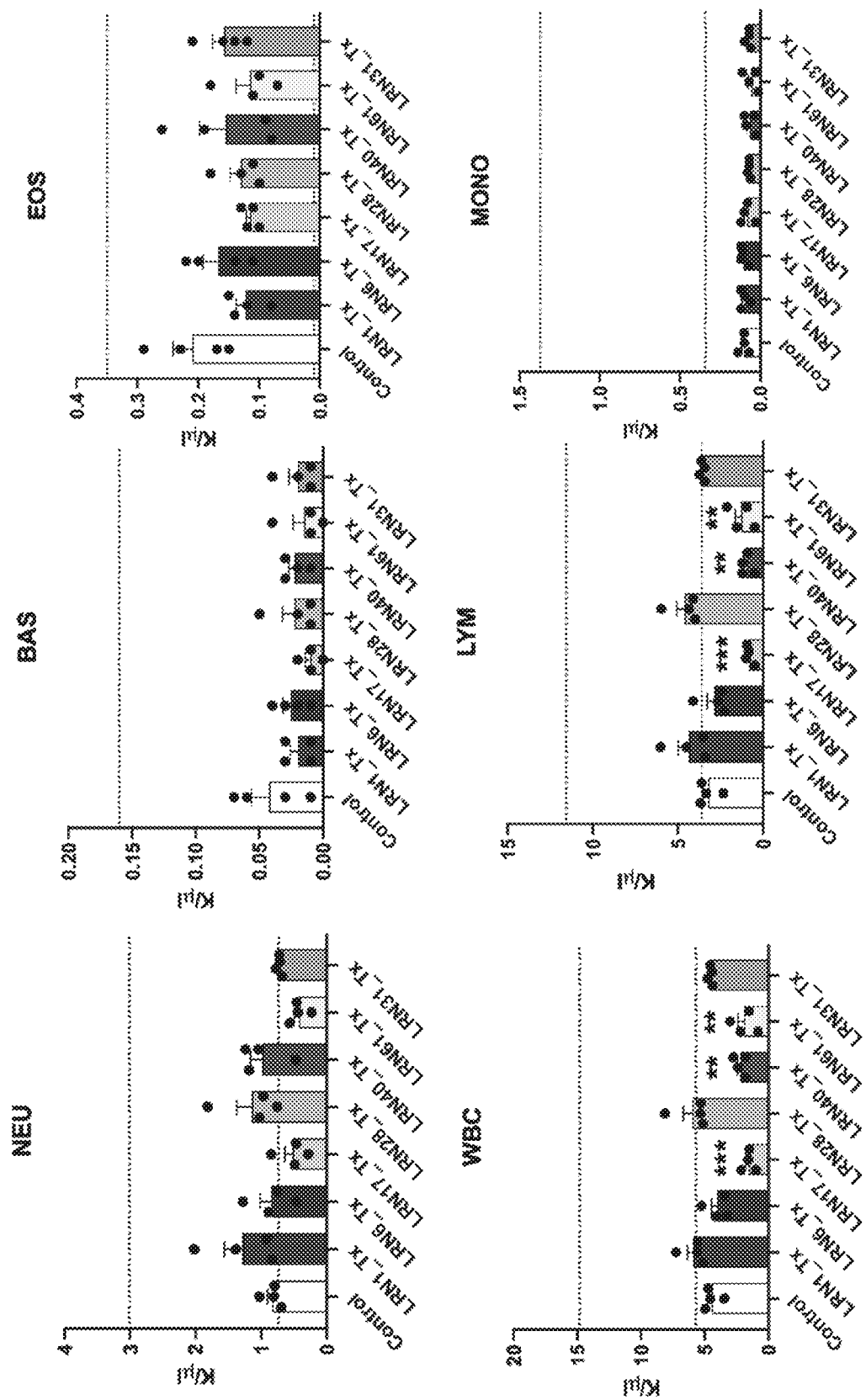


FIG. 4B

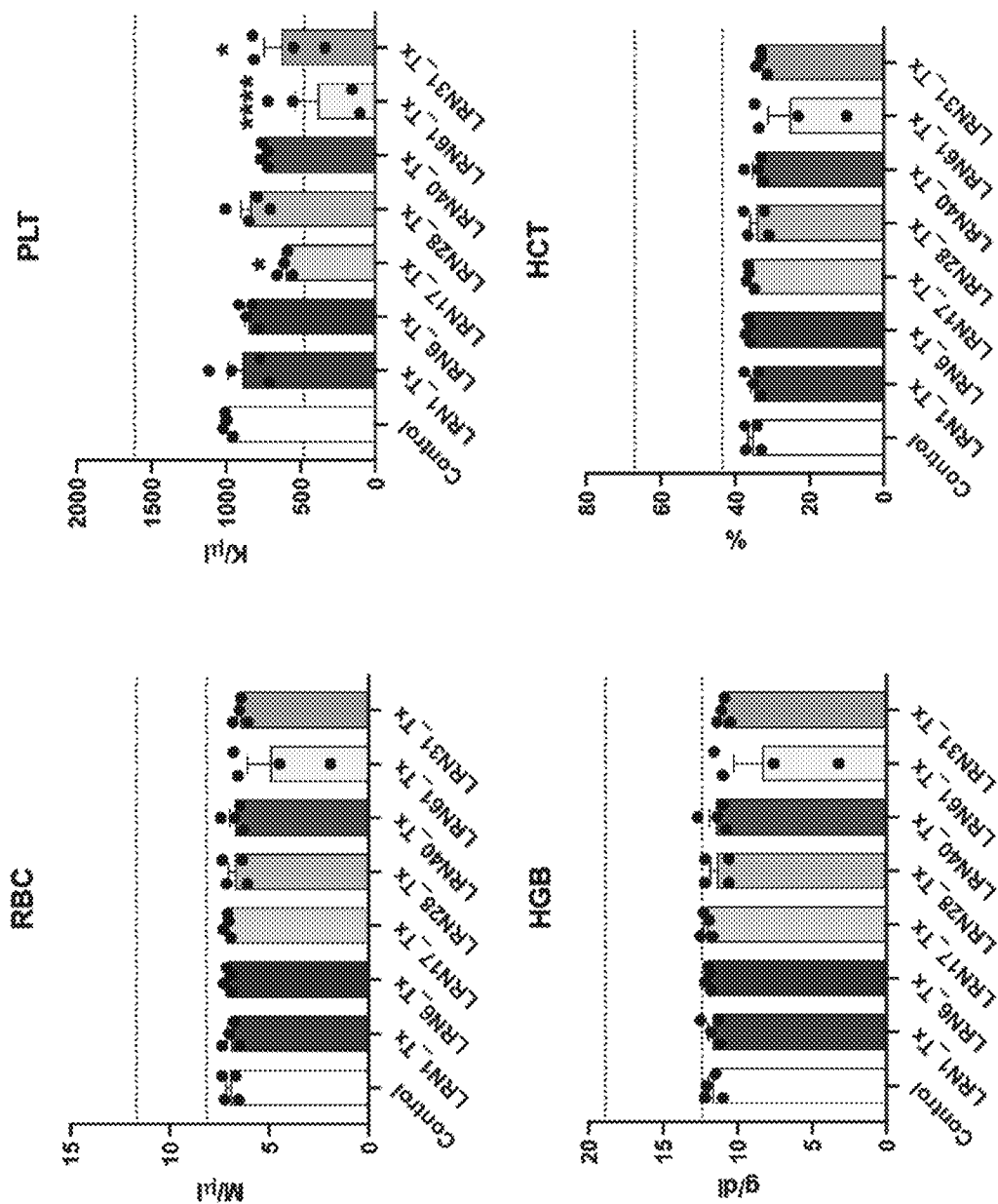


FIG. 4C

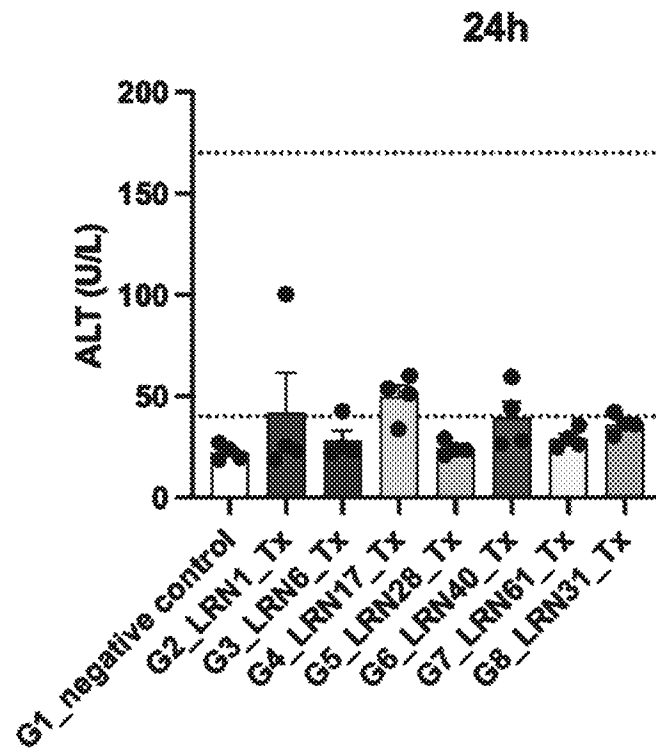


FIG. 4D

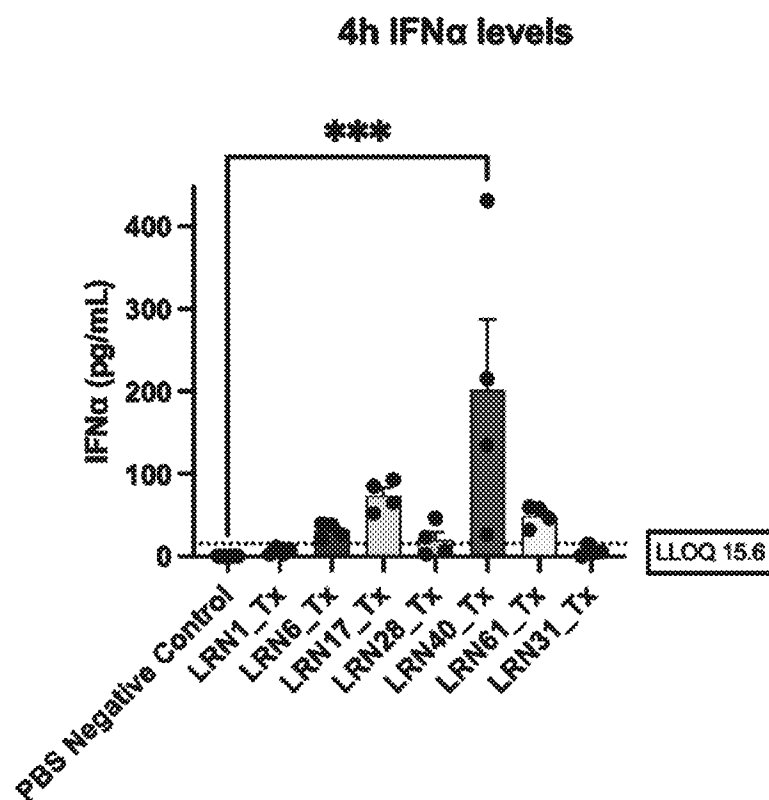


FIG. 4E

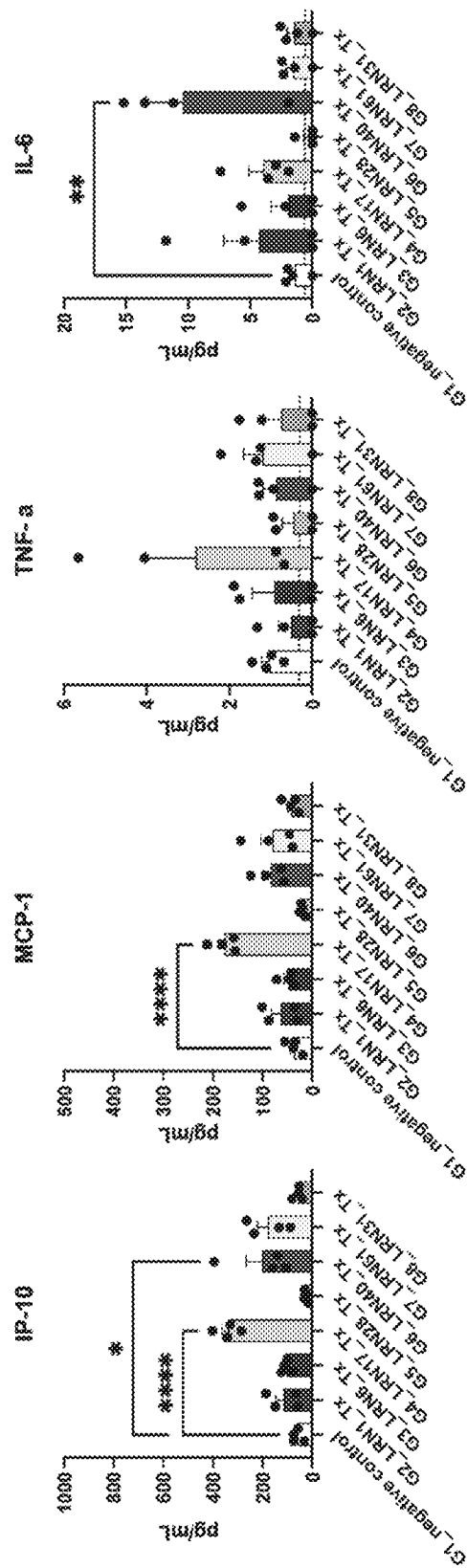


FIG. 4F

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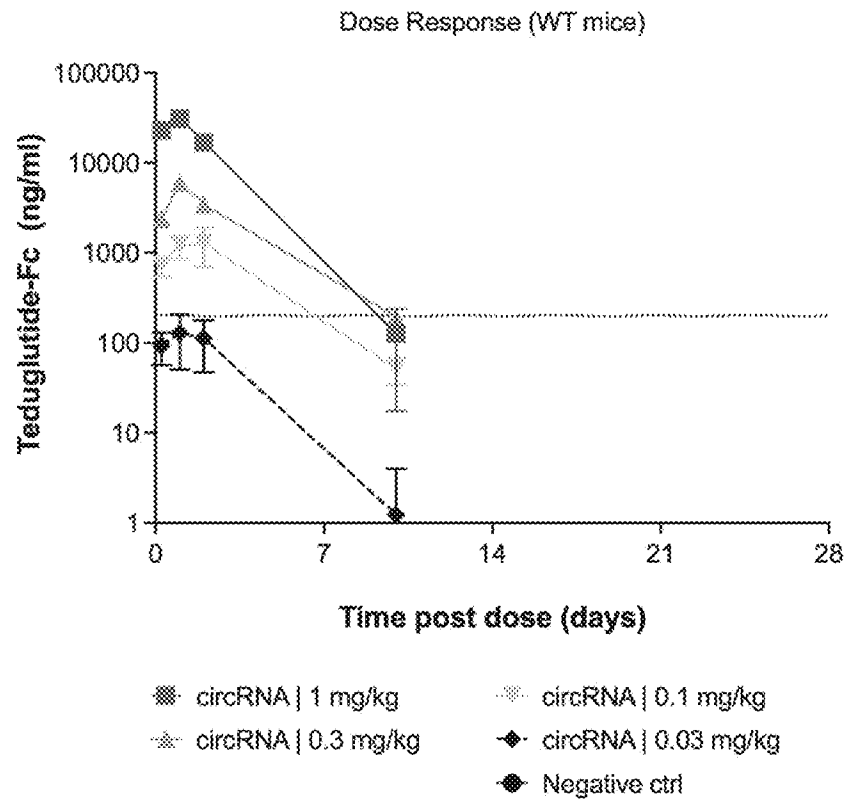


FIG. 5A

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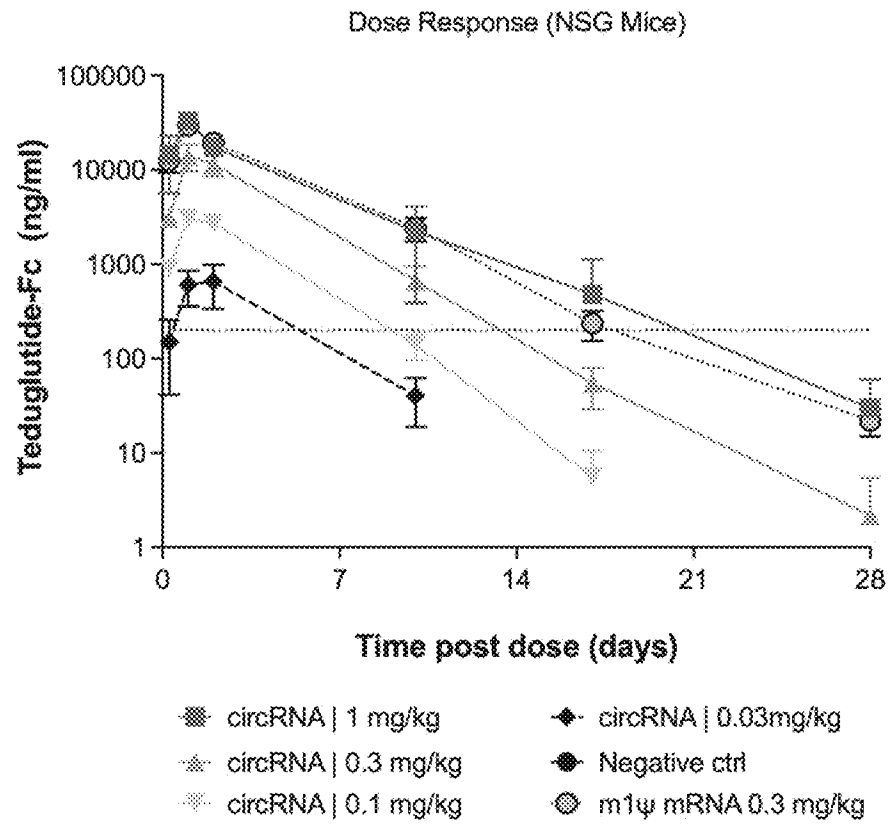


FIG. 5B

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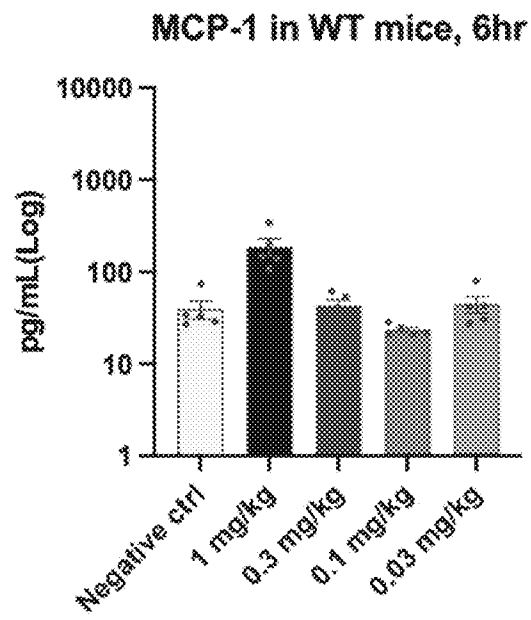


FIG. 5C

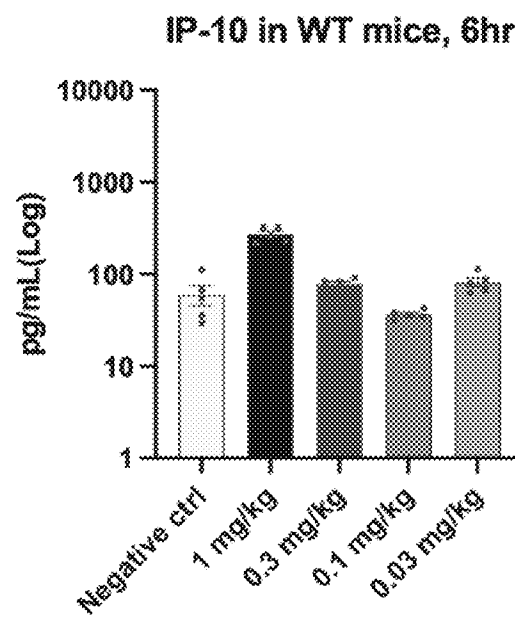


FIG. 5D

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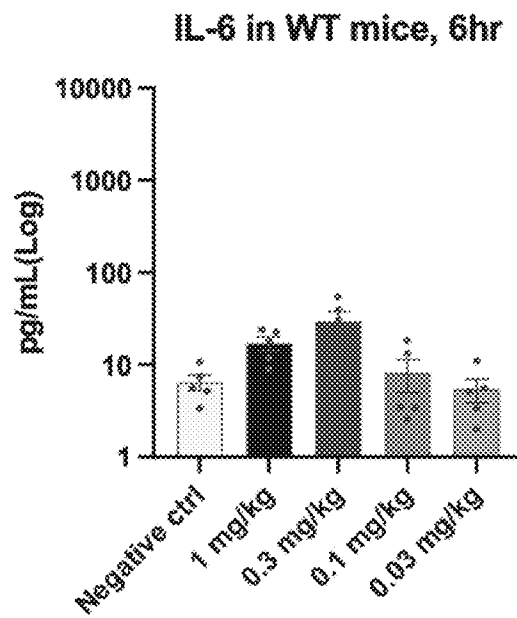


FIG. 5E

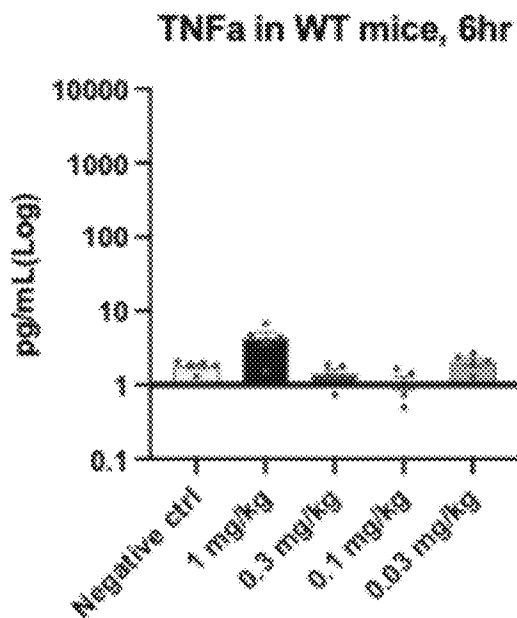


FIG. 5F

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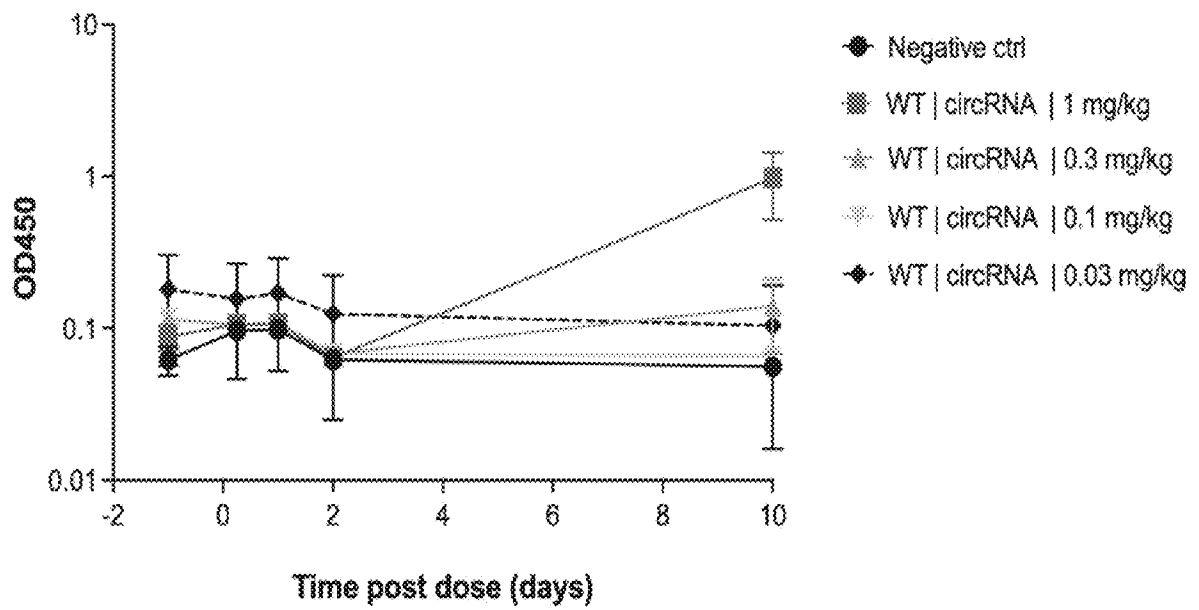


FIG. 5G

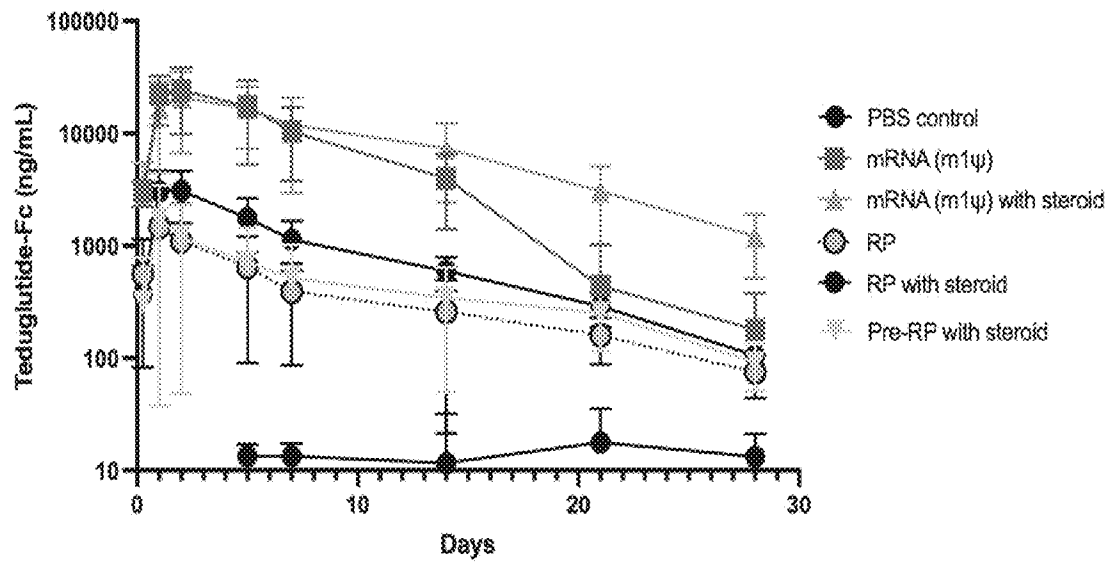
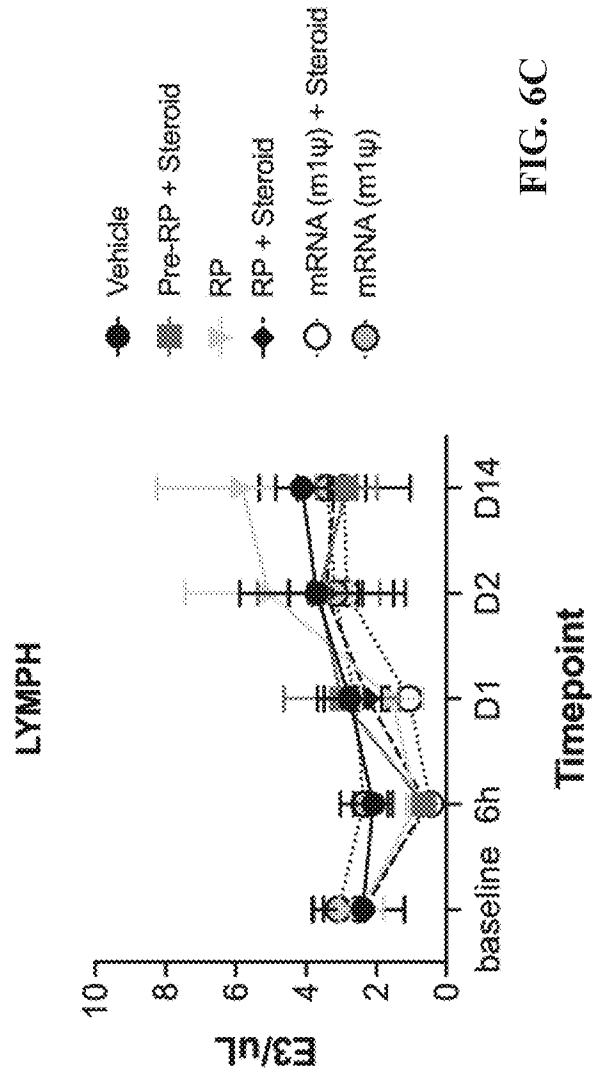
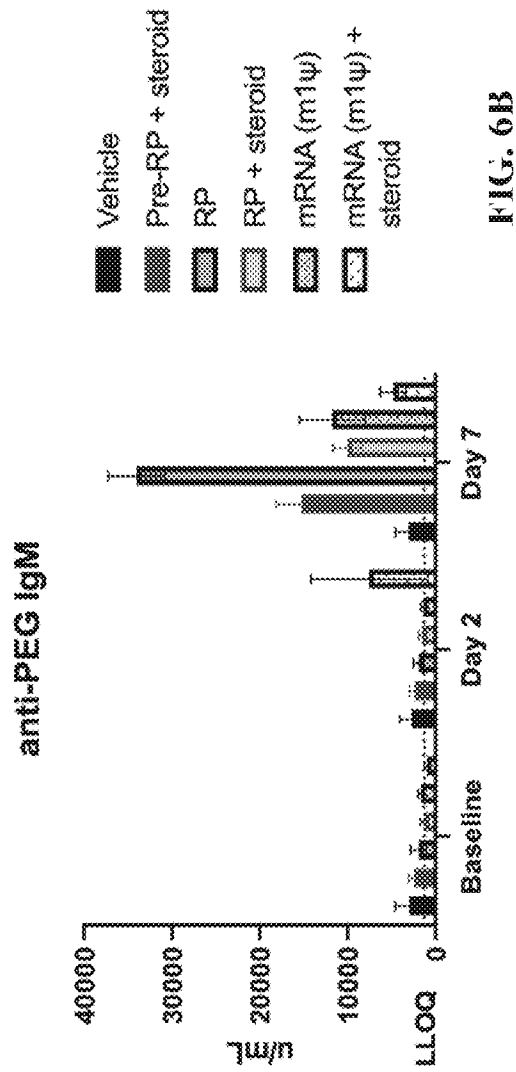


FIG. 6A



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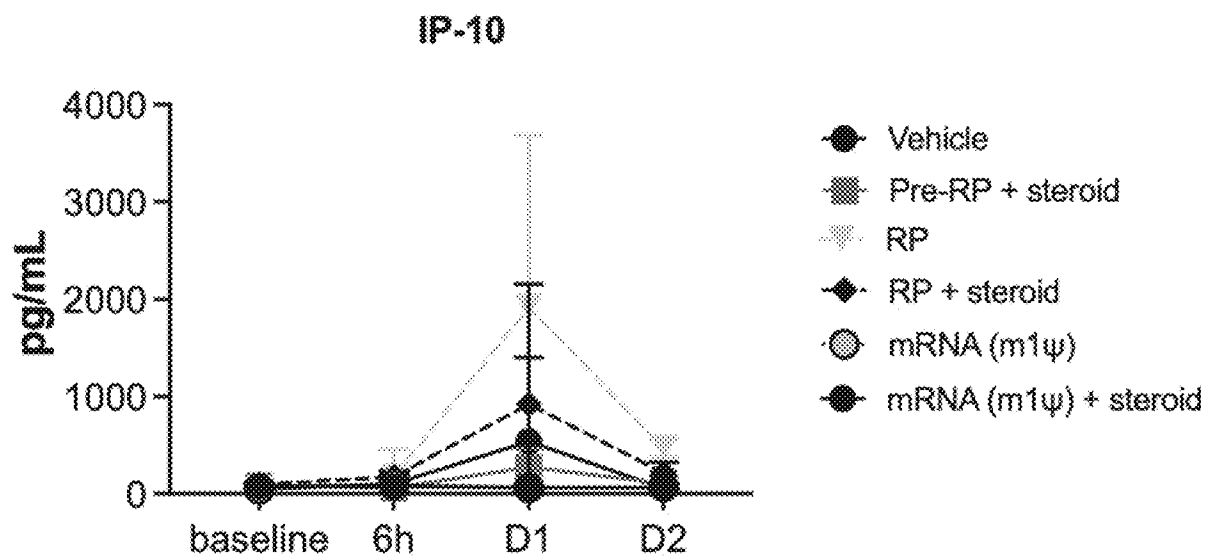
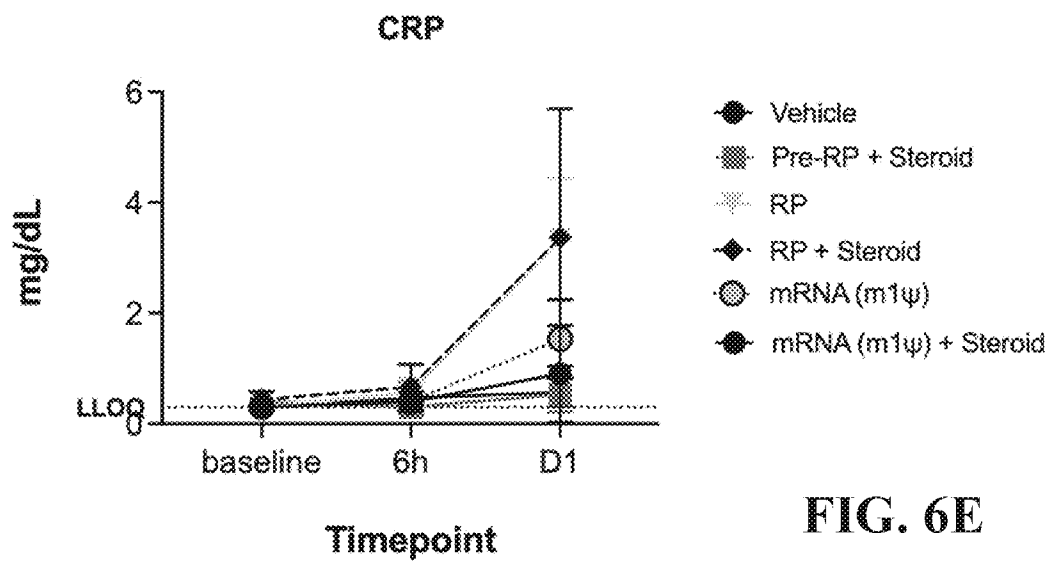
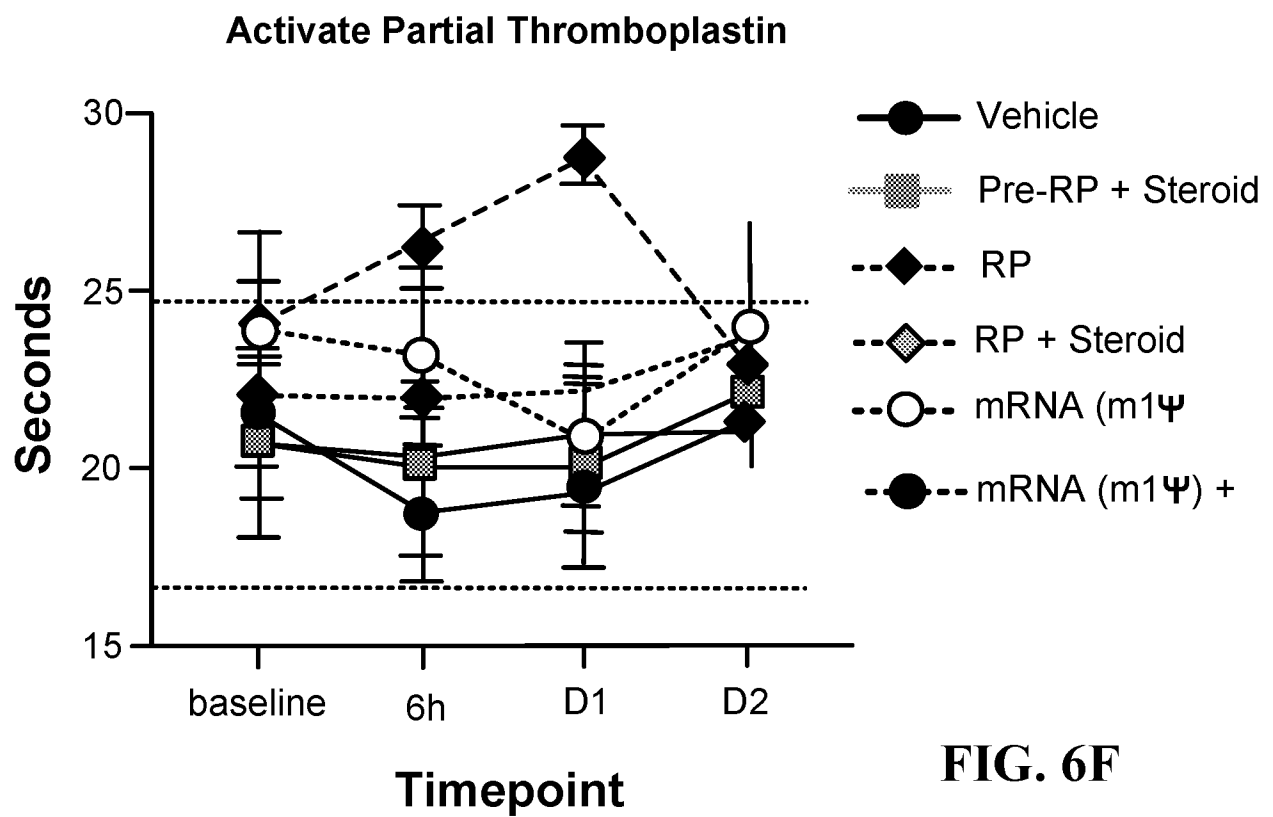


FIG. 6D

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**FIG. 6E****FIG. 6F**

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/056294

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/605

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2023/168405 A2 (UNIV PENNSYLVANIA [US]) 7 September 2023 (2023-09-07) claim 1; sequence 13 -----	1 - 69
Y	R. ALEXANDER WESSELHOEFT ET AL: "Engineering circular RNA for potent and stable translation in eukaryotic cells", NATURE COMMUNICATIONS, vol. 9, no. 1, 6 July 2018 (2018-07-06), XP055622096, DOI: 10.1038/s41467-018-05096-6 the whole document -----	1 - 69
A	WO 2023/122789 A1 (FLAGSHIP PIONEERING INNOVATIONS VI LLC [US]) 29 June 2023 (2023-06-29) the whole document ----- - / - -	1 - 69



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

4 February 2025

Date of mailing of the international search report

04/04/2025

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2

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Fax: (+31-70) 340-3016

Authorized officer

Luo, Xinmei

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2024/056294

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHOI JAEHYUK ET AL: "HM15912, a Novel Long-Acting Glucagon-Like Peptide-2 Analog, Improves Intestinal Growth and Absorption Capacity in a Male Rat Model of Short Bowel Syndrome", THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, vol. 384, no. 2, 1 February 2023 (2023-02-01), pages 277-286, XP093244029, ISSN: 0022-3565, DOI: 10.1124/jpet.122.001381 cited in the application the whole document -----	1 - 69
A	HARGROVE DIANE M. ET AL: "Pharmacological Characterization of Apraglutide, a Novel Long-Acting Peptidic Glucagon-Like Peptide-2 Agonist, for the Treatment of Short Bowel Syndrome", THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, vol. 373, no. 2, 13 April 2020 (2020-04-13), pages 193-203, XP055813396, ISSN: 0022-3565, DOI: 10.1124/jpet.119.262238 cited in the application the whole document -----	1 - 69
A	US 2020/199192 A1 (PAN CLARK [US] ET AL) 25 June 2020 (2020-06-25) paragraph [0186] - paragraph [0187] -----	1 - 69

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/056294

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed.
 - b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/056294

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

1 - 69

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2024/056294

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2023168405 A2	07-09-2023	AU 2023227587 A1	19-09-2024
		CN 119522285 A	25-02-2025
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		EP 4452337 A1	30-10-2024
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		KR 20240118881 A	05-08-2024
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		BR 112020003736 A2	08-09-2020
		CA 3071966 A1	28-02-2019
		CN 111182916 A	19-05-2020
		EP 3672621 A1	01-07-2020
		JP 7249492 B2	31-03-2023
		JP 2020531030 A	05-11-2020
		TW 201920242 A	01-06-2019
		US 2020199192 A1	25-06-2020
		US 2023031280 A1	02-02-2023
		WO 2019040399 A1	28-02-2019
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-69

A circular polyribonucleotide comprising a sequence encoding a polypeptide comprising (i) a glucagon-like peptide-2 (GLP-2) polypeptide or analog thereof, (ii) a half-life extension moiety;

2. claims: 70-72

A modified linear RNA comprising a sequence encoding a polypeptide comprising a glucagon-like peptide-2 (GLP-2) or analog thereof.
