

**[0006]** Recently, there has been an interest in using recombinant circRNAs to express a protein of interest, *in vitro* or *in vivo*. Introduction of an internal ribosome entry sequence (IRES) into a circular RNA allows translation of a protein encoded by a circRNA. However, IRES elements that exist in nature may or may not support translation from engineered circular RNAs, as IRES elements are often evolved in the context of linear RNA genomes.

**[0007]** Accordingly, there is in the need in the art to identify IRES elements that can drive protein translation from recombinant circRNAs. Further, there is a need for engineered IRES elements that improve the amount and/or duration of protein expression from a circRNA.

## **BRIEF SUMMARY**

**[0008]** Described herein are circular RNA molecules comprising an internal ribosome entry sequence (IRES) operably linked to a protein-coding sequence.

**[0009]** In one aspect, the present disclosure refers to a circular RNA molecule comprising an internal ribosome entry site (IRES) sequence operably linked to a protein-coding sequence; wherein the IRES sequence is a modified IRES, and wherein at least 1% of the nucleotides of the circular RNA molecule are 2-thiouridine (2ThioU) or 2'-O-Methylcytidine (2'OMeC). For example, in some examples, a circular RNA molecule comprises an internal ribosome entry site (IRES) sequence operably linked to a protein-coding sequence; wherein the IRES sequence is a viral sequence; and wherein the protein-coding sequence encodes a non-viral protein. In some examples, the molecule comprises a spacer upstream of said IRES.

**[0010]** In some examples, the non-viral protein is a mammalian protein. In some examples, the non-viral protein is a human protein.

**[0011]** In some examples, the IRES is a Type 1 IRES. In some embodiments, the IRES is an enterovirus IRES. In some examples, the IRES is a human rhinovirus (HRV) IRES.

**[0012]** In some examples, the IRES is any one of the IRES listed in Table 7. In some examples, the IRES is any one of the following IRES: iEMCV, iHCV, iCVB5, iSwineVesicular, iHRV-A2, iHRV-C3, iHRV-C11, iCVB1, iPV2, iHRV-B17, iEchoV-E15, iEV71, iHRV-A9, iSimianV4, iEV-D94, iSimianA5, iPV3, iHRV-C54, iHRV-A100, iHRV-B37, iHRV-B4, iHRV-B92, iHRV-B3, iHRV-A1, iEV107, or a fragment or derivative thereof. In some examples, the IRES is any one of the following IRES: iEV-B83, iHRV-A57, iHRV-B35, iHRV-B4, iEV-D68, iHRVB\_R93, iHRV-B5, iHRVB-B52, iHRVB-B93, iHRV-B84, iHRV-B83\_SC2220, iHRV-B72, iHRV-B69, iHRVB\_SC0739, iHRV-B91, iHRV-B42, iHRV-B6, iHRV-B83, iHRV-B48, iHRV-B99, iHRV-B79, iHRV-B97, iHRV-B27, iHRVB\_3039, iHRVB-B14, iCosV-B1, or a fragment or derivative thereof. In some examples, the IRES is

iCVB3, or a fragment or derivative thereof. In some examples, the IRES is iHRV-B3, or a fragment or derivative thereof.

**[0013]** Also described herein is a circular RNA molecule comprising a synthetic internal ribosome entry site (IRES) sequence operably linked to a protein-coding sequence. In some examples, the IRES is upstream of the protein-coding sequence. In some examples, the synthetic IRES sequence comprises an aptamer. In some examples, synthetic IRES sequence comprises an aptamer and a second aptamer.

**[0014]** In some examples, the aptamer is a wildtype aptamer. In some examples, the aptamer is an aptamer was designed and/or evolved to bind one or more DNA sequences. In some examples, the aptamer is a mutant aptamer. In some examples, the aptamer is modified to have an extended stem region.

**[0015]** In some examples, the aptamer is positioned within the secondary structure of the IRES so that is spatially proximal to portion of the IRES responsible for translation initiation. In some examples, the aptamer does not interrupt the native eIF4G binding site of the IRES and does not interrupt a native GRNA tetraloop within the IRES.

**[0016]** In some examples, the aptamer is an eIF4G-binding aptamer. In some examples, the eIF4G-binding aptamer comprises or is encoded by the sequence of SEQ ID NO: 99. In some examples, the IRES is a Type 1 IRES. In some examples, the IRES is a modified enterovirus IRES. In some examples, the IRES is a modified human rhinovirus (HRV) IRES. In some examples, the IRES comprises or is encoded by the sequence of any one of SEQ ID NO: 125-129.

**[0017]** In some examples, synthetic IRES sequence is a modified iCVB3 IRES. In some examples, modified iCVB3 IRES comprises an aptamer inserted in domain I, II, III, IV, V, VI or VII thereof. In some examples, the modified iCVB3 IRES comprises an aptamer inserted in domain IV thereof. In some examples, the modified iCVB3 aptamer is modified to have an extended stem region. In some examples, the modified iCVB3 aptamer is positioned within the secondary structure of the IRES so that is spatially proximal to portion of the IRES responsible for translation initiation. In some examples, the modified iCVB3 aptamer does not interrupt the native eIF4G binding site of the IRES and does not interrupt a native GRNA tetraloop within the IRES.

**[0018]** In some examples, the synthetic IRES sequence is a modified iHRV-B3 IRES. In some examples, the modified iHRV-B3 IRES comprises an aptamer inserted in domain I, II, III, IV, V, or VI thereof. In some examples, the modified iHRV-B3 IRES comprises an aptamer inserted in domain IV thereof. In some examples, the modified iHRV-B3 IRES aptamer is modified to have an extended stem region. In some examples, the modified iHRV-B3 IRES aptamer is positioned within the secondary structure of the IRES so that is spatially proximal to portion of the IRES responsible for translation initiation. In some examples, the modified iHRV-B3 aptamer does not interrupt the native eIF4G binding site of the IRES and does not interrupt a native GRNA tetraloop within the IRES.

**[0019]** In some examples, the circular RNA comprises a least one 2-thiouridine (2ThioU) or at least one 2'-O-methylcitidine (2OMeC). In some examples, the circular RNA molecule comprises about 2% to about 5% 2-thiouridine (e.g., about 2.5% 2-thiouridine). In some examples, the circular RNA molecule comprises about 2% to about 5% 2'-O-methylcitidine (e.g., about 2.5% 2'-O-methylcitidine).

**[0020]** In another aspect, the present disclosure refers to a nucleic acid that encodes one or more of the circular RNA molecules described herein.

**[0021]** In yet another aspect, the present disclosure refers to a composition comprising one or more of the circular RNA molecules and/or the nucleic acids described herein.

**[0022]** In one aspect, the present disclosure refers to host cells comprising one or more of the circular RNA molecules and/or the nucleic acids described herein.

**[0023]** In a further aspect, the present disclosure refers to a method of producing a protein in a cell, the method comprising contacting a cell with the circular RNA molecule as described herein or the nucleic acid as described herein under conditions whereby the protein-coding nucleic acid sequence of the circular RNA is translated and the protein is produced in the cell. Also described are methods for producing a protein in a cell, the method comprising contacting a cell with a circular RNA molecule or a nucleic acid described herein under conditions whereby the protein-coding nucleic acid sequence of the circular RNA is translated and the protein is produced in the cell.

**[0024]** Also described are methods for producing a protein *in vitro*, the method comprising contacting a cell-free extract with a circular RNA molecule or a nucleic acid under conditions whereby the protein-coding nucleic acid sequence of the circular RNA is translated and the protein is produced.

**[0025]** These and other embodiments will be described in further detail below, and in the appended drawings.

## CLAIMS

What is claimed is:

1. A circular RNA molecule comprising an internal ribosome entry site (IRES) sequence operably linked to a protein-coding sequence; wherein the IRES sequence is a modified IRES, and wherein at least 1% of the nucleotides of the circular RNA molecule are 2-thiouridine (2ThioU) or 2'-O-Methylcytidine (2'OMeC).
2. The circular RNA molecule of claim 1, wherein 1% to 10% of the nucleotides of the circular RNA molecule are 2-thiouridine or 2'-O-methylcytidine.
3. The circular RNA molecule of claim 1, wherein the modified IRES comprises at least one modified domain selected from any one of domains I-VII.
4. The circular RNA molecule of claim 3, wherein the at least one modified domain is domain I.
5. The circular RNA molecule of claim 3, wherein the at least one modified domain is domain IV.
6. The circular RNA molecule of any one of claims 1-3, wherein the modified IRES comprises at least one aptamer.
7. The circular RNA molecule of claim 6, wherein the aptamer is a wildtype aptamer.
8. The circular RNA molecule of claim 6, wherein the aptamer is a mutant aptamer.
9. The circular RNA molecule of claim 8, wherein the aptamer is modified to have an extended stem region.
10. The circular RNA molecule of any one of claims 6-9, wherein the aptamer does not interrupt the native eIF4G binding site of the IRES.

11. The circular RNA molecule of any one of claims 6-10, wherein the aptamer does not interrupt a native GRNA tetraloop within the IRES.
12. The circular RNA molecule of any one of claims 6-11, wherein the aptamer is an eIF4G-binding aptamer.
13. The circular RNA molecule of claim 12, wherein the eIF4G-binding aptamer is encoded by the sequence of SEQ ID NO: 99.
14. The circular RNA of any one of claims 1-13, wherein the IRES is a Type 1 IRES.
15. The circular RNA of any one of claims 1-14, wherein the IRES is a modified enterovirus IRES or a modified rhinovirus (HRV) IRES.
16. The circular RNA molecule of claim 3, wherein the modified IRES comprises an aptamer inserted in domain I, II, III, IV, V, VI or VII.
17. The circular RNA molecule of claim 16, wherein the modified IRES comprises an aptamer inserted in domain IV.
18. The circular RNA molecule of claim 17, wherein the aptamer is inserted in the proximal loop of domain IV.
19. The circular RNA molecule of any one of claims 6-18, wherein the aptamer is modified to have an extended stem region.
20. The circular RNA molecule of any one of claims 6-19, wherein the aptamer is positioned within the secondary structure of the IRES so that is spatially proximal to portion of the IRES responsible for translation initiation.
21. The circular RNA molecule of any one of claims 6-20, wherein the aptamer does not interrupt the native eIF4G binding site of the IRES and does not interrupt a native GRNA tetraloop within the IRES.

22. The circular RNA molecule of claim 15, wherein the IRES is a modified iHRV-B3 IRES or a modified iCVB3 IRES.
23. The circular RNA molecule of claim 22, wherein the modified iHRV-B3 IRES or the modified iCVB3 IRES comprises an aptamer inserted in domain I, II, III, IV, V, or VI.
24. The circular RNA molecule of claim 23, wherein the modified iHRV-B3 IRES or the modified iCVB3 IRES comprises an aptamer inserted in domain IV.
25. The circular RNA molecule of claim 24, wherein the modified iHRV-B3 IRES or the modified iCVB3 IRES comprises an aptamer inserted in a proximal loop of domain IV.
26. The circular RNA molecule of any one of claims 1-3, wherein at least 1% of the nucleotides of the circular RNA molecule are 2-thiouridine nucleotides.
27. The circular RNA molecule of claim 26, wherein 2% to 5% of the nucleotides of the circular RNA molecule are 2-thiouridine.
28. The circular RNA molecule of claim 27, wherein 2.5% of the nucleotides of the circular RNA molecule are 2-thiouridine.
29. The circular RNA molecule of any one of claims 1-3, wherein at least 1% of the nucleotides of the circular RNA molecule are 2'-O-methylcytidine.
30. The circular RNA molecule of claim 29, wherein 2% to 5% of the nucleotides of the circular RNA molecule are 2'-O-methylcytidine.
31. The circular RNA molecule of claim 30, wherein 2.5% of the nucleotides of the circular RNA molecule are 2'-O-methylcytidine.

32. The circular RNA molecule of any of the preceding claims, wherein the molecule comprises a nucleic acid spacer upstream of said IRES.
33. The circRNA of any one of claims 1-3, further comprising at least one non-coding functional sequence.
34. The circRNA of claim 33, wherein the non-coding functional sequence is a microRNA (miRNA) sponge.
35. A nucleic acid that encodes the circular RNA molecule of any one of claims 1-34.
36. A composition comprising the circular RNA molecule of any one of claims 1-34 or the nucleic acid of claim 35
37. A host cell comprising the circular RNA molecule of any one of claims 1-34 or the nucleic acid of claim 35.
38. A method of producing a protein in a cell, the method comprising contacting a cell with the circular RNA molecule of any one of claims 1-34 or the nucleic acid of claim 35 under conditions whereby the protein-coding nucleic acid sequence of the circular RNA is translated and the protein is produced in the cell.
39. A method of producing a protein *in vitro*, the method comprising contacting a cell-free extract with the circular RNA molecule of any one of claims 1-34 or the nucleic acid of claim 35 under conditions whereby the protein-coding nucleic acid sequence of the circular RNA is translated and the protein is produced.