RNA SENSORS IN LIVING CELLS UTILIZING ADAR EDITING FOR SENSE-RESPONSE APPLICATIONS

GOVERNMENT RIGHTS

[0001] This invention was made with Government support under contract 1656518 awarded by the National Science Foundation and under contract EB027723 awarded by the National Institutes of Health. The Government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] Pursuant to 35 U.S.C. § 119 (e), this application claims priority to the filing date of United States Provisional Patent Application Serial No. 63/313,423 filed February 24, 2022, the disclosure of which application is herein incorporated by reference.

SEQUENCE LISTING

[0003] A Sequence Listing is provided herewith as a txt file Sequence Listing XML, "STAN-1939WO_SEQ_LIST" created February 24, 2023, and having a size of 13 KB. The contents of the txt file Sequence Listing XML are incorporated by reference herein in their entirety.

INTRODUCTION

[0004] Single-cell transcriptomics often serve as the *de facto* way to define cell types and states, but targeting cells based on their RNA profile has remained challenging. RNA sense-response systems would for example enable the identification and destruction of harmful cells (e.g., in the contexts of cancer and autoimmune disorders), or the experimental manipulation of specific cells in a complex environment (e.g., the nervous and the immune systems). Available RNA sensing technologies are limited to miRNAs, or require careful design around functional RNA structures such as ribozymes, guide RNAs or internal ribosome entry sites. For the latter, an additional confounding factor is the cell's natural response to double-stranded RNA (dsRNA). dsRNA editing by adenosine deaminases acting on RNA (ADARs) allows for the editing of specific RNAs.

[0005] Provided herein are methods and kits for detecting target RNAs and expressing proteins in target cells utilizing ADAR editing.

SUMMARY

[0005a] In one aspect, provided herein is an RNA sensor system comprising:

(a) a single-stranded RNA (ssRNA) sensor comprising a stop codon and a payload; optionally wherein the ssRNA sensor further comprises a normalizing gene; and

(b) an adenosine deaminase acting on RNA (ADAR) deaminase;

wherein the sensor is capable of binding to a ssRNA target to form a double-stranded RNA(dsRNA) duplex that becomes a substrate for the ADAR deaminase;

wherein the substrate comprises a mispairing within the stop codon;

wherein the mispairing is editable by the ADAR deaminase, which editing can effectively remove the stop codon so as to enable translation and expression of the payload.

[0005b] In another aspect, provided herein is a single-stranded RNA (ssRNA) sensor for expressing a protein in a target cell comprising:

(a) a first region comprising: (i) a nucleotide sequence configured to hybridize to a target RNA; and (ii) and a stem-loop sequence comprising one or more editable codons, and

(b) a second region comprising a sequence encoding said protein;

wherein said target RNA is present in said target cell.

[0005c] In another aspect, provided herein is a method for expressing a protein in a target cell, the method comprising combining said target cell with a sensor RNA comprising:

(a) a first region comprising: (i) a nucleotide sequence configured to hybridize to a target RNA; and (ii) a stem-loop sequence comprising one or more editable codons, and

b) a second region comprising a sequence encoding said protein;

wherein said target RNA is present in said target cell.

[0005d] In another aspect, provided herein is use of a sensor RNA in the manufacture of a medicament for expressing a protein in a target cell, wherein said target cell is to be combined with said sensor RNA, wherein said sensor RNA is to be administered to a patient, wherein said sensor RNA comprises:

(a) a first region comprising: (i) a nucleotide sequence configured to hybridize to a target RNA; and (ii) and a stem-loop sequence comprising one or more editable codons, and

(b) a second region comprising a sequence encoding said protein;

wherein said target RNA is present in said target cell.

[0006] The present disclosure provides a method for expressing a protein in a target cell, the method comprising combining the target cell with a sensor RNA comprising the following: (ia) a first nucleotide sequence comprising a sensor nucleotide sequence that is reverse complementary to the target RNA wherein the sensor nucleotide sequence comprises a stem-loop sequence comprising one or more editable codons, or (ib) a first nucleotide sequence comprising a sensor nucleotide sequence that is reverse complementary to the 3' UTR of the target RNA, wherein the sensor nucleotide sequence comprises one or more editable codons, and (ii) a second nucleotide sequence encoding a first cleavage domain, and (iii) a third nucleotide sequence encoding an output protein; wherein the target RNA is present in the target cell.

[0007] The present disclosure provides a method for detecting a target RNA, the method including (a) combining the biological sample with a sensor RNA including the following: (i) a first nucleotide sequence encoding a marker protein, (ii) a second nucleotide sequence including a sensor nucleotide sequence that is reverse complementary to the 3' UTR of the target RNA, wherein the sensor nucleotide sequence includes a stop codon, (iii) a third nucleotide sequence encoding a second cleavage domain, and (iv) a fourth nucleotide sequence encoding an output protein; (b) assaying for the presence of the output protein in the biological sample.

[0008] The present disclosure also provides method for detecting a target RNA in a biological sample, the method including: (a) combining the biological sample with a sensor RNA including the following: (i) a first nucleotide sequence including a stem-loop sequence including one or more stop codons, (ii) a second nucleotide sequence including a sensor nucleotide sequence that is reverse complementary to the target RNA, (iii) a third nucleotide sequence encoding a first cleavage domain, and (iv) a fourth nucleotide sequence encoding an output protein; and (b) assaying for the presence of the output protein in the biological sample.

[0009] The present disclosure provides a method for detecting a target RNA in a biological sample, the method comprising: (a) combining the biological sample with a sensor RNA comprising the following: (i) a first nucleotide sequence comprising a sensor nucleotide sequence that is reverse complementary to the target RNA, wherein the sensor nucleotide sequence comprises a start codon and (ii) a second nucleotide sequence encoding an output protein; and (b) assaying for the presence of the output protein in the biological sample.

[0010] The present disclosure provides a method for detecting a target RNA in a biological sample, the method comprising: (a) combining the biological sample with a sensor RNA comprising the

with β-lactamase inhibitors, cephalosporins, e.g. cefaclor, cefazolin, cefuroxime, moxalactam, etc.:; tetracyclines; cephalosporins; quinolones; lincomycins; macrolides; sulfonamides; glycopeptides including the anti-infective antibiotics vancomycin, teicoplanin, telavancin, ramoplanin and decaplanin. Derivatives of vancomycin include, for example, oritavancin and dalbavancin (both lipoglycopeptides). Telavancin is a semi-synthetic lipoglycopeptide derivative of vancomycin (approved by FDA in 2009). Other vancomycin analogs are disclosed, for example, in WO 2015022335 A1 and Chen et al. (2003) PNAS 100(10): 5658-5663, each herein specifically incorporated by reference. Non-limiting examples of antibiotics include vancomycin, linezolid, azithromycin, daptomycin, colistin, eperezolid, fusidic acid, rifampicin, tetracyclin, fidaxomicin, clindamycin, lincomycin, rifalazil, and clarithromycin.

KITS

[00155] Also provided are kits for practicing the methods described in the present disclosure. In general, subject kits may contain a sensor RNA as described above. The sensor RNA may be contained in a lipid nanoparticle or the sensor RNA may be within a recombinant vector as described above. In some cases, the kit further contains an ADAR protein or a coding sequence thereof. When the kit contains a coding sequence of the ADAR it may be in a recombinant vector as described above. When the kit contains the ADAR protein or the coding sequence thereof, the ADAR protein may be any ADAR protein described above.

[00156] In some cases, the kit may further contain a positive and/or negative control. The positive control may be in the form of a biological sample containing the target RNA, a sensor RNA containing an edited codon (i.e., a stop codon that has been edited to be a non-stop codon or a start codon edited to be a non-start codon or a non-start codon edited to be a start codon) or a sensor RNA containing the nucleotide sequence of the target RNA. The negative control may be in the form of a biological sample that does not contain the target RNA.

[00157] A subject kit can include any combination of components for performing the methods of the present disclosure. The components of a subject kit can be present as a mixture or can be separate entities. In some cases, components are present as a lyophilized mixture. In some cases, the components are present as a liquid mixture. Components of a subject kit can be in the same or separate containers, in any combination.

[00158] The subject kits may further include (in certain embodiments) instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or

20. Uzonyi, A. et al. *Mol. Cell* (2021).doi:10.1016/j.molcel.2021.03.024

21. Biswas, J., Rahman, R., Gupta, V., Rosbash, M. & Singer, R.H. *iScience* **23**, 101318 (2020).

22. Yoshikawa, K. et al. *Biomed. Res.* **31**, 401–411 (2010).

23. Gao, Q. et al. *Cell Rep.* **23**, 227-238.e3 (2018).

24. Dykstra, P.B., Kaplan, M. & Smolke, C.D. *Nat. Rev. Genet.* 1–14 (2022).doi:10.1038/s41576-021-00436-7

25. Groves, B. et al. *Nat. Nanotechnol.* **11**, 287–294 (2016).

[00226] Notwithstanding the appended claims, the disclosure set forth herein is also described by the following clauses:

1. A method for detecting a target RNA in a biological sample, the method comprising:

(a) combining the biological sample with a sensor RNA comprising the following:

(i) a first nucleotide sequence comprising a stem-loop sequence comprising one or more stop codons,

(ii) a second nucleotide sequence comprising a sensor nucleotide sequence that is reverse complementary to the target RNA,

(iii) a third nucleotide sequence encoding a first cleavage domain, and

(iv) a fourth nucleotide sequence encoding an output protein; and

(b) assaying for the presence of the output protein in the biological sample.

2. The method of clause 1, wherein the one or more stop codons comprises at least 1 base that is mismatched with a sequence within the stem loop opposite the one or more stop codons.

3. The method of clauses 1 or 2, further comprising:

(i) a fifth nucleotide sequence comprising a second cleavage domain wherein the fifth nucleotide sequence precedes the first nucleotide sequence and

(ii) a sixth nucleotide sequence comprising a nucleotide sequence encoding a marker protein wherein the sixth nucleotide sequence precedes the fifth nucleotide sequence.

4. The method of any of clauses 1-3, wherein the cleavage domain is a 2A self-cleaving domain.

an adeno-associated virus (AAV) comprising the sensor RNA wherein the sensor RNA in contained with a AAV vector.

16. The method of any of clauses 1-14, wherein the combining with the biological sample comprises transfecting the biological sample with a recombinant vector comprising the sensor RNA.

17. The method of clause 16, wherein the recombinant vector is selected from the group of a plasmid, a viral vector, a cosmid, and an artificial chromosome.

18. The method of any of clauses 1-17, wherein assaying for the presence of the output protein comprises using immunoblotting.

19. The method of any of clauses 1-17, wherein assaying for the presence of the output protein comprises using microscopy.

20. The method of any of clauses 1-17, wherein assaying for the presence of the output protein comprises using flow cytometry.

21. The method of any of clauses 1-20, wherein the sensor RNA comprises one or more MS2 hairpins.

22. The method of any of clauses 1-21, wherein the sensor nucleotide sequence is reverse complementary to two or more non-contiguous sequences within a single target RNA.

23. The method of any of clauses 1-22, wherein the sensor nucleotide sequence is reverse complementary to two or more distinct target RNAs.

24. The method of any of clauses 1-23, wherein the sensor RNA further comprises a nucleotide sequence encoding a second sensor nucleotide sequence that is reverse complementary to a second target RNA wherein the sequences of the first and second target RNAs are different.

36. The method of any of clauses 31-35, wherein the pseudouridine is not adjacent to the adenosine in the stop codon of the sensor nucleotide sequence.

37. A method for detecting a target RNA in a biological sample, the method comprising:

(a) combining the biological sample with a sensor RNA comprising the following:

(i) a first nucleotide sequence comprising a sensor nucleotide sequence that is reverse complementary to the target RNA wherein the sensor nucleotide sequence comprises a stem-loop sequence comprising one or more stop codons,

(ii) a second nucleotide sequence encoding a first cleavage domain, and

(iii) a third nucleotide sequence encoding an output protein; and

(b) assaying for the presence of the output protein in the biological sample.

38. The method of clause 37, further comprising:

(i) a fourth nucleotide sequence comprising a second cleavage domain wherein the fourth nucleotide sequence precedes the first nucleotide sequence and

(ii) a fifth nucleotide sequence comprising a nucleotide sequence encoding a marker protein wherein the fifth nucleotide sequence precedes the fourth nucleotide sequence.

39. The method of clauses 37-38, wherein the one or more stop codons comprises at least 1 base that is mismatched with a sequence within the stem loop opposite the one or more stop codons.

40. The method of any of clauses 37-39, wherein the cleavage domain is a 2A self-cleaving domain.

41. The method of clause 40, wherein the 2A self-cleaving domain is selected from the group of T2A, P2A, E2A and F2A.

42. The method of any of clauses 37-41, wherein the stem loop is a GluR-B stem loop or modified variant thereof.

43. The method of any of clauses 37-42, wherein the stem loop is 9 to 24 base pairs in length.

54. The method of any of clauses 37-53, wherein assaying for the presence of the output protein comprises using immunoblotting.

55. The method of any of clauses 37-54, wherein assaying for the presence of the output protein comprises using microscopy.

56. The method of any of clauses 37-55, wherein assaying for the presence of the output protein comprises using flow cytometry.

57. The method of any of clauses 37-56, wherein the sensor RNA comprises one or more MS2 hairpins.

58. The method of any of clauses 37-57, wherein the sensor nucleotide sequence is reverse complementary to two or more non-contiguous sequences within a single target RNA.

59. The method of any of clauses 37-58, wherein the sensor nucleotide sequence is reverse complementary to two or more distinct target RNAs.

60. The method of any of clauses 37-59, wherein the sensor RNA further comprises a nucleotide sequence encoding a second sensor nucleotide sequence that is reverse complementary to a second target RNA wherein the sequences of the first and second target RNAs are different.

61. The method of clause 60, wherein the second sensor nucleotide sequence comprises a second stop codon or a first start codon.

62. The method of clause 60, wherein the second sensor nucleotide sequence comprises a stem-loop sequence comprising one or more stop or start codons.

63. The method of any of clauses 37-62, further comprising combining the biological sample with an adenosine deaminase acting on RNA (ADAR) protein or coding sequence thereof.

64. The method of clause 63, wherein the ADAR protein is ADAR2 or ADAR^{p150}.

74. The method of clause 73, further comprising:

(i) a fourth nucleotide sequence comprising a second cleavage domain wherein the fourth nucleotide sequence precedes the first nucleotide sequence and

(ii) a fifth nucleotide sequence comprising a nucleotide sequence encoding a marker protein wherein the fifth nucleotide sequence precedes the fourth nucleotide sequence.

75. The method of any of clauses 73-74, wherein the one or more stop codons comprises at least 1 base that is mismatched with the 3' UTR of the target mRNA sequence.

76. The method of any of clauses 73-75, wherein the cleavage domain is a 2A self-cleaving domain.

77. The method of clause 76, wherein the 2A self-cleaving domain is selected from the group of T2A, P2A, E2A and F2A.

78. The method of any of clauses 73-77, further comprising fifth nucleotide sequence comprising a second cleavage domain, wherein the fifth nucleotide sequence precedes the second nucleotide sequence.

79. The method of any of clauses 73-78, wherein the sensor nucleotide sequence is 60 or more nucleotides in length.

80. The method of any of clauses 73-79, wherein the target RNA is encoded by a gene fusion, a splice variant or a gene variant comprising a single nucleotide polymorphism.

81. The method of any of clauses 73-80, wherein the marker protein is a fluorescent protein or a luminescent protein

82. The method of any of clauses 73-81, wherein the output protein is selected from a fluorescent protein, a genomic modification protein, a transcription factor, a killing factor, a toxin, an antigen, a T cell receptor, a therapeutic protein and an enzyme.

83. The method of any of clauses 73-82, wherein the detecting is quantitative or qualitative.

84. The method of any of clauses 73-83, wherein the biological sample is a cell.

85. The method of any of clauses 73-84, wherein the combining with the biological sample comprises contacting the biological sample with a lipid nanoparticle comprising the sensor RNA or an adeno-associated virus (AAV) comprising the sensor RNA wherein the sensor RNA in contained with a AAV vector.

86. The method of any of clauses 73-85, wherein the combining with the biological sample comprises transfecting the biological sample with a recombinant vector comprising the sensor RNA.

87. The method of clause 86, wherein the recombinant vector is selected from the group of a plasmid, a viral vector, a cosmid, and an artificial chromosome.

88. The method of any of clauses 73-87, wherein assaying for the presence of the output protein comprises using immunoblotting.

89. The method of any of clauses 73-88, wherein assaying for the presence of the output protein comprises using microscopy.

90. The method of any of clauses 73-89, wherein assaying for the presence of the output protein comprises using flow cytometry.

91. The method of any of clauses 73-90, wherein the sensor RNA comprises one or more MS2 hairpins.

92. The method of any of clauses 73-91, wherein the sensor nucleotide sequence is reverse complementary to two or more non-contiguous sequences within a single target RNA.

93. The method of any of clauses 73-92, wherein the sensor nucleotide sequence is reverse complementary to two or more distinct target RNAs.

94. The method of any of clauses 73-93, wherein the sensor RNA further comprises a nucleotide sequence encoding a second sensor nucleotide sequence that is reverse complementary to a second target RNA wherein the sequences of the first and second target RNAs are different.

95. The method of clause 94, wherein the second senor nucleotide sequence comprises a second stop codon or a first start codon.

96. The method of clause 94, wherein the second sensor nucleotide sequence comprises a stem-loop sequence comprising one or more stop or start codons.

97. The method of any of clauses 73-96, further comprising combining the biological sample with an adenosine deaminase acting on RNA (ADAR) protein or coding sequence thereof.

98. The method of clause 97, wherein the ADAR protein is ADAR2 or $ADAR^{p150}$.

99. The method of clause 98, wherein the ADAR protein is a modified ADAR comprising an ADAR deaminase domain and a MS2 binding domain.

100. The method of any of clauses 73-99, wherein the biological sample is combined with two or more sensor RNAs that detect two or more target RNAs.

101. The method of any of clauses 73-100, wherein the sensor RNA comprises one or more pseudouridines.

102. The method of clause 101, wherein 75% or less of the uridines in the sensor RNA are pseudouridine.

103. The method of any of clauses 101-102, wherein the pseudouridine is N1-methyl-pseudouridine.

114. The method of any of clauses 107-113, wherein the output protein is selected from a fluorescent protein, a genomic modification protein, a transcription factor, a killing factor, a toxin, an antigen, a T cell receptor, a therapeutic protein and an enzyme.

115. The method of any of clauses 107-114, wherein the detecting is quantitative or qualitative.

116. The method of any of clauses 107-115, wherein the biological sample is a cell.

117. The method of any of clauses 107-116, wherein the combining with the biological sample comprises contacting the biological sample with a lipid nanoparticle comprising the sensor RNA or an adeno-associated virus (AAV) comprising the sensor RNA wherein the sensor RNA in contained with a AAV vector.

118. The method of any of clauses 107-117, wherein the combining with the biological sample comprises transfecting the biological sample with a recombinant vector comprising the sensor RNA.

119. The method of clause 118, wherein the recombinant vector is selected from the group of a plasmid, a viral vector, a cosmid, and an artificial chromosome.

120. The method of any of clauses 107-119, wherein assaying for the presence of the output protein comprises using immunoblotting.

121. The method of any of clauses 107-120, wherein assaying for the presence of the output protein comprises using microscopy.

122. The method of any of clauses 107-121, wherein assaying for the presence of the output protein comprises using flow cytometry.

123. The method of any of clauses 107-122, wherein the sensor RNA comprises one or more MS2 hairpins.

124. The method of any of clauses 107-123, wherein the sensor nucleotide sequence is reverse complementary to two or more non-contiguous sequences within a single target RNA.

125. The method of any of clauses 107-124, wherein the sensor nucleotide sequence is reverse complementary to two or more distinct target RNAs.

126. The method of any of clauses 107-125, wherein the sensor RNA further comprises a nucleotide sequence encoding a second sensor nucleotide sequence that is reverse complementary to a second target RNA wherein the sequences of the first and second target RNAs are different.

127. The method of clause 126, wherein the second sensor nucleotide sequence comprises a second stop codon or a first start codon.

128. The method of clause 126, wherein the second sensor nucleotide sequence comprises a stem-loop sequence comprising one or more stop or start codons.

129. The method of any of clauses 107-128, further comprising combining the biological sample with an adenosine deaminase acting on RNA (ADAR) protein or coding sequence thereof.

130. The method of clause 129, wherein the ADAR protein is ADAR2 or ADAR^{p150}.

131. The method of clause 130, wherein the ADAR protein is a modified ADAR comprising an ADAR deaminase domain and a MS2 binding domain.

132. The method of any of clauses 107-131, wherein the biological sample is combined with two or more sensor RNAs that detect two or more target RNAs.

133. A method for detecting a target RNA in a biological sample, the method comprising:

(a) combining the biological sample with a sensor RNA comprising the following:

143. The method of any of clauses 133-142, wherein the combining with the biological sample comprises transfecting the biological sample with a recombinant vector comprising the sensor RNA.

144. The method of clause 143, wherein the recombinant vector is selected from the group of a plasmid, a viral vector, a cosmid, and an artificial chromosome.

145. The method of any of clauses 133-144, wherein assaying for the presence of the output protein comprises using immunoblotting.

146. The method of any of clauses 133-145, wherein assaying for the presence of the output protein comprises using microscopy.

147. The method of any of clauses 133-146, wherein assaying for the presence of the output protein comprises using flow cytometry.

148. The method of any of clauses 133-147, wherein the sensor RNA comprises one or more MS2 hairpins.

149. The method of any of clauses 133-148, wherein the sensor nucleotide sequence is reverse complementary to two or more non-contiguous sequences within a single target RNA.

150. The method of any of clauses 133-149, wherein the sensor nucleotide sequence is reverse complementary to two or more distinct target RNAs.

151. The method of any of clauses 133-150, wherein the sensor RNA further comprises a nucleotide sequence encoding a second sensor nucleotide sequence that is reverse complementary to a second target RNA the sequences of the first and second target RNAs are different.

152. The method of clause 151, wherein the second senor nucleotide sequence comprises a stop codon or a start codon.

164. The method of any of clauses 158-163, wherein the target RNA is associated with a disease or condition.

165. The method of any of clauses 158-164, wherein the output protein treats the disease or condition.

166. The method of any of clauses 158-165, wherein the target RNA is encoded by a gene fusion, a splice variant or a gene variant comprising a single nucleotide polymorphism.

167. The method of clause 166, wherein the recombinant vector is selected from the group of a plasmid, a viral vector, a cosmid and an artificial chromosome.

168. A recombinant vector comprising the sensor RNA of any of the preceding clauses.

169. The recombinant vector of clause 168, wherein the recombinant vector is selected from the group of a plasmid, a viral vector, a cosmid and an artificial chromosome.

170. A kit, the kit comprising the sensor RNA of any of the preceding clauses.

171. The kit of clause 170, further comprising an ADAR protein or a coding sequence thereof.

172. The kit of clause 171, wherein the ADAR protein is $ADAR^{p150}$.

173. The kit of clause 171, wherein the ADAR protein is a modified ADAR protein comprising an ADAR deaminase domain and a MS2 binding domain.

174. The kit of any of clauses 170-173, further comprising a biological sample with the target RNA and a biological sample without the target RNA.

175. The method of any of clauses 1-36, wherein combining comprises administering to a patient.