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(54) **Title:** RNA CLEAVAGE-INDUCED TRANSCRIPT STABILIZER AND USES THEREOF

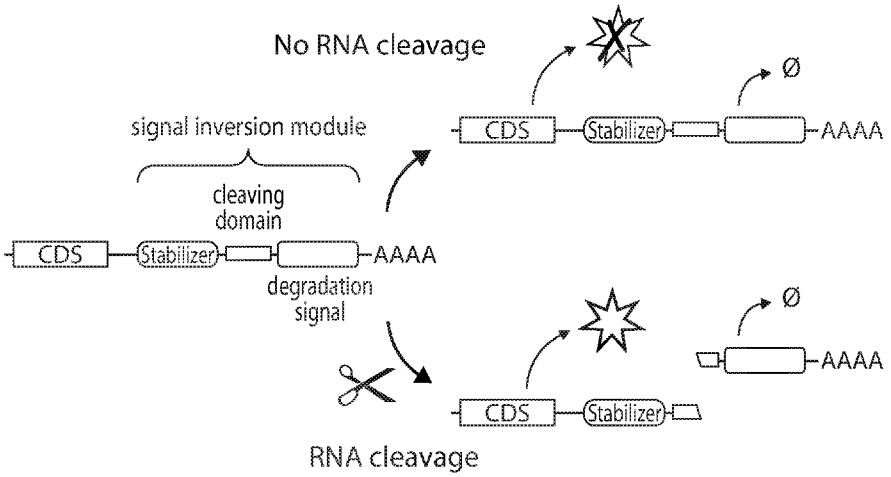

FIG. 1A

(57) **Abstract:** Provided herein are genetic circuits and encoded RNA transcripts that produce an output molecule in response to an RNA cleavage event that removes a degradation signal. In some embodiments, the genetic circuits described herein may be used for detecting RNA cleaver activities (*e.g.*, in a cell). Methods of using the genetic circuits described herein in diagnostic or therapeutic applications are also provided.

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RNA CLEAVAGE-INDUCED TRANSCRIPT STABILIZER AND USES THEREOF

RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/539,375, filed July 31, 2017, and entitled "RNA CLEAVAGE-INDUCED TRANSCRIPT STABILIZER AND USES THEREOF," the entire contents of which are incorporated herein by reference.

BACKGROUND

RNA cleavage is an important process during cellular RNA processing. Existing methods of detecting RNA cleavage activities (*e.g.*, RNA cleavage mediated by a microRNA) usually involve a "double-inversion" strategy, where the RNA cleavage sites are engineered into a transcript encoding a translational or transcriptional repressor of a reporter. As such, high levels of RNA cleavage leads to low repressor expression, which in turn rescues reporter expression. A "time delay" exists between the detection of the RNA cleavage and the production of the reporter.

SUMMARY

The present disclosure, in some aspects, relates to genetic circuits and modules that directly respond to RNA cleavage (*e.g.*, cleavage mediated by RNAi, cis- or trans-acting ribozymes and ribonucleases) and produce an output molecule (*e.g.*, a detectable molecule, a therapeutic molecule, or a functional molecule). Such genetic circuits incorporate RNA degradation signals that lead to the degradation of the RNA molecule. The RNA degradation signals are removed via RNA cleavage, stabilizing the RNA and resulting in expression of the output molecule.

Some aspects of the present disclosure provide cleavage-induced transcript stabilizers, containing: (i) a first promoter operably linked to a nucleotide sequence encoding an output molecule followed, from 5′ to 3′, by an RNA stabilizer, a cleavage site for an RNA cleaver, and a degradation signal. In some embodiments, the cleavage-induced transcript stabilizer further contains (ii) a second promoter operably linked to a nucleotide sequence encoding the RNA cleaver.

In some embodiments, the RNA cleaver is selected from the group consisting of: endoribonucleases, RNAi molecules, and ribozymes. In some embodiments, the RNA cleaver is an endoribonuclease. In some embodiments, the endoribonuclease is selected from the group consisting of: Cse3, Cas6, CasE, and Csy4. In some embodiments, the cleavage site contains a recognition sequence for the endoribonuclease.

In some embodiments, the RNA cleaver is an RNAi molecule. In some embodiments, the RNAi molecule is a microRNA, siRNA, or shRNA. In some embodiments, the cleavage site contains one or more target sites for the RNAi molecule.

In some embodiments, the RNA cleaver is a ribozyme. In some embodiments, the ribozyme is selected from the group consisting of: RNase P, HDV ribozyme, hammerhead ribozyme, hairpin ribozyme, twister ribozyme, twister sister ribozyme, pistol ribozyme, hatchet ribozyme, glmS ribozyme, varkud satellite ribozyme, and spliceozyme. In some embodiments, the ribozyme is a trans-acting ribozyme. In some embodiments, the cleavage site contains a recognition site for the trans-acting ribozyme. In some embodiments, the ribozyme is a cis-acting ribozyme. In some embodiments, the cleavage site contains the cis-acting ribozyme.

In some embodiments, the cleavage-induced transcript stabilizer further contains a third promoter operably linked to a third nucleotide sequence encoding an RNA repressor, and one or more the cleavage sites for the RNA cleaver. In some embodiments, the cleavage-induced transcript stabilizer further contains one or more recognition sites for an RNA repressor operably linked of the nucleotide sequence encoding the output molecule.

In some embodiments, the RNA repressor is an RNA binding protein. In some embodiments, the RNA binding protein is selected from the group consisting of: TetR, CNOT7, DDX6, PPR10, and L7Ae.

In some embodiments, the cleavage-induced transcript stabilizer contains 1-50 repeats of the degradation signal. In some embodiments, the cleavage-induced transcript stabilizer contains 10 repeats of the degradation signal. In some embodiments, the cleavage-induced transcript stabilizer contains 30 repeats of the degradation signal. In some embodiments, the degradation signal contains the nucleotide sequence of TAASTTAT (SEQ ID NO: 1), wherein S is deoxyguanosine or deoxycytosine. In some embodiments, the degradation signal contains the nucleotide sequence of TAAGTTAT (SEQ ID NO: 2). In some embodiments, the degradation signal contains the nucleotide sequence of TAAGACAT (SEQ ID NO: 3).

In some embodiments, the RNA stabilizer is selected from the group consisting of: MALAT1 triplex, MENβ triplex, KSHV PAN triplex, histone stem loop, and a polyA signal. In some embodiments, the RNA stabilizer is a MALAT1 triplex.

In some embodiments, the output molecule is a detectable molecule. In some embodiments, the output molecule is a therapeutic molecule. In some embodiments, the output molecule is a functional molecule. In some embodiments, wherein the functional molecule is selected from the group consisting of: TetR, CNOT7, DDX6, PPR10, L7Ae, Csy4, Cas6, CasE, and Cse3.

In some embodiments, the second promoter of (ii) is an inducible promoter.

An "RNA version" of the cleavage-induced transcript stabilizer is also provided, containing: (i) an RNA transcript containing a ribonucleotide sequence encoding an output molecule followed, in order, by an RNA stabilizer, a cleavage site for an RNA cleaver, and a degradation signal that leads to degradation of the RNA transcript. In some embodiments, the RNA version of the cleavage-induced transcript stabilizer further contains: (ii) a promoter operably linked to a nucleotide sequence encoding an RNA cleaver that cleaves the RNA transcript at the cleavage site. In some embodiments, the promoter of (ii) is an inducible promoter. In some embodiments, the RNA transcript is degraded without in the absence of the RNA cleaver. In some embodiments, the RNA cleaver is expressed in the presence of the RNA cleaver. In some embodiments, the cleavage of the RNA transcript stabilizes the RNA transcript and results in expression of the output molecule.

In some embodiments, the output molecule is a detectable molecule. In some embodiments, the output molecule is a therapeutic molecule. In some embodiments, the output molecule is a functional molecule. In some embodiments, wherein the functional molecule is selected from the group consisting of: TetR, CNOT7, DDX6, PPR10, L7Ae, Csy4, Cas6, CasE, and Cse3.

Cells containing the cleavage-induced transcript stabilizers described herein are provided. In some embodiments, the cell is a prokaryotic cell. In some embodiments, the prokaryotic cell is a bacterial cell. In some embodiments, the cell is a eukaryotic cell. In some embodiments, the eukaryotic cell is a plant cell, an insect cell, or a mammalian cell. In some embodiments, the mammalian cell is a human cell. In some embodiments, the cell is a diseased cell. In some embodiments, the cell is a cancer cell. In some embodiments, the cleavage-induced transcript stabilizer is inserted into the genome of the cell.

Other aspects of the present disclosure provide methods of using the cleavage-induced transcript stabilizer described herein. In some embodiments, the method contains maintaining

the cells containing the cleavage-induced transcript stabilizer. In some embodiments, the method further contains detecting the output molecule. In some embodiments, the method further contains classifying the cell.

In some embodiments, the method contains delivering the cleavage-induced transcript stabilizer described herein to a cell and detecting the output molecule.

In some embodiments, the cleavage-induced transcript stabilizer are used in a method of detecting an RNA cleaver activity, the method contains: delivering the cleavage-induced transcript stabilizer described herein to a cell and detecting the output molecule. In some embodiments, the RNA cleaver is an endoribonuclease, a siRNA transcript, or a ribozyme.

Methods of treating a disease or disorder are provided, the method contains delivering the cleavage-induced transcript stabilizer described herein to a cell, wherein the output molecule is a therapeutic molecule that is effective for treating the disease or disorder. In some embodiments, the method contains administering an effective amount of a composition containing the cleavage-induced transcript stabilizer described herein to a subject in need thereof, wherein the output molecule is a therapeutic molecule that is effective for treating the disease or disorder. In some embodiments, the composition further contains a pharmaceutically acceptable carrier. In some embodiments, the cell is a diseased cell. In some embodiments, the cell is a cancer cell.

Methods of diagnosing a disease or disorder are provided, the method contains delivering the cleavage-induced transcript stabilizer described herein to a cell. In some embodiments, the method contains administering an effective amount of a composition containing the cleavage-induced transcript stabilizer described herein to a subject in need thereof. In some embodiments, the composition further contains a pharmaceutically acceptable carrier. In some embodiments, the cell is a diseased cell. In some embodiments, the cell is a cancer cell. In some embodiments, the method further contains detecting the output molecule. In some embodiments, the lack of expression of the output molecule indicates the disease or disorder. In some embodiments, the expression of the output molecule indicates the disease or disorder.

The summary above is meant to illustrate, in a non-limiting manner, some of the embodiments, advantages, features, and uses of the technology disclosed herein. Other embodiments, advantages, features, and uses of the technology disclosed herein will be apparent from the Detailed Description, the Drawings, the Examples, and the Claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. For purposes of clarity, not every component may be labeled in every drawing.

FIGs. 1A-1B: Inverter module schematic. (**FIG. 1A**) A schematic of the signal inversion module described herein. (**FIG. 1B**) A comparison between a traditional module that detects RNA cleavage and the module described herein. Scissors represent an RNA cleaver such as a ribonuclease, miRNA, or ribozyme. Line plots show sample curves for transgene expression response to RNA cleaver level (FIG. 1B).

FIG. 2: Ultrasensitive switch schematic. Two separate RNA transcripts are shown. A traditional "off" switch controls the expression of an RNA repressor that represses the output molecule. The output molecule is encoded by an RNA transcript that incorporates the "on" switch described herein. The combined effect of the two switches are also shown.

FIGs. 3A-3D: Degradation signals and stabilizers. Solid lines and bars indicate constructs that do not contain the triplex sequence, while dashed lines or bars do contain the triplex sequence. (**FIG. 3A**) Expression response to degradation sequences. "wt1" indicates the wild type Geissler degradation sequence while "mut1" indicates the mutated version. Lines, from top to bottom at right end: EYFP, EYFP-10xmut1, EYFP-10xwt1, background. (**FIG. 3B**) Effects of the triplex on degradation domains. "Trpx" indicates triplex sequence. Lines, from top to bottom at right end: EYFP, EYFP-Trpx, EYFP-Trpx-10xwt1, EYFP-10xwt1, background. (**FIG. 3C**) Effects of Geissler degradation sequence repeat count. Lines, from top to bottom at right end: EYFP-Trpx, EYFP-Trpx-10xwt1, EYFP-Trpx-20xwt1, EYFP-Trpx-30xwt1, background. (**FIG. 3D**) Bar plots of geometric mean normalized to background fluorescence. Colors and patterns match those indicated in line plots (**FIGs. 3A-3C**), from left to right: EYFP, EYFP-10xwt1, EYFP-10xmut1, EYFP-Trpx, EYFP-Trpx-10xwt1, EYFP-Trpx-20xwt1, EYFP-Trpx-30xwt1.

FIGs. 4A-4E: Csy4 signal inverter. (**FIG. 4A**) Titration curves of for Csy4. The line that slopes down from left to right indicates traditional "OFF" switch while the line that slopes up from left to right indicates novel "ON" switch. (**FIGs. 4B-4E**) Response of the cleavage-induced transcript stabilizer described herein cleavage by Csy4, Cse3, Cas6, and CasE respectively.

FIGs. 5A-5D: miRNA signal inverter. (**FIG. 5A**) Response of the cleavage-induced transcript stabilizer described herein to siRNA FF5. Lines, from top to bottom at right end: EYFP-Trpx, EYFP-Trpx-30xwt1, EYFP-Trpx-FF5ts-30xwt1 (+siRFF5), EYFP-Trpx- FF5ts -30xwt1 (-siRFF5), background. (**FIG. 5B**) Bar plots of geometric mean normalized to background fluorescence. Colors and patterns match those indicated in line plots. From left to right: EYFP-Trpx, EYFP-Trpx-30xwt1, EYFP-Trpx-FF5ts-30xwt1 (-siRFF5), EYFP-Trpx-FF5ts -30xwt1 (+siRFF5). (**FIG. 5C**) Response of the cleavage-induced transcript stabilizer described herein to siRNA FF3. An increase in the level of output molecule was observed. (**FIG. 5D**) Response of the cleavage-induced transcript stabilizer described herein to microRNA FF5.

FIGs. 6A-6C: Ribozyme effects. (**FIG. 6A**) Response of the cleavage-induced transcript stabilizer described herein to inactive (iHHR) and active (HHR) hammerhead ribozymes. Lines, from top to bottom at right end: EYFP-iHHR, EYFP, EYFP-HHR, background. (**FIG. 6B**) Bar plots of geometric mean normalized to background fluorescence. Colors and pattern match those indicated in line plots. From left to right: EYFP, EYFP-iHHR, EYFP-HHR. (**FIG. 6C**) In the absence of a polyA tail (the transcript should get degraded) fluorescence is rescued when the mascRNA sequence (which is targeted by RNase P) is added after the triplex.

FIGs. 7A-7B: L7Ae effects. (**FIG. 7A**)Titration curves for L7Ae. (**FIG. 7B**) Background of the "ON"-switch is decreased (as indicated by a shift of the curve to the right) by incorporating the L7Ae construct for an ultrasensitive response.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

Provided herein, in some aspects, are genetic circuits and modules that express an output molecule in response to RNA cleavage (*e.g.*, cleavage mediated by RNAi, cis- or trans-acting ribozymes and ribonucleases). Such genetic circuits incorporates RNA degradation signals that leads to the degradation of the RNA molecule. The RNA degradation signals are removed via RNA cleavage, stabilizing the RNA and resulting in expression of the output molecule. The genetic circuits described herein may be used for the detection of RNA cleaver activities (*e.g.*, in a cell), and for diagnostic or therapeutic applications.

Some aspects of the present disclosure provide a cleavage-induced transcript stabilizer. A "cleavage-induced transcript stabilizer," as used herein, refers to an RNA transcript that is

rapidly degraded due to the presence of a degradation signal in the RNA transcript, but is stabilized upon an RNA cleavage event (*e.g.*, by an endonuclease or a ribozyme) that removes the degradation signal. A genetic circuit that encodes such RNA transcript (*i.e.*, the DNA version of the transcript) is also referred to herein as a "cleavage-induced transcript stabilizer."

The "DNA version" of the cleavage-induced transcript stabilizer comprises a first promoter operably linked to a nucleotide sequence encoding an output molecule followed, from 5′ to 3′, by an RNA stabilizer, a cleavage site for an RNA cleaver, and a degradation signal. The "RNA version" of the cleavage-induced transcript stabilizer comprises a nucleotide sequence encoding an output molecule followed, from 5′ to 3′, by an RNA stabilizer, a cleavage site for an RNA cleaver, and a degradation signal. The order of the RNA stabilizer, the cleavage site for the RNA cleaver and the degradation signal needs to be such that the RNA stabilizer is downstream of and next to the nucleotide sequence encoding the output molecule; the cleavage site for the RNA cleaver is downstream of and next to the RNA stabilizer; and the degradation signal is downstream and next to the cleavage site of the RNA cleaver (*i.e.*, at the 3′ end). An exemplary structure of the cleavage-induced transcript stabilizer is shown in FIG. 1A.

As described herein, a "degradation signal," refers to a cis-acting nucleotide sequence that directs the RNA transcript to degradation, *e.g.*, via the recruitment of enzymes involved in RNA degradation to the RNA molecule. Being "cis-acting" means that the degradation signal is part of the RNA transcript that it directs to degradation. In some embodiments, the degradation signal is present in the 3' untranslated region (3'UTR) or the RNA transcript. In some embodiments, the degradation signals are appended at the 3′ end of the RNA transcript. In some embodiments, appending the degradation signal at the 3′ end of the RNA transcript maximizes its degradative strength.

In some embodiments, the degradation signal is 5-30 nucleotides long. For example, the degradation signal may be 5-30, 5-25, 5-20, 5-15, 5-10, 10-30, 10-25, 10-20, 10-15, 15-30, 15-25, 15-20, 20-30, 20-25, or 25-30 nucleotides long. In some embodiments, the degradation signal is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides long. In some embodiments, longer (*e.g.*, > 30 nt) or shorter (*e.g.*, < 5 nt) degradation signals are used.

In some embodiments, the degradation signal comprises a 8-nt RNA motif that naturally occurs in the 3' UTR of human transcripts and directs the transcripts to degrade (*e.g.*,

as described in Geissler *et al.*, *Genes & Dev.* 2016. 30: 1070-1085, incorporated herein by reference). In some embodiments, in the DNA version of the cleavage-induced transcript stabilizer, the degradation signal comprises the nucleotide sequence of TAASTTAT (SEQ ID NO: 1), wherein S is deoxyguanosine or deoxycytosine. In some embodiments, in the DNA version of the cleavage-induced transcript stabilizer, the degradation signal comprises the nucleotide sequence of TAAGTTAT (SEQ ID NO: 2) or TAACTTAT (SEQ ID NO: 4). In some embodiments, in the DNA version of the cleavage-induced transcript stabilizer, the degradation signal comprises the nucleotide sequence of TAAGACAT (SEQ ID NO: 3), which was shown herein the have induced more robust RNA degradation than the TAAGTTAT (SEQ ID NO: 2) degradation signals (*e.g.*, in FIGs. 3A and 3D).

In some embodiments, in the RNA version of the cleavage-induced transcript stabilizer, the degradation signal comprises the nucleotide sequence of UAASUUAU (SEQ ID NO: 5), wherein S is guanosine or cytosine. In some embodiments, in the RNA version of the cleavage-induced transcript stabilizer, the degradation signal comprises the nucleotide sequence of UAAGUUAU (SEQ ID NO: 6) or UAACUUAU (SEQ ID NO: 7). In some embodiments, in the DNA version of the cleavage-induced transcript stabilizer, the degradation signal comprises the nucleotide sequence of UAAGACAU (SEQ ID NO: 8).

Other known degradation signals that lead to degradation of RNA transcripts (*e.g.*, as described in Matoulkova *et al.*, *RNA Biology*, 9:5, 563-576, 2012, incorporated herein by reference) may also be used in accordance with the present disclosure, including, without limitation: AU-rich elements, GU-rich elements, CA-rich elements, and introns.

In some embodiments, the RNA transcript comprises 1-50 repeats of the degradation signal. For example, the RNA transcript may comprise 1-10, 1-20, 1-30, 1-40, 1-50, 10-50, 10-40, 10-30, 10-20, 20-50, 20-40, 20-30, 30-50, 30-40, or 40-50 repeats of the degradation signal. In some embodiments, the RNA transcript comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 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 repeats of the degradation signal. In some embodiments, the RNA transcript comprises more than 50 (*e.g.*, 60, 70, 80, 90, 100, or more) repeats of the degradation signal.

In some embodiments, the presence of the degradation signal in the RNA transcript reduces the level and/or the half-life of the RNA transcript by at least 30%. For example, the presence of the degradation signal in the RNA transcript may reduce the level and/or the half-

life of the RNA transcript by at least 30%, at least 40%, at least 50%, at least 100%, at least 3-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, at least 100-fold, or more. In some embodiments, the presence of the degradation signal in the RNA transcript reduces the level and/or the half-life of the RNA transcript by 30%, 40%, 50%, 100%, 3-fold, 5-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, or more.

The RNA transcript to which the degradation signal is appended encodes the output molecule. As such, the presence of the degradation signal in the RNA transcript reduces the level and/or activity of the output molecule by at least 30%. For example, the presence of the degradation signal in the RNA transcript may reduce the level and/or activity of the output molecule by at least 30%, at least 40%, at least 50%, at least 100%, at least 3-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, at least 100-fold, or more. In some embodiments, the presence of the degradation signal in the RNA transcript reduces the level and/or activity of the RNA transcript by 30%, 40%, 50%, at least 100%, 3-fold, 5-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, or more. In some embodiments, in the presence of the degradation signal in the RNA transcript, the output molecule does not expression or has a level of expression that is not detectable (*e.g.*, by routine methods such as western blotting).

The present disclosure provides a strategy where the RNA transcript is cleaved between the nucleotide sequence encoding the output molecule and the degradation signal, such that the degradation signal is removed and the RNA transcript is stabilized. The cleavage may be carried out by an RNA cleaver.

An "RNA cleaver," as used herein, refers to a molecule that cleaves the phosphodiester bond between two ribonucleotides, thus resulting two fragments of the RNA transcript, a 5′ fragment and a 3′ fragment. The RNA cleavers of the present disclosure cleaves the RNA transcript in a sequence-specific manner. Exemplary sequence-specific RNA cleavers include, without limitation, certain endoribonucleases, RNA interference (RNAi) molecules, and ribozymes (*e.g.*, cis-acting ribozyme or trans-acting ribozyme). The RNA cleaver of the present disclosure may directly cleave the RNA transcript (*e.g.*, an endoribonuclease or a ribozyme) or indirectly leads to the cleavage of the RNA transcript (*e.g.*, via the recruitment of

other factors that carrier out the cleavage). A non-limiting example of an RNA cleaver that indirectly cleaves the RNA transcript is an RNAi molecule, which is incorporated in a the RNA-induced silencing complex (RISC) that binds and cleaves a target sequence in the RNA transcript.

In some embodiments, the RNA cleaver is an endoribonuclease. An "endoribonuclease," as used herein, refers to a nuclease that cleaves an RNA molecule in a sequence specific manner, *e.g.*, at a recognition site. Sequence-specific endoribonucleases have been described in the art. For example, the *Pyrococcus furiosus* CRISPR-associated endoribonuclease 6 (Cas6) is found to cleave RNA molecules in a sequence-specific manner (Carte *et al.*, *Genes & Dev.* 2008. 22: 3489-3496, incorporated herein by reference). In another example, endoribonucleases that cleave RNA molecules in a sequence-specific manner are engineered, which recognize an 8-nucleotide (nt) RNA sequence and make a single cleavage in the target (Choudhury *et al.*, *Nature Communications* 3, 1147 (2012), incorporated herein by reference).

In some embodiments, the endoribonuclease belongs to the CRISPR-associated endoribonuclease 6 (Cas6) family. Cas6 nucleases from different bacterial species may be used. Non-limiting examples of Cas6 family nucleases include Cas6, Csy4 (also known as Cas6f), Cse3, and CasE.

When an endoribonuclease is used as the RNA cleaver, the recognition site for the RNA cleaver in the RNA transcript comprises one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) recognition sites for the endoribonuclease. A "recognition site for an endoribonuclease" refers to a ribonucleotide sequence that is recognized, bound, and cleaved by the endoribonuclease. The recognition site for an endoribonuclease may be 4-20 nucleotides long. For example, the recognition site may be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides long. In some embodiments, endoribonuclease recognition sites that are shorter than 4 ribonucleotides or longer than 20 nucleotides are used. Table 1 provides the amino acid and nucleotide sequences of exemplary endoribonucleases and their respective recognition sites.

Table 1. Non-limiting, Exemplary Endoribonucleases and Recognition Sites

Endoribonuclease/ Bacterial species	Amino acid sequence	Gene Sequence	Recognition site sequence
Cas6 / Pyrococcus furiosus	MRFLIRLVPEDKDR AFKVPYNHQYYLQ	ATGCGCTTCCTCATTCGTCTCGTGCCT GAGGATAAGGATCGGGCCTTTAAAGT	GTTACAAT AAGACTAA

	GLIYNAIKSSNPKL ATYLHEVKGPKLFT YSLFMAEKREHPK GLPYFLGYKKGFFY FSTCVPEIAEALVN GLLMNPEVRLWDE RFYLHEIKVLREPK KFNGSTFVTLSPIA VTVVRKGKSYDVP PMEKEFYSIIKDDL QDKYVMAYGDKPP SEFEMEVLIAKPKR FRIKPGIYQTAWHL VFRAYGNDDLLKV GYEVGFGEKNSLG FGMVKVEGNKTTK EAEEQEKITFNSRE ELKTGV (SEQ ID NO: 9)	GCCATATAACCATCAGTATTACCTGC AGGGCCTCATCTATAATGCCATCAAA TCCTCCAATCCGAAGCTGGCCACCTA CCTGCATGAGGTGAAGGGTCCCAAAC TGTTCACCTACAGCCTGTTTATGGCCG AAAAACGCGAACACCCTAAGGGGCTG CCTTACTTTTTGGGGTACAAGAAGGG CTTCTTCTACTTTTCTACCTGCGTGCC GGAGATCGCTGAAGCACTGGTCAACG GACTCCTGATGAATCCAGAGGTGCGC CTGTGGGACGAACGCTTCTACCTGCA CGAAATTAAGGTTTTGAGAGAGCCTA AGAAGTTCAACGGCTCTACCTTCGTC ACCCTGTCTCCGATTGCTGTGACTGTC GTGAGGAAGGGTAAGAGTTATGATGT CCCCCCTATGGAGAAGGAGTTTTACA GTATCATCAAAGACGACCTGCAAGAT AAGTATGTGATGGCCTACGGCGACAA GCCCCCATCCGAATTCGAGATGGAGG TGCTGATTGCTAAGCCGAAACGGTTT CGTATTAAGCCTGGCATCTATCAGAC AGCCTGGCACCTGGTTTTTAGGGCCTA CGGAAACGACGACCTGCTGAAGGTTG GTTACGAGGTTGGGTTCGGAGAAAAG AACTCCCTGGGATTCGGCATGGTGAA GGTGGAGGGGAACAAGACCACAAAA GAAGCTGAAGAGCAGGAAAAGATCA CCTTCAACTCTCGCGAGGAGCTGAAG ACCGGCGTGTGA (SEQ ID NO: 10)	ATAGAATT GAAAG (SEQ ID NO: 11)
Csy4 / *Pseudomonas aeruginosa*	MDHYLDIRLRPDPE FPPAQLMSVLFGKL HQALVAQGGDRIG VSFPDLDESRSRLG ERLRIHASADDLRA LLARPWLEGLRDH LQFGEPAVVPHPTP YRQVSRVQAKSNP ERLRRRLMRRHDL SEEEARKRIPDTVA RALDLPFVTLRSQS TGQHFRLFIRHGPL QVTAEEGGFTCYG LSKGGFVPWF (SEQ ID NO: 12)	ATGGACCACTATCTGGACATCAGACT GAGGCCCGATCCTGAGTTCCCTCCCG CCCAGCTGATGAGCGTGCTGTTTGGC AAGCTGCATCAGGCTCTGGTCGCCCA AGGCGGAGACAGAATCGGCGTGTCCT TCCCCGACCTGGACGAGTCCCGGAGT CGCCTGGGCGAGCGGCTGAGAATCCA CGCCAGCGCAGACGATCTGCGCGCCC TGCTGGCCCGGCCTTGGCTGGAGGGC CTGCGGGATCATCTGCAGTTTGGCGA GCCCGCCGTGGTGCCACACCCAACAC CCTACCGCCAGGTGAGCCGCGTGCAG GCCAAGTCAAATCCCGAGAGACTGCG GCGGAGGCTGATGAGGCGACATGATC TGAGCGAGGAGGAGGCCAGAAAGAG AATCCCCGACACAGTGGCCAGAGCCC TGGATCTGCCATTTGTGACCCTGCGGA GCCAGAGCACTGGCCAGCATTTCAGA CTGTTCATCAGACACGGGCCCCTGCA GGTGACAGCCGAGGAGGGCGGATTTA CATGCTATGGCCTGTCTAAAGGCGGC TTCGTGCCCTGGTTCTGA (SEQ ID NO: 13)	GTTCACTG CCGTATAG GCAGCTAA GAAA (SEQ ID NO: 14)
CasE / Escherichia coli	MYLSKIIIARAWSR DLYQLHQELWHLF PNRPDAARDFLFHV EKRNTPEGCHVLL QSAQMPVSTAVAT VIKTKQVEFQLQVG	ATGTACCTCAGTAAGATCATCATCGC CCGCGCTTGGTCCCGTGACCTGTACCA ACTGCACCAAGAGCTCTGGCACCTCT TCCCCAACAGGCCAGATGCCGCTAGA GACTTCCTGTTCCACGTGGAGAAGCG TAACACCCCCGAAGGGTGCCACGTGC	GAGTTCCC CGCGCCAG CGGGGATA AACCG (SEQ ID NO: 17)

	VPLYFRLRANPIKTI LDNQKRLDSKGNI KRCRVPLIKEAEQI AWLQRKLGNAAR VEDVHPISERPQYF SGEGKNGKIQTVCF EGVLTINDAPALID LLQQGIGPAKSMG CGLLSLAPL (SEQ ID NO: 15)	TGTTGCAGAGTGCCCAGATGCCAGTG AGTACCGCTGTTGCCACTGTCATCAA GACTAAACAAGTTGAATTCCAACTGC AAGTGGGCGTCCCTCTGTATTTCCGCC TCAGGGCCAACCCCATCAAAACCATC CTGGACAACCAGAAGCGGCTGGATAG CAAAGGTAATATCAAGAGATGCCGCG TGCCTCTGATCAAGGAGGCCGAGCAG ATCGCTTGGCTGCAACGCAAGCTGGG TAACGCCGCGAGAGTGGAAGATGTGC ACCCAATCTCCGAGCGCCCGCAGTAT TTCTCCGGGGAGGGGAAGAACGGCAA AATTCAGACTGTCTGCTTCGAGGGGG TGCTCACTATTAACGACGCCCCTGCTC TGATCGACCTCCTGCAGCAGGGCATT GGGCCCGCGAAGAGCATGGGATGCGG ATTGTTGAGCCTGGCACCCCTG (SEQ ID NO: 16)	
Cse3/ Thermus thermophilus	MWLTKLVLNPASR AARRDLANPYEMH RTLSKAVSRALEEG RERLLWRLEPARG LEPPVVLVQTLTEP DWSVLDEGYAQVF PPKPFHPALKPGQR LRFRLRANPAKRLA ATGKRVALKTPAE KVAWLERRLEEGG FRLLEGERGPWVQI LQDTFLEVRRKKD GEEAGKLLQVQAV LFEGRLEVVDPERA LATLRRGVGPGKA LGLGLLSVAP (SEQ ID NO: 18)	ATGTGGTTGACCAAATTGGTTCTGAAT CCTGCGAGCCGCGCAGCACGGCGCGA TTTGGCTAACCCTTACGAGATGCATCG GACTCTTTCAAAAGCGGTTAGCAGGG CTTTGGAAGAAGGGCGCGAGCGCCTT TTGTGGAGGCTGGAGCCAGCTCGGGG ACTGGAGCCCCCTGTCGTCCTGGTGC AGACCCTCACTGAGCCTGATTGGTCC GTACTTGATGAAGGTTACGCACAGGT CTTTCCTCCTAAGCCTTTCCACCCAGC ATTGAAGCCGGGCCAGCGGCTCCGCT TTAGGCTCCGGGCGAATCCCGCCAAA CGGTTGGCTGCCACCGGAAAGCGAGT TGCGTTGAAAACGCCCGCCGAAAAAG TGGCGTGGCTTGAGAGGCGGCTGGAG GAGGGTGGTTTTCGACTCCTTGAAGG GGAAAGGGGACCATGGGTACAGATAC TTCAAGATACGTTCCTGGAGGTGCGG AGAAAAAAAGACGGAGAAGAGGCAG GCAAGCTGCTTCAAGTCCAAGCCGTC TTGTTCGAGGGGAGACTCGAAGTTGT TGATCCTGAGAGAGCACTTGCGACAC TGAGACGAGGGGTGGGACCTGGTAAA GCGCTGGGTCTTGGACTTCTTAGTGTT GCACCATGA (SEQ ID NO: 19)	GTAGTCCC CACGCGTG TGGGGATG GACCG (SEQ ID NO: 20)

In some embodiments, the RNA cleaver is a ribozyme (*e.g.*, a *cis*-acting ribozyme or a *trans*-acting ribozyme). A "ribozyme" is an RNA molecule that is capable of catalyzing specific biochemical reactions, similar to the action of protein enzymes. In some embodiments, the ribozyme is a *cis*-acting ribozyme. A "*cis*-acting ribozyme" refers to a ribozyme that catalyzes self-cleavage (intramolecular or "in-*cis*" catalysis) from the RNA molecule that contains the ribozyme itself. In these instances, the cleavage site for the RNA cleaver in the RNA transcript of the present disclosure comprises the *cis*-acting ribozyme,

which upon cleavage, excises itself and leaving two separated fragments of the RNA transcript. In some embodiments, the ribozyme is a trans-acting ribozyme. A "trans-acting ribozyme," as used herein, refers to a ribozyme that cleaves an external substrate in a specific-manner. In these instances, the cleavage site for the RNA cleaver in the RNA transcript of the present disclosure comprises the recognition and cleavage sites for the trans-acting ribozyme. Suitable ribozymes that may be used in accordance with the present disclosure and their respective sequences include, without limitation: RNase P, hammerhead ribozymes, Hepatitis delta virus ribozymes, hairpin ribozymes, twister ribozymes, twister sister ribozymes, pistol ribozymes, hatchet ribozymes, glmS ribozymes, varkud satellite ribozymes, and spliceozyme. Naturally occurring ribozymes may be used. Further, ribozymes engineered such that they cleave their substrates in cis or in trans, *e.g.*, as described in Carbonell *et al. Nucleic Acids Res.* 2011 Mar; 39(6): 2432–2444, incorporated herein by reference. Minimal ribozymes (*i.e.*, the minimal sequence a ribozyme needs for its function, *e.g.*, as described in Scott *et al.*, *Prog Mol Biol Transl Sci.* 2013; 120: 1–23, incorporated herein by reference) may also be used in accordance with the present disclosure. Non-limiting, exemplary ribozymes and their sequences are provided in Table 2.

Table 2. Non-limiting, Exemplary Ribozymes and Sequences

Ribozyme	*Cis* or *trans*	Nucleotide sequence
Hammerhead	*Cis*	CACCACGAACCTGATGAGTCCGTGAGGACGAAACGAGCTAGCTCGTCGTTC GTGCTG (SEQ ID NO: 21)
Schistosoma-like hammerhead ribozyme	*Cis*	GGCGTCGGAGTATCCAATCAGTGGATGTACTACTCCCTGATGAGTCCCAAAT AGGACGAAACGCC (SEQ ID NO: 22)
Satellite virus hammerhead ribozyme	*Cis*	GGGTGCCCTGTCGGAGGATGTGCTTTCCTCCCTGATGAGTCCGTGAGGACGA AACAGGGCACCC (SEQ ID NO: 23)
Hepatitis delta virus ribozymes	*Cis*	GGCCGGCATGGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATGCTTC GGCATGGCGAATGGGAC (SEQ ID NO: 24)
RNase P	*trans*	ATAGGGCGGAGGGAAGCTCATCAGTGGGGCCACGAGCTGAGTGCGTCCTGT CACTCCACTCCCATGTCCCTTGGGAAGGTCTGAGACTAGGGCCAGAGGCGG CCCTAACAGGGCTCTCCCTGAGCTTCGGGGAGGTGAGTTCCCAGAGAACGG GGCTCCGCGCGAGGTCAGACTGGGCAGGAGATGCCGTGGACCCCGCCCTTC GGGGAGGGGCCCGGCGGATGCCTCCTTTGCCGGAGCTTGGAACAGACTCAC GGCCAGCGAAGTGAGTTCAATGGCTGAGGTGAGGTACCCCGCAGGGGACCT CATAACCCAATTCAGACTACTCTCCTCCGCCCATT (SEQ ID NO: 25) Exemplary RNase P recognition sequence: GACGCTGGTGGCTGGCACTCCTGGTTTCCAGGACGGGGTTCAAGTCCCTGCG GTGTCT (SEQ ID NO: 26)

In some embodiments, the RNA cleaver is an RNA interference (RNAi) molecule. An "RNAi molecule" refers to an RNA molecule that inhibit gene expression or translation, by recruiting RNA degradation factors to targeted mRNA molecules to degrade the mRNA. As the RNA cleaver of the present disclosure, RNAi molecules do not directly cleave the RNA transcript, but rather binds to their target sites in the mRNA transcript and induces cleavage of the RNA transcript by the RNA-induced silencing complex (RISC), which contains multiple proteins that can cleave and degrade the RNA transcript. Non-limiting examples of RNAi molecules include: microRNAs, small interfering RNAs (siRNA), and short hairpin RNAs (shRNA). These RNAi molecules vary in their origin and structure, but function in a similar manner in cleaving their target RNA transcript and gene silencing.

A "microRNA" or "miRNA" is a small non-coding RNA molecule that functions in RNA silencing and post-transcriptional regulation of gene expression (*e.g.*, as described in Ambros *et al.*, *Nature* 431 (7006): 350–5, 2004; and Bartel *et al.*, *Cell.* 136 (2): 215–33, 2004). A microRNA may be 15-30 nucleotides in length. For example, a microRNA may be 15-30, 15-25, 15-20, 20-30, 20-25, or 25-30 nucleotides in length. In some embodiments, a microRNA may be 16-24 nucleotides in length. In some embodiments, a microRNA may be 20-24 nucleotides in length. In some embodiments, a microRNA may be 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

The microRNAs that may be used as the RNA cleavers of the present disclosure may be naturally occurring or synthetic. Information about the sequences, origins, and functions of known microRNAs maybe found in publically available databases (*e.g.*, mirbase.org/, all versions, as described in Kozomara *et al.*, *Nucleic Acids Res* 2014 42:D68-D73; Kozomara *et al.*, *Nucleic Acids Res* 2011 39:D152-D157; Griffiths-Jones *et al.*, *Nucleic Acids Res* 2008 36:D154-D158; Griffiths-Jones *et al.*, *Nucleic Acids Res* 2006 34:D140-D144; and Griffiths-Jones *et al.*, *Nucleic Acids Res* 2004 32:D109-D111, including the most recently released version miRBase 21, which contains "high confidence" microRNAs). Non-limiting examples of microRNAs that may be used as the RNA cleaver of the present disclosure are: FF4, FF5, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, miR-100, miR-103, miR-106a, miR-107, miR-10a, miR-10b, miR-122, miR-125a, miR-125b, miR-126, miR-126*, miR-127-3p, miR-128a, miR-129, miR-133b, miR-135b, miR-137, miR-141, miR-143, miR-145, miR-146a, miR-146b, miR-148a, miR-149, miR-150, miR-155, miR-15a, miR-17-3p, miR-17-5p, miR-181a, miR-

181b, miR-181c, miR-183, miR-184, miR-186, miR-187, miR-189, miR-18a, miR-190, miR-191, miR-192, miR-195, miR-197, miR-199a, miR-199a*, miR-19a, miR-19b, miR-200a, miR-200a*, miR-200b, miR-200c, miR-202, miR-203, miR-205, miR-20a, miR-21, miR-210, miR-216, miR-218, miR-22, miR-221, miR-222, miR-223, miR-224, miR-23a, miR-23b, miR-24, miR-25, miR-26a, miR-26b, miR-27a, miR-27b, miR-29a, miR-29b, miR-296-5p, miR-301, miR-302a, miR-302a*, miR-30a, miR-30b, miR-30c, miR-30d, miR-30e-3p, miR-30e-5p, miR-31, miR-320, miR-323, miR-324-5p, miR-326, miR-330, miR-331, miR-335, miR-346, miR-34a, miR-370, miR-372, miR-373, miR-373*, miR-497, miR-498, miR-503, miR-92, miR-93, miR-96, and miR-99a.

In some embodiments, the microRNA used as the RNA cleaver is selected from: hsa-let-7a-2-3p, hsa-let-7a-3p, hsa-let-7a-5p, hsa-let-7b-3p, hsa-let-7b-5p, hsa-let-7c-5p, hsa-let-7d-3p, hsa-let-7d-5p, hsa-let-7e-3p, hsa-let-7e-5p, hsa-let-7f-1-3p, hsa-let-7f-2-3p, hsa-let-7f-5p, hsa-let-7g-3p, hsa-let-7g-5p, hsa-let-7i-5p, hsa-miR-1, hsa-miR-1-3p, hsa-miR-1-5p, hsa-miR-100-3p, hsa-miR-100-5p, hsa-miR-101-3p, hsa-miR-101-5p, hsa-miR-103a-2-5p, hsa-miR-103a-3p, hsa-miR-105-3p, hsa-miR-105-5p, hsa-miR-106a-3p, hsa-miR-106a-5p, hsa-miR-106b-3p, hsa-miR-106b-5p, hsa-miR-107, hsa-miR-10a-3p, hsa-miR-10a-5p, hsa-miR-10b-3p, hsa-miR-10b-5p, hsa-miR-1185-1-3p, hsa-miR-1185-2-3p, hsa-miR-1185-5p, hsa-miR-122a-5p, hsa-miR-1249-3p, hsa-miR-1249-5p, hsa-miR-124a-3p, hsa-miR-125a-3p, hsa-miR-125a-5p, hsa-miR-125b-1-3p, hsa-miR-125b-2-3p, hsa-miR-125b-5p, hsa-miR-126-3p, hsa-miR-126-5p, hsa-miR-127-3p, hsa-miR-1271-3p, hsa-miR-1271-5p, hsa-miR-1278, hsa-miR-128-1-5p, hsa-miR-128-2-5p, hsa-miR-128-3p, hsa-miR-1285-3p, hsa-miR-1285-5p, hsa-miR-1287-3p, hsa-miR-1287-5p, hsa-miR-129-1-3p, hsa-miR-129-2-3p, hsa-miR-129-5p, hsa-miR-1296-3p, hsa-miR-1296-5p, hsa-miR-1304-3p, hsa-miR-1304-5p, hsa-miR-1306-3p, hsa-miR-1306-5p, hsa-miR-1307-3p, hsa-miR-1307-5p, hsa-miR-130a-3p, hsa-miR-130b-3p, hsa-miR-130b-5p, hsa-miR-132-3p, hsa-miR-132-5p, hsa-miR-133a-3p, hsa-miR-133a-5p, hsa-miR-133b, hsa-miR-134-3p, hsa-miR-134-5p, hsa-miR-135a-3p, hsa-miR-135a-5p, hsa-miR-135b-3p, hsa-miR-135b-5p, hsa-miR-136-3p, hsa-miR-136-5p, hsa-miR-138-1-3p, hsa-miR-138-5p, hsa-miR-139-3p, hsa-miR-139-5p, hsa-miR-140-3p, hsa-miR-140-5p, hsa-miR-141-3p, hsa-miR-141-5p, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-143-3p, hsa-miR-143-5p, hsa-miR-144-3p, hsa-miR-144-5p, hsa-miR-145-5p, hsa-miR-146a-3p, hsa-miR-146a-5p, hsa-miR-147a, hsa-miR-148a-3p, hsa-miR-148a-5p, hsa-miR-148b-3p, hsa-miR-148b-5p, hsa-miR-149-3p, hsa-miR-144-3p, hsa-miR-150-3p, hsa-miR-150-5p, hsa-miR-151a-3p, hsa-miR-

151a-5p, hsa-miR-152-3p, hsa-miR-152-5p, hsa-miR-154-3p, hsa-miR-154-5p, hsa-miR-155-3p, hsa-miR-155-5p, hsa-miR-15a-3p, hsa-miR-15a-5p, hsa-miR-15b-3p, hsa-miR-15b-5p, hsa-miR-16-1-3p, hsa-miR-16-2-3p, hsa-miR-16-5p, hsa-miR-17-3p, hsa-miR-17-5p, hsa-miR-181a-3p, hsa-miR-181a-5p, hsa-miR-181b-2-3p, hsa-miR-181b-5p, hsa-miR-181c-5p, hsa-miR-181d-3p, hsa-miR-181d-5p, hsa-miR-182-3p, hsa-miR-182-5p, hsa-miR-183-3p, hsa-miR-183-5p, hsa-miR-185-3p, hsa-miR-185-5p, hsa-miR-186-3p, hsa-miR-186-5p, hsa-miR-188-3p, hsa-miR-188-5p, hsa-miR-18a-3p, hsa-miR-18a-5p, hsa-miR-18b-5p, hsa-miR-1908-3p, hsa-miR-1908-5p, hsa-miR-190a-3p, hsa-miR-190a-5p, hsa-miR-191-3p, hsa-miR-191-5p, hsa-miR-1910-3p, hsa-miR-1910-5p, hsa-miR-192-3p, hsa-miR-192-5p, hsa-miR-193a-3p, hsa-miR-193a-5p, hsa-miR-193b-3p, hsa-miR-193b-5p, hsa-miR-194-3p, hsa-miR-194-5p, hsa-miR-195-3p, hsa-miR-195-5p, hsa-miR-196a-3p, hsa-miR-196a-5p, hsa-miR-196b-3p, hsa-miR-196b-5p, hsa-miR-197-3p, hsa-miR-197-5p, hsa-miR-199a-3p, hsa-miR-199a-5p, hsa-miR-199b-3p, hsa-miR-199b-5p, hsa-miR-19a-3p, hsa-miR-19a-5p, hsa-miR-19b-1-5p, hsa-miR-19b-2-5p, hsa-miR-19b-3p, hsa-miR-200a-3p, hsa-miR-200a-5p, hsa-miR-200b-3p, hsa-miR-200b-5p, hsa-miR-200c-3p, hsa-miR-200c-5p, hsa-miR-202-3p, hsa-miR-202-5p, hsa-miR-203a-3p, hsa-miR-203a-5p, hsa-miR-204-5p, hsa-miR-208b-3p, hsa-miR-208b-5p, hsa-miR-20a-3p, hsa-miR-20a-5p, hsa-miR-20b-3p, hsa-miR-20b-5p, hsa-miR-21-5p, hsa-miR-210-3p, hsa-miR-210-5p, hsa-miR-211-3p, hsa-miR-211-5p, hsa-miR-2116-3p, hsa-miR-2116-5p, hsa-miR-212-3p, hsa-miR-214-3p, hsa-miR-215-5p, hsa-miR-217, JG_miR-218-1-3p, hsa-miR-218-5p, hsa-miR-219a-1-3p, hsa-miR-219a-2-3p, hsa-miR-219a-5p, hsa-miR-219b-3p, hsa-miR-219b-5p, hsa-miR-22-3p, hsa-miR-22-5p, hsa-miR-221-3p, hsa-miR-221-5p, hsa-miR-222-3p, hsa-miR-222-5p, hsa-miR-223-3p, hsa-miR-223-5p, hsa-miR-23a-3p, hsa-miR-23a-5p, hsa-miR-23b-3p, hsa-miR-24-1-5p, hsa-miR-25-3p, hsa-miR-25-5p, hsa-miR-26a-1-3p, hsa-miR-26a-2-3p, hsa-miR-26a-5p, hsa-miR-26b-5p, hsa-miR-27a-3p, hsa-miR-27a-5p, hsa-miR-27b-3p, hsa-miR-27b-5p, hsa-miR-28-3p, hsa-miR-28-5p, hsa-miR-296-3p, hsa-miR-296-5p, hsa-miR-299-3p, hsa-miR-299-5p, hsa-miR-29a-3p, hsa-miR-29a-5p, hsa-miR-29b-1-5p, hsa-miR-29b-3p, hsa-miR-29c-3p, hsa-miR-301a-3p, hsa-miR-301a-5p, hsa-miR-301b-3p, hsa-miR-301b-5p, hsa-miR-302a-3p, hsa-miR-302a-5p, hsa-miR-302b-5p, hsa-miR-302c-3p, hsa-miR-302c-5p, hsa-miR-3065-3p, hsa-miR-3065-5p, hsa-miR-3074-3p, hsa-miR-3074-5p, hsa-miR-30a-3p, hsa-miR-30a-5p, hsa-miR-30b-3p, hsa-miR-30b-5p, hsa-miR-30c-1-3p, hsa-miR-30c-2-3p, hsa-miR-30c-5p, hsa-miR-30d-3p, hsa-miR-30d-5p, hsa-miR-30e-3p, hsa-miR-30e-5p, hsa-miR-31-3p, hsa-miR-31-5p, hsa-miR-3130-3p, hsa-miR-

3130-5p, hsa-miR-3140-3p, hsa-miR-3140-5p, hsa-miR-3144-3p, hsa-miR-3144-5p, hsa-miR-3158-3p, hsa-miR-3158-5p, hsa-miR-32-3p, hsa-miR-32-5p, hsa-miR-320a, hsa-miR-323a-3p, hsa-miR-323a-5p, hsa-miR-324-3p, hsa-miR-324-5p, hsa-miR-326, hsa-miR-328-3p, hsa-miR-328-5p, hsa-miR-329-3p, hsa-miR-329-5p, hsa-miR-330-3p, hsa-miR-330-5p, hsa-miR-331-3p, hsa-miR-331-5p, hsa-miR-335-3p, hsa-miR-335-5p, hsa-miR-337-3p, hsa-miR-337-5p, hsa-miR-338-3p, hsa-miR-338-5p, hsa-miR-339-3p, hsa-miR-339-5p, hsa-miR-33a-3p, hsa-miR-33a-5p, hsa-miR-33b-3p, hsa-miR-33b-5p, hsa-miR-340-3p, hsa-miR-340-5p, hsa-miR-342-3p, hsa-miR-342-5p, hsa-miR-345-3p, hsa-miR-345-5p, hsa-miR-34a-3p, hsa-miR-34a-5p, hsa-miR-34b-3p, hsa-miR-34b-5p, hsa-miR-34c-3p, hsa-miR-34c-5p, hsa-miR-3605-3p, hsa-miR-3605-5p, hsa-miR-361-3p, hsa-miR-361-5p, hsa-miR-3613-3p, hsa-miR-3613-5p, hsa-miR-3614-3p, hsa-miR-3614-5p, hsa-miR-362-3p, hsa-miR-362-5p, hsa-miR-363-3p, hsa-miR-363-5p, hsa-miR-365a-3p, hsa-miR-365a-5p, hsa-miR-365b-3p, hsa-miR-365b-5p, hsa-miR-369-3p, hsa-miR-369-5p, hsa-miR-370-3p, hsa-miR-370-5p, hsa-miR-374a-3p, hsa-miR-374a-5p, hsa-miR-374b-3p, hsa-miR-374b-5p, hsa-miR-375, hsa-miR-376a-2-5p, hsa-miR-376a-3p, hsa-miR-376a-5p, hsa-miR-376c-3p, hsa-miR-376c-5p, hsa-miR-377-3p, hsa-miR-377-5p, hsa-miR-378a-3p, hsa-miR-378a-5p, hsa-miR-379-3p, hsa-miR-379-5p, hsa-miR-381-3p, hsa-miR-381-5p, hsa-miR-382-3p, hsa-miR-382-5p, hsa-miR-409-3p, hsa-miR-409-5p, hsa-miR-411-3p, hsa-miR-411-5p, hsa-miR-412-3p, hsa-miR-421, hsa-miR-423-3p, hsa-miR-423-5p, hsa-miR-424-3p, hsa-miR-424-5p, hsa-miR-425-3p, hsa-miR-425-5p, hsa-miR-431-3p, hsa-miR-431-5p, hsa-miR-432-5p, hsa-miR-433-3p, hsa-miR-433-5p, hsa-miR-449a, hsa-miR-449b-5p, hsa-miR-450a-1-3p, hsa-miR-450a-2-3p, hsa-miR-450a-5p, hsa-miR-450b-3p, hsa-miR-450b-5p, hsa-miR-451a, hsa-miR-452-3p, hsa-miR-4524a-3p, hsa-miR-4524a-5p, hsa-miR-4536-3p, hsa-miR-4536-5p, hsa-miR-454-3p, hsa-miR-454-5p, hsa-miR-4707-3p, hsa-miR-4707-5p, hsa-miR-4755-3p, hsa-miR-4755-5p, hsa-miR-4787-3p, hsa-miR-4787-5p, hsa-miR-483-3p, hsa-miR-483-5p, hsa-miR-484, hsa-miR-485-3p, hsa-miR-485-5p, hsa-miR-487b-3p, hsa-miR-487b-5p, hsa-miR-488-3p, hsa-miR-488-5p, hsa-miR-489-3p, hsa-miR-490-3p, hsa-miR-490-5p, hsa-miR-491-3p, hsa-miR-491-5p, hsa-miR-493-3p, hsa-miR-493-5p, hsa-miR-494-3p, hsa-miR-494-5p, hsa-miR-495-3p, hsa-miR-495-5p, hsa-miR-497-3p, hsa-miR-497-5p, hsa-miR-498, hsa-miR-5001-3p, hsa-miR-5001-5p, hsa-miR-500a-3p, hsa-miR-500a-5p, hsa-miR-5010-3p, hsa-miR-5010-5p, hsa-miR-503-3p, hsa-miR-503-5p, hsa-miR-504-3p, hsa-miR-504-5p, hsa-miR-505-3p, hsa-miR-505-5p, hsa-miR-506-3p, hsa-miR-506-5p, hsa-miR-508-3p, hsa-miR-508-5p, hsa-miR-509-3-5p, hsa-miR-509-3p, hsa-miR-509-5p,

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miR-942-3p, hsa-miR-942-5p, hsa-miR-96-3p, hsa-miR-96-5p, hsa-miR-98-3p, hsa-miR-98-5p, hsa-miR-99a-3p, hsa-miR-99a-5p, hsa-miR-99b-3p, and hsa-miR-99b-5p.

A "siRNA" is a class of double-stranded RNA molecules, which, similar to miRNA, interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription. A siRNA may be 20-25 base pairs (*e.g.*, 20, 21, 22, 23, 24, or 25 base pairs) in length. Typical, siRNA is a synthetic RNA molecule that may be signed to target any target genes of interest.

A "shRNA" an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNAi. Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors. shRNA is an advantageous mediator of RNAi in that it has a relatively low rate of degradation and turnover.

In some embodiments, the cleavage site for the RNA cleaver in the RNA transcript of the present disclosure comprises one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) target sites for the RNAi molecule.

A "target site for an RNAi molecule (*e.g.*, a microRNA, siRNA, or shRNA)" is a nucleotide sequence that is complementary to a core nucleotide sequence (the sequence that binds to the target) of the RNAi molecule. Naturally, microRNA targeting sites exist in messenger RNAs (mRNA), typically in the 3' untranslated regions of mRNAs. Binding of the microRNA to its target site in via sequence complementarity leads to silencing of an output molecule either via degrading the mRNA or suppressing translation of the mRNA (*e.g.*, as described in Bartel *et al.*, *Cell* 136 (2): 215–33 (2009), incorporated herein by reference) containing the microRNA binding sites. Herein, when microRNA target sites are referred in the context of DNA, it means the nucleotide sequence that encodes the microRNA target sites in the mRNA that is produced from the genetic circuit. Non-limiting, exemplary microRNA and respective target site sequences are provided in Table 3.

Table 3. Non-limiting, Exemplary Synthetic microRNA and Target Sites

microRNA Name	Nucleotide Sequence Encoding microRNA	SEQ ID NO:	Target Sequence	SEQ ID NO:
FF3	TTTGTATTCAGCCCATATCG	27	AACGATATGGGCTGAATACAAA	32
FF4	TTTAATTAAAGACTTCAAGCG	28	CCGCTTGAAGTCTTTAATTAAA	33
FF5	TAATTGTCAAATCAGAGTGC	29	AAGCACTCTGATTTGACAATTA	34
FF6	TTTATGAGGAATCTCTTTGG	30	AACCAAAGAGATTCCTCATAAA	35
T1	TTCGAAGTATTCCGCGTACG	31	CACGTACGCGGAATACTTCGAA	36

In some embodiments, the RNA cleaver is naturally expressed in the context where cleavage-induced transcript stabilizer is used. For example, the cleavage-induced transcript stabilizer may be used in a cell, and the cell naturally expresses any of the RNA cleavers described herein (*e.g.*, microRNA, endoribonuclease, or ribozyme). In some embodiments, the RNA cleaver is not naturally expressed but is provided (*e.g.*, to a cell) via any known methods in the art, *e.g.*, transfection of a microRNA, siRNA, or ribozyme, delivering of a nucleic acid encoding any of the RNA cleavers. Accordingly, in some embodiments, the cleavage-induced transcript stabilizer further comprises a second promoter operably linked to a nucleotide sequence encoding an RNA cleaver that cleaves at the cleavage site in the RNA transcript. In some embodiments, the second promoter may be a constitutive promoter. In some embodiments, the second promoter is an inducible promoter. In some embodiments, the expression of the RNA cleaver is coupled with an upstream signal, *e.g.*, an environment signal or a cellular event, such that the cleavage of the RNA transcript and the expression of the output molecule can be used to "sense" the signal.

As described herein, cleavage of the RNA transcript by the RNA cleaver removes the degradation signal from the RNA transcript, which in turn stabilizes the RNA transcript. However, cleavage of the RNA transcript generates RNA fragments with free and unprotected 3′ ends (in the 5′ fragment) and 5′ ends (in the 3′ fragment), which are rapidly degraded if unprotected. The present disclosure further provides strategies of protecting the 5′ fragment of the RNA transcript containing the nucleotide sequence encoding the output molecule. In some embodiments, the RNA transcript of the present disclosure further comprises an RNA stabilizer between the nucleotide sequence encoding the output molecule and the cleavage site for the RNA cleaver.

An "RNA stabilizer," refers to an RNA sequence that, when present in an RNA molecule (*e.g.*, at the 5′ end or 3′ end), protects the RNA molecule from degradation. In some embodiments, the RNA stabilizer sequence forms secondary structures that blocks access of exoribonucleases to the unprotected ends of the RNA molecule. The RNA stabilizer of the present disclosure is at the 3′ end of the 5′ fragment (the fragment that contains the nucleotide sequence encoding the output molecule) and prevents degradation of the 5′ fragment. Non-limiting examples of RNA stabilizers that may be used in accordance with the present disclosure include: synthetic poly-adenylated tails, and stabilizing RNA triple helix structures such as MALAT1 (*e.g.*, as described in Brown *et al.*, *Nature Structural & Molecular Biology*

21, 633–640, 2014, incorporated herein by reference), MENβ triplex, KSHV PAN triplex, and histone stem loop. The nucleotide sequences of non-limiting, exemplary RNA stabilizer sequences are provided in Table 4.

Table 4. Non-limiting, Exemplary RNA Stabilizers

3′ RNA stabilizer	Nucleotide sequence
MALAT1	GAUUCGUCAGUAGGGUUGUAAAGGUUUUUCUUUUCCUGAGAAAACAA CCUUUUGUUUUCUCAGGUUUUGCUUUUUGGCCUUUCCCUAGCUUUAA AAAAAAAAAAGCAAAA (SEQ ID NO: 37)
MENβ triplex	GCCGCCGCAGGUGUUUCUUUUACUGAGUGCAGCCCAUGGCCGCACUC AGGUUUUGCUUUUCACCUUCCCAUCUG (SEQ ID NO: 38)
KSHV PAN triplex	GCUGGGUUUUUCCUUGUUCGCACCGGACACCUCCAGUGACCAGACGG CAAGGUUUUAUCCCAGU (SEQ ID NO: 39)
histone stem loop	AAAAAGGCUCUUUUCAGAGCACCCA (SEQ ID NO: 40)

The RNA stabilizer stabilizes the RNA fragment containing nucleotide sequence encoding the output molecule, generated by cleavage of the RNA transcript by the RNA cleaver. An RNA fragment is considered to be stabilized when the half-life of the RNA fragment is at least 20% longer with of the RNA stabilizer, compared to without the RNA stabilizer. For example, an RNA fragment is considered to be stabilized when the half-life of the RNA fragment is increased by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 2-fold, at least 5-fold, at least 10-fold, at least 50-fold, at least 100-fold or more, compared to without the RNA stabilizer. In some embodiments, the half-life of the RNA fragment is increased by 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 5-fold, 10-fold, 50-fold, 100-fold or more, with the RNA stabilizer, compared to without the RNA stabilizer.

In some embodiments, the stabilizer further contributes to the stabilization of the RNA fragment containing nucleotide sequence encoding the output molecule, generated by cleavage of the RNA transcript by the RNA cleaver. In some embodiments, the half-life of the RNA transcript is increased by at least 30%, with the RNA stabilizer, compared to without the RNA stabilizer. For example, the half-life of the RNA transcript may be increased by at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 2-fold, at least 5-fold, at least 10-fold, at least 50-fold, at least 100-fold or more, with the RNA stabilizer, compared to without the RNA stabilizer. In some embodiments, the half-life of the RNA fragment is increased by 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%,

2-fold, 5-fold, 10-fold, 50-fold, 100-fold or more, with the RNA stabilizer, compared to without the RNA stabilizer.

In some embodiments, stabilization of the RNA transcript leads to increased expression of the output molecule. In some embodiments, the expression level of the output molecule is increased by at least 20%, when the degradation signal is cleaved, compared to before it was cleaved. For example, the expression level of the output molecule may be increased by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 2-fold, at least 5-fold, at least 10-fold, at least 50-fold, at least 100-fold or more, when the degradation signal is cleaved, compared to before it was cleaved. In some embodiments, the expression level of the output molecule is increased by 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 5-fold, 10-fold, 50-fold, 100-fold or more, when the degradation signal is cleaved, compared to before it was cleaved.

In some embodiments, additional regulatory elements and genetic circuits are added to the cleavage-induced transcript stabilizer described herein to enhance its performance (*e.g.*, sensitivity). For example, the expression of the output molecule may further be repressed by an RNA repressor. An "RNA repressor," as used herein, refers to a protein that inhibits the expression of the output molecule. Inhibition of output molecule expression may be achieved via different methods. For example, one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) recognition sites of an RNA binding protein may be placed upstream of and are operably linked to the nucleotide sequence encoding the output molecule, and binding of RNA binding proteins to the recognition sites can block translation. The one or more recognition sites of the RNA binding protein are "operably linked to" the nucleotide sequence encoding the output molecule, when binding of the RNA binding protein to the recognition sites can inhibit the expression of the output molecule.

In some embodiments, the RNA repressor is an RNA binding protein. An "RNA binding protein," as used herein, refers to a protein that binds to an RNA molecule. The binding of an RNA binding protein to RNA may be dependent on the RNA sequence, or the structure of the RNA. As such, the targets sites of the RNA binding protein, may comprise a specific sequence motif, or form a specific structure (*e.g.*, a stem-loop structure). Any RNA binding protein may be used as the RNA repressor of the present disclosure. Non-limiting examples of RNA binding proteins and their respective recognition site sequences are provided in Table 5.

Table 5. Non-limiting Examples of RNA Binding Proteins and Target Sites

RNA binding protein	Amino acid sequence	Gene sequence	Target site sequence
TetR	MSRLDKSKVINSALELLNE VGIEGLTTRKLAQKLGVE QPTLYWHVKNKRALLDA LAIEMLDRHHTHFCPLEGE SWQDFLRNNAKSFRCALL SHRDGAKVHLGTRPTEKQ YETLENQLAFLCQQGFSLE NALYALSAVGHFTLGCVL EDQEHQVAKEERETPTTD SMPPLLRQAIELFDHQGAE PAFLFGLELIICGLEKQLKC ESGS (SEQ ID NO: 41)	ATGTCAAGACTCGACAAGAGCAAGGTGATT AACAGTGCACTGGAACTTCTCAATGAAGTT GGGATCGAGGGGCTGACTACTAGAAAACTC GCACAGAAACTGGGGGTTGAGCAGCCCACC TTGTACTGGCACGTTAAAAACAAAAGGGCC CTGCTGGATGCTCTGGCCATCGAGATGCTGG ATAGGCATCATACCCACTTCTGCCCTCTGGA AGGAGAATCCTGGCAGGATTTCCTTAGAAA CAACGCCAAGTCCTTTCGCTGTGCTCTTCTT AGCCACCGGGATGGTGCTAAAGTCCATCTC GGCACACGACCAACTGAGAAGCAGTACGAA ACTCTCGAGAACCAGCTGGCCTTTCTCTGTC AACAGGGCTTTTCTCTTGAAAACGCCCTGTA CGCACTGAGTGCAGTTGGGCACTTTACACTC GGATGTGTTCTGGAGGACCAAGAACATCAG GTGGCAAAGGAAGAGAGGGAGACCCCTAC GACTGACTCCATGCCCCCTCTCTTGAGGCAG GCAATAGAATTGTTCGACCATCAGGGCGCA GAACCCGCCTTTCTGTTTGGGCTGGAACTGA TTATCTGCGGTCTTGAGAAACAGCTGAAGT GCGAGTCCGGGAGC (SEQ ID NO: 42)	ATCCAGG CAGAGAA AGGTCGA TACGGAC GGAATGT GGTGGCC TGGATCA ACAACAA CAAAATC CAGGCAG AGAAAGG TCGATAC GGACGGA ATGTGGT GGCCTGG ATCAACA ACAACAA CACTG (SEQ ID NO: 43)
PPR10	MLPLDSLLLHLTAPAPAPA PAPRRSHQTPTPPHSFLSP DAQVLVLAISSHPLPTLAA FLASRRDELLRADITSLLK ALELSGHWEWALALLRW AGKEGAADASALEMVVR ALGREGQHDAVCALLDET PLPPGSRLDVRAYTTVLH ALSRAGRYERALELFAEL RRQGVAPTLVTYNVVLDV YGRMGRSWPRIVALLDE MRAAGVEPDGFTASTVIA ACCRDGLVDEAVAFFEDL KARGHAPCVVTYNALLQ VFGKAGNYTEALRVLGE MEQNGCQPDAVTYNELA GTYARAGFFEEAAR (SEQ ID NO: 44)	ATGCTCCCCTTGGACAGTCTCCTGCTGCATC TCACCGCCCCCGCCCCCGCCCCAGCCCCTGC TCCAAGAAGGTCTCATCAAACGCCGACCCC CCCTCACAGCTTCCTGTCCCCTGATGCTCAG GTGTTGGTACTCGCAATCAGTTCTCACCCTC TGCCTACCCTGGCTGCTTTCCTCGCTAGCAG GCGGGATGAGTTGCTGAGGGCCGATATCAC CTCTCTCCTTAAGGCACTTGAGCTGTCTGGG CACTGGGAATGGGCATTGGCCCTGCTGCGA TGGGCAGGTAAGGAGGGAGCTGCCGATGCT AGCGCTTTGGAGATGGTCGTAAGAGCACTC GGTAGAGAAGGCCAGCATGACGCAGTCTGT GCTCTGCTGGACGAAACTCCATTGCCTCCAG GCAGCAGACTGGACGTACGGGCCTACACCA CCGTGCTTCACGCCCTCTCAAGAGCCGGTAG GTACGAGAGAGCTCTCGAGCTGTTCGCTGA ACTCAGAAGACAGGGCGTGGCCCCAACCTT GGTAACTTATAACGTGGTACTGGACGTCTAC GGCCGAATGGGGAGAAGTTGGCCGCGCATC GTCGCATTGCTCGACGAAATGCGGGCCGCA GGCGTCGAGCCAGATGGGTTTACCGCAAGC ACGGTGATCGCTGCTTGCTGCCGGGATGGTT TGGTGGATGAAGCCGTGGCCTTCTTTGAGG ACTTGAAGGCCAGGGGTCACGCACCTTGTG TCGTAACCTATAACGCACTGTTGCAGGTGTT CGGCAAGGCTGGGAATTATACTGAGGCCCT GAGAGTTCTTGGCGAAATGGAGCAGAACGG GTGCCAGCCAGATGCTGTGACATATAATGA GCTGGCCGGAACCTACGCACGCGCCGGCTT	ATTGTAT CCTTAAC CATTTCTT TTATTGTA TCCTTAA CCATTTCT TT (SEQ ID NO: 46)

		CTTTGAGGAGGCCGCCCGGTGTCTGGACAC GATGGCCAGTAAGGGCCTGCTTCCTAACGC ATTCACATACAATACCGTGATGACAGCATA TGGAAATGTGGGGAAGGTCGACGAAGCTCT CGCCCTTTTCGATCAGATGAAAAAGACTGG CTTCGTTCCCAACGTGAACACGTACAACCTG GTCCTGGGGATGCTGGGAAAGAAATCAAGA TTCACGGTAATGTTGGAAATGTTGGGCGAA ATGAGCAGGTCAGGATGTACCCCTAACAGG GTTACTTGGAATACTATGCTCGCTGTGTGTG GAAAGCGAGGGATGGAAGATTACGTGACAC GGGTTCTGGAGGGCATGCGGAGTTGCGGTG TCGAGCTGTCCCGAGACACATACAACACCC TCATCGCTGCTTATGGGAGGTGCGGTAGCC GGACAAATGCTTTTAAGATGTATAACGAAA TGACGTCCGCAGGGTTCACTCCCTGCATCAC TACATATAACGCTCTGCTGAATGTGCTCTCT CGGCAAGGAGACTGGTCCACTGCTCAGTCA ATCGTTTCAAAGATGCGGACTAAGGGCTTT AAGCCCAACGAGCAATCTTACTCACTCCTCC TGCAGTGTTACGCAAAGGGGGGCAATGTGG CAGGAATTGCAGCCATCGAAAACGAAGTTT ACGGGTCCGGCGCTGTTTTCCCATCTTGGGT GATCCTGAGGACTCTTGTAATCGCTAATTTC AAATGTCGCCGCTTGGACGGCATGGAAACT GCTTTCCAGGAGGTAAAGGCCAGGGGGTAT AATCCTGATTTGGTGATATTCAACTCAATGC TTTCCATCTACGCTAAGAATGGTATGTATAG CAAAGCAACTGAGGTCTTCGACTCAATTAA GAGGTCAGGTCTGTCCCCAGACCTTATAACT TACAATTCCTTGATGGATATGTATGCCAAGT GTAGCGAGTCCTGGGAAGCTGAAAAGATTC TTAATCAGCTGAAATGTTCCCAGACTATGAA GCCCGATGTTGTTAGCTATAATACAGTTATC AACGGATTCTGCAAACAGGGCCTTGTGAAA GAAGCCCAGAGAGTGCTGTCCGAAATGGTC GCCGACGGCATGGCTCCTTGCGCTGTGACCT ACCATACATTGGTCGGCGGCTATTCCTCTCT CGAGATGTTCTCCGAGGCCAGGGAGGTCAT CGGCTACATGGTGCAACATGGACTGAAACC TATGGAACTGACCTATAGGAGGGTGGTGGA ATCATACTGCAGAGCCAAGCGATTCGAGGA AGCTCGGGGTTTCCTGTCCGAAGTGTCTGAG ACTGATCTGGACTTCGACAAAAAAGCTTTG GAAGCATACATCGAGGACGCTCAATTTGGG CGCTA (SEQ ID NO: 45)	
MS2CP	MASNFTQFVLVDNGGTG DVTVAPSNFANGVAEWIS SNSRSQAYKVTCSVRQSS AQKRKYTIKVEVPKVATQ TVGGEELPVAGWRSYLN MELTIPIFATNSDCELIVKA MQGLLKDGNPIPSAIAANS GIY (SEQ ID NO: 47)	ATGGCTTCTAACTTTACTCAGTTCGTTCTCG TCGACAATGGCGGAACTGGCGACGTGACTG TCGCCCCAAGCAACTTCGCTAACGGGGTCG CTGAATGGATCAGCTCTAACTCGCGTTCACA GGCTTACAAAGTAACCTGTAGCGTTCGTCA GAGCTCTGCGCAGAAGCGCAAATACACCAT CAAAGTCGAGGTGCCTAAAGTGGCAACCCA GACTGTTGGTGGTGAGGAGCTTCCTGTAGCC GGTTGGCGTTCGTACTTAAATATGGAACTAA CCATTCCAATTTTCGCCACGAATTCCGACTG CGAGCTTATTGTTAAGGCAATGCAAGGCCT CCTAAAAGATGGAAACCCGATTCCCTCGGC	ACATGAG GATCACC CATGTCT GCAGGTC GACTCTA GAAAACA TGAGGAT CACCCAT GTCCTGC AGGTCGA CTCTAGA AA (SEQ ID

		CATCGCAGCAAACTCCGGCATCTAC (SEQ ID NO: 48)	NO: 49)
L7Ae	MYVRFEVPEDMQNEALSL LEKVRESGKVKKGTNETT KAVERGLAKLVYIAEDVD PPEIVAHLPLLCEEKNVPYI YVKSKNDLGRAVGIEVPC ASAAIINEGELRKELGSLV EKIKGLQK (SEQ ID NO: 50)	ATGTACGTGAGATTTGAGGTTCCTGAGGAC ATGCAGAACGAAGCTCTGAGTCTGCTGGAG AAGGTTAGGGAGAGCGGTAAGGTAAAGAA AGGTACCAACGAGACGACAAAGGCTGTGGA GAGGGGACTGGCAAAGCTCGTTTACATCGC AGAGGATGTTGACCCGCCTGAGATCGTTGC TCATCTGCCCCTCCTCTGCGAGGAGAAGAAT GTGCCGTACATTTACGTTAAAAGCAAGAAC GACCTTGGAAGGGCTGTGGGCATTGAGGTG CCATGCGCTTCGGCAGCGATAATCAACGAG GGAGAGCTGAGAAAGGAGCTTGGAAGCCTT GTGGAGAAGATTAAAGGCCTTCAGAAG (SEQ ID NO: 51)	GGGCGTG ATCCGAA AGGTGAC CCGGATC TGGGGCG TGATCCG AAAGGTG ACCCGGA TCCACCG GTC (SEQ ID NO: 52)

In some embodiments, to repress translation, the recognition sites of RNA binding proteins are placed upstream of the coding sequence. For example, in some embodiments, the one or more (*e.g.*, 1, 2, 3, 4, ,5 ,6 ,7, 8, 9, 10 or more) recognition sites of the RNA binding protein is placed immediately upstream (no spacer between them) of the nucleotide sequence encoding the output molecule. The start of the coding sequence is marked by a start codon, usually AUG. In some embodiments, the one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) recognition sites of the RNA binding protein is placed upstream of the nucleotide sequence encoding the output molecule and is separated by a ribonucleotide spacer. The ribonucleotide spacer may be 2-30 nucleotides long. For example, the ribonucleotide spacer may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides long). Shorter and longer ribonucleotide spacers may also be used. In some embodiments, the binding of RNA binding proteins to the recognition sites blocks translation. In some embodiments, translation is blocked via inhibition of translation initiation.

In some embodiments, the RNA repressor is fused to a modifying domain. A "modifying domain" as used herein, refers to a protein or polypeptide, or a functional domain thereof, that is capable of modifying a ribonucleoprotein complex formed between the RNA molecule and the RNA binding protein. The modification may be to the ribonucleotide bases (with or without changing the ribonucleotide sequence), to the structure of the RNA molecule containing the RNA binding protein target sites, or the remodeling of the ribonucleoprotein complex. Such modifying domains have been described in the art. For example, Cooke *et al.* (*J Biol Chem.* 285(37): 28506–28513, 2010, incorporated herein by reference) describes a CCR4-CAF1-NOT deadenylation complex that, when associated with RNA binding proteins, represses translation in mammalian cells. Cooke further demonstrates that CAF1 (also known

as CNOT7) represses translation independent of deadenylation. In another example, Weston *et al.* (*Nucleic Acids Res.* 34(10): 3082–3094, 2006, incorporated herein by reference) demonstrates that DEAD-box RNA helicase family proteins (*e.g.*, DDX6, Xp54, etc.) play key roles in mRNA degradation and in earlier remodeling of messenger ribonucleoprotein complexes during translation initiation. Accordingly, in some embodiments, the RNA binding protein is fused to a CNOT7 protein. In some embodiments, the RNA binding protein is fused a DEAD-box RNA helicase protein (*e.g.*, DDX6, or Xp54). The amino acid and nucleotide sequences of non-limiting, exemplary modifying domains are provided in Table 6.

Table 6. Non-limiting, Exemplary Modifying Domains for Translation Repression

Modifying domain	Amino acid sequence	Nucleotide sequence (cDNA)
CNOT7	MPAATVDHSQRICEVWACN LDEEMKKIRQVIRKYNYVA MDTEFPGVVARPIGEFRSNA DYQYQLLRCNVDLLKIIQLG LTFMNEQGEYPPGTSTWQF NFKFNLTEDMYAQDSIELLT TSGIQFKKHEEEGIETQYFAE LLMTSGVVLCEGVKWLSFH SGYDFGYLIKILTNSNLPEEE LDFFEILRLFFPVIYDVKYLM KSCKNLKGGLQEVAEQLEL ERIGPQHQAGSDSLLTGMAF FKMREMFFEDHIDDAKYCG HLYGLGSGSSYVQNGTGNA YEEEANKQSV (SEQ ID NO: 53)	ATGCCAGCGGCAACTGTAGATCATAGCCAAAGAATT TGTGAAGTTTGGGCTTGCAACTTGGATGAAGAGATG AAGAAAATTCGTCAAGTTATCCGAAAATATAATTAC GTTGCTATGGACACCGAGTTTCCAGGTGTGGTTGCA AGACCCATTGGAGAATTCAGGAGCAATGCTGACTAT CAATACCAACTATTGCGGTGTAATGTAGACTTGTTAA AGATAATTCAGCTAGGACTGACATTTATGAATGAGC AAGGAGAATACCCTCCAGGAACTTCAACTTGGCAGT TTAATTTTAAATTTAATTTGACGGAGGACATGTATGC CCAGGACTCTATAGAGCTACTAACAACATCTGGTAT CCAGTTTAAAAAACATGAGGAGGAAGGAATTGAAA CCCAGTACTTTGCAGAACTTCTTATGACTTCTGGAGT GGTCCTCTGTGAAGGGGTCAAATGGTTGTCATTTCAT AGCGGTTACGACTTTGGCTACTTAATCAAAATCCTAA CCAACTCTAACTTGCCTGAAGAAGAACTTGACTTCTT TGAGATCCTTCGATTGTTTTTTCCTGTCATTTATGATG TGAAGTACCTCATGAAGAGCTGCAAAAATCTCAAAG GTGGATTACAGGAGGTGGCAGAACAGTTAGAGCTGG AACGGATAGGACCACAACATCAGGCAGGATCTGATT CATTGCTCACAGGAATGGCCTTTTTCAAAATGAGAG AAATGTTCTTTGAAGATCATATTGATGATGCCAAATA TTGTGGTCATTTGTATGGCCTTGGTTCTGGTTCATCCT ATGTACAGAATGGCACAGGGAATGCATATGAAGAG GAAGCCAACAAGCAGTCAGTT (SEQ ID NO: 54)
DDX6	MSTARTENPVIMGLSSQNGQ LRGPVKASAGPGGGGTQPQ PQLNQLKNTSTINNGTPQQA QSMAATIKPGDDWKKTLKL PPKDLRIKTSDVTSTKGNEFE DYCLKRELLMGIFEMGWEK PSPIQEESIPIALSGRDILARA KNGTGKSGAYLIPLLERLDL KKDNIQAMVIVPTRELALQV SQICIQVSKHMGGAKVMAT TGGTNLRDDIMRLDDTVHV VIATPGRILDLIKKGVAKVD HVQMIVLDEADKLLSQDFV QIMEDIILTLPKNRQILLYSA	ATGAGCACAGCTCGCACCGAGAACCCGGTGATTATG GGCCTGTCCAGCCAGAACGGACAGCTCAGAGGGCCT GTAAAGGCTTCAGCAGGCCCCGGCGGAGGCGGCACA CAACCACAACCACAGCTTAATCAGCTTAAGAATACT AGCACTATTAATAACGGAACACCGCAGCAGGCCCAA AGCATGGCTGCCACAATTAAACCCGGAGATGACTGG AAGAAGACCCTGAAGCTCCCTCCAAAAGATCTCAGG ATTAAAACTAGCGATGTTACTTCAACAAAGGGAAAT GAGTTCGAAGACTACTGTCTGAAGCGAGAGTTGCTG ATGGGGATTTTCGAAATGGGCTGGGAGAAGCCCTCT CCTATTCAAGAAGAGAGCATCCCCATCGCTCTGTCC GGGAGGGACATCCTTGCCAGGGCTAAAAATGGGACC GGAAAATCAGGAGCTTACTTGATCCCACTCCTTGAA AGGCTTGATCTCAAGAAGGACAACATCCAAGCTATG

	TFPLSVQKFMNSHLQKPYEI NLMEELTLKGVTQYYAYVT ERQKVHCLNTLFSRLQINQSI IFCNSSQRVELLAKKISQLGY SCFYIHAKMRQEHRNRVFH DFRNGLCRNLVCTDLFTRGI DIQAVNVVINFDFPKLAETY LHRIGRSGRFGHLGLAINLIT YDDRFNLKSIEEQLGTEIKPI PSNIDKSLYVAEYHSEPAED EKP (SEQ ID NO: 55)	GTTATCGTGCCAACTAGAGAACTCGCCCTCCAGGTC AGCCAGATTTGCATCCAGGTGAGTAAGCACATGGGC GGAGCTAAGGTGATGGCTACAACTGGAGGGACTAAC CTGCGAGACGACATAATGAGACTTGATGACACAGTC CATGTGGTCATCGCTACACCTGGGAGGATTCTGGAT CTGATCAAAAAAGGAGTGGCAAAGGTGGATCATGTG CAGATGATAGTCTTGGACGAGGCCGACAAACTGCTG AGCCAAGACTTTGTGCAGATCATGGAGGATATCATC TTGACACTCCCCAAGAACCGACAGATTCTGCTGTACT CCGCAACATTTCCTCTTTCCGTTCAGAAATTCATGAA CTCACATCTCCAGAAACCTTATGAGATCAATTTGATG GAAGAACTGACACTGAAGGGCGTGACCCAGTATTAT GCCTACGTTACTGAGAGGCAAAAGGTCCACTGCCTG AATACTCTCTTCTCCAGGCTCCAGATCAACCAGTCTA TCATCTTTTGCAATAGCTCCCAGCGAGTCGAGCTGCT GGCTAAGAAGATCTCACAGCTTGGATATTCCTGTTTC TACATCCATGCTAAGATGAGACAAGAGCACAGAAAC CGCGTCTTTCATGATTTCCGGAACGGACTCTGTCGCA ACCTGGTTTGCACAGATCTTTTTACTAGAGGCATCGA TATCCAAGCAGTGAACGTGGTTATCAACTTCGACTTT CCCAAACTCGCCGAGACTTATCTTCATAGAATTGGCC GATCCGGTAGGTTTGGGCACCTGGGGCTCGCCATCA ATCTCATTACGTATGATGATAGGTTCAACCTCAAGTC AATAGAAGAGCAGTTGGGGACCGAGATCAAACCAA TCCCGAGCAATATTGACAAATCACTCTATGTGGCCG AATACCATTCAGAGCCTGCCGAGGATGAGAAGCCT (SEQ ID NO: 56)

In some embodiments, the expression of the output molecule is considered to be "repressed" by the RNA repressor if the expression of the gene is at least 20% lower in the presence of the RNA repressor, compared to without the RNA repressor. For example, the expression of the output molecule is considered to be repressed by the RNA repressor if the expression of the genes is at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99% or lower in the presence of the RNA repressor, compared to without the RNA repressor. In some embodiments, the expression of the output molecule is considered to be repressed by the RNA repressor if the expression of the genes is 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or even 100% in the presence of the RNA repressor, compared to without the RNA repressor.

In some embodiments, expression of the RNA repressor can be controlled by the RNA cleaver, *e.g.*, by incorporating one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) cleavage sites for the RNA cleaver into the transcript encoding the RNA repressor. The RNA cleaver cleaves at the cleavage sites, leading to the degradation of the transcript encoding the RNA repressor and no expression of the RNA repressor.

In the absence of the RNA cleaver ("off" state), the RNA transcript encoding the output molecule is degraded, and the RNA repressor expresses, further repressing the expression of the output molecule. This leads to very low or no expression of the output molecule. Conversely, in the presence of the RNA cleaver ("on" state), the transcript encoding the RNA repressor is cleaved and degraded, leading to no expression of the RNA repressor. Further, the RNA cleaver removes the degradation signal from the RNA transcript encoding the output molecule, stabilizing the RNA transcript and allowing expression of the output molecule. In the absence of the RNA repressor, the translation of the output molecule is not repressed, further ensuring its expression. In some embodiments, the level of the RNA repressor may be modulated (*e.g.*, by modulating the strength of the promoter that controls its expression) such that the threshold of the cleavage-induced transcript stabilizer is modulated, allowing it to detect a range of RNA cleaving activities.

An "output molecule," as used herein, refers to a signal produced by the cleavage-induced transcript stabilizer in the presence of the RNA cleaver. In some embodiments, the output molecule has a basal expression level and the expression level increases (*e.g.*, by at least 20%) when an RNA cleaver is present, compared to when the RNA cleaver is not present. For example, the expression level of the output molecule may be at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 2-fold, at least 5-fold, at least 10-fold, at least 100-fold, at least 1000-fold, or higher when the RNA cleaver is present, compared to when the RNA cleaver is not present. In some embodiments, the expression level of the output molecule is 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 5-fold, 10-fold, 100-fold, 1000-fold, or higher when an RNA cleaver is present, compared to when the RNA cleaver is not present.

The output molecule, in some embodiments, is a detectable protein. In some embodiments, a detectable protein is a fluorescent protein. A fluorescent protein is a protein that emits a fluorescent light when exposed to a light source at an appropriate wavelength (*e.g.*, light in the blue or ultraviolet range). Suitable fluorescent proteins that may be used in accordance with the present disclosure include, without limitation, eGFP, eYFP, eCFP, mKate2, mCherry, mPlum, mGrape2, mRaspberry, mGrape1, mStrawberry, mTangerine, mBanana, and mHoneydew. In some embodiments, a detectable protein is an enzyme that hydrolyzes an substrate to produce a detectable signal (*e.g.*, a chemiluminescent signal). Such enzymes include, without limitation, beta-galactosidase (encoded by LacZ), horseradish

peroxidase, or luciferase. In some embodiments, the output molecule is a fluorescent RNA. A fluorescent RNA is an RNA aptamer that emits a fluorescent light when bound to a fluorophore and exposed to a light source at an appropriate wavelength (*e.g.*, light in the blue or ultraviolet range). Suitable fluorescent RNAs that may be used as an output molecule in the sensor circuit of the present disclosure include, without limitation, Spinach and Broccoli (*e.g.*, as described in Paige *et al.*, *Science* Vol. 333, Issue 6042, pp. 642-646, 2011, incorporated herein by reference).

In some embodiments, the output molecule is a therapeutic molecule. A "therapeutic molecule" is a molecule that has therapeutic effects on a disease or condition, and may be used to treat a diseases or condition. Therapeutic molecules of the present disclosure may be nucleic acid-based or protein or polypeptide-based.

In some embodiments, nucleic acid-based therapeutic molecule may be an RNA interference (RNAi) molecule (*e.g.*, a microRNA, siRNA, or shRNA) or an nucleic acid enzyme (*e.g.*, a ribozyme). RNAi molecules and there use in silencing gene expression are familiar to those skilled in the art. In some embodiments, the RNAi molecule targets an oncogene. An oncogene is a gene that in certain circumstances can transform a cell into a tumor cell. An oncogene may be a gene encoding a growth factor or mitogen (*e.g.*, c-Sis), a receptor tyrosine kinase (*e.g.*, EGFR, PDGFR, VEGFR, or HER2/neu), a cytoplasmic tyrosine kinase (*e.g.*, Src family kinases, Syk-ZAP-70 family kinases, or BTK family kinases), a cytoplasmic serine/threonine kinase or their regulatory subunits (*e.g.*, Raf kinase or cyclin-dependent kinase), a regulatory GTPase (*e.g.*, Ras), or a transcription factor (*e.g.*, Myc). In some embodiments, the oligonucleotide targets Lipocalin (Lcn2) (*e.g.*, a Lcn2 siRNA). One skilled in the art is familiar with genes that may be targeted for the treatment of cancer.

Non-limiting examples of protein or polypeptide-based therapeutic molecules include enzymes, regulatory proteins (*e.g.*, immuno-regulatory proteins), antigens, antibodies or antibody fragments, and structural proteins. In some embodiments, the protein or polypeptide-based therapeutic molecules are for cancer therapy.

Suitable enzymes (for operably linking to a synthetic promoter) for some embodiments of this disclosure include, for example, oxidoreductases, transferases, polymerases, hydrolases, lyases, synthases, isomerases, and ligases, digestive enzymes (*e.g.*, proteases, lipases, carbohydrases, and nucleases). In some embodiments, the enzyme is selected from the group consisting of lactase, beta-galactosidase, a pancreatic enzyme, an oil-degrading enzyme,

mucinase, cellulase, isomaltase, alginase, digestive lipases (*e.g.*, lingual lipase, pancreatic lipase, phospholipase), amylases, cellulases, lysozyme, proteases (*e.g.*, pepsin, trypsin, chymotrypsin, carboxypeptidase, elastase,), esterases (*e.g.* sterol esterase), disaccharidases (*e.g.*, sucrase, lactase, beta-galactosidase, maltase, isomaltase), DNases, and RNases.

Non-limiting examples of antibodies and fragments thereof include: bevacizumab (AVASTIN®), trastuzumab (HERCEPTIN®), alemtuzumab (CAMPATH®, indicated for B cell chronic lymphocytic leukemia,), gemtuzumab (MYLOTARG®, hP67.6, anti-CD33, indicated for leukemia such as acute myeloid leukemia), rituximab (RITUXAN®), tositumomab (BEXXAR®, anti-CD20, indicated for B cell malignancy), MDX-210 (bispecific antibody that binds simultaneously to HER-2/neu oncogene protein product and type I Fc receptors for immunoglobulin G (IgG) (Fc gamma RI)), oregovomab (OVAREX®, indicated for ovarian cancer), edrecolomab (PANOREX®), daclizumab (ZENAPAX®), palivizumab (SYNAGIS®, indicated for respiratory conditions such as RSV infection), ibritumomab tiuxetan (ZEVALIN®, indicated for Non-Hodgkin's lymphoma), cetuximab (ERBITUX®), MDX-447, MDX-22, MDX-220 (anti-TAG-72), IOR-C5, IOR-T6 (anti-CD1), IOR EGF/R3, celogovab (ONCOSCINT® OV103), epratuzumab (LYMPHOCIDE®), pemtumomab (THERAGYN®), Gliomab-H (indicated for brain cancer, melanoma). In some embodiments, the antibody is an antibody that inhibits an immune check point protein, *e.g.*, an anti-PD-1 antibody such as pembrolizumab (Keytruda®) or nivolumab (Opdivo®), or an anti-CTLA-4 antibody such as ipilimumab (Yervoy®). Other antibodies and antibody fragments may be operably linked to a synthetic promoter, as provided herein.

A regulatory protein may be, in some embodiments, a transcription factor or a immunoregulatory protein. Non-limiting, exemplary transcriptional factors include: those of the NFkB family, such as Rel-A, c-Rel, Rel-B, p50 and p52; those of the AP-1 family, such as Fos, FosB, Fra-1, Fra-2, Jun, JunB and JunD; ATF; CREB; STAT-1, -2, -3, -4, -5 and -6; NFAT-1, -2 and -4; MAF; Thyroid Factor; IRF; Oct-1 and -2; NF-Y; Egr-1; and USF-43, EGR1, Sp1, and E2F1. Other transcription factors may be operably linked to a synthetic promoter, as provided herein.

As used herein, an immunoregulatory protein is a protein that regulates an immune response. Non-limiting examples of immunoregulatory include: antigens, adjuvants (*e.g.*, flagellin, muramyl dipeptide), cytokines including interleukins (*e.g.*, IL-2, IL-7, IL-15 or superagonist/mutant forms of these cytokines), IL-12, IFN-gamma, IFN-alpha, GM-CSF,

FLT3-ligand), and immunostimulatory antibodies (*e.g.*, anti-CTLA-4, anti-CD28, anti-CD3, or single chain/antibody fragments of these molecules). Other immunoregulatory proteins may be operably linked to a synthetic promoter, as provided herein.

As used herein, an antigen is a molecule or part of a molecule that is bound by the antigen-binding site of an antibody. In some embodiments, an antigen is a molecule or moiety that, when administered to or expression in the cells of a subject, activates or increases the production of antibodies that specifically bind the antigen. Antigens of pathogens are well known to those of skill in the art and include, but are not limited to parts (coats, capsules, cell walls, flagella, fimbriae, and toxins) of bacteria, viruses, and other microorganisms. Examples of antigens that may be used in accordance with the disclosure include, without limitation, cancer antigens, self-antigens, microbial antigens, allergens and environmental antigens. Other antigens may be operably linked to a synthetic promoter, as provided herein.

In some embodiments, the antigen of the present disclosure is a cancer antigen. A cancer antigen is an antigen that is expressed preferentially by cancer cells (*i.e.*, it is expressed at higher levels in cancer cells than on non-cancer cells) and, in some instances, it is expressed solely by cancer cells. Cancer antigens may be expressed within a cancer cell or on the surface of the cancer cell. Cancer antigens that may be used in accordance with the disclosure include, without limitation, MART-1/Melan-A, gp100, adenosine deaminase-binding protein (ADAbp), FAP, cyclophilin b, colorectal associated antigen (CRC)--C017-1A/GA733, carcinoembryonic antigen (CEA), CAP-1, CAP-2, etv6, AML1, prostate specific antigen (PSA), PSA-1, PSA-2, PSA-3, prostate-specific membrane antigen (PSMA), T cell receptor/CD3-zeta chain and CD20. The cancer antigen may be selected from the group consisting of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4 and MAGE-C5. The cancer antigen may be selected from the group consisting of GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8 and GAGE-9. The cancer antigen may be selected from the group consisting of BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin, γ-catenin, p120ctn, gp100Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 ganglioside, GD2 ganglioside, human papilloma virus proteins, Smad family of tumor

antigens, lmp-1, P1A, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-3, SSX-4, SSX-5, SCP-1 and CT-7, CD20 and c-erbB-2. Other cancer antigens may be operably linked to a synthetic promoter, as provided herein.

In some embodiments, a protein or polypeptide-based therapeutic molecule is a fusion protein. A fusion protein is a protein comprising two heterologous proteins, protein domains, or protein fragments, that are covalently bound to each other, either directly or indirectly (*e.g.*, via a linker), via a peptide bond. In some embodiments, a fusion protein is encoded by a nucleic acid comprising the coding region of a protein in frame with a coding region of an additional protein, without intervening stop codon, thus resulting in the translation of a single protein in which the proteins are fused together.

In some embodiments, the output molecule is a functional molecule. A "function molecule" refers to a molecule that is able to interact with other molecules or circuits to exert a function (e.g., transcription regulation, DNA or RNA cleavage, or any enzymatic activities). Exemplary functional molecules include, without limitation, enzymes (*e.g.*, without limitation, nucleases), transcriptional regulators (*e.g.*, without limitation, activators and repressors), RNAi molecules (*e.g.*, without limitation, siRNA, miRNA, shRNA), and antibodies. In some embodiments, the functional molecule is a nuclease (*e.g.*, a site-specific nuclease such as Csy4, Cas6, CasE, and Cse3). In some embodiments, the functional molecule is a transcriptional repressor (*e.g.*, without limitation, TetR, CNOT7, DDX6, PPR10, and L7Ae). In some embodiments, having a functional molecule as the output molecule of the cleavage-induced transcript stabilizers described herein allows the cleavage-induced transcript stabilizer to further interact with downstream genetic circuits that contain elements responsive to the functional molecule produced by the cleavage-induced transcript stabilizer. Thus, "layering" of genetic circuits can be achieved, allowing multiple levels of complex regulation.

A "promoter" refers to a control region of a nucleic acid sequence at which initiation and rate of transcription of the remainder of a nucleic acid sequence are controlled. A promoter drives expression or drives transcription of the nucleic acid sequence that it regulates. A promoter may also contain sub-regions at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors. Promoters may be constitutive, inducible, activatable, repressible, tissue-specific or any combination thereof. A promoter is considered to be "operably linked" when it is in a correct functional location and orientation in

relation to a nucleic acid sequence it regulates to control ("drive") transcriptional initiation and/or expression of that sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment of a given gene or sequence. Such a promoter can be referred to as "endogenous."

In some embodiments, a coding nucleic acid sequence may be positioned under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with the encoded sequence in its natural environment. Such promoters may include promoters of other genes; promoters isolated from any other cell; and synthetic promoters or enhancers that are not "naturally occurring" such as, for example, those that contain different elements of different transcriptional regulatory regions and/or mutations that alter expression through methods of genetic engineering that are known in the art. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including polymerase chain reaction (PCR) (see U.S. Pat. No. 4,683,202 and U.S. Pat. No. 5,928,906).

In some embodiments, a promoter is an "inducible promoter," which refer to a promoter that is characterized by regulating (*e.g.*, initiating or activating) transcriptional activity when in the presence of, influenced by or contacted by an inducer signal. An inducer signal may be endogenous or a normally exogenous condition (*e.g.*, light), compound (*e.g.*, chemical or non-chemical compound) or protein that contacts an inducible promoter in such a way as to be active in regulating transcriptional activity from the inducible promoter. Thus, a "signal that regulates transcription" of a nucleic acid refers to an inducer signal that acts on an inducible promoter. A signal that regulates transcription may activate or inactivate transcription, depending on the regulatory system used. Activation of transcription may involve directly acting on a promoter to drive transcription or indirectly acting on a promoter by inactivation a repressor that is preventing the promoter from driving transcription. Conversely, deactivation of transcription may involve directly acting on a promoter to prevent transcription or indirectly acting on a promoter by activating a repressor that then acts on the promoter.

The administration or removal of an inducer signal results in a switch between activation and inactivation of the transcription of the operably linked nucleic acid sequence.

Thus, the active state of a promoter operably linked to a nucleic acid sequence refers to the state when the promoter is actively regulating transcription of the nucleic acid sequence (*i.e.*, the linked nucleic acid sequence is expressed). Conversely, the inactive state of a promoter operably linked to a nucleic acid sequence refers to the state when the promoter is not actively regulating transcription of the nucleic acid sequence (*i.e.*, the linked nucleic acid sequence is not expressed).

An inducible promoter of the present disclosure may be induced by (or repressed by) one or more physiological condition(s), such as changes in light, pH, temperature, radiation, osmotic pressure, saline gradients, cell surface binding, and the concentration of one or more extrinsic or intrinsic inducing agent(s). An extrinsic inducer signal or inducing agent may comprise, without limitation, amino acids and amino acid analogs, saccharides and polysaccharides, nucleic acids, protein transcriptional activators and repressors, cytokines, toxins, petroleum-based compounds, metal containing compounds, salts, ions, enzyme substrate analogs, hormones or combinations thereof.

Inducible promoters of the present disclosure include any inducible promoter described herein or known to one of ordinary skill in the art. Examples of inducible promoters include, without limitation, chemically/biochemically-regulated and physically-regulated promoters such as alcohol-regulated promoters, tetracycline-regulated promoters (*e.g.*, anhydrotetracycline (aTc)-responsive promoters and other tetracycline-responsive promoter systems, which include a tetracycline repressor protein (tetR), a tetracycline operator sequence (tetO) and a tetracycline transactivator fusion protein (tTA)), steroid-regulated promoters (*e.g.*, promoters based on the rat glucocorticoid receptor, human estrogen receptor, moth ecdysone receptors, and promoters from the steroid/retinoid/thyroid receptor superfamily), metal-regulated promoters (*e.g.*, promoters derived from metallothionein (proteins that bind and sequester metal ions) genes from yeast, mouse and human), pathogenesis-regulated promoters (*e.g.*, induced by salicylic acid, ethylene or benzothiadiazole (BTH)), temperature/heat-inducible promoters (*e.g.*, heat shock promoters), and light-regulated promoters (*e.g.*, light responsive promoters from plant cells).

In some embodiments, an inducer signal of the present disclosure is an N-acyl homoserine lactone (AHL), which is a class of signaling molecules involved in bacterial quorum sensing. Quorum sensing is a method of communication between bacteria that enables the coordination of group based behavior based on population density. AHL can diffuse across

cell membranes and is stable in growth media over a range of pH values. AHL can bind to transcriptional activators such as LuxR and stimulate transcription from cognate promoters.

In some embodiments, an inducer signal of the present disclosure is anhydrotetracycline (aTc), which is a derivative of tetracycline that exhibits no antibiotic activity and is designed for use with tetracycline-controlled gene expression systems, for example, in bacteria.

In some embodiments, an inducer signal of the present disclosure is isopropyl β-D-1-thiogalactopyranoside (IPTG), which is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon, and it is therefore used to induce protein expression where the gene is under the control of the lac operator. IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon, such as the gene coding for beta-galactosidase, a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides. The sulfur (S) atom creates a chemical bond which is non-hydrolyzable by the cell, preventing the cell from metabolizing or degrading the inducer. IPTG is an effective inducer of protein expression, for example, in the concentration range of 100 μM to 1.0 mM. Concentration used depends on the strength of induction required, as well as the genotype of cells or plasmid used. If lacIq, a mutant that over-produces the lac repressor, is present, then a higher concentration of IPTG may be necessary. In blue-white screen, IPTG is used together with X-gal. Blue-white screen allows colonies that have been transformed with the recombinant plasmid rather than a non-recombinant one to be identified in cloning experiments.

Other inducible promoter systems are known in the art and may be used in accordance with the present disclosure.

In some embodiments, inducible promoters of the present disclosure are from prokaryotic cells (*e.g.*, bacterial cells). Examples of inducible promoters for use prokaryotic cells include, without limitation, bacteriophage promoters (*e.g.* Pls1con, T3, T7, SP6, PL) and bacterial promoters (*e.g.*, Pbad, PmgrB, Ptrc2, Plac/ara, Ptac, Pm), or hybrids thereof (*e.g.* PLlacO, PLtetO). Examples of bacterial promoters for use in accordance with the present disclosure include, without limitation, positively regulated E. coli promoters such as positively regulated σ70 promoters (*e.g.*, inducible pBad/araC promoter, Lux cassette right promoter, modified lambda Prm promoter, plac Or2-62 (positive), pBad/AraC with extra REN sites,

pBad, P(Las) TetO, P(Las) CIO, P(Rhl), Pu, FecA, pRE, cadC, hns, pLas, pLux), σS promoters (*e.g.*, Pdps), σ32 promoters (*e.g.*, heat shock) and σ54 promoters (*e.g.*, glnAp2); negatively regulated E. coli promoters such as negatively regulated σ70 promoters (*e.g.*, Promoter (PRM+), TetR - TetR-4C P(Las) TetO, P(Las) CIO, P(Lac) IQ, RecA_DlexO_DLacO1, dapAp, FecA, Pspac-hy, pcI, plux-cI, plux-lac, CinR, CinL, glucose controlled, modified Pr, modified Prm+, FecA, Pcya, rec A (SOS), Rec A (SOS), EmrR_regulated, BetI_regulated, pLac_lux, pTet_Lac, pLac/Mnt, pTet/Mnt, LsrA/cI, pLux/cI, LacI, LacIQ, pLacIQ1, pLas/cI, pLas/Lux, pLux/Las, pRecA with LexA binding site, reverse BBa_R0011, pLacI/ara-1, pLacIq, rrnB P1, cadC, hns, PfhuA, pBad/araC, nhaA, OmpF, RcnR), σS promoters (*e.g.*, Lutz-Bujard LacO with alternative sigma factor σ38), σ32 promoters (*e.g.*, Lutz-Bujard LacO with alternative sigma factor σ32), and σ54 promoters (*e.g.*, glnAp2); negatively regulated *B. subtilis* promoters such as repressible *B. subtilis* σA promoters (*e.g.*, Gram-positive IPTG-inducible, Xyl, hyper-spank) and σB promoters. Other inducible microbial promoters may be used in accordance with the present disclosure.

The cleavage-induced transcript stabilizer may be included in one or more (*e.g.*, 2, 3 or more) nucleic acid molecules (*e.g.*, vectors) and introduced into a cell. A "nucleic acid" is at least two nucleotides covalently linked together, and in some instances, may contain phosphodiester bonds (*e.g.*, a phosphodiester "backbone"). A nucleic acid may be DNA, both genomic and/or cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribonucleotides and ribonucleotides (*e.g.*, artificial or natural), and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine and isoguanine. Nucleic acids of the present disclosure may be produced using standard molecular biology methods (*see*, *e.g.*, *Green and Sambrook, Molecular Cloning*, A Laboratory Manual, 2012, Cold Spring Harbor Press).

In some embodiments, nucleic acids are produced using GIBSON ASSEMBLY® Cloning (*see*, *e.g.*, Gibson, D.G. *et al. Nature Methods*, 343–345, 2009; and Gibson, D.G. *et al. Nature Methods*, 901–903, 2010, each of which is incorporated by reference herein). GIBSON ASSEMBLY® typically uses three enzymatic activities in a single-tube reaction: 5′ exonuclease, the 3′ extension activity of a DNA polymerase and DNA ligase activity. The 5′ exonuclease activity chews back the 5′ end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps on the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The

overlapping sequence of adjoining fragments is much longer than those used in Golden Gate Assembly, and therefore results in a higher percentage of correct assemblies.

In some embodiments, the cleavage-induced transcript stabilizer are is delivered to a cell a vector. A "vector" refers to a nucleic acid (*e.g.*, DNA) used as a vehicle to artificially carry genetic material (*e.g.*, an engineered nucleic acid) into a cell where, for example, it can be replicated and/or expressed. In some embodiments, a vector is an episomal vector (see, *e.g.*, Van Craenenbroeck K. *et al. Eur. J. Biochem.* 267, 5665, 2000, incorporated by reference herein). A non-limiting example of a vector is a plasmid. Plasmids are double-stranded generally circular DNA sequences that are capable of automatically replicating in a host cell. Plasmid vectors typically contain an origin of replication that allows for semi-independent replication of the plasmid in the host and also the transgene insert. Plasmids may have more features, including, for example, a "multiple cloning site," which includes nucleotide overhangs for insertion of a nucleic acid insert, and multiple restriction enzyme consensus sites to either side of the insert. Another non-limiting example of a vector is a viral vector (*e.g.*, retroviral, adenoviral, adeno-association, helper-dependent adenoviral systems, hybrid adenoviral systems, herpes simplex, pox virus, lentivirus, Epstein–Barr virus). In some embodiments, the viral vector is derived from an adeno-associated virus (AAV). In some embodiments, the viral vector is derived from an herpes simplex virus (HSV).

The nucleic acids or vectors containing the genetic circuits of the cleavage-induced transcript stabilizer may be delivered to a cell by any methods known in the art for delivering nucleic acids. For example, for delivering nucleic acids to a prokaryotic cell, the methods include, without limitation, transformation, transduction, conjugation, and electroporation. For delivering nucleic acids to a eukaryotic cell, methods include, without limitation, transfection, electroporation, and using viral vectors.

Cells containing the cleavage-induced transcript stabilizer are also provided herein. A "cell" is the basic structural and functional unit of all known independently living organisms. It is the smallest unit of life that is classified as a living thing. Some organisms, such as most bacteria, are unicellular (consist of a single cell). Other organisms, such as humans, are multicellular.

In some embodiments, a cell for use in accordance with the present disclosure is a prokaryotic cell, which may comprise a cell envelope and a cytoplasmic region that contains the cell genome (DNA) and ribosomes and various sorts of inclusions. In some embodiments,

the cell is a bacterial cell. As used herein, the term "bacteria" encompasses all variants of bacteria, for example, prokaryotic organisms and cyanobacteria. Bacteria are small (typical linear dimensions of around 1 micron), non-compartmentalized, with circular DNA and ribosomes of 70S. The term bacteria also includes bacterial subdivisions of *Eubacteria* and *Archaebacteria*. *Eubacteria* can be further subdivided into gram-positive and gram-negative *Eubacteria*, which depend upon a difference in cell wall structure. Also included herein are those classified based on gross morphology alone (*e.g.*, cocci, bacilli). In some embodiments, the bacterial cells are gram-negative cells, and in some embodiments, the bacterial cells are gram-positive cells. Examples of bacterial cells that may be used in accordance with the invention include, without limitation, cells from *Yersinia spp.*, *Escherichia spp.*, *Klebsiella spp.*, *Bordetella spp.*, *Neisseria spp.*, *Aeromonas spp.*, *Franciesella spp.*, *Corynebacterium spp.*, *Citrobacter spp.*, *Chlamydia spp.*, *Hemophilus spp.*, *Brucella spp.*, *Mycobacterium spp.*, *Legionella spp.*, *Rhodococcus spp.*, *Pseudomonas spp.*, *Helicobacter spp.*, *Salmonella spp.*, *Vibrio spp.*, *Bacillus spp.*, *Erysipelothrix spp.*, *Salmonella spp.*, *Stremtomyces spp.* In some embodiments, the bacterial cells are from *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium butyricum*, *Brevibacterium lactofermentum*, *Streptococcus agalactiae*, *Lactococcus lactis*, *Leuconostoc lactis*, *Streptomyces*, *Actinobacillus actinobycetemcomitans*, *Bacteroides*, *cyanobacteria*, *Escherichia coli*, *Helobacter pylori*, *Selnomonas ruminatium*, *Shigella sonnei*, *Zymomonas mobilis*, *Mycoplasma mycoides*, *Treponema denticola*, *Bacillus thuringiensis*, *Staphlococcus lugdunensis*, *Leuconostoc oenos*, *Corynebacterium xerosis*, *Lactobacillus planta rum*, *Streptococcus faecalis*, *Bacillus coagulans*, *Bacillus ceretus*, *Bacillus popillae*, *Synechocystis strain PCC6803*, *Bacillus liquefaciens*, *Pyrococcus abyssi*, *Selenomonas nominantium*, *Lactobacillus hilgardii*, *Streptococcus ferus*, *Lactobacillus pentosus*, *Bacteroides fragilis*, *Staphylococcus epidermidis*, , *Zymomonas mobilis*, *Streptomyces phaechromogenes*, *Streptomyces ghanaenis*, *Halobacterium strain GRB*, or *Halobaferax sp.* strain Aa2.2.

In some embodiments, a cell for use in accordance with the present disclosure is a eukaryotic cell, which comprises membrane-bound compartments in which specific metabolic activities take place, such as a nucleus. Examples of eukaryotic cells for use in accordance with the invention include, without limitation, mammalian cells, insect cells, yeast cells (*e.g.*, *Saccharomyces cerevisiae*) and plant cells. In some embodiments, the eukaryotic cells are from a vertebrate animal. In some embodiments, the cell is a mammalian cell. In some

embodiments, the cell is a human cell. In some embodiments, the cell is from a rodent, such as a mouse or a rat. Examples of vertebrate cells for use in accordance with the present disclosure include, without limitation, reproductive cells including sperm, ova and embryonic cells, and non-reproductive cells, including kidney, lung, spleen, lymphoid, cardiac, gastric, intestinal, pancreatic, muscle, bone, neural, brain and epithelial cells. Stem cells, including embryonic stem cells, can also be used.

In some embodiments, the cell is a diseased cell. A "diseased cell," as used herein, refers to a cell whose biological functionality is abnormal, compared to a non-diseased (normal) cell. In some embodiments, the diseased cell is a cancer cell.

In some embodiments, the cleavage-induced transcript stabilizer is inserted into the genome of the cell. Methods of inserting genetic circuits into the genome of a cell are known to those skilled in the art (*e.g.*, via site-specific recombination, using any of the known genome-editing tools, or using other recombinant DNA technology). In some instances, integrating the cleavage-induced transcript stabilizer into the genome of a cell is advantageous for its applications (*e.g.*, therapeutic application or biomanufacturing application), compared to a cell engineered to simply express a transgene (*e.g.*, via transcription regulation). It is known that genetically engineered cells suffer from epigenetic silencing of the integrated transgene. However, continuous transcription of transgenes helps to prevent their silencing, which is not possible with transcriptionally-regulated gene circuits relying on transcriptional repression. In contrast, the cleavage-induced transcript stabilizer described herein relies on RNA-level regulation and can achieve continuous transcription of the transgenes.

Applications

Further provided herein are the functionality of the cleavage-induced transcript stabilizer and methods of using them. In some embodiments, the methods comprising delivering the cleavage-induced transcript stabilizers described herein into a cell (*e.g.*, by any of the methods described herein and known to one skilled in the art). In some embodiments, the methods comprises maintaining the cell containing the cleavage-induced transcript stabilizer. In some embodiments, the maintaining is carried out under conditions to allow the cleavage-induced transcript stabilizer to function.

In some embodiments, the cleavage-induced transcript stabilizer described herein is used in a method for detecting an RNA cleaver activity (*e.g.*, in a cell). The RNA cleaver may

be any RNA cleavers described herein, *e.g.*, an endoribonuclease, an RNAi molecule such as a microRNA, or a ribozyme. In some embodiments, the RNA cleaver is naturally expressed by the cell. In some embodiments, the RNA cleaver is introduced into the cell, *e.g.*, on an expression vector. As described herein, expression of the RNA cleaver leads to the expression of the output molecule. Accordingly, the expression of the output molecule indicates the presence of the RNA cleaver (*e.g.*, in a cell). Thus, in some embodiments, the method for detecting an RNA cleaver activity further comprises detecting the output molecule.

The cleavage-induced transcript stabilizer described herein may be used for a variety of applications. In some embodiments, the cleavage-induced transcript stabilizer is used for diagnostic purposes. The presence of certain RNA cleavers (*e.g.*, microRNAs), in some embodiments, may be used for determining the cell type. For example, diseased cells such as cancer cells may express cancer-cell specific RNA cleavers (e.g., microRNAs). The present disclosure further contemplates the use of the cleavage-induced transcript stabilizer in classifying cell types. For example, the cleavage-induced transcript stabilizer may be designed to detect an RNA cleaver (*e.g.*, microRNA) that is specific to a diseased cell (*e.g.*, cancer cell), and in the presence of the RNA cleaver (*e.g.*, microRNA), the cleavage-induced transcript stabilizer expresses the output molecule. For diagnostic purposes, the output molecules of the cleavage-induced transcript stabilizer is typically a detectable molecule (*e.g.*, a fluorescent protein or chemiluminescent protein). Depending on the specific RNA cleaver (*e.g.*, microRNA) a diseased cell produces, in some embodiments, detection of the output molecule indicates that the cell is a diseased cell (*e.g.*, cancer cell). In some embodiments, the lack of expression of the output molecule indicates a diseased cell.

In another example, the cleavage-induced transcript stabilizer is used for therapeutic purposes. For example, in some embodiments, the cleavage-induced transcript stabilizer is designed to detect an RNA cleaver (e.g., a microRNA) in a diseased cell (*e.g.*, a cancer cell) and to produce an output molecule that is a therapeutic molecule (*e.g.*, a therapeutic protein or RNA). Upon detecting of the RNA cleaver in the diseased cell, the cleavage-induced transcript stabilizer produces the therapeutic molecule, thus treating the disease. Such therapeutic methods are highly specific to the diseased cell and have low impact on healthy cells because the cleavage-induced transcript stabilizer will not detect the RNA cleaver in a healthy cell and thus will not produce the output molecule. Further, the therapeutic effect of the cleavage-induced transcript stabilizer is long lasting. For example, the cleavage-induced transcript

stabilizer will continuing to produce the therapeutic molecule until the diseased cell no longer expresses the RNA cleaver that is specific to the disease (*e.g.*, cancer). Once therapeutic effects have taken place, the cleavage-induced transcript stabilizer can sense the change in the expression of the RNA cleaver and stop the production of the therapeutic molecule.

For either diagnostic or treatment purposes, the cell may be *in vitro* (*e.g.*, cultured cell), *ex vivo* (*e.g.*, isolated from a subject), or *in vivo* in a subject. For *in vivo* applications, in some embodiments, the method comprises administering an effective amount of a composition comprising the cleavage-induced transcript stabilizer described herein to a subject in need thereof. The composition can further comprise additional agents (*e.g.* for specific delivery, increasing half-life, or other therapeutic agents). In some embodiments, the composition further comprises a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. A "pharmaceutically acceptable carrier" is a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

Some examples of materials which can serve as pharmaceutically-acceptable carriers include, without limitation: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters,

polycarbonates and/or polyanhydrides; (22) bulking agents, such as peptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (24) C2-C12 alcohols, such as ethanol; and (25) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient," "carrier," "pharmaceutically acceptable carrier" or the like are used interchangeably herein.

An "effective amount" refers to the amount of the cleavage-induced transcript stabilizer or composition comprising such required to confer therapeutic effect on the subject, either alone or in combination with one or more other therapeutic 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 subject parameters including age, physical condition, size, gender and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. 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 subject may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

Empirical considerations, such as the half-life, generally will contribute to the determination of the dosage. Frequency of administration may 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 disorder. Alternatively, sustained continuous release formulations of agent may be appropriate. Various formulations and devices for achieving sustained release are known in the art.

An effective amount of the cleavage-induced transcript stabilizer or composition comprising such may be administered repeatedly to a subject (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 times or more). In some embodiments, dosage is daily, every other day, every three days, every four days, every five days, or every six days. In some embodiments, dosing frequency is once every week, every 2 weeks, every 4 weeks, every 5 weeks, every 6 weeks, every 7 weeks, every 8 weeks, every 9 weeks, or every 10 weeks; or once every month, every 2 months, or

every 3 months, or longer. The progress of this therapy is easily monitored by conventional techniques and assays. The dosing regimen (including the agents used) can vary over time.

In some embodiments, for an adult subject of normal weight, doses ranging from about 0.01 to 1000 mg/kg may be administered. In some embodiments, the dose is between 1 to 200 mg. The particular dosage regimen, *i.e.*, dose, timing and repetition, will depend on the particular subject and that subject's medical history, as well as the properties of the agent (such as the half-life of the agent, and other considerations well known in the art).

For the purpose of the present disclosure, the appropriate dosage of the cleavage-induced transcript stabilizer or compositions comprising such will depend on the specific agent (or compositions thereof) employed, the formulation and route of administration, the type and severity of the disorder, previous therapy, the subject's clinical history and response to the agents, and the discretion of the attending physician. Typically the clinician will administer an agent until a dosage is reached that achieves the desired result. Administration can be continuous or intermittent, depending, for example, upon the recipient's physiological condition, and other factors known to skilled practitioners. The administration of an agent may be essentially continuous over a preselected period of time or may be in a series of spaced dose, *e.g.*, either before, during, or after developing a disorder.

A "subject" refers to human and non-human animals, such as apes, monkeys, horses, cattle, sheep, goats, dogs, cats, rabbits, guinea pigs, rats, and mice. In one embodiment, the subject is human. In some embodiments, the subject is an experimental animal or animal substitute as a disease model. A "subject in need thereof" refers to a subject who has or is at risk of a disease or disorder (*e.g.*, cancer).

The cleavage-induced transcript stabilizer of the present disclosure may be delivered to a subject (*e.g.*, a mammalian subject, such as a human subject) by any *in vivo* delivery method known in the art. For example, the cleavage-induced transcript stabilizer may be delivered intravenously. In some embodiments, cleavage-induced transcript stabilizer is delivered in a delivery vehicle (*e.g.*, non-liposomal nanoparticle or liposome). In some embodiments, the cleavage-induced transcript stabilizer is delivered systemically to a subject having a cancer or other disease and produces a therapeutic molecule specifically in cancer cells or diseased cells of the subject. In some embodiments, cleavage-induced transcript stabilizer is delivered to a site of the disease or disorder (*e.g.*, site of cancer).

Non-limiting examples of cancers that may be treated using the cleavage-induced transcript stabilizer methods described herein include: premalignant neoplasms, malignant tumors, metastases, or any disease or disorder characterized by uncontrolled cell growth such that it would be considered cancerous or precancerous. The cancer may be a primary or metastatic cancer. Cancers include, but are not limited to, ocular cancer, biliary tract cancer, bladder cancer, pleura cancer, stomach cancer, ovary cancer, meninges cancer, kidney cancer, brain cancer including glioblastomas and medulloblastomas, breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, esophageal cancer, gastric cancer, hematological neoplasms including acute lymphocytic and myelogenous leukemia, multiple myeloma, AIDS-associated leukemias and adult T-cell leukemia lymphoma, intraepithelial neoplasms including Bowen's disease and Paget's disease, liver cancer, lung cancer, lymphomas including Hodgkin's disease and lymphocytic lymphomas, neuroblastomas, oral cancer including squamous cell carcinoma, ovarian cancer including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells, pancreatic cancer, prostate cancer, rectal cancer, sarcomas including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma, and osteosarcoma, skin cancer including melanoma, Kaposi's sarcoma, basocellular cancer, and squamous cell cancer, testicular cancer including germinal tumors such as seminoma, non-seminoma, teratomas, choriocarcinomas, stromal tumors and germ cell tumors, thyroid cancer including thyroid adenocarcinoma and medullar carcinoma, and renal cancer including adenocarcinoma and Wilms' tumor. Commonly encountered cancers include breast, prostate, lung, ovarian, colorectal, and brain cancer. In some embodiments, the tumor is a melanoma, carcinoma, sarcoma, or lymphoma.

EXAMPLES

Example 1: A signal inverter module for RNA cleavers: detecting and evaluating miRNA, ribozymes, and ribonucleases

Introduction:

Cellular RNAs are processed in many ways; one important mechanism is by RNA cleavage. Cleavage is mediated by several factors including cis- or trans-acting ribozymes and ribonucleases. For example, a well-known factor for regulating expression is through the RNAi pathway: in some instances (*i.e.* perfect complementarity) miRNA or siRNA targeting can cleave mRNA, which usually leads to rapid degradation of the transcript. This mechanism

in particular has been especially useful for the field of synthetic biology for two reasons, (1) miRNAs have been shown to serve as biomarkers since they tend to vary drastically across different cell types and disease states [1] and (2) building sensors for a given miRNA is as straight forward as engineering the complementary sequence into a reporter transcript[2], where the reporter expression is then inversely related to the miRNA level (*i.e.* a high miRNA level would lead to degradation and low reporter expression). To detect states where miRNAs of interest are low, this is ideal since the reporter transcript will not be targeted and expression will stay high, however sensors to detect miRNAs that are expected to be at high levels are more complex. They usually involve a double-inversion strategy where the miRNA target sites are engineered into a transcript encoding a translational or transcriptional repressor instead, therefore high levels of miRNA targeting leads to low repressor expression and rescues reporter expression. This method has some drawbacks including failure modes cause by time delays, and the requirement of more than one transcriptional unit and transcript. The need for a mechanism to turn on expression in response to RNA cleavage, which would only require one transcript and would therefore avoid these drawbacks, exists. Such a mechanism would likewise find utility in the activity detection of ribonucleases or ribozymes, which typically lead to transcript cleavage and rapid degradation, and would therefore normally result in reporter expression that is inversely proportional to activity. A mechanism to turn reporter expression "ON" instead of "OFF" in response to RNA cleavage would enable monitoring of *in vivo* activity of these species in varying contexts, something which is poorly understood.

Herein a novel module that can be added to transgenes to induce expression upon an RNA cleavage event is described. This allows for the inversion of signal and also a monitoring of cleavage activity where increased output indicates increased activity. The transgene may be a fluorescent protein for direct reporting or another protein to link to downstream genetic logic or therapeutic output.

Design:

As depicted in **FIG. 1A**, this novel signal inverter relies on the ability to cleave a degradation signal from the 3' end of a transcript. The cleavage product would be stable and could be translated to produce a protein of interest. Therefore, in the absence of the RNA cleaver or when the cleaver is inactive, the entire transcript is targeted for rapid degradation leading to low output expression. When the RNA cleaver is present or active the degradation

signal is removed allowing for transgene expression due to an upstream stabilizer that prevents transcript degradation in the absence of a poly-A tail. This module will reverse the logic of an RNA cleaver that normally results in transcript degradation and inversely proportional transgene expression to one that results in transcript stabilization after cleavage (**FIG. 1B**).

Degradation signal selection

The degradation signal should be potent enough to decrease expression levels substantially and should ideally function without the need of another synthetic unit. A short 8-nt segment discovered by Geissler et. al.[3] is present in many endogenous transcripts across many mammalian cell types and is shown to bind hnRNPs which recruit deadenylases to degrade the transcript. To ensure maximum repression these signals were repeated in the 3'UR of a reporter transcript.

Stabilizer selection

The stabilizer portion of this module should act to stabilize the transcript once cleaved. It should not inhibit degradation via the signals mentioned above in the absence of cleavage and once the transcript is cleaved, translation should be facilitated. A triple helix structure has been shown to stabilize the 3' end of transcripts that lack a poly-A tail[4]. When appended to the end of mRNAs, translation was facilitated with high expression. Additionally, it has been shown to stabilize transcripts after Csy4 cleavage.

Ultrasensitive switch

A typical cleavage "OFF" switch can control the expression of a translational repressor where expression is inversely proportional to the cleaver; this new cleavage "ON" switch controls the expression of an output protein proportionally to the cleaver activity. Therefore, as depicted in **FIG. 2**, an ultrasensitive cleavage "ON" switch can be achieved by introducing these switches at different levels of a cascade[5]. A cleavage "ON" switch, as described herein, can be added to an output transgene while a cleavage "OFF" switch can be added to a repressor of the transgene. In the absence of the RNA cleaver, transgene expression remains low both because expression is blocked by the repressor and the degradation domains signal transcript degradation. However, in the presence of the cleaver, the repressor transcript is cleaved, relieving the expression inhibition while the degradation domains on the output transcript are

also removed to rescue expression. By changing the level of the repressor component, it is possible to modulate the threshold of the ultrasensitive switch to enable a more digital response.

Results:

When 10 copies of the Geissler degradation sequence were cloned into the 3'UTR after the CDS of EYFP driven by a constitutive CMV promoter, expression was 24 fold less than the same construct with 10 repeated copies of a mutated version of this sequence and 42 fold less than a constitutive EYFP (see **FIGs. 3A** and **3D**).

The triplex sequence was cloned after EYFP CDS. As seen in **FIG. 3B**, the EYFP-triplex construct expressed highly, while adding 10 repeats of the Geissler degradation sequence after the triplex enabled knock-down (**FIGs. 3B** and **3D**). When additional repeats of the Geissler degradation sequence were added after the triplex, expression was further decreased, with 30x repeat performing best enabling a 125 fold decrease compared to constitutive EYFP (see **FIGs. 3C** and **3D**).

Ribonuclease inverter

When a Csy4 recognition site (Csy4rec) was inserted into the 5' UTR of a gene constitutively expressing EYFP, the Csy4 inhibited EYFP expression leading to a signal that was inversely proportional to amount of Csy4 (**FIG. 4A**, blue curve).

Csy4rec was inserted into the cassette between the triplex and 30 repeats of the Geissler degradation signal. Adding the recognition site alone did not disrupt the degradation of the reporter, and when constitutive Csy4, Cse3, Cas6, or CasE plasmid was transfected into the same cells, expression was rescued (see **FIGs. 4B** to **4E**). This therefore inverts the signal of the ribonuclease so that output expression is proportional to Csy4 amount (**FIG. 4A**, magenta curve).

miRNA inverter

It may also be possible to invert the signal due to miRNA cleavage. FF5 target sites were inserted into the cassette between the triplex and 30 repeats of the Geissler degradation signal. Adding the target site alone did not disrupt the degradation of the reporter, and when siRNA FF5 was transfected into the same cells, expression was rescued 2 fold (see **FIGs. 5A-**

5B). An increase in the output signal in response to siRNA FF3 was also observed (**FIG. 5C**). An increase in the output signal in response to microRNA FF5 was also observed (**FIG. 5D**).

Ribozyme inverter

It may also be possible to invert the signal resulting from ribozyme cleavage. An inactive hammerhead ribozyme (iHHR) placed in the 3' UTR of a transgene constitutively expressing EYFP has little effect on its expression, however when the ribozyme is active, the transcript is cleaved resulting in degradation and 89 fold decreased expression (see **FIGs. 6A-6B**). When the same inactive and active ribozymes are placed in this signal inverter (between the triplex and degradation sequences) the effect should be reversed: when the ribozyme is inactive, the degradation sequences remain intact and the transcript expressing EYFP is quickly degraded resulting in low fluorescence, while when the ribozyme is active, the degradation signals are cleaved off resulting in a stable transcript and high expression. Further, in the absence of a polyA tail (the transcript should get degraded) fluorescence is rescued when the mascRNA sequence (which is targeted by RNase P) is added after the triplex (**FIG. 6C**).

Enabling an ultrasensitive switch

It may be possible to achieve an ultrasensitive switch by interacting at two levels of a cascade. One manifestation of this mechanism might be through the use of L7Ae, which has been shown to inhibit translation by binding a k-turn RNA motif in a transcript [6]. Two repeats of the k-turn motif were inserted in the 5'UTR of EYFP and expression was controlled by the level of plasmid expressing L7Ae (see **FIG. 7A**).

By inserting a cleaving target site in the transcript encoding L7Ae and also between a signal inverter module downstream of EYFP (containing 2 k-turn motifs upstream) as described, it may be possible to control the fluorescence response to the cleaver by varying the level of L7Ae. The background of the "ON"-switch is decreased (as indicated by a shift of the curve to the right) by incorporating the L7Ae construct for an ultrasensitive response (**FIG. 7B**).

Methods:

Cell Culture and Transfection

HEK293FT cells used in this study were maintained in Dulbecco's modified Eagle medium (DMEM, Corning) supplemented with 10% FBS (VWR), 1% penicillin/streptomycin/L-Glutamine (Corning) and 1% non-essential amino acids (Corning) at 37 ºC and 5% CO_2.

Transfections were carried out in 24-well plate format with Lipofectamine 3000 transfection reagent (Invitrogen). Cells were harvested by trypsinization and $1.5x10^6$ cells were seeded in 500uL culture medium in each well. Immediately following, 600 ng total DNA was diluted in 25uL Opti-MEM (Thermo Fisher) and 1.2uL of P300 was added to the dilution. 1.2uL of Lipoectamine 3000 was diluted in 25uL Opti-MEM and this dilution was added to the DNA dilution and mixed well. The complexes were incubated for 5-10 minutes before being added dropwise to the freshly seeded cells, followed by gentle rocking.

Flow Cytometry & Data Analysis

Cells were analyzed by flow cytometry 48 hours after transfection using the LSR-II Fortessa Flow Cytometer. 20,000 events were collected per sample.

For each sample, data were segmented by constitutive transfection marker fluorescence in the Pacific Blue channel into bins and geometric mean and variance computed for the data points in each bin. For bar plots the geometric mean of these bin values was calculated for bins greater than the autoflourescence cutoff which was calculated from the 99^{th} percentile Pacific Blue value of non-transfected cells.

DNA cloning and plasmid construction

Plasmid vectors were created using the Golden Gate cloning system. DNA oligos were ordered as necessary from IDT.

Conclusions:

Herein the Geissler domain is shown to be a potent "degradation domain" for transcripts which alone may be a mechanism for designing mRNA stability. The triplex sequence can stabilize the 3' end of transcripts that do not contain a polyA tail, but does not block the degradation mechanism utilized by the Geissler sequences. When used in combination within a transcript, these sequences represent an "inverter module": cleaving target sites such as ribonuclease recognition sites, miRNA target sites, or ribozymes can be

inserted between the triplex and degradation signals to induce expression only in a cleavage event. This mechanism can be useful in the detection of disease-specific miRNAs or for the activity monitoring of ribonucleases and ribozymes. When combined with the traditional "OFF" switch of RNA cleavers, it is shown that it may also be possible to create an RNA-level ultrasensitive switch.

References:

1. Lu, J. *et al.* MicroRNA expression profiles classify human cancers. *Nature* **435,** 834–838 (2005).
2. Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R. & Benenson, Y. Multi-Input RNAi-Based Logic Circuit for Identification of Specific Cancer Cells. *Science (80-.).* **333,** 1307–1311 (2011).
3. Geissler, R. *et al.* A widespread sequence-specific mRNA decay pathway mediated by hnRNP A1 and A2 / B1. 1–23 (2016). doi:10.1101/gad.277392.116
4. Wilusz, J. E. *et al.* A triple helix stabilizes the 3 9 ends of long noncoding RNAs that lack poly (A) tails. *Genes \& Dev.* **26,** 2392–2407 (2012).
5. Zhang, Q., Bhattacharya, S. & Andersen, M. E. Ultrasensitive response motifs: basic amplifiers in molecular signalling networks. *Open Biol.* **3,** 130031 (2013).
6. Saito, H. *et al.* Synthetic translational regulation by an L7Ae–kink-turn RNP switch. *Nat. Chem. Biol.* **6,** 71–78 (2010).

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding,"

"composed of," and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

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What is claimed is:

CLAIMS

1. A cleavage-induced transcript stabilizer, comprising:

(i) a first promoter operably linked to a nucleotide sequence encoding an output molecule followed, from 5′ to 3′, by an RNA stabilizer, a cleavage site for an RNA cleaver, and a degradation signal.

2. The cleavage-induced transcript stabilizer of claim 1, further comprising:

(ii) a second promoter operably linked to a nucleotide sequence encoding the RNA cleaver.

3. The cleavage-induced transcript stabilizer of claim 1 or claim 2, wherein the RNA cleaver is selected from the group consisting of: endoribonucleases, RNAi molecules, and ribozymes.

4. The cleavage-induced transcript stabilizer of claim 3, wherein the RNA cleaver is an endoribonuclease.

5. The cleavage-induced transcript stabilizer of claim 4, wherein the endoribonuclease is selected from the group consisting of: Cse3, Cas6, CasE, and Csy4.

6. The cleavage-induced transcript stabilizer of any one of claims 3-5, wherein the cleavage site comprises a recognition sequence for the endoribonuclease.

7. The cleavage-induced transcript stabilizer of claim 1 or 2, wherein the RNA cleaver is an RNAi molecule.

8. The cleavage-induced transcript stabilizer of claim 7, wherein the RNAi molecule is a microRNA, siRNA, or shRNA.

9. The cleavage-induced transcript stabilizer of claim 7 or claim 8, wherein the cleavage site comprises one or more target sites for the RNAi molecule.

10. The cleavage-induced transcript stabilizer of claim 1 or claim 2, wherein the RNA cleaver is a ribozyme.

11. The cleavage-induced transcript stabilizer of claim 10, wherein the ribozyme is selected from the group consisting of: RNase P, hammerhead ribozymes, Hepatitis delta virus ribozymes, hairpin ribozymes, twister ribozymes, twister sister ribozymes, pistol ribozymes, hatchet ribozymes, glmS ribozymes, varkud satellite ribozymes, and spliceozyme.

12. The cleavage-induced transcript stabilizer of claim 10 or claim 11, wherein the ribozyme is a trans-acting ribozyme.

13. The cleavage-induced transcript stabilizer of claim 12, wherein the cleavage site comprises a recognition site for the trans-acting ribozyme.

14. The cleavage-induced transcript stabilizer of claim 10 or claim 11, wherein the ribozyme is a cis-acting ribozyme.

15. The cleavage-induced transcript stabilizer of claim 14, wherein the cleavage site comprises the cis-acting ribozyme.

16. The cleavage-induced transcript stabilizer of any one of claims 1-15, further comprising a third promoter operably linked to a third nucleotide sequence encoding an RNA repressor, and one or more the cleavage sites for the RNA cleaver.

17. The cleavage-induced transcript stabilizer of claim 16, further comprising one or more recognition sites for an RNA repressor operably linked of the nucleotide sequence encoding the output molecule.

18. The cleavage-induced transcript stabilizer of claim 10 or claim 11, wherein the RNA repressor is an RNA binding protein.

19. The cleavage-induced transcript stabilizer of claim 18, wherein the RNA binding protein is selected from the group consisting of: TetR, MS2CP, PPR10, and L7Ae.

20. The cell state classifier of claim 18 or claim 19, wherein the RNA binding protein is fused to a modifying domain.

21. The cell state classifier of claim 20, wherein the modifying domain is CNOT7 or DDX6.

22. The cleavage-induced transcript stabilizer of any one of claims 1-19, wherein the cleavage-induced transcript stabilizer comprises 1-50 repeats of the degradation signal.

23. The cleavage-induced transcript stabilizer of any one of claim 1-22, wherein the degradation signal comprises the nucleotide sequence of TAASTTAT (SEQ ID NO: 1), wherein S is deoxyguanosine or deoxycytosine.

24. The cleavage-induced transcript stabilizer of claim 23, wherein the degradation signal comprises the nucleotide sequence of TAAGTTAT (SEQ ID NO: 2).

25. The cleavage-induced transcript stabilizer of any one of claims 1-22, wherein the degradation signal comprises the nucleotide sequence of TAAGACAT (SEQ ID NO: 3).

26. The cleavage-induced transcript stabilizer of any one of claims 1-25, wherein the RNA stabilizer is selected from the group consisting of: MALAT1 triplex, MENβ triplex, KSHV PAN triplex, histone stem loop, and a polyA signal.

27. The cleavage-induced transcript stabilizer of claim 26, wherein the RNA stabilizer is a MALAT1 triplex.

28. The cleavage-induced transcript stabilizer of any one of claims 1-27, wherein the output molecule is a detectable molecule.

29. The cleavage-induced transcript stabilizer of any one of claims 1-27, wherein the output molecule is a therapeutic molecule.

30. The cleavage-induced transcript stabilizer of any one of claims 1-27, wherein the output molecule is a functional molecule.

31. The cleavage-induced transcript stabilizer of claim 30, wherein the functional molecule is selected from the group consisting of: TetR, CNOT7, DDX6, PPR10, L7Ae, Csy4, Cas6, CasE, and Cse3.

32. The cleavage-induced transcript stabilizer of any one of claims 1-31, wherein the second promoter of (ii) is an inducible promoter.

33. A cleavage-induced transcript stabilizer comprising:

(i) an RNA transcript comprising a ribonucleotide sequence encoding an output molecule followed, in order, by an RNA stabilizer, a cleavage site for an RNA cleaver, and a degradation signal that leads to degradation of the RNA transcript.

34. The cleavage-induced transcript stabilizer of claim 33, further comprising:

(ii) a promoter operably linked to a nucleotide sequence encoding an RNA cleaver that cleaves the RNA transcript at the cleavage site.

35. The cleavage-induced transcript stabilizer of claim 34, wherein the promoter of (ii) is an inducible promoter.

36. The cleavage-induced transcript stabilizer of any one of claims 33-35, wherein the RNA transcript is degraded without in the absence of the RNA cleaver.

37. The cleavage-induced transcript stabilizer of any one of claims 33-35, wherein the RNA cleaver is expressed in the presence of the RNA cleaver.

38. The cleavage-induced transcript stabilizer of claim 37, wherein the cleavage of the RNA transcript stabilizes the RNA transcript and results in expression of the output molecule.

39. The cleavage-induced transcript stabilizer of any one of claims 33-38, wherein the output molecule is a detectable molecule.

40. The cleavage-induced transcript stabilizer of any one of claims 33-38, wherein the output molecule is a therapeutic molecule.

41. The cleavage-induced transcript stabilizer of any one of claims 33-38, wherein the output molecule is a functional molecule.

42. The cleavage-induced transcript stabilizer of claim 41, wherein the functional molecule is selected from the group consisting of: TetR, CNOT7, DDX6, PPR10, L7Ae, Csy4, Cas6, CasE, and Cse3.

43. A cell comprising the cleavage-induced transcript stabilizer of any one of claims 1-42.

44. The cell of claim 43, wherein the cell is a prokaryotic cell.

45. The cell of claim 44, wherein the prokaryotic cell is a bacterial cell.

46. The cell of claim 43, wherein the cell is a eukaryotic cell.

47. The cell of claim 46, wherein the eukaryotic cell is a plant cell, an insect cell, or a mammalian cell.

48. The cell of claim 47, wherein the mammalian cell is a human cell.

49. The cell of claim 43, wherein the cell is a diseased cell.

50. The cell of claim 49, wherein the cell is a cancer cell.

51. The cell of any one of claims 43-50, wherein the cleavage-induced transcript stabilizer is inserted into the genome of the cell.

52. A method comprising maintaining the cell of any one of claims 43-51.

53. The method of claim 52, further comprising detecting the output molecule.

54. The method of claim 52 or claim 53, further comprising classifying the cell.

55. A method comprising delivering the cleavage-induced transcript stabilizer of any one of claims 1-42 to a cell and detecting the output molecule.

56. A method of detecting an RNA cleaver activity, comprising: delivering the cleavage-induced transcript stabilizer of any one of claims 1-42 to a cell and detecting the output molecule.

57. The method of claim 56, wherein the RNA cleaver is an endoribonuclease, a siRNA transcript, or a ribozyme.

58. A method of treating a disease or disorder comprising delivering the cleavage-induced transcript stabilizer of any one of claims 1-42 to a cell, wherein the output molecule is a therapeutic molecule that is effective for treating the disease or disorder.

59. The method of claim 58, wherein the cell is a diseased cell.

60. The method of claim 58 or claim 59, wherein the cell is a cancer cell.

61. A method of diagnosing a disease or disorder comprising delivering the cleavage-induced transcript stabilizer of any one of claims 1-42 to a cell.

62. The method of claim 61, wherein the cell is a diseased cell.

63. The method of claim 61 or claim 62, wherein the cell is a cancer cell.

64. The method of any one of claims 61-63, the method further comprising detecting the output molecule.

65. The method of claim 64, wherein the lack of expression of the output molecule indicates the disease or disorder.

66. The method of claim 64, wherein the expression of the output molecule indicates the disease or disorder.

67. A method of treating a disease or disorder comprising administering an effective amount of a composition comprising the cleavage-induced transcript stabilizer of any one of claims 1-42 to a subject in need thereof, wherein the output molecule is a therapeutic molecule that is effective for treating the disease or disorder.

68. A method of diagnosing a disease or disorder comprising administering an effective amount of a composition comprising the cleavage-induced transcript stabilizer of any one of claims 1-42 to a subject in need thereof and detecting the output molecule.

69. The method of 67 or 68, wherein the composition further comprises a pharmaceutically acceptable carrier.

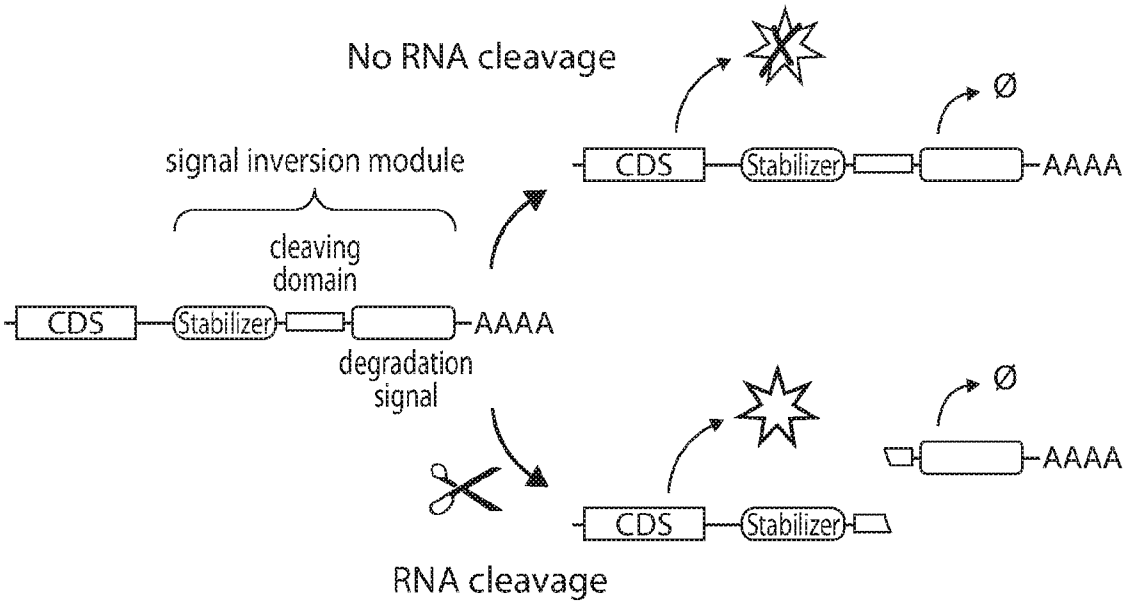
No RNA cleavage
signal inversion module
cleaving domain
CDS
Stabilizer
AAAA
degradation signal
Ø
RNA cleavage

FIG. 1A

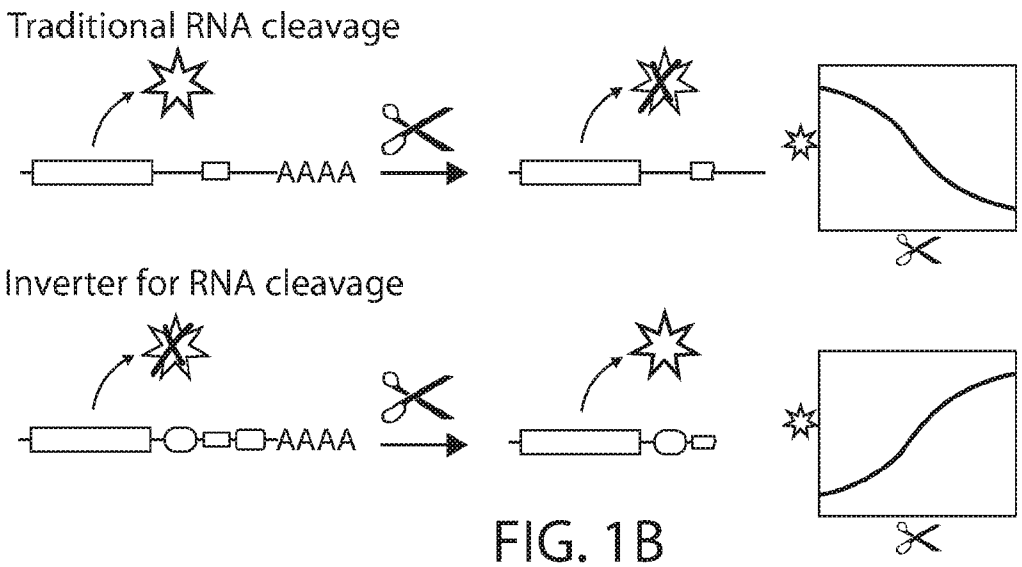
Traditional RNA cleavage
AAAA
Inverter for RNA cleavage

FIG. 1B

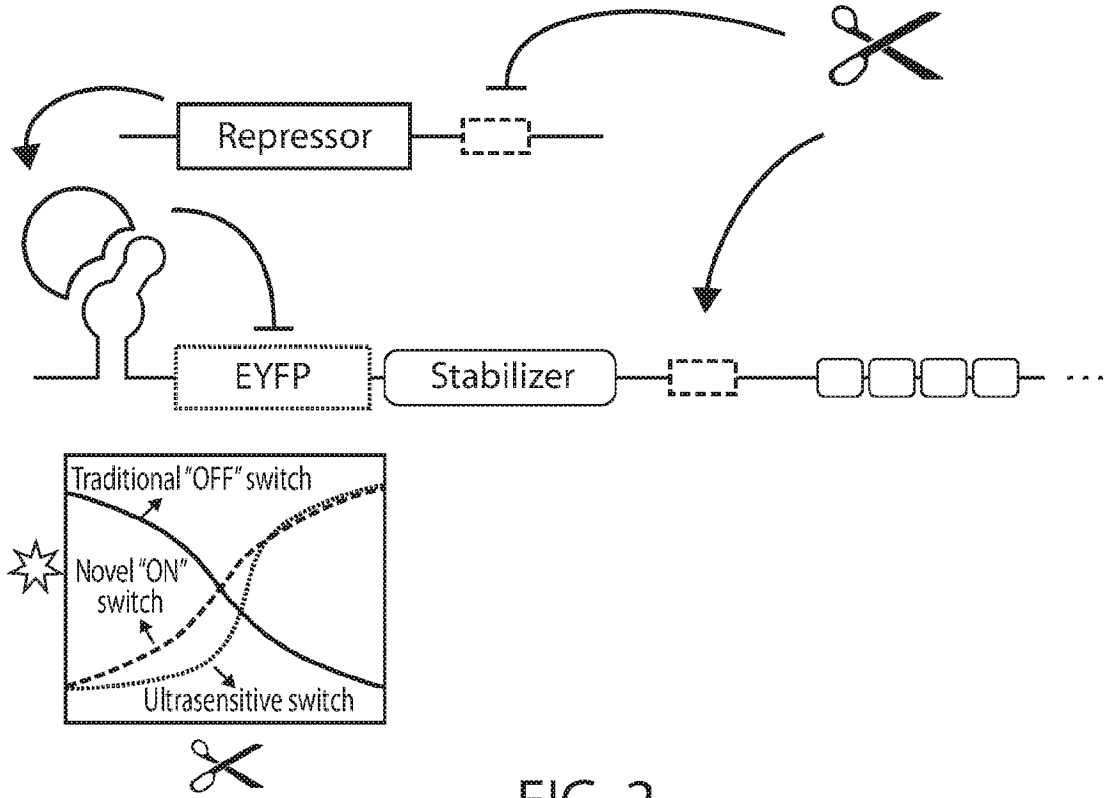
Repressor
EYFP
Stabilizer
Traditional "OFF" switch
Novel "ON" switch
Ultrasensitive switch

FIG. 2

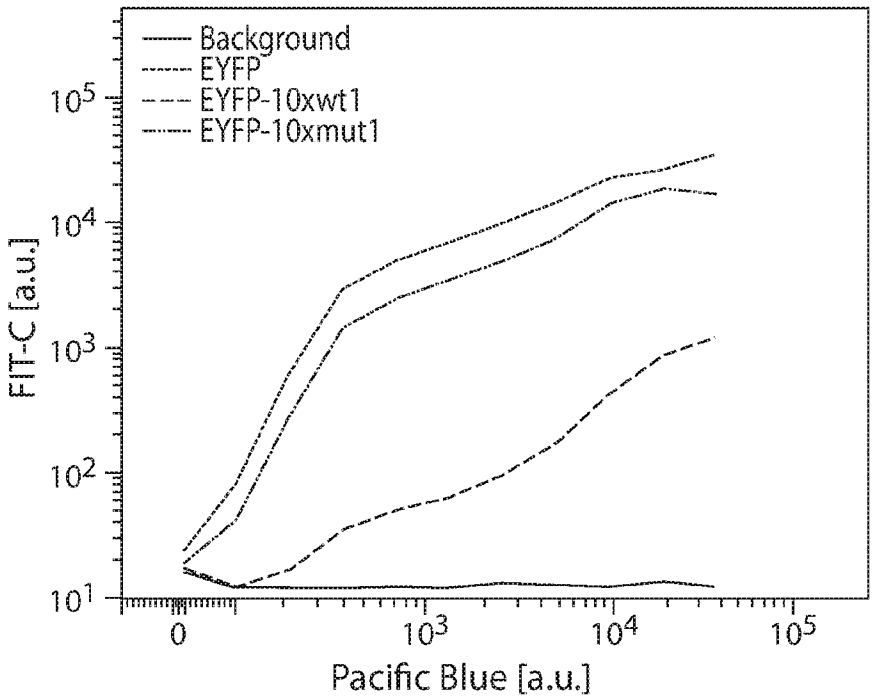
Background
EYFP
EYFP-10xwt1
EYFP-10xmut1
FIT-C [a.u.]
10^5
10^4
10^3
10^2
10^1
0
10^3
10^4
10^5
Pacific Blue [a.u.]

FIG. 3A

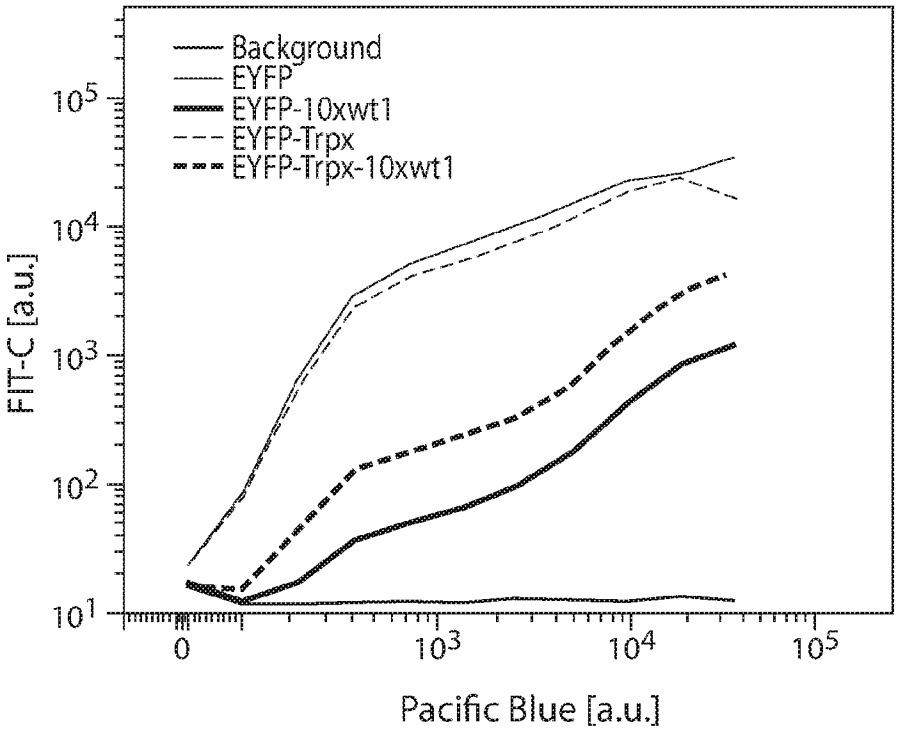
Background
EYFP
EYFP-10xwt1
EYFP-Trpx
EYFP-Trpx-10xwt1
FIT-C [a.u.]
10^5
10^4
10^3
10^2
10^1
0
10^3
10^4
10^5
Pacific Blue [a.u.]

FIG. 3B

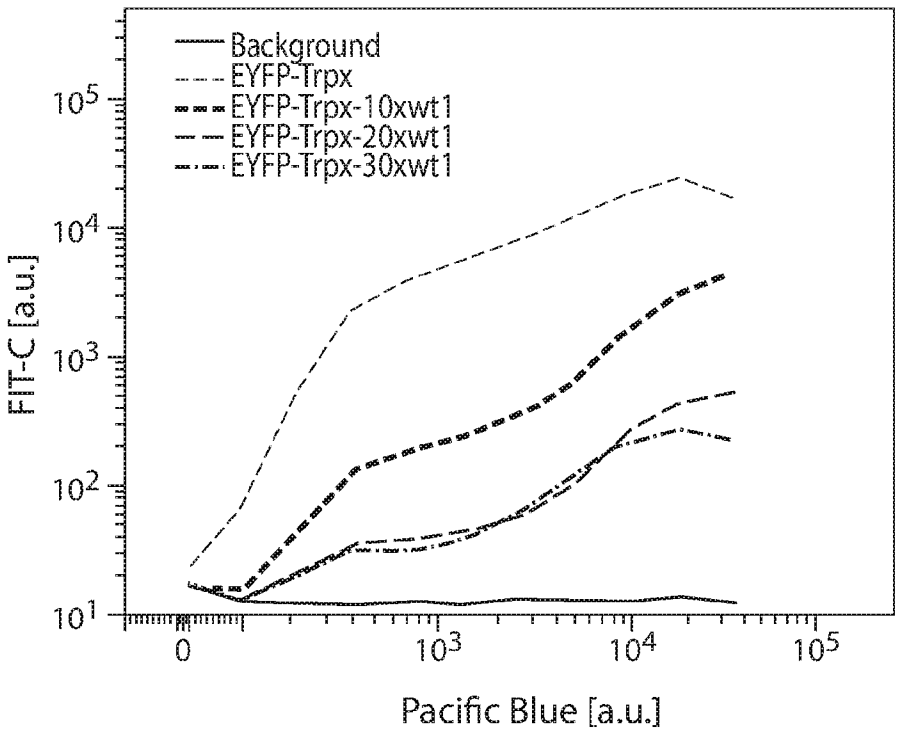
Background
EYFP-Trpx
EYFP-Trpx-10xwt1
EYFP-Trpx-20xwt1
EYFP-Trpx-30xwt1
FIT-C [a.u.]
10^5
10^4
10^3
10^2
10^1
0
10^3
10^4
10^5
Pacific Blue [a.u.]

FIG. 3C

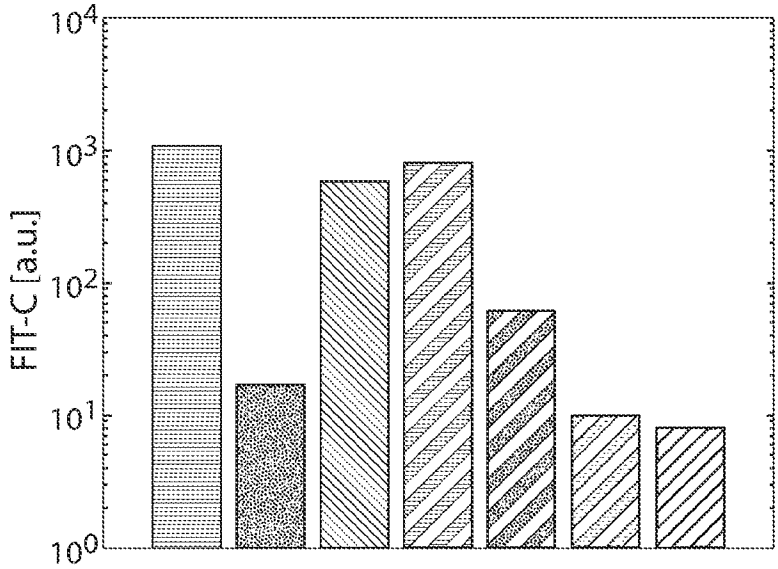
FIT-C [a.u.]
10^4
10^3
10^2
10^1
10^0

FIG. 3D

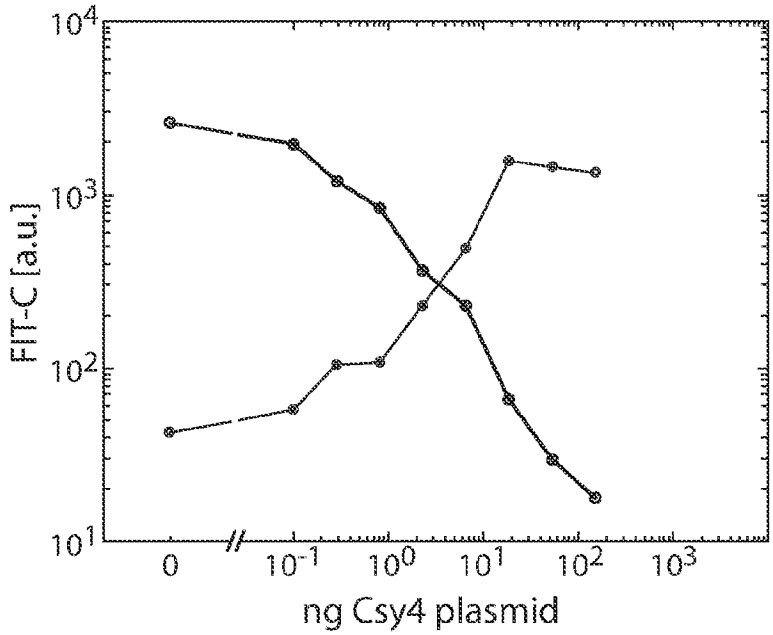
FIT-C [a.u.]
10^4
10^3
10^2
10^1
0
10^{-1}
10^0
10^1
10^2
10^3
ng Csy4 plasmid

FIG. 4A

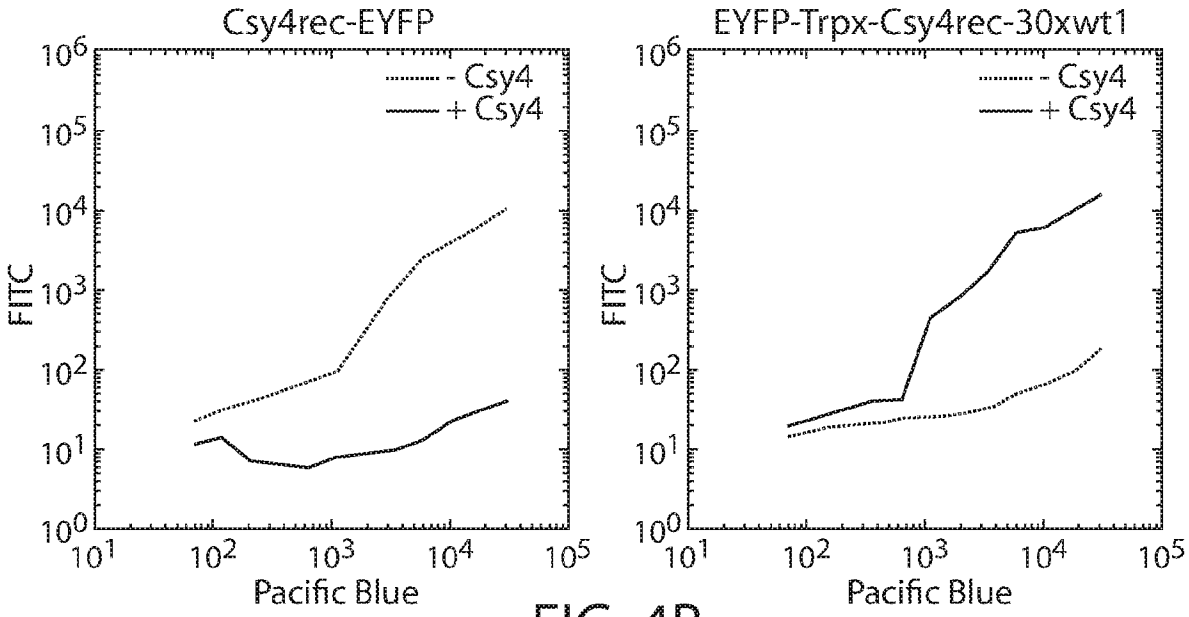
Csy4rec-EYFP
EYFP-Trpx-Csy4rec-30xwt1
- Csy4
+ Csy4
FITC
Pacific Blue
10^0
10^1
10^2
10^3
10^4
10^5
10^6

FIG. 4B

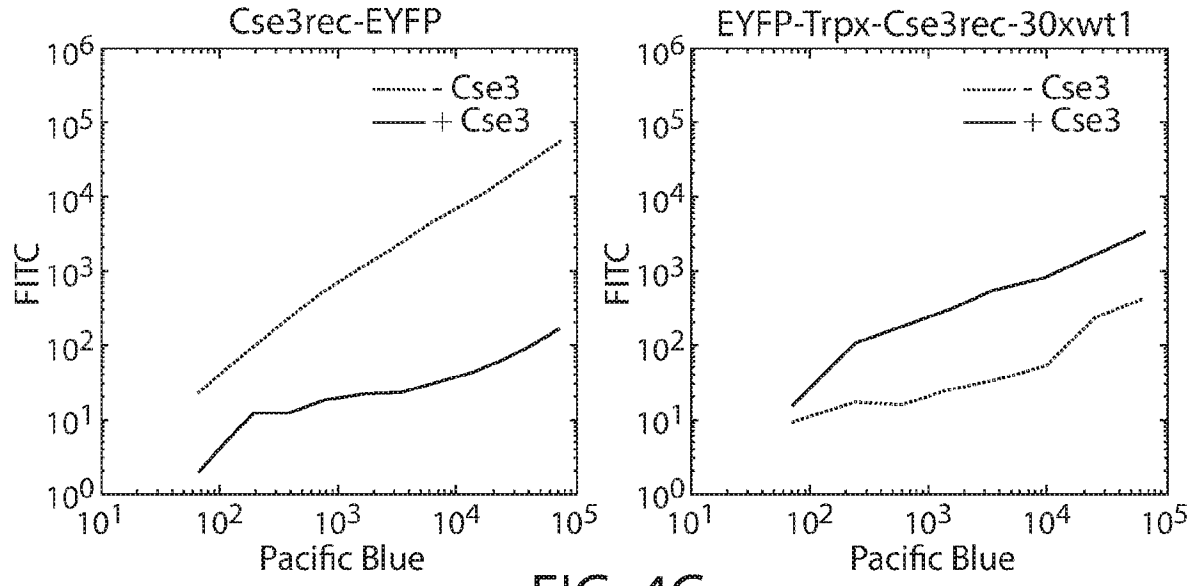
Cse3rec-EYFP
EYFP-Trpx-Cse3rec-30xwt1
- Cse3
+ Cse3
FITC
Pacific Blue
10^0
10^1
10^2
10^3
10^4
10^5
10^6

FIG. 4C

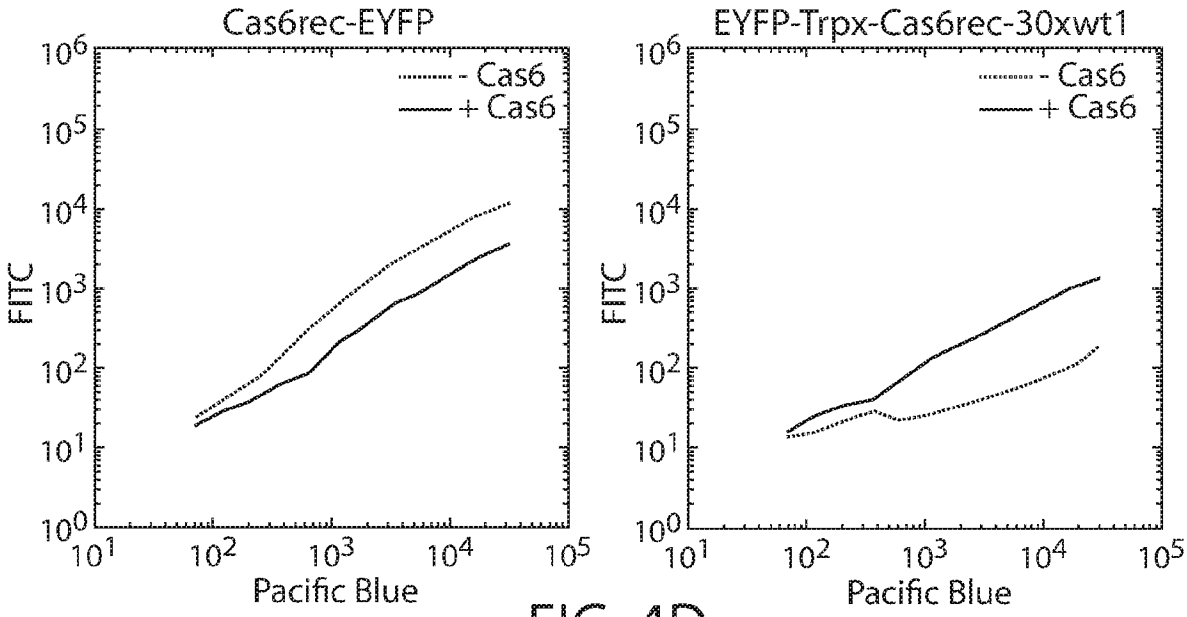
Cas6rec-EYFP
- Cas6
+ Cas6
FITC
Pacific Blue
EYFP-Trpx-Cas6rec-30xwt1
- Cas6
+ Cas6
FITC
Pacific Blue

FIG. 4D

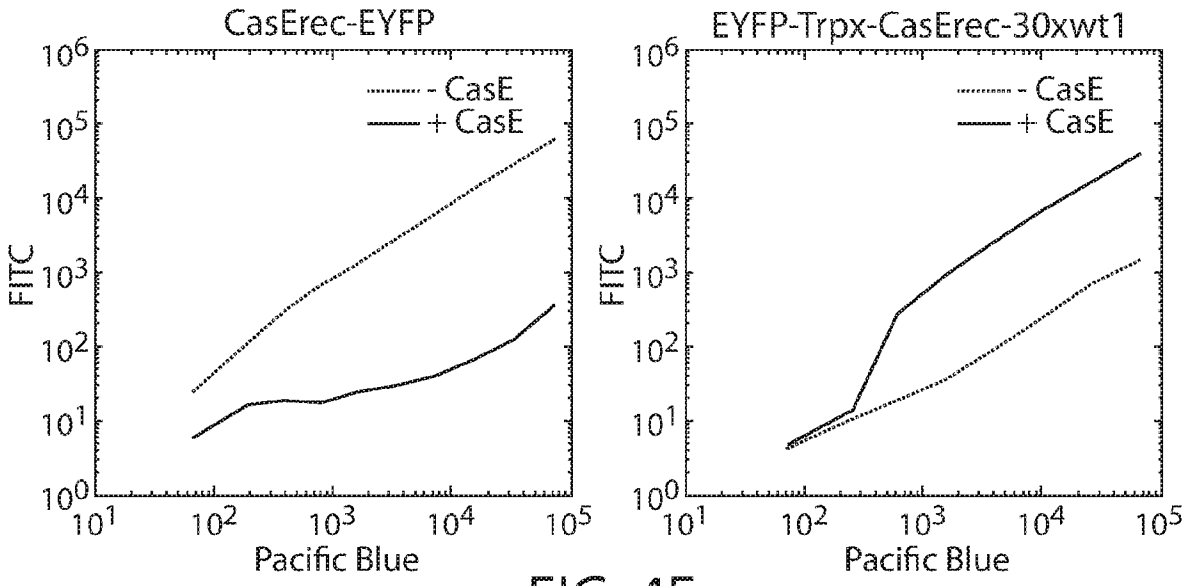
CasErec-EYFP
- CasE
+ CasE
FITC
Pacific Blue
EYFP-Trpx-CasErec-30xwt1
- CasE
+ CasE
FITC
Pacific Blue

FIG. 4E

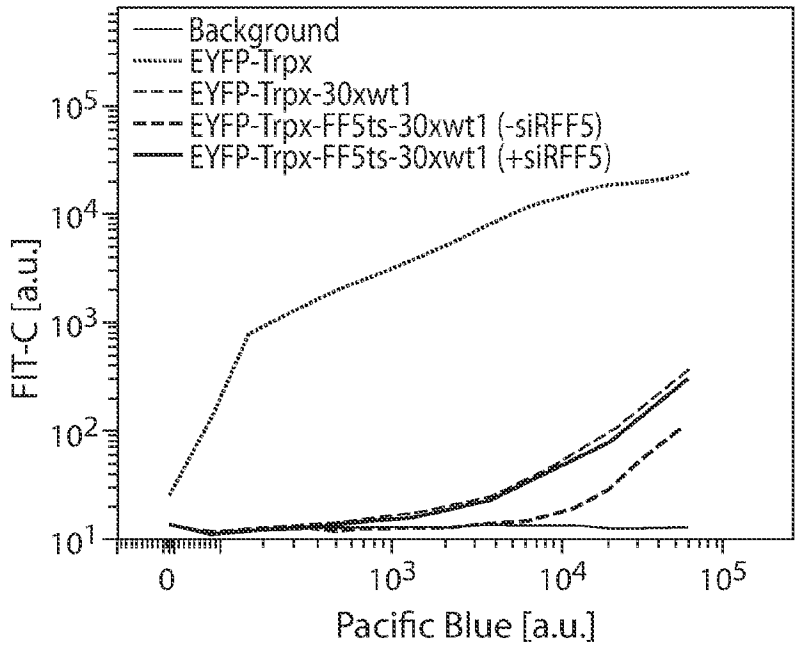
Background
EYFP-Trpx
EYFP-Trpx-30xwt1
EYFP-Trpx-FF5ts-30xwt1 (-siRFF5)
EYFP-Trpx-FF5ts-30xwt1 (+siRFF5)
FIT-C [a.u.]
10^5
10^4
10^3
10^2
10^1
0
10^3
10^4
10^5
Pacific Blue [a.u.]

FIG. 5A

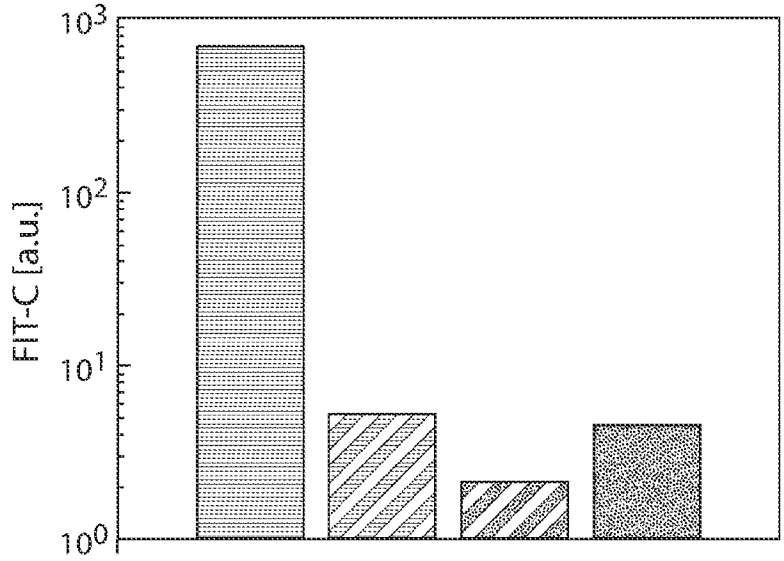
FIT-C [a.u.]
10^3
10^2
10^1
10^0

FIG. 5B

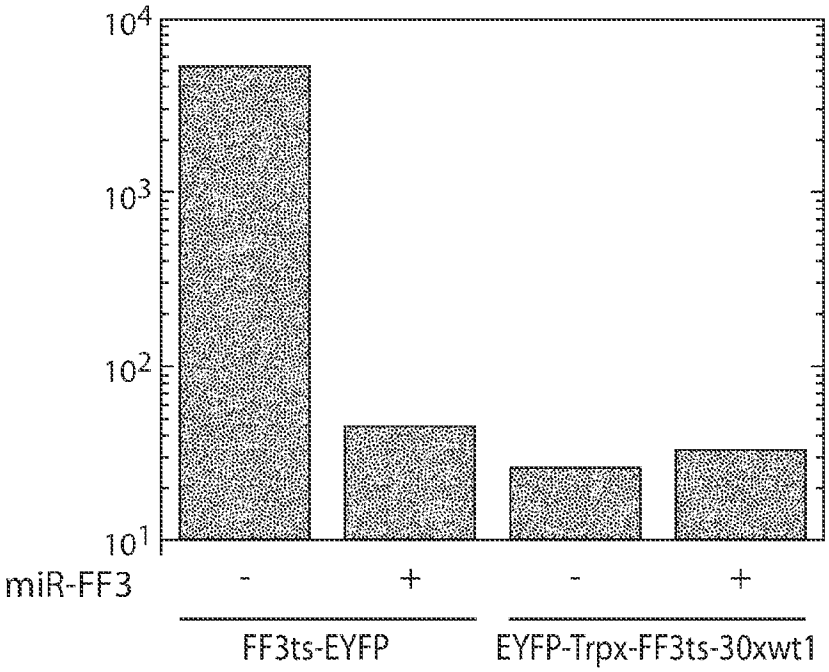
10^4
10^3
10^2
10^1
miR-FF3
-
+
-
+
FF3ts-EYFP
EYFP-Trpx-FF3ts-30xwt1

FIG. 5C

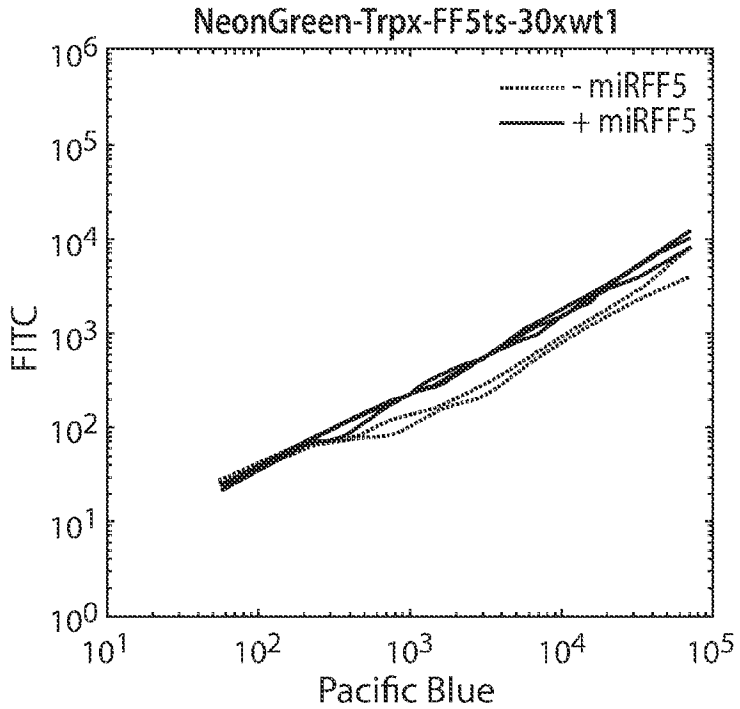
NeonGreen-Trpx-FF5ts-30xwt1
- miRFF5
+ miRFF5
FITC
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^1
10^2
10^3
10^4
10^5
Pacific Blue

FIG. 5D

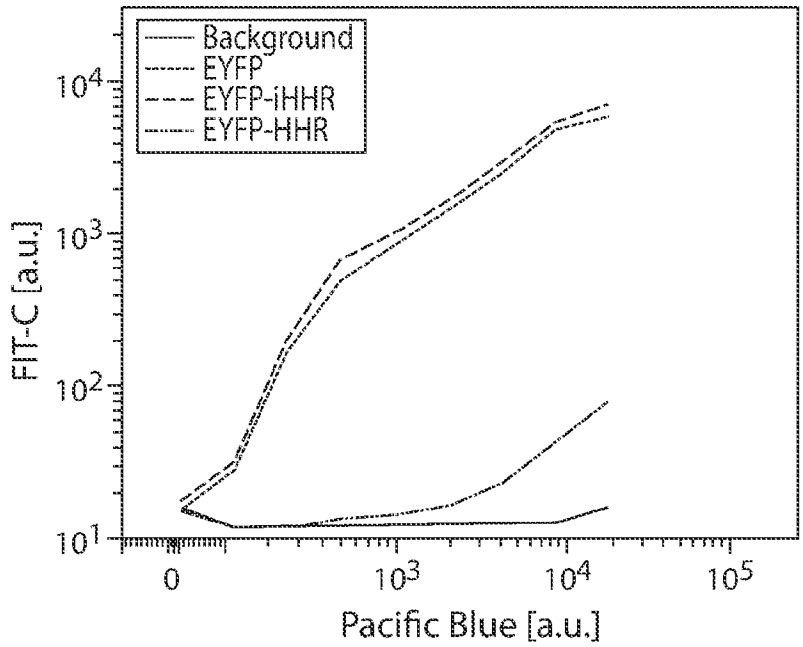
Background
EYFP
EYFP-iHHR
EYFP-HHR
FIT-C [a.u.]
10^4
10^3
10^2
10^1
0
10^3
10^4
10^5
Pacific Blue [a.u.]

FIG. 6A

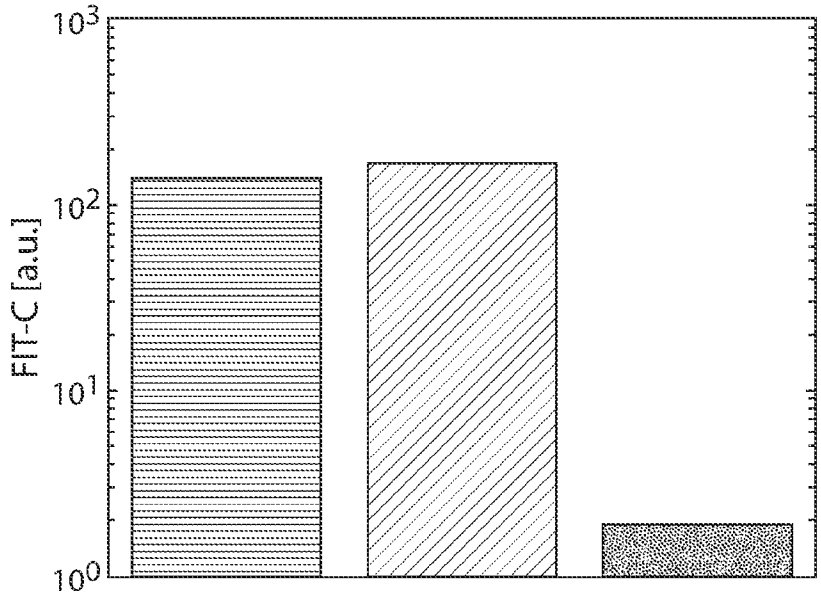
FIT-C [a.u.]
10^3
10^2
10^1
10^0

FIG. 6B

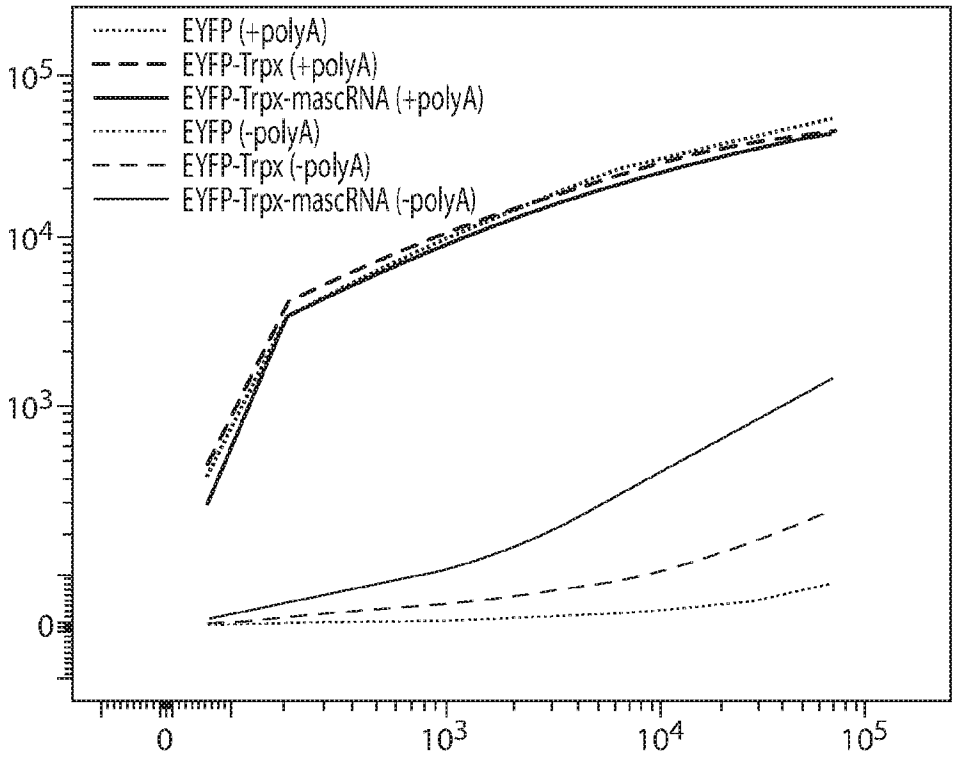
EYFP (+polyA)
EYFP-Trpx (+polyA)
EYFP-Trpx-mascRNA (+polyA)
EYFP (-polyA)
EYFP-Trpx (-polyA)
EYFP-Trpx-mascRNA (-polyA)
10^5
10^4
10^3
0
0
10^3
10^4
10^5

FIG. 6C

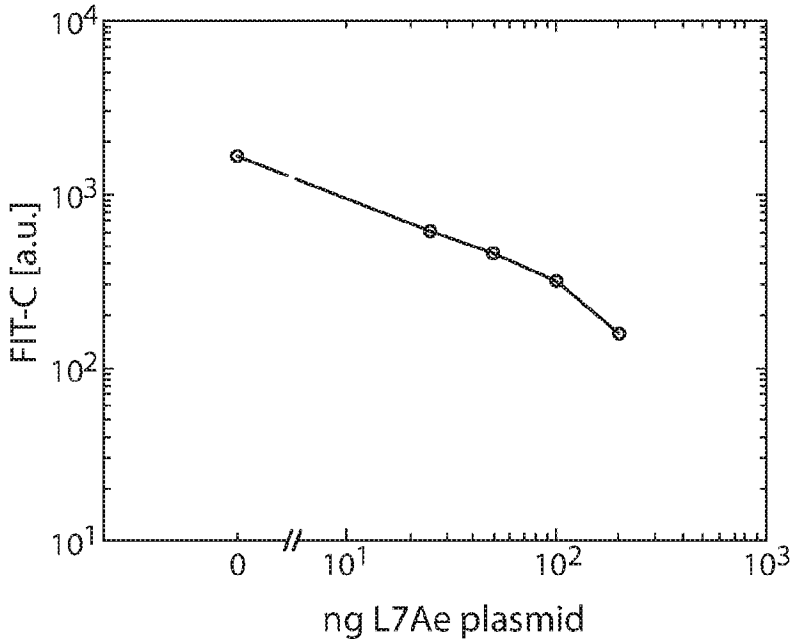
FIT-C [a.u.]
10^4
10^3
10^2
10^1
0
10^1
10^2
10^3
ng L7Ae plasmid

FIG. 7A

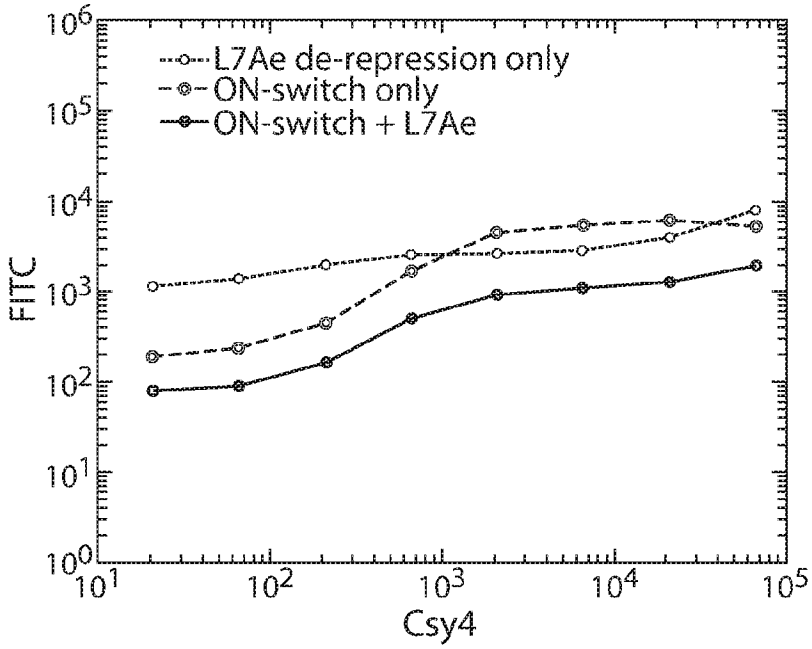
L7Ae de-repression only
ON-switch only
ON-switch + L7Ae
FITC
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^1
10^2
10^3
10^4
10^5
Csy4

FIG. 7B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/044309

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/63 C12Q1/6897
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)
C12N C12Q

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, COMPENDEX, 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.
X	ERIN K. BORCHARDT ET AL: "Controlling mRNA stability and translation with the CRISPR endoribonuclease Csy4", RNA, vol. 21, no. 11, 9 September 2015 (2015-09-09), pages 1921-1930, XP055513753, US ISSN: 1355-8382, DOI: 10.1261/rna.051227.115 figures 2,4 ----- -/--	1-6,22, 26-28, 30, 32-36, 38,39, 41,43, 46-57

[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
25 October 2018	02/01/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Piret, Bernard

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/044309

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/056440 A1 (NANODOC LTD [IL]; ABITBOL GUY [IL]) 3 May 2012 (2012-05-03) figure 11D -----	1,3,7-9, 22,28, 30,33, 36,38, 39,41, 43, 46-50,52
A	NIKOLAY KANDUL ET AL: "A positive readout single transcript reporter for site-specific mRNA cleavage", PEERJ, vol. 5, 20 July 2017 (2017-07-20), page e3602, XP055513726, DOI: 10.7717/peerj.3602 figures 2,3 -----	1-9,22, 26-28, 30, 32-36, 38,39, 41,43, 46-57

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/044309

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.:

7-9(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43
46-57(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 7-9(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43, 46-57(partially)

   A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the RNA cleaver is a RNAi molecule; methods and reagents derived therefrom.

   ---

2. claims: 10-15(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43, 46-57(partially)

   A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the RNA cleaver is a ribozyme; methods and reagents derived therefrom.

   ---

3. claims: 16-21(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43, 46-57(partially)

   A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, further comprising a second promoter linked to the nucleotide sequence encoding the RNA cleaver, and a third promoter linked to a third sequence encoding a RNA repressor; methods and reagents derived therefrom.

   ---

4. claims: 23-25(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43, 46-57(partially)

   A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, where the degradation signal comprises a sequence TAA[G/C][T/A][T/C]AT (SEQ ID NO:1-3); methods and reagents derived therefrom.

   ---

5. claims: 29, 40, 58-69(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43, 46-57(partially)

   A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, for use in diagnosis or therapy.

   ---

6. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all

partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is TetR.

---

7. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is CNOT7.

---

8. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is DDX6.

---

9. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is PPR10.

---

10. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is L7Ae.

---

11. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is Csy4.

---

12. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is Cas6.

---

13. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is CasE.

---

14. claims: 1-6, 22, 26-28, 30-36, 38, 39, 41-43, 46-57(all partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the output molecule is Cse3.

---

15. claims: 37(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43, 46-57(partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, wherein the RNA cleaver is expressed in the presence of the RNA cleaver.

---

16. claims: 44, 45(completely); 1-6, 22, 26-28, 30, 32-36, 38, 39, 41, 43, 46-57(partially)

A construct encoding a cleavage-inducible transcript, comprising a promoter, a RNA stabilizer, a cleavage site for a RNA cleaver, and a degradation signal, for use in a prokaryotic cell.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2018/044309

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012056440 A1	03-05-2012	AU 2011322114 A1	30-05-2013
		CA 2824604 A1	03-05-2012
		CN 103314003 A	18-09-2013
		EP 2632932 A2	04-09-2013
		JP 2013544511 A	19-12-2013
		US 2013245096 A1	19-09-2013
		WO 2012056440 A1	03-05-2012
		WO 2012056457 A2	03-05-2012