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(71) Applicant: TSINGHUA UNIVERSITY [CN/CN]; No. 1, Qinghuayuan, Haidian District, Beijing 100084 (CN).

(72) Inventor: WANG, Jianwei; No. 1, Qinghuayuan, Haidian District, Beijing 100084 (CN).

(74) Agent: TSINGYIHUA INTELLECTUAL PROPERTY LLC; Room 806, Block B, Yuhui Building, No. 73 Fucheng Road, Haidian District, Beijing 100142 (CN).

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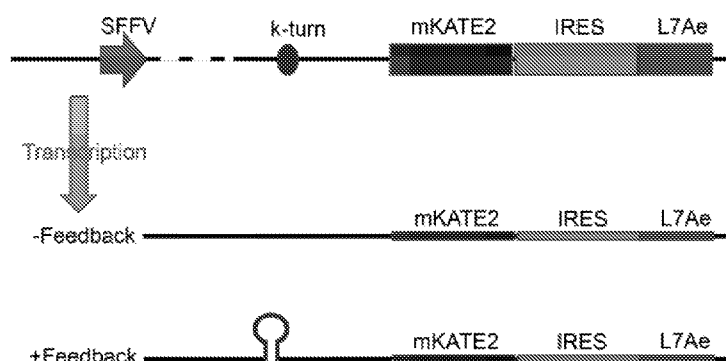


Fig. 3

(57) Abstract: Provided is a method for manipulating the expression level of a candidate gene and uses thereof. Provided is the method for manipulating the expression level of a candidate gene, comprising: controlling the strength of the feedback loop to manipulate the expression level of the candidate gene. It is revealed that the location and quantity of the motifs which can form one or more RNA hairpin structures after transcription are the key factors determining the strength of feedback circuit, which resulting in the achievement of precise gene expression. Briefly, the method for the first time establishes a technique of quantitative gene expression and provides a novel technical approach for the study of gene function and clinical relevance precisely.



METHODS FOR MANIPULATING QUANTITATIVE GENE EXPRESSION AND USES THEREOF

TECHNICAL FIELD

[0001] The present disclosure is in the field of molecular biology. Specifically, it relates to methods for manipulating quantitative gene expression and uses thereof.

BACKGROUND OF THE INVENTION

[0002] The study for gene function has flourished for decades. Cells live in a complex environment and respond to external and internal signals by producing appropriate proteins. The difference of protein quantity often reflects the difference of cell function. From a higher perspective, many of the differences between animal species lie in the differences of protein level, rather than the differences in their genes. Gene delivery by using virus-derived vectors into the host genome maintains stable long-term transgene expression, which is frequently utilized for scientific research and therapeutic applications (see *e.g.*, Dunbar CE, et al. Gene therapy comes of age. *Science* **359**, eaan4672 (2018). Milone MC, O'Doherty U. Clinical use of lentiviral vectors. *Leukemia* **32**, 1529–1541 (2018)). While, current induction systems often exhibit excessive gene expression which is not consistent with physiological or pathological alteration. Therefore, manipulating quantitative gene expression *in vitro* and *in vivo* is an essential tool for life science and clinical application.

[0003] In recent years, synthetic biology has aimed to revolutionize biological research with modulating precise spatial and temporal control of gene expression. One study reported that the incorporation of TATA box mutations allows for effective method to control gene expression noise (see *e.g.*, Murphy KF, Adams RM, Wang X, Balázsi G, Collins JJ. Tuning and controlling gene expression noise in synthetic gene networks. *Nucleic Acids Res* **38**, 2712-2726 (2010)). Segall-Shapiro et al. engineered an incoherent feedforward loop into *Escherichia coli* promoters using transcription-activator like effectors (TALEs) to achieve near-identical expression in different genome locations and plasmids (see Segall-Shapiro TH, Sontag ED, Voigt CA. Engineered promoters enable constant gene expression at any copy number in bacteria. *Nat Biotechnol* **36**, 352-358 (2018)). Frei et al. engineered natural and synthetic miRNA-based incoherent feedforward loop (iFFL) circuits that mitigate gene expression burden (see Frei T, et al. Characterization and mitigation of gene expression burden in mammalian cells. *Nat Commun* **11**, 4641 (2020)). Murphy et al. observed that the position and number of operator sites together determined the dose–response curve and gene expression noise (see Murphy KF, Balázsi G, Collins JJ. Combinatorial promoter design for engineering noisy gene expression. *Proc Natl Acad Sci U S A* **104**, 12726-12731 (2007)). Nevozhay et al. constructed a “linearizer” circuit by adding TetR autoregulation loop and observed significant reduction of noise at intermediate induction and linearization of dose–response before saturation (see Nevozhay D, Adams RM, Murphy KF, Josic K, Balázsi G. Negative autoregulation linearizes the dose-response and suppresses the heterogeneity of gene expression. *Proc Natl Acad Sci U S A* **106**, 5123-5128 (2009)). A very recent review summarized the application of CRISPR/Cas9 system in modulating gene expression (see *e.g.*, Du P, et al. CRISPR-Based Genetic Switches and Other Complex Circuits: Research and Application. *Life (Basel)* **11**, 1255 (2021)). Lillacci et al. developed a novel gene expression control systems including incoherent feedforward circuit, negative feedback circuit and hybrid circuit integrating negative feedback and incoherent feedforward (see Lillacci G, Benenson Y, Khammash M. Synthetic control systems for high performance gene expression in mammalian cells. *Nucleic Acids Res* **46**, 9855-9863 (2018)). One more study implement feedback control of endogenous pathways and synthetic gene circuits by using degronLOCKR system (see Ng AH, et al. Modular and tunable biological feedback control using a de novo protein switch. *Nature* **572**, 265-269 (2019)). Previous studies reported that RNA–protein (RNP) interaction is a natural tool for regulating protein expression (see *e.g.*, Saito H, Inoue T. Synthetic biology with RNA motifs. *Int J Biochem Cell Biol* **41**, 398-404 (2009)). Wieland M, Fussenegger M. Ligand-dependent regulatory RNA parts for Synthetic Biology in eukaryotes. *Curr Opin Biotechnol* **21**, 760-765 (2010). Liang JC,

Bloom RJ, Smolke CD. Engineering biological systems with synthetic RNA molecules. *Mol Cell* 43, 915-926 (2011)). Two studies developed effective TALE and TALERS repressor system for targeted inhibition of transcriptional and microRNA-mediated post-transcriptional regulation (see Cong L, Zhou R, Kuo YC, Cunniff M, Zhang F. Comprehensive interrogation of natural TALE DNA-binding modules and transcriptional repressor domains. *Nat Commun* 3, 968 (2012)). Li Y, et al. Modular construction of mammalian gene circuits using TALE transcriptional repressors. *Nat Chem Biol* 11, 207-213(2015)). Moreover, Kelly et al. developed a sophisticated feedback loop by using small RNA to modulate gene expression (see Kelly CL, et al. Synthetic negative feedback circuits using engineered small RNAs. *Nucleic Acids Res* 46, 9875-9889(2018)).

[0004] Although previous studies have made significant progress in synthetic field, there are still some unsolved issues, including: 1) manipulating quantitative expression of genes according to experimental requirements, 2) achieving such purpose by using a relatively simple system.

SUMMARY OF THE INVENTION

[0005] In order to find a method for manipulating the expression level of a candidate gene and a method for predicting the expression level of a candidate gene, a quantitative expression system by utilizing a feedback loop was introduced, which is composed of L7Ae and its binding partner (*e.g.*, the box C/D RNA kink-turn (k-turn)) to form an RNA-protein complex to regulate gene translation. Further study revealed that the location and quantity of the motifs which can form one or more RNA hairpin structures after transcription are the key factors determining the strength of feedback circuit, which resulting in the achievement of precise gene expression. Briefly, the present invention for the first time establishes a technique of quantitative gene expression and provides a novel technical approach for the study of gene function and clinical relevance precisely.

[0006] In one aspect, provided herein is a method for manipulating the expression level of a candidate gene comprising:

controlling the strength of the feedback loop to manipulate the expression level of the candidate gene, wherein the feedback loop comprises a sequence encoding L7Ae, a sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription, and a sequence encoding the candidate gene, and

L7Ae can bind with the RNA hairpin structure to form an RNA-protein complex to regulate the translation of the candidate gene.

[0007] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription, the sequence encoding the candidate gene and the sequence encoding L7Ae share the same promoter.

[0008] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is between the promoter and the sequence encoding the candidate gene, and the sequence encoding the candidate gene is between the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription and the sequence encoding L7Ae.

[0009] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is linked with the promoter and the sequence encoding the candidate gene.

[0010] In some embodiments, the RNA hairpin structure comprises a k-turn.

[0011] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription further comprises some random sequences.

[0012] In some embodiments, the feedback loop further comprises a sequence which functions as translation initiation.

[0013] In some embodiments, the sequence which functions as translation initiation is selected from at least one of Kozak sequence, IRES sequence and the sequence encoding 2A peptides.

[0014] In some embodiments, wherein the sequence which functions as translation initiation is between the sequence encoding the candidate gene and the sequence encoding L7Ae.

5 [0015] In some embodiments, the strength of the feedback loop is influenced by the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs.

[0016] In some embodiments, the method comprising:
normalizing the distance of the motif which can form RNA hairpin structure after transcription to the center of
10 the promoter and the sequence encoding the candidate gene and/or the number of the motifs to obtain the quantitative relationship between the strength of the feedback loop and the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs.

[0017] In some embodiments, the method comprising:
15 regulating the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs to obtain the desired expression level of the candidate gene.

[0018] In some embodiments, the candidate gene is an exogenous gene.

[0019] In another aspect, provided herein is a method for predicting the expression level of a
20 candidate gene, comprising utilizing the method of said above to obtain the strength of the feedback loop to predict the quantitative expression level of the candidate gene.

[0020] In another aspect, provided herein is use of the method of said above in manipulating or predicting the expression level of a candidate gene.

[0021] In another aspect, provided herein is a nucleic acid molecule comprising a promoter, a
25 sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription, a sequence encoding a candidate gene, a sequence which functions as translation initiation and a sequence encoding L7Ae,

wherein the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is linked with the promoter and the sequence encoding the candidate gene, and located
30 between the promoter and the sequence encoding the candidate gene, and the sequence which functions as translation initiation is linked with the sequence encoding the candidate gene and the sequence encoding L7Ae, and located between the sequence encoding the candidate gene and the sequence encoding L7Ae, and

L7Ae can bind with the RNA hairpin structure to form an RNA-protein complex to regulate the translation of
35 the candidate gene.

[0022] In some embodiments, the sequence which functions as translation initiation is selected from at least one of Kozak sequence, IRES sequence and the sequence encoding 2A peptides.

[0023] In another aspect, provided herein is an expression vector comprising the nucleic acid molecule of any one of said above.

40 [0024] In another aspect, provided herein is a recombinant cell carrying the nucleic acid molecule of any one of said above and/or the expression vector of said above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] FIG. 1 is a graph depicting the comparison of coding sequences of original L7Ae and
45 optimized L7Ae.

[0026] FIG. 2 is a graph depicting a lentiviral vector containing the optimized cDNA of L7Ae, IRES

(Internal Ribosome Entry Site), red fluorescent protein (mKATE2), wherein, the optimized cDNA of L7Ae is at the downstream of the red fluorescence protein (mKATE2) connected by IRES. Meanwhile, the k-turn motif is inserted into the same vector between promoter SFFV (Spleen Focus Forming Virus) and mKATE2.

[0027] FIG. 3 is a graph depicting the transcriptional results of a portion of lentiviral vectors with and without the k-turn motif respectively.

[0028] FIG. 4 is a graph depicting the expression of mKATE2 mRNA in NIH-3T3 cells infected by feedback loop-carrying (+Feedback) and feedback loop-free (-Feedback) lentivirus respectively, and the expression level of mKATE2 mRNA is evaluated by RT-PCR, wherein the left column is +Feedback group, and the right column is -Feedback group.

[0029] FIG. 5A-5B depict the expression of mKATE2 protein in NIH-3T3 cells infected by feedback loop-carrying (+Feedback), feedback loop-free (-Feedback) lentivirus and wildtype (also referred to as WT, which is not infected by lentivirus) respectively by FACS (Fluorescence-Activated Cell Sorting) and fluorescence microscope. FIG. 5A is a graph depicting the expression of mKATE2 protein by FACS. FIG. 5B is a graph depicting the expression of mKATE2 protein by fluorescence microscope.

[0030] FIG. 6 is a graph depicting the transcriptional results of a portion of lentiviral vectors with different location of the k-turn motif between SFFV promoter and mKATE2, including K43, K65, K85, K163 and K178, wherein the numbers represent the distance between k-turn motif and the promoter, and KC represents the vector without the k-turn motif.

[0031] FIG. 7 is a graph depicting the expression of mKATE2 mRNA in NIH-3T3 cells infected by feedback loop-carrying lentivirus with different location of the k-turn motif between SFFV promoter and mKATE2, and the expression level of mKATE2 mRNA is evaluated by RT-PCR, wherein the columns are numbered KC, K43, K65, K85, K163, K178 groups from left to right.

[0032] FIG. 8A-8B depict the expression of mKATE2 protein in NIH-3T3 cells infected by feedback loop-carrying lentivirus with different location of the k-turn motif between SFFV promoter and mKATE2 respectively by FACS and fluorescence microscope. FIG. 8A is a graph depicting the expression of mKATE2 protein by FACS. FIG. 8B is a graph depicting the expression of mKATE2 protein by fluorescence microscope.

[0033] FIG. 9 is a graph depicting the relative fluorescence intensity in NIH-3T3 cells infected by feedback loop-carrying lentivirus with different location of the k-turn motif between SFFV promoter and mKATE2 respectively.

[0034] FIG. 10 is a graph depicting a mathematical model obtained from different red fluorescence intensities in NIH-3T3 cells infected by feedback loop-carrying lentivirus with different location of the k-turn motif between SFFV promoter and mKATE2 respectively.

[0035] FIG. 11 is a graph depicting the transcriptional results of a portion of lentiviral vector with K148, wherein the number represents the distance between k-turn motif and the promoter, and KC represents the vector without the k-turn motif.

[0036] FIG. 12A-12B depict the expression of mKATE2 protein in NIH-3T3 cells infected by feedback loop-carrying lentivirus with K148 k-turn motif or without the k-turn motif respectively by FACS and fluorescence microscope. FIG. 12A is a graph depicting the expression of mKATE2 protein by FACS. FIG. 12B is a graph depicting the expression of mKATE2 protein by fluorescence microscope.

[0037] FIG. 13 is a graph depicting the relative fluorescence intensity in NIH-3T3 cells infected by feedback loop-carrying lentivirus with K148 k-turn motif or without the k-turn motif.

[0038] FIG. 14 is a graph depicting the predicted intensity of red fluorescence in NIH-3T3 cells infected by feedback loop-carrying lentivirus with K148.

[0039] FIG. 15 is a graph depicting the transcriptional results of a portion of lentiviral vectors with one or two k-turn motifs between SFFV promoter and EGFP, including K-turn1, K-turn2, and KC, wherein the numbers represent the number of k-turn motif between SFFV promoter and EGFP, and KC represents the vector

without the k-turn motif.

[0040] FIG. 16A-16B depict the expression of EGFP protein in NIH-3T3 cells infected by feedback loop-carrying lentivirus with one or two k-turn motifs or without the k-turn motif respectively by FACS and fluorescence microscope, wherein one or two k-turn motifs called K-turn1, K-turn2, respectively. FIG. 16A is a graph depicting the expression of EGFP protein by FACS. FIG. 16B is a graph depicting the expression of EGFP protein by fluorescence microscope.

[0041] FIG. 17 is a graph depicting the relative fluorescence intensity in NIH-3T3 cells infected by feedback loop-carrying lentivirus with K-turn1, K-turn2 or without the k-turn motif, wherein the columns are numbered KC, K-turn1, K-turn2 groups from left to right.

[0042] FIG. 18 is a graph depicting a vector containing the optimized cDNA of L7Ae, IRES (Internal Ribosome Entry Site), red fluorescent protein (mKATE2), wherein, the optimized cDNA of L7Ae is at the downstream of the red fluorescence protein (mKATE2) connected by IRES. Meanwhile, the k-turn motif is inserted into the same vector between promoter CAG and mKATE2.

[0043] FIG. 19 is a graph depicting the process of the linearization and microinjection of the plasmids CAG-KC (control) and CAG-K77 into mouse zygotes respectively to obtain the transgenic mice.

[0044] FIG. 20 is a graph depicting the results of PCR identification of positive transgenic mice.

[0045] FIG. 21A-21B is a graph depicting the intensity of red fluorescence in the brain, kidney, thymus and ileum of CAG-K77 mice and CAG-KC mice.

DETAILED DESCRIPTION

[0046] The invention is intended to cover all alternatives, modifications, and equivalents which may be included within the scope of the present invention as defined by the claims. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described herein. In the event that one or more of the incorporated literature, patents, and similar materials differ from or contradict this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls.

[0047] It is further appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment, can also be provided separately or in any suitable subcombination.

[0048] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one skilled in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference in their entirety. Although many methods and materials similar or equivalent to those described herein could be used in the practice or test of the present invention, the preferred methods, equipment and materials are described in the invention.

General

[0049] Practice of the methods, as well as preparation and use of the compositions disclosed herein employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, chromatin structure and analysis, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. *See, for example, Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL*, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel et al., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John Wiley & Sons, New York, 1987 and periodic updates; the series *METHODS IN ENZYMOLOGY*, Academic Press, San Diego; Wolffe, *CHROMATIN STRUCTURE AND FUNCTION*, Third edition,

Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol. 304, "Chromatin" (P.M. Wassarman and A. P. Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, "Chromatin Protocols" (P.B. Becker, ed.) Humana Press, Totowa, 1999.

[0050] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

A method for manipulating quantitative gene expression

[0051] Although previous studies have made significant progress in synthetic field, there are still some unsolved issues, including: 1) manipulating quantitative expression of genes according to experimental requirements, 2) achieving such purpose by using a relatively simple system, and 3) generating a mouse model with quantitative expression of interested gene.

[0052] In this study, a quantitative expression system by utilizing a feedback loop, which comprises L7Ae and its binding partner (for example, the box C/D RNA kink-turn (k-turn) motif), was introduced to form an RNA-protein complex to regulate gene translation. Further study revealed that the location and quantity of the motifs which can form one or more RNA hairpin structures after transcription are the key factors determining the strength of feedback circuit, which resulting in the achievement of precise gene expression. Briefly, the present invention for the first time establishes a technique of quantitative gene expression and provides a novel technical approach for the study of gene function and clinical relevance precisely.

[0053] L7Ae is a ribosomal protein of *Archaeoglobus fulgidus*, it can bind to the box C/D RNA kink-turn (k-turn) motif or other RNA hairpin structures, then they form an RNA-protein complex and this RNA-protein complex increases the steric hindrance, and then prevent ribosomes from scanning the downstream, thereby reducing the efficiency of ribosomes reaching the initiation codon, that is, reducing the translation efficiency of proteins.

[0054] In some embodiments, provided herein is a method for manipulating the expression level of a candidate gene, comprising:

controlling the strength of the feedback loop to manipulate the expression level of the candidate gene, wherein the feedback loop comprises a sequence encoding L7Ae, a sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription, and a sequence encoding the candidate gene, and

L7Ae can bind with the RNA hairpin structure to form an RNA-protein complex to regulate the translation of the candidate gene. In the present invention, the feedback loop is also known as feedback circuit.

[0055] The strength of the feedback loop is inversely proportional to the expression level of the candidate gene, which means the stronger of the feedback loop, the lower of the expression level of candidate gene.

[0056] The sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is a nucleic acid sequence, and a motif can form an RNA hairpin structures after transcription. The RNA hairpin structure binds to L7Ae to form an RNA-protein complex to regulate the translation of the candidate gene.

[0057] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is located between the promoter and the sequence encoding the candidate gene, and the sequence encoding the candidate gene is between the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription and the sequence encoding L7Ae.

[0058] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription, the sequence encoding the candidate gene and the sequence encoding L7Ae share the same promoter. But it not means the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription, the candidate gene and the gene

encoding L7Ae co-express.

[0059] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is linked with the promoter and the sequence encoding the candidate gene.

[0060] In some embodiments, the RNA hairpin structure comprises a k-turn. In some embodiments, the k-turn is a hairpin structure which could bind with L7Ae. For the sequence of the k-turn, it refers to the sequences have been reported in the literature or patents known in the art, *e.g.*, DJ Klein, TM Schmeing, PB Moore, TA Steitz. The kink-turn: a new RNA secondary structure motif, *The EMBO Journal* 20(15) pp.4214-4221 (2001), and the like.

[0061] In the present invention, a box C/D RNA kink-turn (k-turn) motifs also known as k-turn motif or k-turn, they are equally interchangeable.

[0062] In some embodiments, the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription further comprises some random sequences. These sequences are random and cannot affect the binding of the RNA hairpin structures and L7Ae. These random sequences can contribute to obtain different distances of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene.

[0063] In fact, the feedback loop further comprises a sequence which functions as translation initiation, which is located between the sequence encoding the candidate gene and the sequence encoding L7Ae. In some embodiments, the sequence which functions as translation initiation is selected from at least one of Kozak sequence, IRES (Internal Ribosome Entry Site) sequence and the sequence encoding 2A peptides. But it does not exclude other translation initiation sequences known in the art, and they are all included in the protection scope of the present invention. In some embodiments, the promoter can be any promoter known in the art, for example, CAG promoter or SFFV (Spleen Focus Forming Virus) promoter and so on.

[0064] In some embodiments, the gene encoding L7Ae can be wildtype sequence, or optimized the coding sequence of L7Ae according to the principle of codon bias in mammals or other species.

[0065] In some embodiments, the strength of the feedback loop is influenced by the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs.

[0066] In some embodiments, both the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and the number of the motifs can affect the strength of the feedback loop.

[0067] In some embodiments, the method for manipulating the expression level of a candidate gene, comprising:

normalizing the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs to obtain the quantitative relationship between the strength of the feedback loop and the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs.

[0068] In some embodiments, the method for manipulating the expression level of a candidate gene, comprising:

normalizing the distance of the k-turn to the center of the promoter and the candidate gene to obtain the quantitative relationship between the strength of the feedback loop and the distance of the k-turn to the center of the promoter and the candidate gene.

[0069] In some embodiments, the method for manipulating the expression level of a candidate gene, comprising:

normalizing the number of k-turns to obtain the quantitative relationship between the strength of the feedback loop and the number of k-turns.

[0070] In some embodiments, the method for manipulating the expression level of a candidate gene, comprising:

regulating the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs to obtain the desired expression level of the candidate gene.

[0071] In some embodiments, the method for manipulating the expression level of a candidate gene, comprising:

regulating the distance of the k-turn to the center of the promoter and the candidate gene and/or the number of k-turns to obtain the desired expression level of the candidate gene.

[0072] In some embodiments, the method for manipulating the expression level of a candidate gene, comprising:

regulating the distance of the k-turn to the center of the promoter and the candidate gene to obtain the desired expression level of the candidate gene.

[0073] In some embodiments, the method for manipulating the expression level of a candidate gene, comprising:

regulating the number of k-turns to obtain the desired expression level of the candidate gene.

[0074] In some embodiments, the strength of the feedback loop is influenced by the power of the promoter. In fact, the power of the promoter can also affect the strength of the feedback loop. But generally on the basis of a defined promoter, and then by regulating the distance of the k-turn to the center of the promoter and the candidate gene and/or the number of k-turns to obtain the desired expression level of the candidate gene.

[0075] In some embodiments, the candidate gene is an exogenous gene. For example, the candidate gene can be any interested gene. Also, the candidate gene can be some functional genes, or marker genes (for example, the gene encoding red fluorescent protein (mKATE2)).

[0076] In some embodiments, when there is a need to quantitatively express an interested gene, the method for manipulating the expression level of a candidate gene provided by the present invention can be used to determine the distance of the k-turn to the center of the promoter and the interested gene and/or the number of k-turns, thereby realizing the quantitative expression of the interested gene. And the following examples of the present invention further demonstrate the method for manipulating the expression level of a candidate gene provided by the present invention can accurately manipulate the expression of candidate genes both *in vitro* and *in vivo*.

[0077] In the present invention, the k-turn is also known as k-turn, they are equally interchangeable.

[0078] In the present invention, all data are shown as mean $\pm$ SEM. statistical significance analysis was calculated by Two-tailed unpaired Student's t-test after testing for normal distribution and data were plotted and nonlinear regression curves was fitted by GraphPad Prism 9 software. *: $p < 0.05$, **: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$ and n.s. represents not significant. All experiments were repeated twice or three times independently.

EXAMPLES

Example 1: Achieving stable gene suppression by using L7Ae and k-turn feedback loop

[0079] Given that L7Ae is a ribosomal protein of *Archaeoglobus fulgidus*, then optimized the coding sequence of L7Ae to produce more functional protein according to the principle of codon bias in mammals (see FIG. 1). As shown in FIG. 1, depicting the comparison of coding sequences of original L7Ae and optimized L7Ae.

[0080] In order to achieve stable expression of candidate gene, inserted the optimized cDNA of L7Ae at the downstream of a red fluorescence protein (mKATE2) connected by IRES (Internal Ribosome Entry Site) of a lentiviral vector (see FIG. 2 and FIG. 3). Meanwhile, the k-turn motif was inserted into the same vector between promoter SFFV (Spleen Focus Forming Virus) and mKATE2 (see FIG. 2 and FIG. 3). Lentivirus was produced in 293T cells according to standard procedures (see He, H. et al. Aging-induced IL27Ra Signaling

Impairs Hematopoietic Stem Cells. *Blood* 136, 183-198(2020)).

[0081] NIH-3T3 cells were infected by feedback loop-carrying (+Feedback) and feedback loop-free (-Feedback) lentivirus respectively. Three days later, the expression of mKATE2 mRNA was evaluated by RT-PCR (Real-time PCR) and the result revealed that it is comparable between these two groups (see FIG. 4).

5 NIH-3T3 Cell culture

[0082] NIH-3T3 cells were cultured under 5% CO₂ at 37 °C in DMEM medium (Gibco, C11995500BT) that supplement with 10% FBS (GEMINI, 900-108) and 1% Penicillin-Streptomycin (Hyclone, SV30010). Cells were passaged every 2-3 days using 0.25% trypsin-EDTA (Hyclone, SH300042.01).

Quantitative Real-time PCR

10 [0083] Total RNA was extracted using TRIzol (Invitrogen, 15596018) according to the manufacturer's instructions. Total RNA was subjected to reverse transcription using PrimeScript™RT reagent Kit with gDNA Eraser (Takara, RR047A). Quantitative real-time PCR was performed on QuantStudio-3 Real-time PCR System (Applied Biosystems) using the 2× SYBR Green master mix (Applied Biosystems, A25780). The primer information is listed in Table1.

15 **Table1: Exemplary primer designs**

primers	Forward	Reverse
mKATE2 primer 1	5'-ggtgagcgagctgattaagg-3'(SEQ ID NO: 1)	5'-cgccctgattctcatggtct-3'(SEQ ID NO: 2)
mKATE2 primer 2	5'-ccacctgatctgcaactga-3'(SEQ ID NO: 3)	5'-tgctaggagggtcgagctat-3'(SEQ ID NO: 4)
Actin	5'-ggctgtattccctccatcg-3'(SEQ ID NO: 5)	5'-ccagttggtaacaatgccatgt-3'(SEQ ID NO: 6)

[0084] Then sought out to evaluate the expression of mKATE2 protein by FACS (Fluorescence-Activated Cell Sorting) and fluorescence microscope. The intensity of red fluorescence is significantly lower in the feedback loop-carrying group than control was observed (see FIG. 5A-5B). These results indicate that the feedback loop of L7Ae and k-turn is able to repress the translation, but not the transcription of candidate gene.

Detection of mKATE2 by Flow Cytometry.

20 [0085] NIH-3T3 cells were infected by lentivirus, and three days later, cells were collected by trypsinization and centrifugation at 500g for 5 min. The supernatant was then removed and the cells were then resuspended. The cell suspensions were identified by flow cytometry using BD LSRFortessa (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Detection of mKATE2 by Confocal microscope.

[0086] NIH-3T3 cells were infected by lentivirus, and three days later, cells were observed under a FV1200 Confocal microscope (Olympus), using a ×10 objective.

30 [0087] KC and K77 positive mice were anesthetized and collected different tissues after heart perfusion. Tissues were rinsed in ice cold PBS once and fixed in 4% PFA 2-4h at 4°C. Then these tissues were rinsed in ice cold PBS for 5 minutes, dehydrate using graded sucrose and embed in OCT stored in -80°C. These tissues were cut into 15mm pieces on slide, DAPI staining for 15 minutes and observed under a FV1200 Confocal microscope (Olympus), using a ×40 objective. Image analysis was performed with Fiji/ImageJ and imaris viewer.

Example 2: Quantitative Gene Delivery by using L7Ae and k-turn feedback loop

40 [0088] A hypothesis was then made whether quantitative gene expression could be achieved according to the experimental requirements by using L7Ae and k-turn. To address this question, the k-turn motif to different location between SFFV promoter and mKATE2 was cloned, including K43, K65, K85, K163 and

K178, wherein the numbers represent the distance between k-turn motif and the promoter (see **FIG. 6**). NIH-3T3 cells were infected by these lentiviruses and 72 hours later, the expression of mKATE2 mRNA by RT-PCR was evaluated and the result showed that it is comparable between all situations (see **FIG. 7**), indicating that the location of k-turn does not affect the transcription of candidate gene.

5 **[0089]** Refer to Example 1 for the experimental procedures of NIH-3T3 cell culture, RT-PCR, primers for RT-PCR, detection of mKATE2 by Flow Cytometry, and detection of mKATE2 by Confocal microscope.

[0090] Next, the expression levels of mKATE2 protein by FACS and fluorescence microscope were examined. As shown in **FIG. 8A-8B**, the result shows that the intensity of red fluorescence is arranged in the following order: K43, K178, K65, K163 and K85, which indicates that the location of k-turn motif indeed influences the feedback strength. Then the intensity of red fluorescence of each group was normalized and the result reveals that: the feedback strength is strong when k-turn locates close to promoter or mKATE2, and it gradually decreases as it gets closer to the center (see **FIG. 9**).

Example 3: Mathematical equation to predict the feedback strength

15 **[0091]** To facilitate to calculate the feedback strength of k-turn at different locations, the distance of k-turn to promoter was normalized (Table 2). The mathematical modeling to fit the changes of the intensity of red fluorescence was performed (see **FIG. 10**) and the following equation was generated: $Y = -145.1 + 8.812X - 0.08134X^2$, wherein Y represents the theoretical expression of candidate gene, X represents the relative distance of k-turn to promoter.

20 **Table 2:**

Distance of k-turn to promoter/bp(A _n)	feedback intensity (B _n)	Distance of promoter to target gene/bp(C _n)	no feedback intensity(D _n)	Relative Distance calculation	Relative Distance value (X)	Relative intensity percentage calculation	Relative intensity percentage(%) (Y)
43	319	214	2920	$=A_n/C_n * 100$	20.09345794	$=B_n/D_n * 100$	10.92465753
65	717				30.37383178		24.55479452
85	2488				39.71962617		85.20547945
163	1959				76.1682243		67.0890411
178	427				83.17757009		14.62328767

[0092] In order to verify whether this mathematical equation can accurately predict the expression of candidate gene, a K148 vector was generated, wherein the k-turn motif was cloned at the 148th base pair downstream of promoter (see **FIG. 11**). NIH-3T3 cells were infected by K148 and KC lentiviruses respectively and 72 hours later, the red fluorescence by FACS and fluorescence microscope were examined. The result shows that intensity of red fluorescence of K148 is reduced to 66% of KC (see **FIG. 12A-12B, FIG. 13**). Interestingly, the predicted intensity of red fluorescence of K148 is 75% of KC (**FIG. 14, Table 3**). This result demonstrates that the predicted value generated by this mathematical equation is consistent with the experimental value.

30 **Table 3**

Distance of k-turn to promoter/bp(A _n)	148
Distance of promoter to target gene/bp(C _n)	214
Relative Distance calculation	$=A_n/C_n * 100$
Relative Distance value(X)	69.1588785

equation	$Y = -145.1 + 8.812X - 0.08134X^2$
Relative intensity percentage% (Y)	75.28284566
no feedback intensity	9559
Relative intensity percentage calculation	=percentage%*KC
feedback intensity	7196.287217

[0093] Refer to Example 1 for the experimental procedures of NIH-3T3 cell culture, RT-PCR, primers for RT-PCR, detection of mKATE2 by Flow Cytometry, and detection of mKATE2 by Confocal microscope.

5 Example 4: The quantify of k-turn affects the feedback strength

[0094] A hypothesis was then made whether the quantity of k-turn affects the strength of feedback loop. To test this hypothesis, two lentiviral vectors containing one and two k-turn motifs were generated (see FIG. 15). NIH-3T3 cells were infected by one and two k-turn motif-carrying lentivirus. The intensity of green fluorescence is significantly lower in two k-turn motif-carrying group was observed (see FIG. 16A-16B). While the mRNA of EGFP is comparable between them (see FIG. 17). The result indicates that the feedback strength is proportional to the number of k-turn motifs.

NIH-3T3 Cell culture

[0095] NIH-3T3 cells were cultured under 5% CO₂ at 37 °C in DMEM medium (Gibco, C11995500BT) that supplement with 10% FBS (GEMINI, 900-108) and 1% Penicillin-Streptomycin (Hyclone, SV30010). Cells were passaged every 2-3 days using 0.25% trypsin-EDTA (Hyclone, SH300042.01).

Quantitative Real-time PCR

[0096] Total RNA was extracted using TRIzol (Invitrogen, 15596018) according to the manufacturer's instructions. Total RNA was subjected to reverse transcription using PrimeScript™RT reagent Kit with gDNA Eraser (Takara, RR047A). Quantitative real-time PCR was performed on QuantStudio-3 Real-time PCR System (Applied Biosystems) using the 2× SYBR Green master mix (Applied Biosystems, A25780). The primer information is listed in Table4.

Table 4: Exemplary primer designs

primers	Forward	Reverse
EGFP primer1	5'-acgtaaacggccacaagttcA-3'(SEQ ID NO: 7)	5'-aagtcgtgctgcttcatgtg-3'(SEQ ID NO: 8)
EGFP primer2	5'-agaacggcatcaaggtgaac-3'(SEQ ID NO: 9)	5'-tgctcaggtagtggtgtcg-3'(SEQ ID NO: 10)
Actin	5'-ggctgtattccctccatcg -3'(SEQ ID NO: 5)	5'-ccagttggaataatgccatgt-3'(SEQ ID NO: 6)

Detection of EGFP by Flow Cytometry.

[0097] NIH-3T3 cells were infected by lentivirus, and three days later, cells were collected by trypsinization and centrifugation at 500g for 5 min. The supernatant was then removed and the cells were then resuspended. The cell suspensions were identified by flow cytometry using BD LSRFortessa (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

Detection of EGFP by Confocal microscope.

[0098] NIH-3T3 cells were infected by lentivirus, and three days later, cells were observed under a FV1200 Confocal microscope (Olympus), using a ×10 objective.

[0099] KC and K77 positive mice were anesthetized and collected different tissues after heart perfusion. Tissues were rinsed in ice cold PBS once and fixed in 4% PFA 2-4h at 4°C. Then these tissues were rinsed in ice cold PBS for 5 minutes, dehydrate using graded sucrose and embed in OCT stored in -80°C. These tissues were cut into 15mm pieces on slide, DAPI staining for 15 minutes and observed under a FV1200 Confocal microscope (Olympus), using a ×40 objective. Image analysis was performed with Fiji/ImageJ and

imaris viewer.

[00100] In order to depict the logical relationship between the output and each parameter, the output can be modeled as

$$F(\text{gene}) \propto w \cdot \frac{P}{D_{k\text{-turn}} \cdot N_{k\text{-turn}}}$$

wherein w represents weight coefficient, which is context-dependent, for example, cell type, environmental factor; P represents the power of promoter; $D_{k\text{-turn}}$ represents the distance of k-turn to the center of the promoter and candidate gene; $N_{k\text{-turn}}$ represents the number of k-turns. The function indicates that the feedback strength is positively correlated with the strength of the promoter, positively correlated with the specific cellular context, and negatively correlated with the location and quantity of k-turn motif.

Example 5: Generating quantitative gene expression mice model

Generation of transgenic mice

[00101] The reformed CAG promoter, CAG-K77, containing k-turn were designed. The k-turn was located 77bp downstream from the transcription start site and L7Ae was connected with mKATE2 by IRES. The CAG-KC-mKATE2-IRES-L7Ae and CAG-K77-mKATE2-IRES-L7Ae plasmids were constructed by connecting CAG promoter, CAG-KC or CAG-K77 with mKATE2-IRES-L7Ae-SV40PA cassette separately(see FIG. 18).

[00102] The recombinant plasmids CAG-KC-mKATE2-IRES-L7Ae and CAG-K77-mKATE2-IRES-L7Ae were identified by sequencing and linearized by ScaI. Then the two linearized plasmids were diluted to the final concentration of 5ng/μl with TE buffer and microinjected into the pronucleus of C57BL/6J fertilized oocytes separately. The fertilized oocytes were subsequently implanted in the oviduct of pseudopregnant recipient ICR mice(see FIG. 19). The plasmids CAG-KC (control) and CAG-K77 were linearized and micro-injected into murine zygotes respectively. CAG-K77 mice were delivered successfully, indicating that L7Ae and k-turn have no effect on embryonic development.

[00103] PCR was used to detect the genotype of DNA separated from the newborn mouse tail tissues. CAG-KC and CAG-K77 littermates were genotyped by PCR with primers CAG seq F1: 5'-TATGCCAAGTACGCCCCCTATTGA-3'(SEQ ID NO: 11), CAG seq R1: 5'-CCTCGCCATAAAAGGAACTTTCG-3'(SEQ ID NO: 12), using the following parameters: 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and 72°C for 5 min to final extension. PCR product for positive band is 363 bp (see FIG. 20).

[00104] As shown in FIG.20, the single bands (363bp) in the CAG-KC-11# and CAG-K77-23# mice are obtained, which indicates that they are positive ones.

[00105] To investigate whether the feedback loop functions *in vivo*, the organs of both mice were collected, and the intensity of red fluorescence were detected. As shown in FIG.21A and FIG.21B, the intensity of red fluorescence indeed drops to 63%-76% in the brain (72.9%), kidney (75.9%), thymus (66.2%) and ileum (62.8%) of CAG-K77 mice compared to CAG-KC mice. The result of CAG-K77 theoretic calculation by the equation mentioned above (in Example 3) is 65.1%, which agrees with the experimental finding in several organs. The mouse data substantiate that the model which fitted by NIH-3T3 cells statistics is optimal to multiple systems.

[00106] In a word, the above results show that the system for quantitative expression of candidate gene provided by the present invention can accurately manipulate the expression of candidate genes both *in vitro* and *in vivo* by controlling the distance of k-turn to the center of the promoter and candidate gene and/or the number of k-turns.

[00107] All mice were housed in specific pathogen-free conditions.

[00108] All patents, patent applications and publications mentioned herein are hereby incorporated by reference for all purposes in their entirety.

[00109] Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and
5 modifications can be practiced without departing from the spirit or scope of the disclosure. Accordingly, the foregoing descriptions and examples should not be construed as limiting.

CLAIMS

1. A method for manipulating the expression level of a candidate gene comprising:

controlling the strength of the feedback loop to manipulate the expression level of the candidate gene,

wherein the feedback loop comprises a sequence encoding L7Ae, a sequence that comprises one or more motifs
5 which can form one or more RNA hairpin structures after transcription, and a sequence encoding the candidate gene, and

L7Ae can bind with the RNA hairpin structure to form an RNA-protein complex to regulate the translation of the candidate gene.

2. The method of claim 1, wherein the sequence that comprises one or more motifs which can form one or more
10 RNA hairpin structures after transcription, the sequence encoding the candidate gene and the sequence encoding L7Ae share the same promoter.

3. The method of any one of claims 1-2, wherein the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is between the promoter and the sequence encoding the candidate gene, and the sequence encoding the candidate gene is between the sequence that comprises one
15 or more motifs which can form one or more RNA hairpin structures after transcription and the sequence encoding L7Ae.

4. The method of any one of claims 1-3, wherein the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is linked with the promoter and the sequence encoding the candidate gene.

5. The method of any one of claims 1-4, wherein the RNA hairpin structure comprises a k-turn.

6. The method of any one of claims 1-5, wherein the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription further comprises some random sequences.

7. The method of any one of claims 1-6, wherein the feedback loop further comprises a sequence which functions as translation initiation.

8. The method of any one of claims 1-7, wherein the sequence which functions as translation initiation is selected from at least one of Kozak sequence, IRES sequence and the sequence encoding 2A peptides.

9. The method of any one of claims 1-8, wherein the sequence which functions as translation initiation is between the sequence encoding the candidate gene and the sequence encoding L7Ae.

10. The method of any one of claims 1-9, wherein the strength of the feedback loop is influenced by the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the
30 sequence encoding the candidate gene and/or the number of the motifs.

11. The method of any one of claims 1-10, wherein the method comprising:

normalizing the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs to obtain the
35 quantitative relationship between the strength of the feedback loop and the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs.

12. The method of any one of claims 1-11, wherein the method comprising:

regulating the distance of the motif which can form RNA hairpin structure after transcription to the center of the promoter and the sequence encoding the candidate gene and/or the number of the motifs to obtain the desired
40 expression level of the candidate gene.

13. The method of any one of claims 1-12, wherein the candidate gene is an exogenous gene.

14. A method for predicting the expression level of a candidate gene, comprising utilizing the method of any one of claims 1-13 to obtain the strength of the feedback loop to predict the quantitative expression level of the
45 candidate gene.

15. Use of the method of any one of claims 1-13 in manipulating or predicting the expression level of a

candidate gene.

16. A nucleic acid molecule comprising a promoter, a sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription, a sequence encoding a candidate gene, a sequence which functions as translation initiation and a sequence encoding L7Ae,

5 wherein the sequence that comprises one or more motifs which can form one or more RNA hairpin structures after transcription is linked with the promoter and the sequence encoding the candidate gene, and located between the promoter and the sequence encoding the candidate gene, and

10 the sequence which functions as translation initiation is linked with the sequence encoding the candidate gene and the sequence encoding L7Ae, and located between the sequence encoding the candidate gene and the sequence encoding L7Ae, and

L7Ae can bind with the RNA hairpin structure to form an RNA-protein complex to regulate the translation of the candidate gene.

17. The nucleic acid molecule of claim 16, wherein the sequence which functions as translation initiation is selected from at least one of Kozak sequence, IRES sequence and the sequence encoding 2A peptides.

15 18. An expression vector comprising the nucleic acid molecule of any one of claims 16-17.

19. A recombinant cell carrying the nucleic acid molecule of any one of claims 16-17 and/or the expression vector of claim 18.

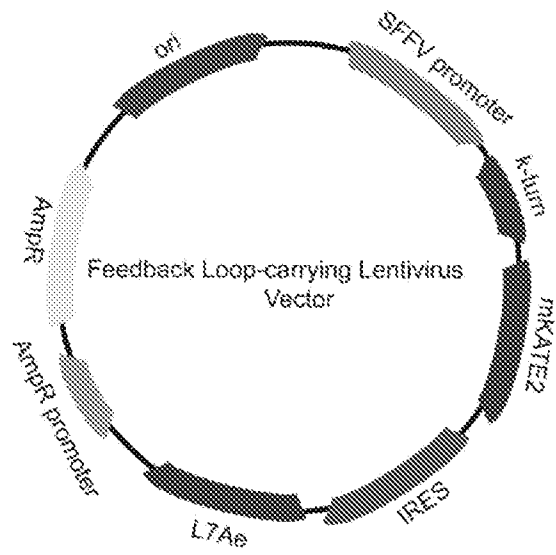


Fig. 2

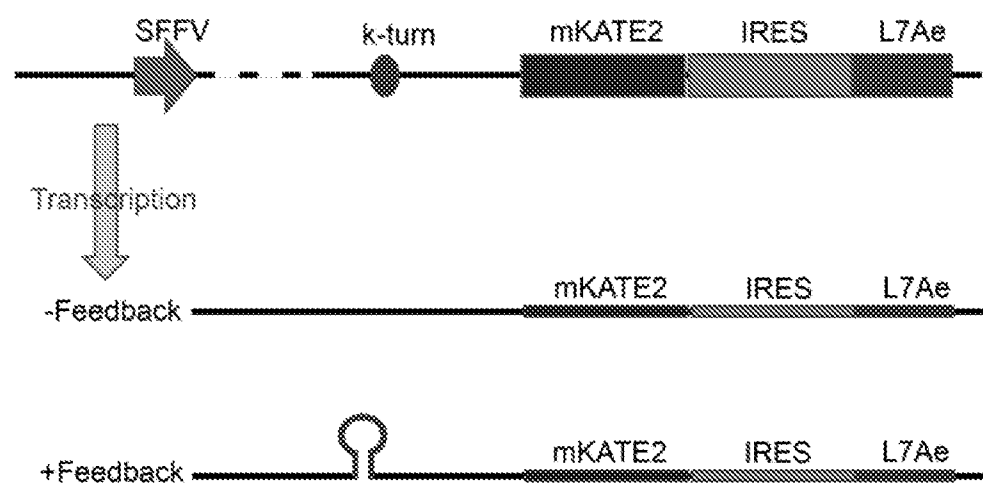


Fig. 3

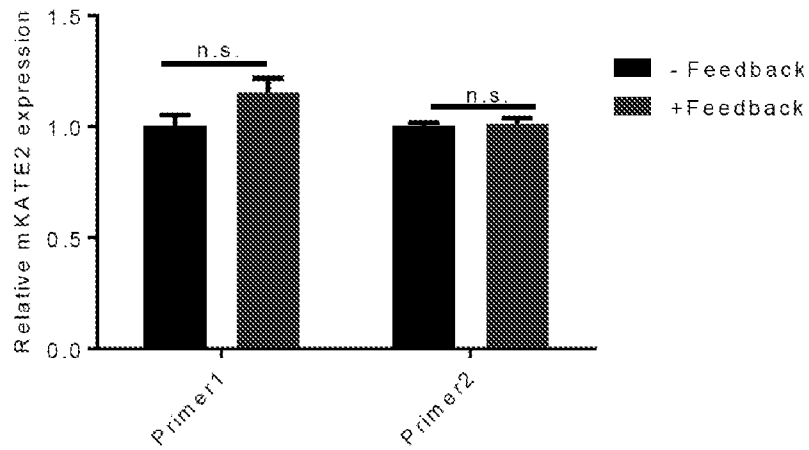


Fig. 4

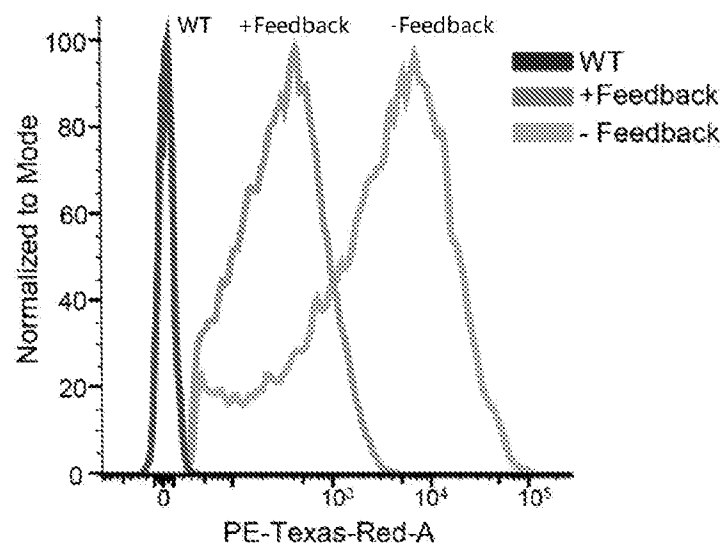


Fig. 5A

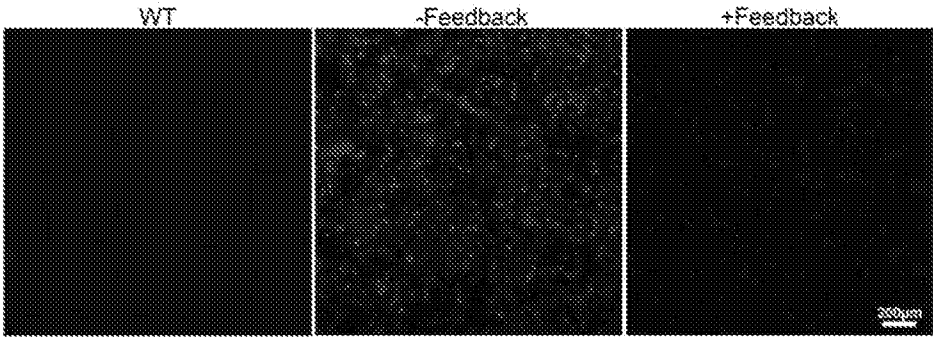


Fig. 5B

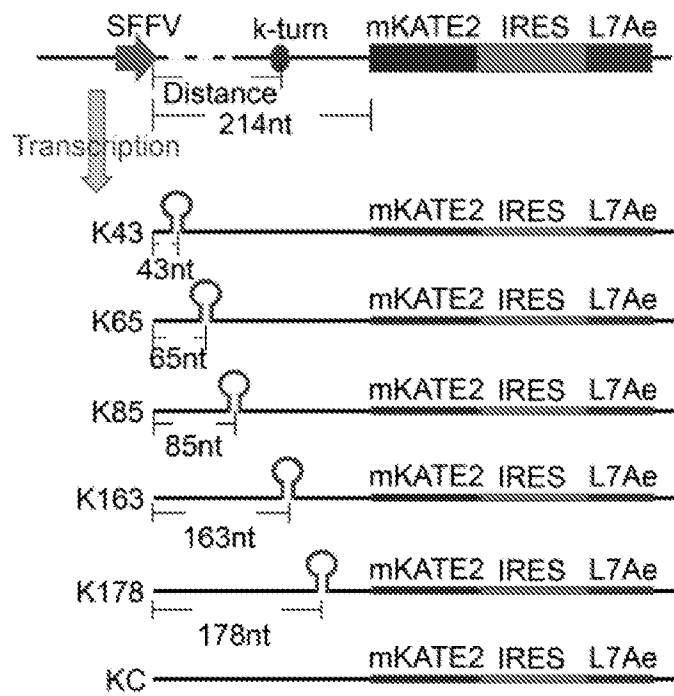


Fig. 6

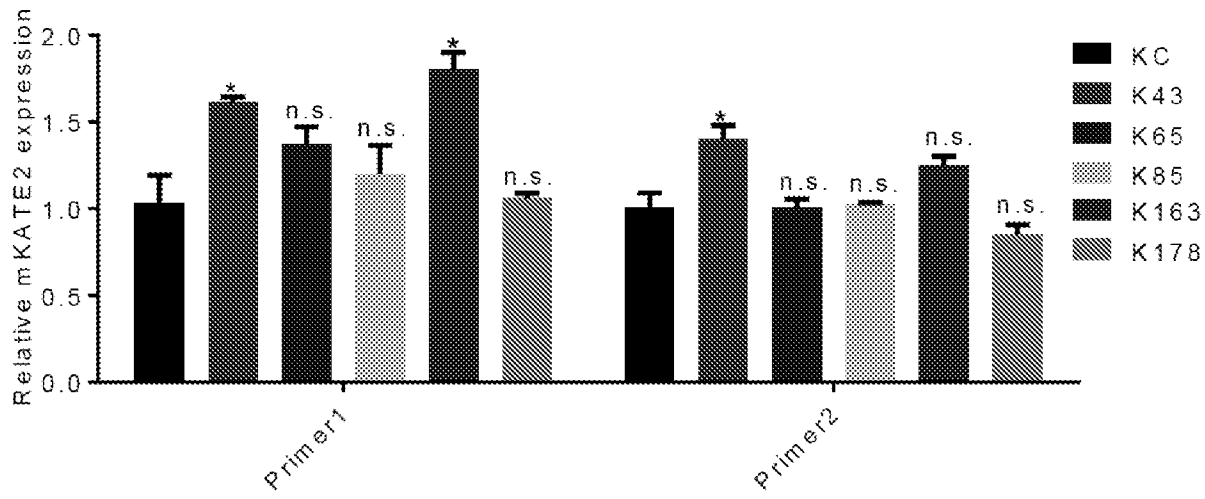


Fig. 7

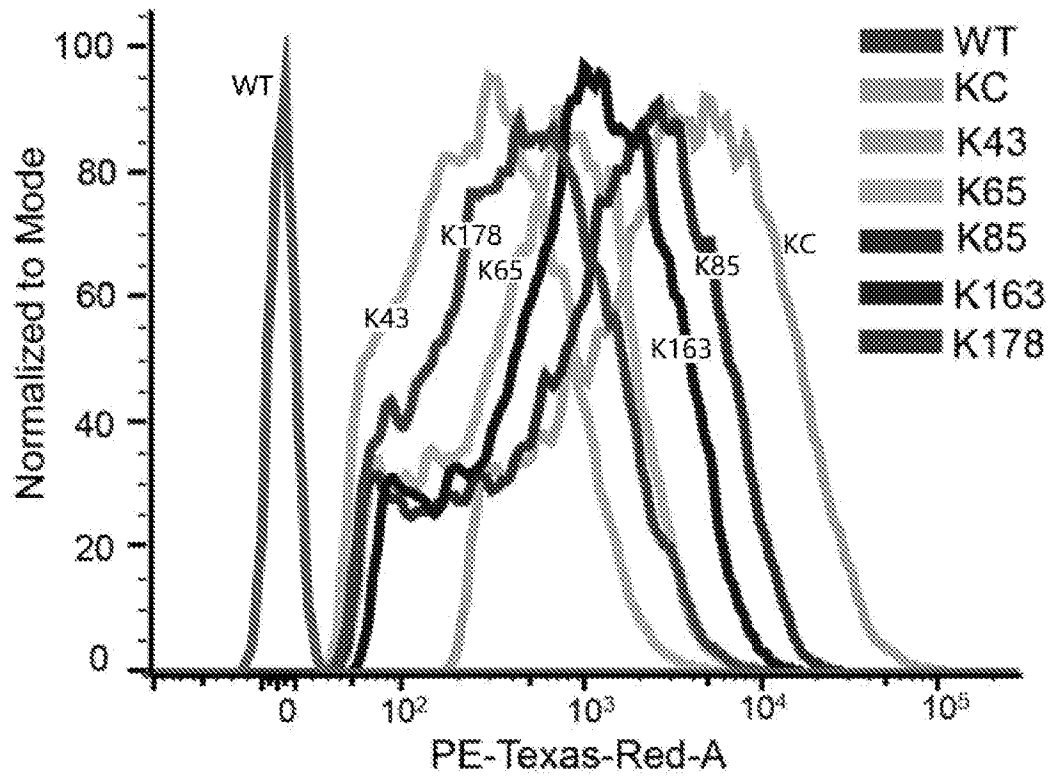


Fig. 8A

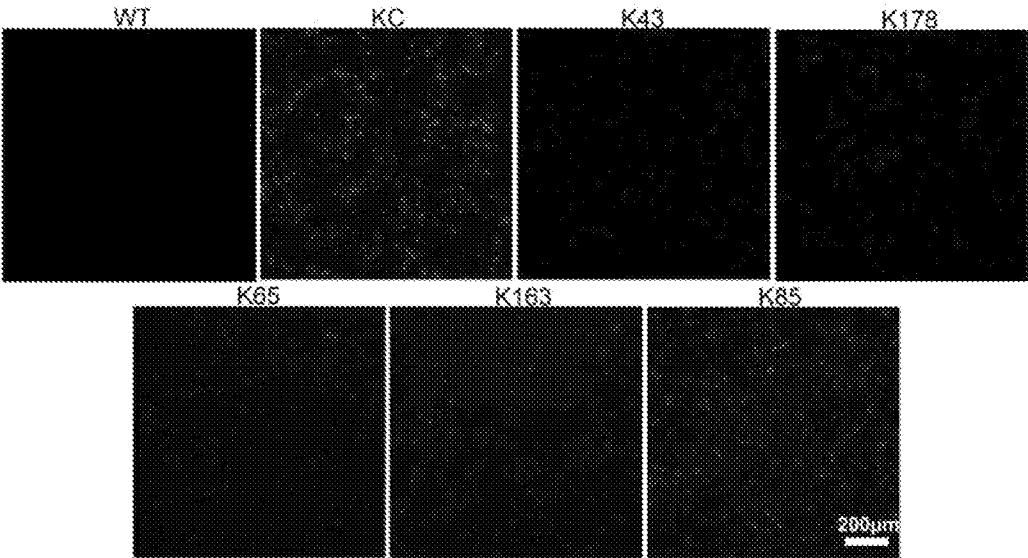


Fig. 8B

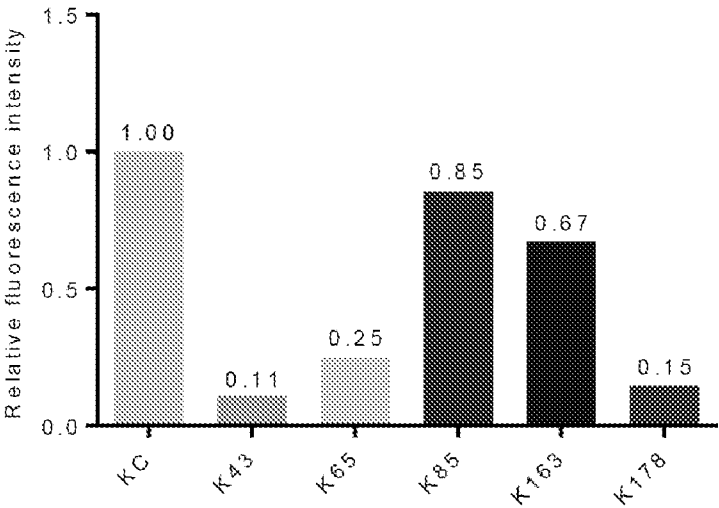


Fig. 9

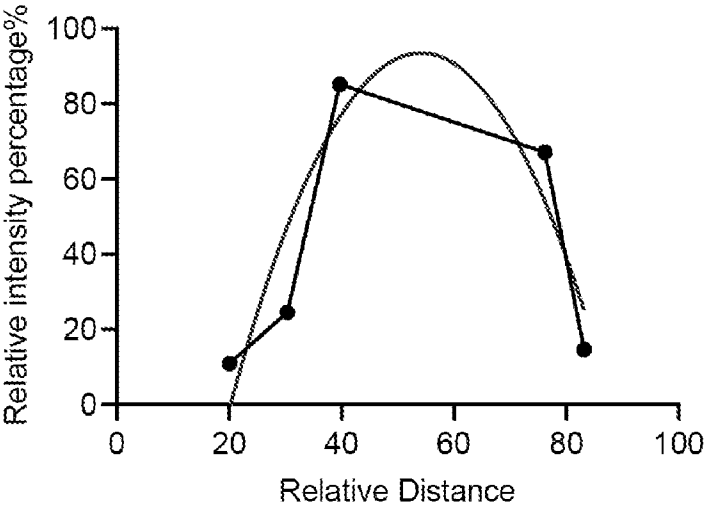


Fig. 10

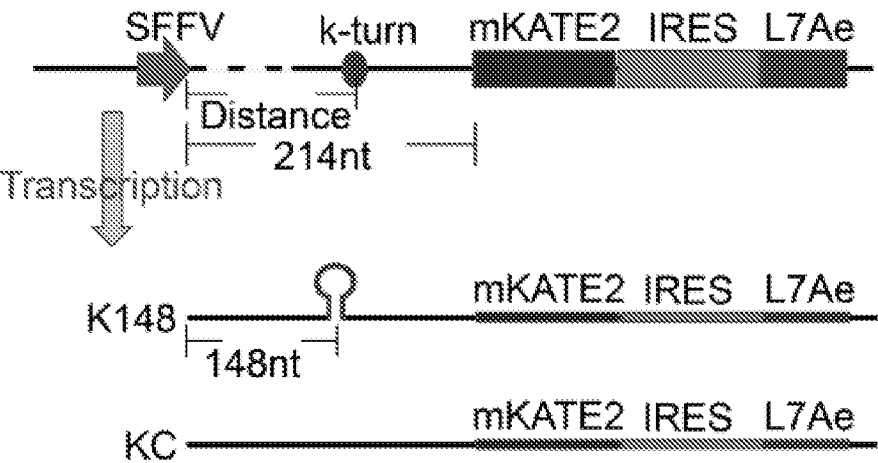


Fig. 11

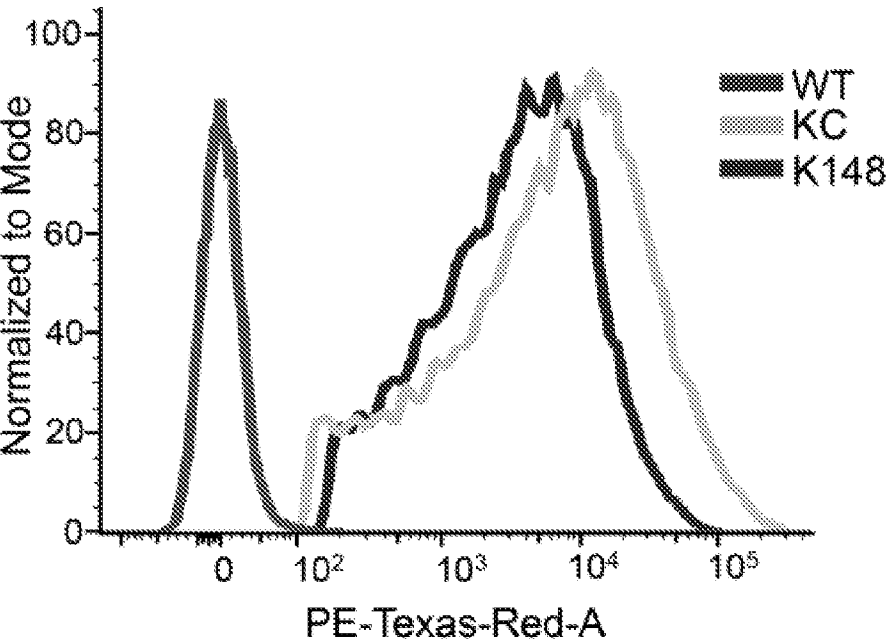


Fig. 12A

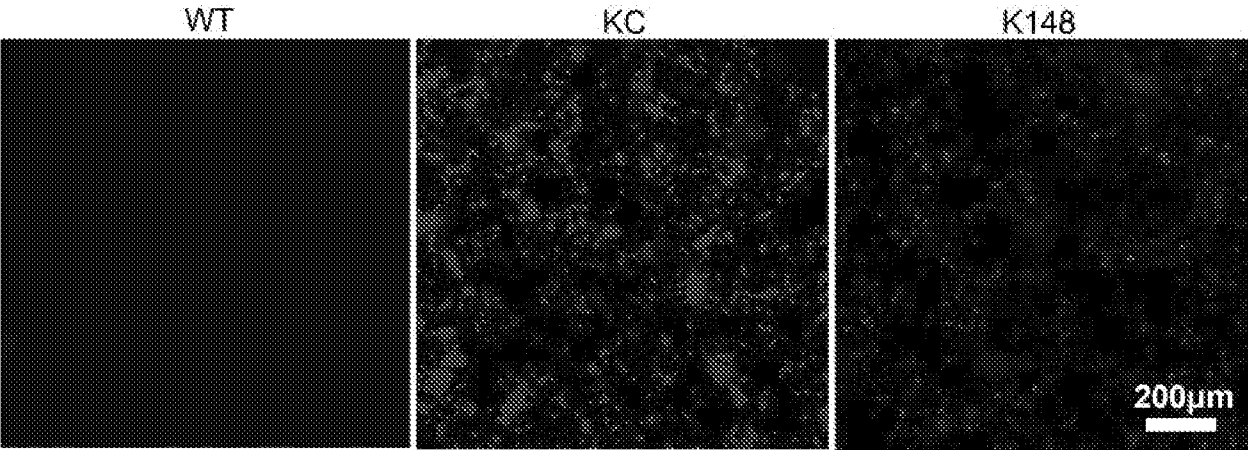


Fig. 12B

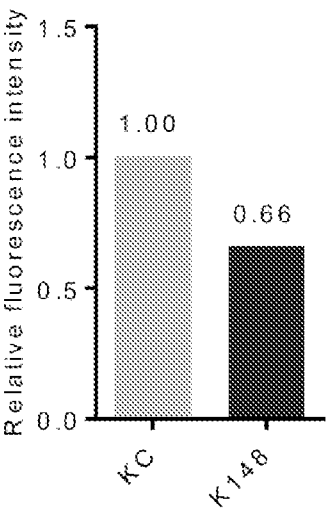


Fig. 13

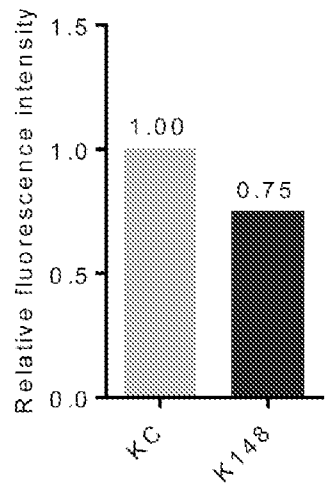


Fig. 14

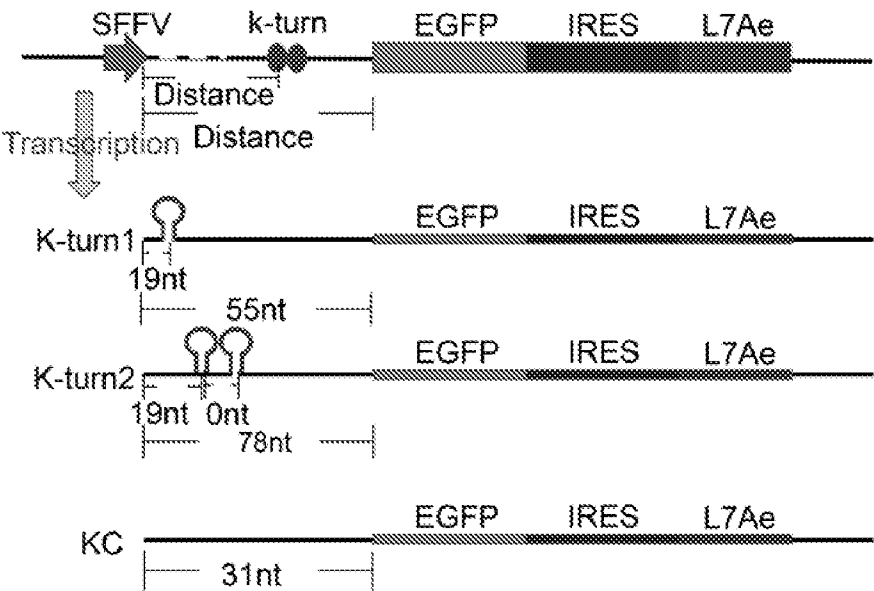


Fig. 15

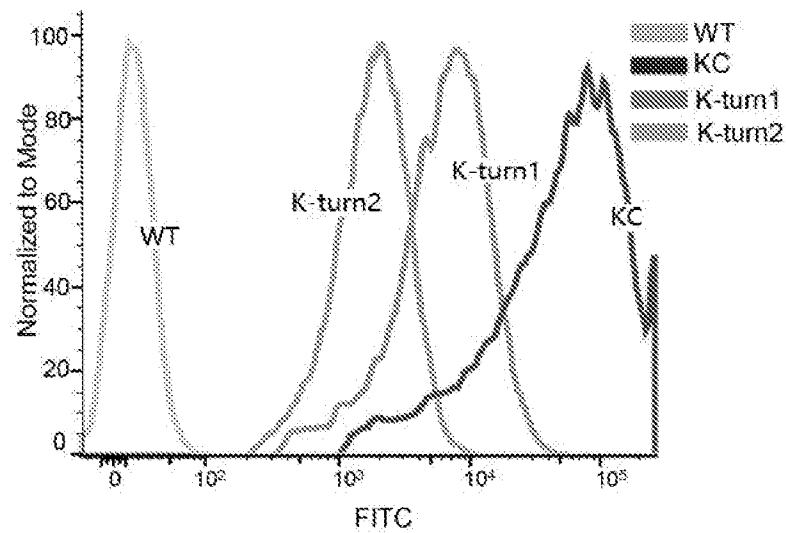


Fig. 16A

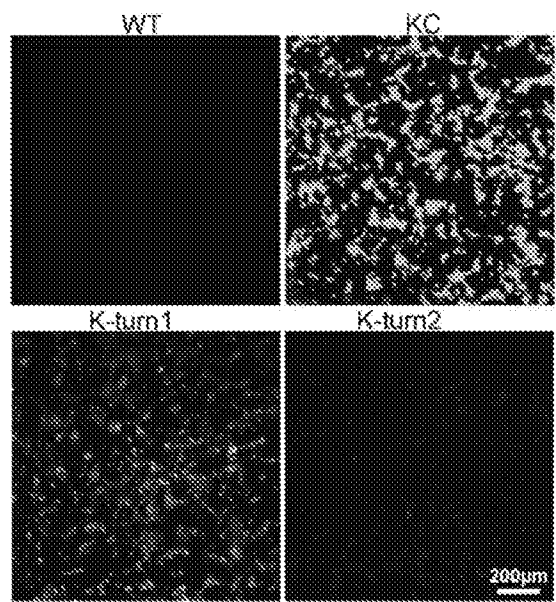


Fig. 16B

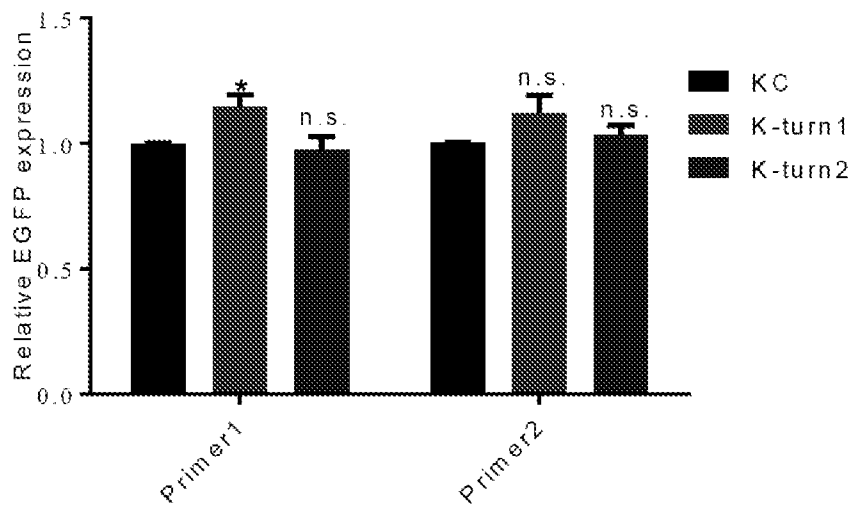


Fig. 17

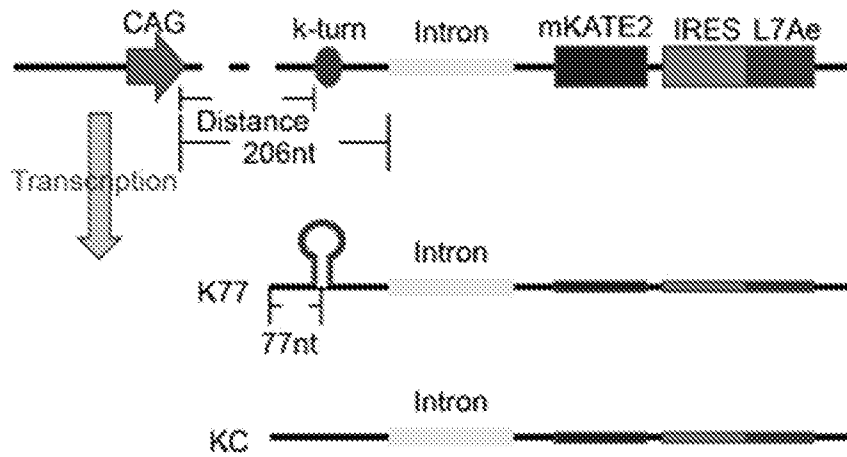


Fig. 18

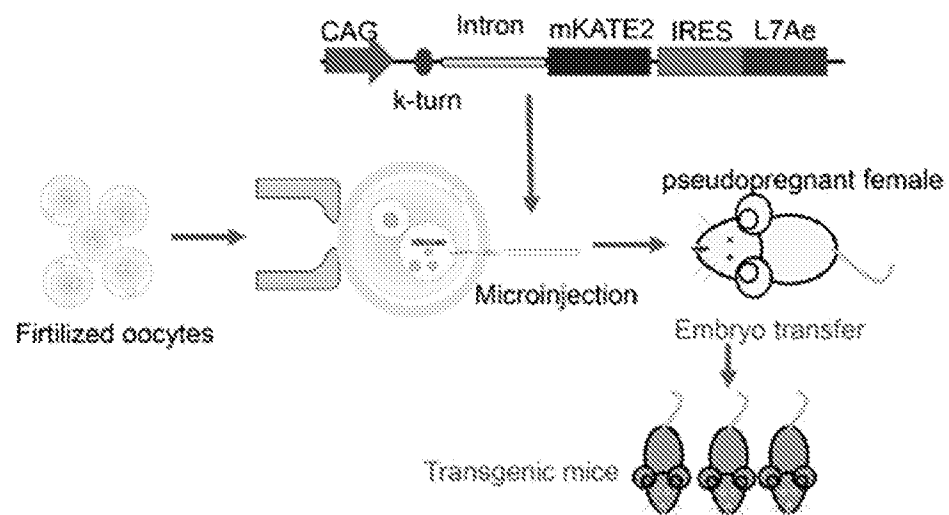


Fig. 19

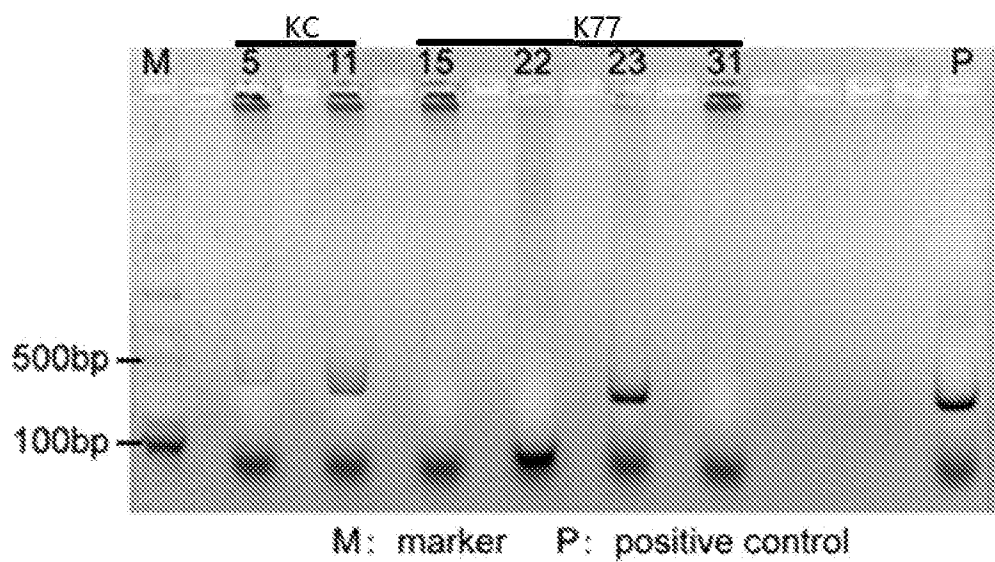


Fig. 20

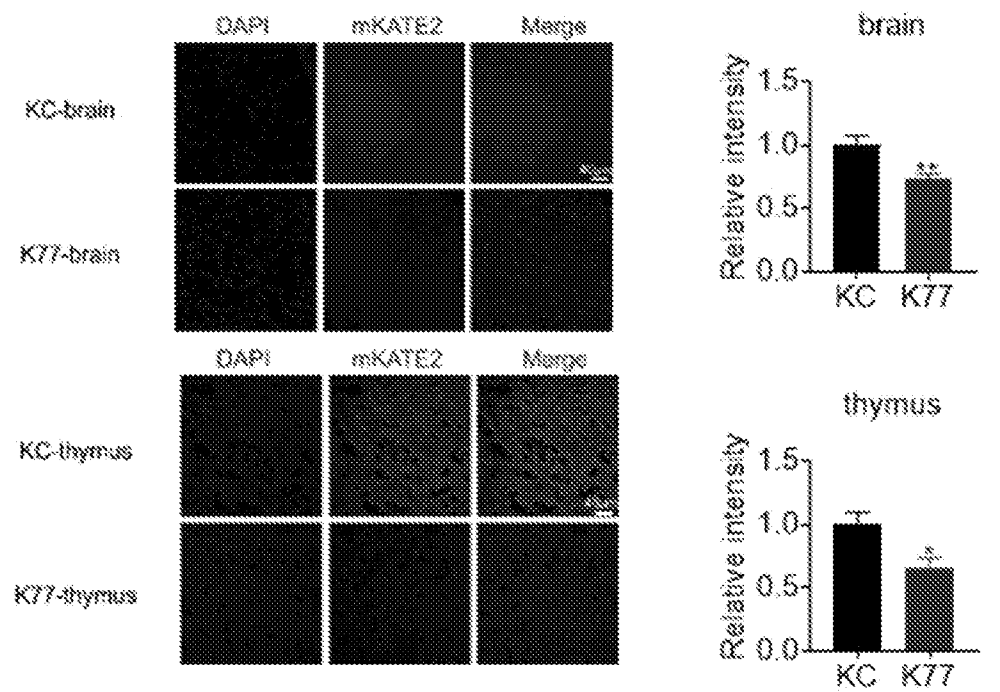


Fig. 21A

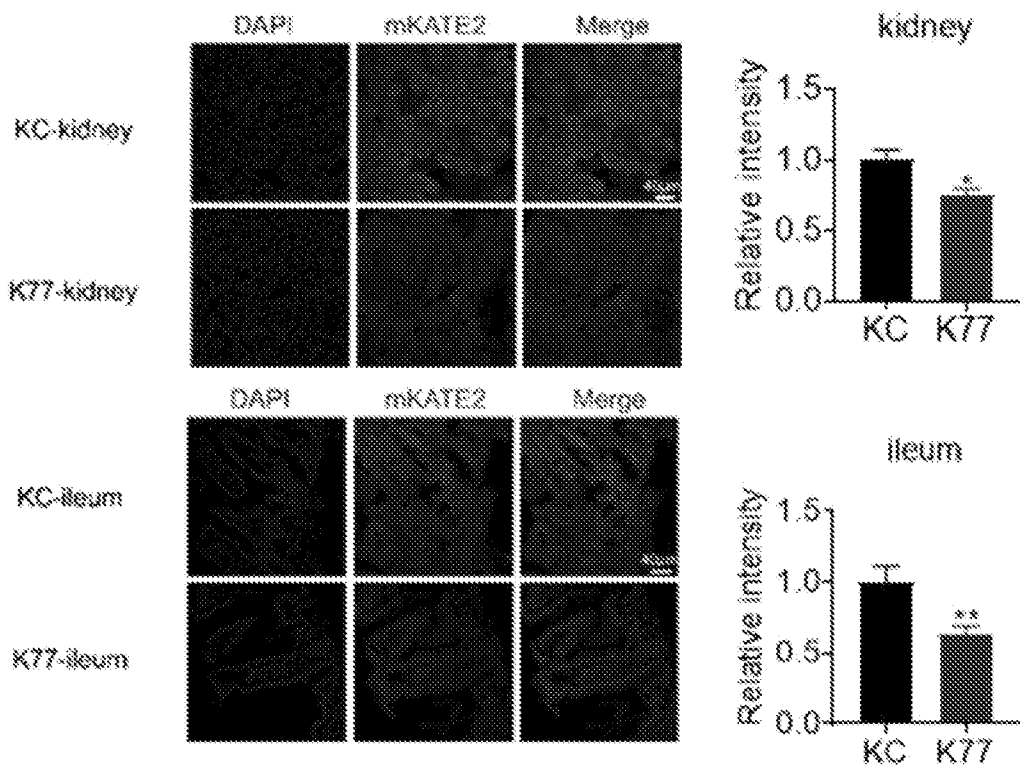


Fig. 21B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2022/088203

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/85(2006.01)i; A61K 48/00(2006.01)i; C12N 15/63(2006.01)i; C12N 15/11(2006.01)i

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; A61K

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)

CNKI,PUBMED,ISI web of Knowledge,CNTXT,OETXT,DWPI,SIPOABS:SAITO HIROHIDE, L7AE,Kink-turn, k-turn, random sequence,distance, number, RNA hairpin, BOX C/D, Quantitative expression, Feedback loop; circuit

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STAPLETON, J.A. et al. "Feedback Control of Protein Expression in Mammalian Cells by Tunable Synthetic Translational Inhibition" <i>ACS Synth. Biol.</i> , Vol. 1, No. 3, 30 November 2011 (2011-11-30), abstract, pages 84-87, Figures 1, 3	1-9, 13-19
Y	STAPLETON, J.A. et al. "Feedback Control of Protein Expression in Mammalian Cells by Tunable Synthetic Translational Inhibition" <i>ACS Synth. Biol.</i> , Vol. 1, No. 3, 30 November 2011 (2011-11-30), abstract, pages 84-87, Figures 1, 3	10-15
Y	ENDO, K. et al. "Quantitative and simultaneous translational control of distinct mammalian mRNAs" <i>Nucleic Acids Research</i> , Vol. 41, No. 13, 18 May 2013 (2013-05-18), abstract, pages 2, 5-7, 9, Figures 3-5	10-15
Y	SAITO, H. et al. "Synthetic translational regulation by an L7Ae-kink-turn RNP switch" <i>NATURE CHEMICAL BIOLOGY</i> , Vol. 6, 31 January 2010 (2010-01-31), abstract, pages 72-77	1-19



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search

09 December 2022

Date of mailing of the international search report

22 December 2022

Name and mailing address of the ISA/CN

National Intellectual Property Administration, PRC
6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing
100088, China

Facsimile No. (86-10)62019451

Authorized officer

XU, Dan

Telephone No. 53961985

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2022/088203

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2018296702 A1 (MASSACHUSETTS INST TECHNOLOGY et al.) 18 October 2018 (2018-10-18) abstract, description, paragraphs 108, 113, 139, example 2, figures 5, 10, 37	1-19
Y	US 2017051292 A1 (UNIV KYOTO) 23 February 2017 (2017-02-23) abstract, claims 1-8, description, paragraphs 23-27, figures 4-8.	1-19
A	US 2011040077 A1 (JAPAN SCIENCE & TECH AGENCY) 17 February 2011 (2011-02-17) whole document	1-19
A	AUSLANDER, S. et al. "Synthetic RNA-based switches for mammalian gene expression control" <i>Current Opinion in Biotechnology</i> , Vol. 48, 04 April 2017 (2017-04-04), pages 54-60	1-19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2022/088203

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2022/088203

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
US	2018296702	A1	18 October 2018	WO	2016040395	A1	17 March 2016
US	2017051292	A1	23 February 2017	US	2019144873	A1	16 May 2019
US	2011040077	A1	17 February 2011	WO	2009066757	A1	28 May 2009
				EP	2221367	A1	25 August 2010
				JP	2009142259	A	02 July 2009