Quantitative and simultaneous translational control of distinct mammalian mRNAs

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Received June 26, 2012; Accepted April 11, 2013

ABSTRACT

The introduction of multiple genes into cells is increasingly required for understanding and engineering biological systems. Small-molecule–responsive transcriptional regulation has been widely used to control transgene expression. In contrast, methods for specific and simultaneous regulation of multiple genes with a single regulatory protein remain undeveloped. In this report, we describe a method for quantitatively tuning the expression of multiple transgenes with a translational regulatory protein. A protein that binds a specific RNA motif inserted in the 5′-untranslated region (UTR) of an mRNA modulates the translation of that message in mammalian cells. We provide two independent mechanisms by which to rationally fine-tune the output: the efficiency of translation correlates well with the distance between the inserted motif and the 5′ terminus of the mRNA and is further modulated by the tandem insertion of multiple RNA motifs. The combination of these two approaches allowed us to fine-tune the translational efficiency of target mRNAs over a wide dynamic range. Moreover, we controlled the expression of two transgenes simultaneously and specifically by engineering each cis-regulatory 5′-UTR. The approach provides a useful alternative regulatory layer for controlling gene expression in biological research and engineering.

INTRODUCTION

Construction of synthetic gene regulatory systems that quantitatively and simultaneously control expression of multiple genes is important for both understanding and engineering biological processes. Given that the complexity of the gene regulatory systems being constructed and integrated into cells is increasing rapidly (1–4), the expression of the exogenous genes that comprise these synthetic circuits must be controlled precisely to ensure optimal system performance. The independent regulation of multiple exogenous genes in a single cell is particularly gaining in importance. For example, ectopic expression of four transcription factors can induce pluripotency in adult somatic cells (5). Another specific set of factors can convert exocrine cells in the mouse pancreas into insulin-producing endocrine cells (6). The stoichiometry of these factors has an impact on the efficiency of reprogramming (7). However, artificially introduced transgenes are generally uncontrolled in comparison with their strictly programmed, genomically encoded counterparts. Thus, there is a need for a method of controlling the expression of multiple transgenes in a cell.

Thus far, especially in higher eukaryotes, quantitative regulation of protein expression from exogenous genes has largely depended on transcription factors that respond to supplemented small molecules, such as tetracycline (1,2,4) (Figure 1A). The concentration of the supplement determines the activity of the factors (8,9), each of which can evenly adjust the transcription levels of multiple target genes in a cell (Figure 1A). In one study, doxycycline modulated the activities of two transcriptional regulators to enable reversible control of two target genes (10). In another instance, several trans-acting regulatory factors were used to regulate multiple transgenes under the control of a single stimulus (11,12). However, although only one small molecule stimulant was used in these systems, multiple trans-acting regulatory proteins or RNAs were required to control multiple transgenes in these cases. Simultaneous and specific regulation of multiple transgenes with a single regulatory protein (Figure 1B) could ameliorate the acute
shortage of well-characterized regulatory proteins (especially in mammalian systems) and minimize the risk of cross-talk among transgenes and intrinsic gene regulatory networks.

It has been previously reported that libraries of synthetic promoters with a range of activity have been developed as cis transcriptional regulatory elements (13,14), enabling independent control of multiple transgenes. However, the activity using these synthetic promoter libraries was constitutively determined by endogenous transcription factors, resulting in difficulty in controlling expression of transgenes with an internal or external stimulus. Moreover, it is difficult to rationally determine expression levels of multiple genes with these library-based screening approaches.

Recently, in addition to transcriptional regulation, means for fine-tuning translational efficiency have gained in importance. For example, programmable mammalian biocomputers have been developed using both transcriptional and translational regulatory components (15). So far, few methods have been developed for translational regulation of genes of interest in mammalian cells (16,17). We have reported a translational OFF switch that strongly represses the translation of a target mRNA designed to contain the binding motif [termed the kink-turn RNA motif (Kt)] of an archaeal ribosomal protein, L7Ae (18,19). However, the binary switch-like behavior of the system has limited its use for quantitative regulation.

In a previous report, we took the first step toward a quantitative system by demonstrating tunable feedback regulation by a set of modified L7Ae proteins with engineered binding properties (20). In this study, we aimed to develop complementary approaches for quantitatively tuning translation of distinct mRNAs by an RNA-binding protein. We describe two independent methods for finely tuning protein expression without modification of the RNA–protein interaction. It has been reported that protein-binding RNA motifs inserted close to the 5′-end of an mRNA effectively repress translation in the presence of their corresponding proteins (21–23). We examined the dependence of translational repression in our system on the placement of the inserted RNA motif, and we found that the efficiency of translation adjusts continuously in a manner that correlates well with the distance between the binding motif and the 5′ terminus of the mRNA. Tandem insertion of multiple RNA motifs into one mRNA further modifies the inhibition. Combining these two mechanisms allows us to fine-tune the translational efficiency of the target mRNAs over a wide range. Simultaneous and specific control of two different transgenes was rationally designed and experimentally demonstrated by engineering each cis-regulatory 5′-UTR. Tuning cis-regulatory elements instead of trans-acting effectors enables simultaneous, specific and quantitative control of target genes (Figure 1B).

**MATERIALS AND METHODS**

**Plasmid constructions**

Reporter plasmids that express enhanced green fluorescent protein (EGFP) or enhanced cyan fluorescent protein (ECFP) following engineered 5′-UTRs were derived from pKt-EGFP and pdKt-EGFP, previously described as p1-box C/D-EGFP and p1-box C/D mut-EGFP (18),...
respectively. The region encoding EGFP was replaced by ECFP to generate pKt-ECFP and pdKt-ECFP, referred to as 32 nt in the length of the 5'-UTR. Spacer sequences were amplified by polymerase chain reaction (PCR) from LacZ gene using primer sets (5'-CCGGATCCGATCC CTCGTTTTTACAC-3'/5'-AGATCTACCGGTACG CTCGCAACGACCGGATACA CCGCAC-3'/5'-ACTAGTATGCTCTAATGCGAGAT CCCAG-3'), digested and ligated between the BamH– viol sites and the NheI–BglII sites of pKt-EGFP to create plasmids pKt-Sp-ECFP and pSp-Kt-ECFP, respectively. Similarly, pdKt-Sp-ECFP and pSp-dKt-EGFP were generated from pdKt-EGFP.

pKt-ECFP and pdKt-ECFP were digested with NheI and BamHI, bluntly by Klenow fragment (Takara Bio, Otsu, Japan) and self-ligated to create the shortest 5'-UTR (18 nt). The longest spacer sequence (320 nt) was generated by concatenation of the two spacer fragments described earlier in the text and inserted between the NheI–BglII sites of pKt-ECFP and pdKt-ECFP. The remaining series of spacers were amplified with proper primer sets and inserted into the 5'-UTRs of the reporter plasmids in a similar manner. All the sequences of the 5'-UTRs from the reporter plasmids containing Kt are available in Supplementary Table S1.

Pairs of oligonucleotides [Kl (20): 5'-CATGGGATGCC GGTGATGACGGTGTACCCGCGA-3'/5'-CATCTC GGTGATCCAGCTCTGCTCAGTCA GGTGACCGTACA-3'/5'-GATCTCGCCGATCTGC ATCCCGGTCACGTAGTCGGATC-3'], Kl2: 5'-CATGGGATGCC GGTGATGACGGTGTACCCGCGA-3'/5'-CATCTC GGTGATCCAGCTCTGCTCAGTCA GGTGACCGTACA-3', and MS2SL (24): 5'-CATGGGATGCC GGTGATGACGGTGTACCCGCGA-3'/5'-CATCTC GGTGATCCAGCTCTGCTCAGTCA GGTGACCGTACA-3'] were annealed and cloned into a cloning vector. Fr15 (25) was amplified by PCR using a primer set (5'-GAATCCATGGG ATCCATGTAAGTGAATGGATCCCCGCT-3'/5'-CACCC AGATCTCTCTGAAAGCCCTTTATTACCTTTC-3', 5'-CACCATGGGATCCGCTTTACTTTAATCAGTTC GTCCTC-3'/5'-TATGAGATCGTAGTAGGCCCAGT TGGC-3' and 5'-GACACCATTGGGATCCGATTTGAC GCAAGAGCGG-3'/5'-TATGAGATCGTAGTAGGCCCAGT TGGC-3'] from plasmids pL7S and MS2-EGFP (27) and a newly synthesized plasmid based on the past study (28), respectively.

Cell culture and transfections

HeLa cells were cultured at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (Nihirehi Biosciences, Tokyo, Japan) and 1% antibiotic–antimycotic solution (Sigma-Aldrich, St Louis, MO, USA). In all, 5 × 104 cells were seeded in 24-well plates, and after 24 h, 70–90% confluent cells were transiently transfected with plasmids using 1 μl of Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. In the double-transfection experiments (Figures 2–6), 0.1 μg of a reporter plasmid and 0.5 μg of a trigger plasmid were transfected into cells. In the triple-transfection experiment (Figure 7), 0.1 μg each of two reporter plasmids and 0.3 μg of a trigger protein plasmid were used. Media were changed 4 h after transfection.

Flow cytometric measurement

Twenty-four hours after transfection, cells were washed with phosphate-buffered saline and incubated in 100 μl of 0.25% Trypsin–ethylenediaminetetraacetic acid (GIBCO) for 2 min at 37°C. After addition of 100 μl of the medium, cells were passed through a 35-μm strainer (BD Biosciences, San Jose, CA, USA) and then analyzed with a FACS Aria (BD Biosciences) and a FACSCalibur (BD Biosciences) with a 488-nm semiconductor laser for excitation and a 450/40-nm filter for emission. The median fluorescence of ECFP, and a 685/40-nm filter for emission was used to measure the fluorescence of ECFP. A 488-nm semiconductor laser and 530/30 and 695/40 nm filters were used for EGFP and DsRed-Express, respectively.

Flow cytometric analysis

Dead cells were gated out by forward and side scatter properties. Translational efficiency (e) is defined in this study as the ratio of an average of the EGFP or ECFP fluorescence in the presence of cognate RNA-binding
protein divided by that of non-cognate RNA-binding protein from cells expressing an arbitrarily chosen
1000 ± 100 a.u. of DsRed-Express. Different cut-off of DsRed-Express (500 ± 50 a.u.) did not change the trans-
lational efficiencies throughout the experiments performed in Figures 2–7 (Supplementary Figure S1). In double-
transfection experiments using the EGFP and DsRed-Express (Figure 2), and the triple-transfection experiments
(Figure 7), the levels of fluorescence were compensated in a similar manner as described in the previous study
(29). Black boxes in plots indicate cut-off used to calculate the translational efficiency (e) of the switch. The right most panels show histograms in the cut-off, and the bar in the panels indicates the change of the average EGFP fluorescence. The number of events in plots is shown in the right panels. Representative results are shown out of three independent experiments, except translational efficiencies, which were the average of the three experiments.

Isolation of total RNA and complementary DNA (cDNA) synthesis
Twenty-four hours after transfection, cells were washed with chilled phosphate-buffered saline. Total RNA was
isolated using the RNAqueous-4PCR Kit (Ambion,
of protein production among the cells (30), the concentration ratio between the incorporated plasmids in the transfected cells seems constant. To normalize variations in transfection efficiency, we chose cells for analysis according to the fluorescence intensity (1000 ± 100 a.u.) of DsRed-Express, which was synthesized from an internal ribosome entry site on the mRNA encoding the RNA-binding proteins (Figure 2C–E, black boxes in plots). This range was chosen because the fluorescence intensity of cells with lower DsRed-Express was more sensitive to noise from autofluorescence. The translational efficiency values were similar among cells expressing DsRed-Express from 500 to 1000 a.u. (Supplementary Figure S1). We determined the translational efficiency of the Kt-EGFP and Sp-Kt-EGFP constructs. Insertion of the spacer upstream of Kt increased the translational efficiency from 0.017 (Kt-EGFP; Figure 2C) to 0.20 (Sp-Kt-EGFP; Figure 2D).

To enable the design of mRNAs that express proteins at any desired level, we more closely examined the relationship between the efficiency of translation and the distance...
between the 5'-end of the mRNA and Kt. We constructed a series of reporter mRNAs with Kt located at various sites between the 18th and 320th nt from the 5'-end (Figure 3A and Supplementary Table S1) and evaluated the effect on protein synthesis. To avoid fluorescence spill over, we replaced EGFP with ECFP in the following experiments. In the presence of the regulatory protein L7Ae, the translational efficiency correlates well with the distance of Kt from the 5'-end (Kt; Figure 3B). When Kt was replaced with a defective Kt (dKt) to which L7Ae cannot bind in vitro, the protein expression levels were almost constant irrespective of the presence of L7Ae (dKt; Figure 3B). The results indicate that the binding between the mRNA and L7Ae, and not the distance between the open reading frame and the 5'-end of the mRNA, is responsible for the translational regulation. Interestingly, the translational efficiency continuously increased with the length of the spacer despite the constant expression level of L7Ae (Figure 3B and Supplementary Figure S2), indicating that translation can be tuned quantitatively by adjusting the distance between the RNA motif and the 5' terminus of the mRNA.

Two-dimensional approach to tune translational efficiency

The translational efficiency under repression by Kt and L7Ae ranged between 0.017 and 0.24, reaching a plateau when Kt was positioned beyond the 164th nt from the 5'-end of the mRNA (Figure 3B). To expand the versatility of the method by widening the tuning range, we designed 5'-UTRs that contained multiple protein-binding motifs (Figure 4A). Multiple RNA–protein interaction motifs have been used in series to enhance the binding between the RNA molecule and the corresponding protein. For example, tandem arrangements of protein-binding RNA motifs have been used as tools for visualizing target mRNA molecules in living cells (26), detecting RNA–protein interactions (31) or regulating the stability of mRNA (32). We first substituted Kt with a K-loop RNA motif (Kl) (33) that binds to L7Ae less tightly than does Kt (20) (Figure 4B and C). Translation from the mRNA containing Kl showed slight repression in the presence of MS2 coat protein (red) or L7Ae (blue). All the data are shown as in Figure 2. Representative results are shown out of the three independent experiments, except translational efficiencies, which were the average of the three experiments.

Figure 4. Inserting multiple K-loop motifs enhanced the reactivity of the switch. (A) Construction of engineered mRNAs, which were tuned by altering the number of motifs. (B) Secondary structure of K-loop (Kl) used in this study. Non-canonical base pairing is shown in dotted lines. (C) Modified K-loop (Kl2) used only in this experiment. (D and E) Results of the flow cytometric analysis of cells expressing switches containing one Kl2 (D) or two tandemly repeated Kl2 (2× Kl2; E) in the presence of MS2 coat protein (red) or L7Ae (blue). All the data are shown as in Figure 2. Representative results are shown out of the three independent experiments, except translational efficiencies, which were the average of the three experiments.
presence of L7Ae (Figure 4D). We next designed an OFF mRNA with two repeated Kl motifs. The mRNA showed 9-fold stronger repression than an mRNA with a single Kl (Figure 4D and E), demonstrating that the translational efficiency can be tuned by altering the number of the RNA motif at the 5' UTR.

We combined the two strategies described earlier in the text to allow fine-tuning of the OFF mRNA system over a wide dynamic range. Specifically, the continuous downward tuning provided by adjusting the distance of Kl from the 5'-end of the mRNA was used together with the upward discrete tuning resulting from using multiple Kls (Figure 5A). We constructed mRNAs containing one to four copies of Kl (1/C2 to 4/C2 Kl) in the 5'-UTR and placed the first motif at either the 18th, 67th, 120th or 164th nt from the 5'-end (Supplementary Table S2). Sixteen constructs were transiently expressed in HeLa cells and analyzed for their translational efficiency. As anticipated, the translational efficiency of the mRNAs increased or decreased as the distance of the first motif from the 5'-end of the mRNA or the number of RNA motifs increased, respectively (Figure 5B and C). The combination of the two tuning strategies successfully extended the range of the translational efficiencies of the mRNAs (from 0.039 to 0.67).

To see whether the reporter gene expression was regulated at the level of translation, the amount of designed mRNAs transiently expressed in the cells was analyzed by real-time quantitative PCR (Figure 5D). No significant difference was observed in the amount of the mRNAs with one or four repeated Kls, the first motif of which was located at the 18th or 164th nt from the 5'-end.

To assess the versatility of our method, we replaced Kl/L7Ae regulatory pair with another RNA–protein pair. We constructed two series of 2D-tuned mRNAs containing a MS2 stem–loop, to which MS2 coat proteins bind (24,27,31) (Figure 6A), or a binding motif for Bacillus ribosomal protein S15 (25,28) (Figure 6B). Translational efficiencies of these constructs in HeLa cells responded to tuning similarly to those containing Kl, although the sequence around the motif might weakly affect the translational efficiency (Figure 6C and D and Supplementary Table S2). Thus, it is conceivable that any desired RNA–protein interaction pair can be used for controlling translation. Taken together, our results show that 2D engineering of cis-regulatory motifs in the 5'-UTR of mRNAs (i.e. engineering both the position and the number of protein-binding motifs on the mRNA) enables quantitative tuning of transgene expressions over a wide range.

### Simultaneous control of two differently tuned mRNAs

We next tested whether a single, unmodulated effector protein is able to both simultaneously and specifically regulate multiple genes containing differently tuned cis-regulatory elements (Figure 1B). Two sets of differently tuned reporter plasmids were designed and transfected into cells in the presence and absence of the cognate RNA-binding protein. The first set encoded EGFP mRNA with a single Kt in the 5'-UTR (Figure 2),
whereas the second set encoded ECFP mRNA with 2D arranged Kls in the 5′-UTR (Figure 5). Each set consisted of three differently tuned variants: low- (Kt-EGFP, 18 nt-3/C2 Kl-ECFP), high- (dKt-EGFP, 120 nt-1/C2 Kl-ECFP) and intermediate-expression constructs (Sp-Kt-EGFP, 67 nt-3/C2 Kl-ECFP) (Figure 7A). In the absence of L7Ae, EGFP and ECFP were uniformly expressed in all nine cell populations regardless of their engineered 5′-UTR (Figure 7B). In contrast, in the presence of L7Ae, the expression levels of EGFP and ECFP varied depending on the nature of the constructs, resulting in nine different fluorescence profiles (Figure 7C and D).

Figure 7 shows that the protein output of each mRNA is unaffected by translation of the other. To further investigate whether our method can control translation of multiple genes independently, we calculated the translational efficiencies of EGFP (Table 1) and ECFP (Table2) constructs under the co-transfectional conditions (Supplementary Figure S3). Obtained values were consistent with those in the single-reporter experiments (Figures 2 and 5), indicating that our system can achieve simultaneous and specific translational regulation of the two distinct mRNAs using a unique regulatory protein, L7Ae.

**DISCUSSION**

We have developed a method for independently regulating protein production from distinct target mRNAs in mammalian cells. The translation from each mRNA can be
controlled across a wide dynamic range with a regulatory RNA-binding protein. We found that the location of the protein-binding motif in the 5'-UTR determines the translational efficiency. Translational repression was maximized when the motif was located close to the 5' terminus of mRNA, and gradually decreased to become constant when the motif was located away from the 5'-end (Figure 3). We believe that our mRNA design-based approach could be advantageous compared with library screening-based approaches because expression levels of multiple genes can be rationally and quantitatively tuned by engineering both the position and the number of protein-binding motifs on the mRNA in a predictable manner.

Interestingly, the translational efficiency of the constructs was not affected in the presence of another construct in a cell (Figure 7, Tables 1 and 2 and Supplementary Figure S3), indicating that L7Ae has no preference for RNA motifs located at any particular position on the mRNA. Together with our previous study, which showed the expression level of L7Ae correlates with the translational efficiency (19), these results suggest that the ratio of L7Ae to the total of all the Kt-containing mRNAs in a cell determines the efficiency. If the amount of L7Ae is indeed the limiting factor, it is conceivable that a large number of distinct mRNAs can be simultaneously and specifically controlled provided that a sufficient amount of L7Ae is present.

Translational regulation using a protein-binding site in the 5'-UTR has been shown in a yeast system (22), indicating that the method is applicable to a variety of eukaryotes and RNA-binding proteins. Indeed, we found that MS2 coat protein or Bacillus ribosomal protein S15 with their corresponding RNA motifs can be used as alternative pairs (Figure 6), demonstrating the versatility of our method. Accordingly, an endogenous RNA-binding protein expressed in a specific cell could be a trigger for the control of target transgene expression. It is also conceivable that a synthetic RNA aptamer for a specific protein can be adapted to our system (34). In addition, we expect that the method described in this study can be applied for a wide range of protein outputs (e.g. fluorescent reporter proteins and apoptosis regulator proteins) (Supplementary Table S3).

In prokaryotes, the level of translation can be modulated in cis with engineered ribosome-binding sites (35,36). To express target proteins at desired levels, the sequences of ribosome-binding sites have been rationally designed based on a biophysical model of translation initiation affected by RNA folding and hybridization, combined with an optimization algorithm (37). Furthermore, by using a multiplex genome engineering method, simultaneous optimization of the translational efficiency of 24 genes in the same metabolic pathway enabled a biosynthesis circuit in Escherichia coli to overproduce industrially important chemical compounds (38). However, alternative approaches are required to control translation in mammalian cells, which do not share the ribosome-binding sites used in

**Figure 7.** Simultaneous control of two independently tuned output proteins. (A) Pairs of tuned constructs were co-transfected into cells. Kt-EGFP and 18 nt-3' Kl-ECFP were tuned to low-protein expression levels, dKt-EGFP and 120 nt-1' Kl-ECFP were tuned for high expression and Sp-Kt-EGFP and 67 nt-3' Kl-ECFP were tuned for medium expression. (B and C) Flow cytometric analysis of cells expressing two tuned mRNAs in the absence (B) and in the presence (C) of L7Ae. Plots are shown in the color scheme listed in A. Representative data out of three independent experiments are shown. Plots of EGFP and ECFP as a function of DsRed-Express are available in Supplementary Figure S3. (D) Schematic representation of the plots shown in C. The oval with the black dotted outline indicates all the pairs shown in B.
prokaryotic cells. Our approach, which enables us to tune the translation of multiple genes specifically, may allow genetic circuits to be engineered and optimized in a eukaryotic system.

Our method for quantitatively tuning the expression of multiple transgenes can be used as an alternative tool for studying complex cellular processes and engineering cells for therapeutic uses. For example, it could be used to study the expression balance of multiple disease-related genes, or for controlling cellular phenotypes determined by threshold dose responses (1). The method can also be useful for controlling differentiation or self-renewal of stem cells, where the optimal stoichiometry of multiple transcription factors governs the fates of the cells (7). Direct injection of synthetic mRNAs into mammalian cells could serve as a powerful tool for gene therapy (39) and regenerative medicine (40) because transferred mRNAs do not integrate into the genome and pose no risk of causing cellular damage such as might lead to tumor formation. Thus, the combined use of RNA injection and our method could allow quantitative regulation of gene function in a cell.

We have previously shown that an L7Ae gene integrated into the genome under tetracycline-inducible transcriptional control regulates the translation of the anti-apoptotic protein Bcl-xL to allow control of mammalian cell fate (19). That result demonstrated that our translational regulation method can be used cooperatively with conventional transcriptional control systems because they exist as two completely different layers. In transcriptional control systems, transgenes are placed under specific promoters that determine the timing and location of their expression. In the translational control method described in this study, the translational efficiency of the target is determined by the RNA-binding protein produced in a cell. Accordingly, quantitative and simultaneous regulation of the production of multiple proteins should be temporally and spatially inducible in a cell containing a single regulatory protein (e.g. L7Ae)-coding gene under the control of a specific promoter.

A recent genome-wide analysis has revealed that post-transcriptional control of gene expression predominantly determines the amounts of proteins in mammalian cells (41). Moreover, it has been reported that translational control together with the localization of mRNAs significantly contributes to many important cellular processes, such as the establishment of polarity, asymmetric division, synaptic plasticity and memory consolidation (42). In the near future, our method may enable translational control to play the same leading role in synthetic regulatory systems as it does in natural ones.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3 and Supplementary Figures 1–3.

ACKNOWLEDGEMENTS

The authors thank Y. Fujita (Kyoto University) for many helpful discussions, Y. Kodama (ICORP, JST) for constructing vectors, H. Kawabata (ICORP, JST) for performing quantitative PCR analysis and S. Masui (Kyoto University) for critical reading of the manuscript. They also thank A. Hüttenhofer (Innsbruck Medical University) and T. S. Rozhdestvensky (University of Muenster), J. Ellenberg (EMBL), T. Funatsu (University of Tokyo) and J. R. Williamson (The Scrips Research Institute) for providing the plasmids.

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*aTranslational efficiency from the ECFP construct (32 nt-dKt) shown in Figure 3 and Supplementary Figure S2.

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