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Bidirectional Regulation of mRNA Translation in Mammalian Cells by Using PUF Domains**

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Abstract: *The regulation of gene expression is crucial in diverse areas of biological science, engineering, and medicine. A genetically encoded system based on the RNA binding domain of the Pumilio and FBF (PUF) proteins was developed for the bidirectional regulation (i.e., either upregulation or downregulation) of the translation of a target mRNA. PUF domains serve as designable scaffolds for the recognition of specific RNA elements and the specificity can be easily altered to target any 8-nucleotide RNA sequence. The expression of a reporter could be varied by over 17-fold when using PUF-based activators and repressors. The specificity of the method was established by using wild-type and mutant PUF domains. Furthermore, this method could be used to activate the translation of target mRNA downstream of PUF binding sites in a light-dependent manner. Such specific bidirectional control of mRNA translation could be particularly useful in the fields of synthetic biology, developmental biology, and metabolic engineering.*

Considerable work has focused on developing systems for the exogenous control of gene expression. These efforts have been motivated both by a fundamental interest in understanding biological systems and by the need for such control in applications ranging from stem cell manipulation to metabolic engineering.[1–7] Several unidirectional systems have been developed that can be used either to upregulate or to downregulate gene expression. For instance, "ON" switches can be used to increase gene expression through the application of an activator or other signals. Similarly, "OFF" switches can be used to decrease gene expression through the application of a repressor or other signals. In contrast, other gene regulatory systems, such as the *lac* operon, allow both upregulation and downregulation.

Tuning the concentration of a protein of interest would benefit from the ability to both activate and repress translation of a target mRNA. Approaches for upregulating or downregulating translation in mammalian cells have been previously reported. For instance, translational activators recruit ribosomes to activate translation.[8–11] Alternatively, translation can be repressed by the binding of proteins or small molecules to the 5′-untranslated region (5′-UTR) of mRNA, which can prevent ribosome binding or scanning.[7,12–15] To expand upon this prior work, we aimed to design a translational regulation system for mammalian cells with: 1) the ability to target endogenous genes and to both activate and repress translation, and 2) inducers that are orthogonal, nontoxic to cells, and can in principle be regulated by using exogenous signals.

We reasoned that Pumilio and FBF (PUF) domains would be attractive components of such a system. PUF domains, which are comprised of multiple modular RNA-base-recognition repeats, can be rationally designed to target any 8-nucleotide RNA sequence and have been successfully used to target endogenous mRNAs.[16–21] Human Pumilio1 homology domain [PUF(wt)] consists of eight repeats, each binding to a single RNA base, and recognizes the sequence UGUAUAUA, known as the Nanos response element (NRE; Figure 1a).[17] However, the binding specificity of each repeat can be engineered independently.[17,22–24] Specifically, three amino acids on an individual repeat participate in RNA base recognition: two of the amino acids make contact with the Watson–Crick edge of the corresponding base and

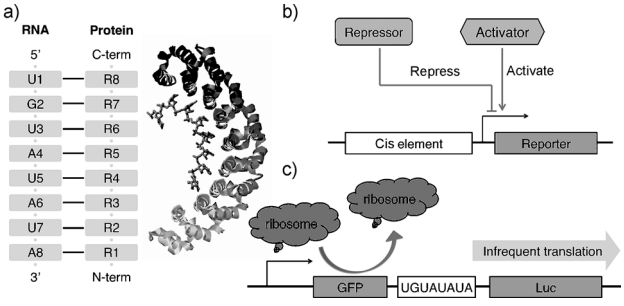

Figure 1. Design of a system that can be used to both upregulate and downregulate translation. a) The interaction between the PUF domain of Human Pumilio1 (PDB ID: 1m8W) and RNA is illustrated as a diagram (left) and structure (right).[17] Eight RNA-recognition repeats of the PUF domain (R1–R8) recognize eight consecutive RNA nucleotides (A8–U1; the NRE). b) A schematic illustrating the mechanism of translational activation and repression. The activator and repressor act on the cis element of the reporter plasmid. c) A schematic presentation of the reporter plasmid. Most ribosomes translate the GFP ORF; the Luc ORF is infrequently translated.

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the third stacks between two bases. In principle, a PUF domain could be engineered to target any combination of eight consecutive RNA nucleotides through rational design of the amino acid sequence of each repeat.[21,23,24] PUF domains thus offer a powerful tool for regulating RNA post-transcriptional events with designed sequence specificity and they have accordingly been harnessed successfully to target reporter or endogenous RNA to control RNA splicing and cleavage.[18,20] In an elegant study, Cooke et al. used PUF domains to influence the translation of microinjected RNAs in *Xenopus laevis* oocytes by controlling poly(A) addition or removal.[19] The use of microinjection, however, limits the application of this approach for translational control in mammalian cells.

In this work, we engineered a genetically encoded system based on PUF domains that can be used for the bidirectional regulation of target mRNA translation in mammalian cells (Figure 1b). In particular, we used the PUF domain to tether a translational activation domain to enhance translation, or the PUF domain alone to repress translation. The specificity of this method was tested by using both wild-type and mutant PUF domains. Finally, we demonstrated the ability to control target mRNA translation in a light-responsive manner by using PUF domains.

To test whether this system would enable both activation and repression of translation (Figure 1b), we designed a plasmid in which the translation of the reporter gene luciferase occurred infrequently in the absence of a translational activator (Figure 1c). The reporter plasmid consists of two open reading frames (ORFs) separated by a regulatory binding site (Figure 1c). An ORF encoding firefly luciferase (Luc) was placed downstream of a primary ORF encoding green fluorescent protein (GFP). The primary ORF was long enough to prevent translation re-initiation and nonsense-mediated decay (NMD).[25,26] With this construct alone, basal protein translation occurred infrequently and thus yielded relatively low levels of luciferase protein (Figure S1 in the Supporting Information).

We first tested the ability to enhance the translation of target mRNA by using the PUF–eIF4E fusion protein (Figure 2a). The translation-initiation protein eukaryotic initiation factor 4E (eIF4E) drives 5′-cap-dependent mRNA translation, and eIF4E is therefore generally considered to be the rate-limiting factor in translation initiation.[9–11,27,28] Briefly, eIF4E binds to the 5′ 7-methyl guanosine cap of a mature mRNA and then recruits other initiation factors and the ribosome 40S subunit to form the 43S pre-initiation complex. The 43S pre-initiation complex then scans the mRNA from the 5′ end until it finds the first appropriate start codon. The 60S subunit joins, the initiation factors are released, and the resulting 80S ribosome initiates translation. Importantly, previous studies have shown that tethering eIF4E upstream of the gene of interest is sufficient to activate translation.[9–11]

To test the ability of the fusion protein to activate mRNA translation, we cotransfected human embryonic kidney 293T (HEK 293T) cells with plasmids encoding PUF(wt)–eIF4E and the bicistronic reporter containing the PUF-binding sites (Figure 1c). PUF(wt)–eIF4E increased the expression of luciferase from the bicistronic reporter but had no effect on

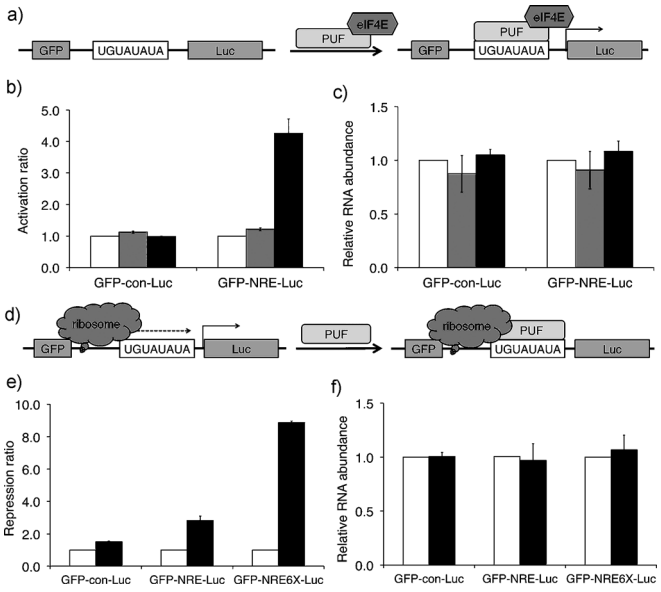

Figure 2. It is possible to either activate or repress mRNA translation by using PUF–eIF4E and PUF, respectively. a–c) PUF–eIF4E enhances mRNA translation. a) A schematic illustrating the mechanism of translational activation. The PUF–eIF4E fusion protein localizes to the PUF binding sites upstream of the reporter mRNA, thereby resulting in initiation of translation. b) The influence of the activator (PUF–eIF4E) or control (eIF4E) on the expression of luciferase. The reporter plasmid GFP-NRE-Luc contains PUF binding sites whereas the control reporter plasmid GFP-con-Luc does not. The experiments were performed without regulator (white bars); with eIF4E (gray bars); and with PUF(wt)–eIF4E (black bars). Activation ratios were calculated as the ratio of luciferase activity in the presence of PUF–eIF4E to that for the reporter alone without PUF–eIF4E. c) The influence of the expression of the activator (PUF–eIF4E) or control protein (eIF4E) on the transcript level of the reporter mRNA. d–f) PUF inhibits mRNA translation. d) A schematic illustrating the mechanism of translational repression. e) The influence of the repressor (PUF) and the number of repeats of the PUF binding site on the expression of luciferase. The reporter plasmid GFP-con-Luc has no PUF binding site, GFP-NRE-Luc has one PUF binding site, and the GFP-NRE6X has six PUF binding sites. The experiments were performed with (black bars) and without (white bars) PUF(wt). Repression ratios were calculated as the ratio of the luciferase activity for the reporter alone to that in the presence of PUF. f) The influence of the expression of the repressor (PUF) on the transcript level of the reporter mRNA.

a reporter lacking a wild-type NRE (Figure 2b). Activation was not enhanced by increasing the number of NREs in the reporter mRNA (Figure S2 in the Supporting Information). Moreover, eIF4E alone had little effect on luciferase expression. Quantitative real-time polymerase chain reaction (Q-RT-PCR) results revealed that the amount of the reporter mRNA remained essentially unchanged for all transfected cells (Figure 2c; relative abundance was calculated as the ratio of the normalized reporter transcript level in the presence of regulator to that for the reporter alone). These results confirm that the increase in luciferase activity was not due to an increase in the level of reporter mRNA and are consistent with target-specific translational activation by PUF(wt)–eIF4E. We note that the efficacy of this system could potentially be further enhanced by the generation of

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stable cell lines expressing these constructs, especially for cells that are difficult to transfect.

Next, we investigated translational repression of the same reporter by PUF(wt) alone. Previous reports show that mRNA translation can be repressed by tethering proteins or small molecules to the 5′-UTR of a reporter mRNA through aptamers.[12,13,15] Given that PUF domains bind tightly to their corresponding RNA binding sites, we reasoned that tethering PUF domains upstream of the luciferase reporter might also inhibit its translation (Figure 2d).

We cotransfected HEK 293T cells with the bicistronic reporter used above and plasmids encoding PUF(wt). Consistent with our prediction, PUF(wt) effectively inhibited translation of the reporter containing an NRE. We also found that increasing the number of NRE repeats significantly enhanced translational repression (Figure 2e). Furthermore, the amount of reporter mRNA remained unchanged, thus indicating that the repressor functioned through translational repression rather than by decreasing the level of target mRNA (Figure 2f).

The data in Figure 2 validate our strategy to design a system that can either upregulate or downregulate the translation of target mRNA. In contrast to the *lac* operon, which has two regulators (activator and repressor) interacting with two different cis elements, our system uses a single cis element for both activation and repression (Figure 2a,d). Additionally, increasing the number of NRE repeats on the reporter plasmid significantly enhanced the translational repression in the presence of PUF (Figure 2e), thereby allowing the expression level of the luciferase reporter to be varied by more than 17-fold when using the regulatory system (Table 1).

Table 1: Control of luciferase expression by using the PUF-based translational regulation system.

Reporter	Regulation range[a]
GFP-con-Luc	1.5
GFP-NRE-Luc	12.2
GFP-NRE6X-Luc	17.3

[a] Regulation ranges were calculated as the ratio of the luciferase activity in the presence of PUF–eIF4E to that in the presence of PUF.

To demonstrate the generality and specificity of the PUF-based approach for translational regulation, we tested the ability to activate translation by using two other PUF mutant domains derived from human Pumilio1 homology domain, PUF(3-2) and PUF(6-2/7-2), which have been well characterized in previous studies (Figure 3a).[18,22] Fusion proteins PUF(3-2)–eIF4E and PUF(6-2/7-2)–eIF4E were constructed and their corresponding reporters, GFP-A6G-Luc and GFP-GU32UG-Luc, were generated by replacing the wild-type NRE by UGUAUGUA and UUGAUAUA, respectively.

We found that the cells cotransfected with matched PUF–eIF4E and reporter showed significantly higher activation of luciferase expression than those cotransfected with mismatched pairs (Figure 3b). These results demonstrate that the system has a high specificity for the target mRNA and can

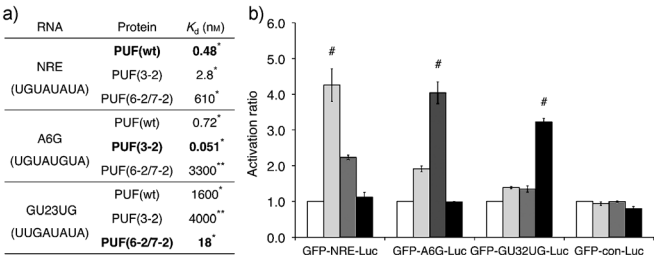

Figure 3. PUF domains provide specificity. a) The affinities of wild type and mutant PUF domains for different RNA sequences.[18,22] The table entries in bold represent data for matched PUF domain and RNA pairs. *The data are from Ref. [22]. **The data are from Ref. [18]. b) The specificity of translational activation when using wild-type and mutant PUF domains. Reporter plasmids containing wild-type or mutant PUF binding sites were cotransfected with activator plasmids encoding wild-type or mutant PUF domains. The activation was measured without activator (white bars); with PUF(wt)–eIF4E (light gray bars); with PUF(3-2)–eIF4E (dark gray bars); and with PUF(6-2/7-2)-eIF4E (black bars). Activation ratios were calculated as the ratio of luciferase activity in the presence of PUF–eIF4E to that for the reporter alone without PUF–eIF4E. The bars marked # represent data for matched reporter and activator plasmids.

distinguish mRNAs with one or two point mutations. We observed a small amount of cross-activations between PUF(wt)–eIF4E and PUF(3-2)–eIF4E since these two PUF domains can also bind to each other's binding sites (Figure 3a,b). This type of cross-interaction was also observed in previous reports.[18,19]

Having shown the ability of PUF domains to control the translation of target mRNA with high specificity, we next demonstrated the control of translational activation by using light. Light is an orthogonal signal that doesn't interfere with cellular ligands in most mammalian cells and has been successfully used to provide spatiotemporal control over cellular function.[11,29–32] Cryptochrome 2 (CRY2) from *Arabidopsis thaliana* binds to its partner protein cryptochrome-interacting basic-helix-loop-helix 1 (CIB1) upon blue-light illumination.[33,34] We used the N-terminal photolyase homology region (PHR; 1-498) of CRY2, which lacks a nuclear localization signal (NLS), and an NLS-deficient truncated version of the CIB1 protein (CIBN; 1-170) because mRNA translation occurs in the cytoplasm.[11,34] We made two fusion proteins, one in which PUF(wt) was fused to CIBN and another in which eIF4E was fused to CRY2PHR (Figure 4a,b). We reasoned that by separately fusing PUF domains and the translation initiation protein eIF4E to the two light-sensitive protein partners, light would induce the translocation of eIF4E to the PUF binding sites upstream of the gene of interest, thereby activating translation.

HEK 293T cells were cotransfected with plasmids encoding CRY2PHR–eIF4E and PUF(wt)–CIBN, and the bicistronic reporter containing six NRE repeats. We observed a 2.1-fold increase in luciferase activity in the cells exposed to blue light compared to cells in the dark (Figure 4c). No significant change in luciferase levels was observed in control experiments in which CRY2PHR–eIF4E was replaced by CRY2PHR or PUF(wt)–CIBN was replaced by CIBN. In principle, this system could be used to target endogenous

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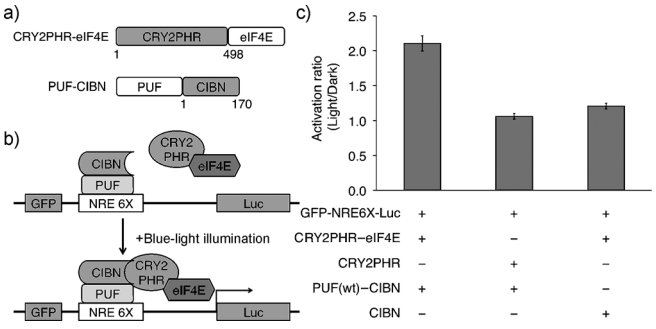

Figure 4. Light-inducible activation of gene expression by using PUF domains. a) A schematic representation of the effectors with numbers indicating the amino acid residues of CRY2PHR and CIBN. b) A schematic illustrating the mechanism of light-inducible translation initiation. The PUF–CIBN fusion protein is tethered to an NRE upstream of the luciferase ORF and blue-light illumination induces the heterodimerization of CRY2PHR and CIBN, thereby translocating eIF4E to the target mRNA and initiating translation. c) The influence of blue light on the luciferase activity of HEK 293T cells transfected with 3 plasmids: 1) reporter (GFP-NRE6X-Luc) containing 6 NREs; 2) plasmid encoding CRY2PHR–eIF4E or CRY2PHR; and 3) plasmid encoding PUF(wt)–CIBN or CIBN. Activation ratios were calculated as the ratio of luciferase activity of cells under light illumination to that of cells placed in dark.

mRNA for light-inducible gene regulation using PUF domains.

In mammalian cells, the binding of proteins or small molecules to aptamers has been shown to repress mRNA translation mostly when located close to the 5′ cap.[13,15] Our results showed that PUF domains could inhibit downstream mRNA translation even when the NRE was approximately 2000 nucleotides away from the 5′ cap of the mRNA (Figure 2e). To test whether ligand binding to aptamers could also repress translation from a cap-distal position, we constructed reporters by replacing the PUF binding sites with the boxB aptamer (Figure S3 in the Supporting Information). The boxB aptamer is a 19-nucleotide RNA hairpin that recognizes the N-terminal 22 amino acids of the lambda phage antiterminator protein (λN) with a K_d of 1.3 nM (Figure S3a).[8,9] Reporters containing 0, 1, 6, or 10 boxB aptamers were cotransfected with constructs encoding either the fusion protein λ–eIF4E to activate translation or λ–LacZ to repress translation of the gene of interest. β-galactosidase (encoded by *lacZ*) was fused to the λ peptide to enhance its stability in cells (Figure S3b). Consistent with previous reports, the ligand could activate translation as a tether,[9,11] whereas tethering had little effect on translational repression at this cap-distal site (Figure S3c).[15]

Hence, while tethering a translational activator like eIF4E to the intercistronic region of two ORFs or to a cap-distal location through ligand–aptamer interactions can be used to upregulate gene expression, a PUF-based approach appears particularly well suited to systems in which both upregulation and downregulation may be required. For instance, alternative translation from downstream start codons is pervasive in eukaryotic cells.[25,26,35] Our system enables the regulation of mRNA translation from alternative start codons, a kind of regulation that is rarely achieved .

In summary, our system enables both translational activation and translational repression of a single genetically encoded mRNA in mammalian cells. Since PUF domains can be redesigned to target any RNA sequence, our approach could be used to regulate the expression of endogenous cellular mRNAs. We note that although the PUF repeats of Human Pumilio1 can recognize eight consecutive RNA bases, artificial PUF domains recognizing sixteen RNA bases have been reported.[23] It should therefore be feasible to use a PUF-based approach to target endogenous mRNAs with higher affinity and specificity. Moreover, the combination of light sensitive proteins and PUF domains provides a method for regulating cellular mRNA translation in a light-inducible manner. Such an approach to regulating protein expression has applications ranging from the characterization of cellular signaling networks to optimizing the expression of enzymes in metabolic pathways to control metabolic flux. In the future, this approach may even be applied to characterizing cellular responses to changes in mRNA translation at the single-cell level.[36]

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