ACS SyntheticBiology

Research Article

pubs.acs.org/synthbio

Engineering Translational Activators with CRISPR-Cas System

Pei Du,[†] Chensi Miao,[†,‡] Qiuli Lou,[⊥] Zefeng Wang,[*,§,#] and Chunbo Lou[*,†]

[†]CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences; Beijing, 100101, China

[‡]College of Life Science, University of Science and Technology of China, Hefei 230027, China

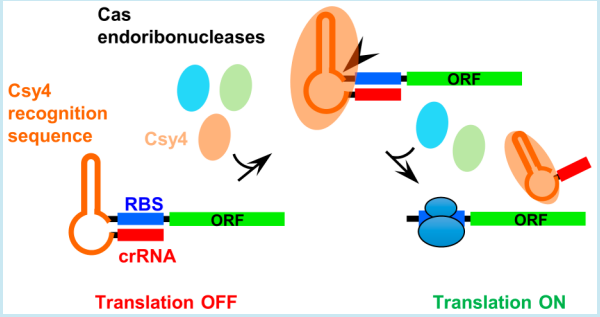
[⊥]College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China

[§]Department of Pharmacology, UNC−CH School of Medicine, Chapel Hill, North Carolina 27599, United States

[#]CAS-MPG Partner Instituter for Computational Biology, Shanghai 200031, China

S *Supporting Information*

ABSTRACT: RNA parts often serve as critical components in genetic engineering. Here we report a design of translational activators which is composed of an RNA endoribonuclease (Csy4) and two exchangeable RNA modules. Csy4, a member of Cas endoribonuclease, cleaves at a specific recognition site; this cleavage releases a cis-repressive RNA module (crRNA) from the masked ribosome binding site (RBS), which subsequently allows the downstream translation initiation. Unlike small RNA as a translational activator, the endoribonuclease-based activator is able to efficiently unfold the perfect RBS-crRNA pairing. As an exchangeable module, the crRNA-RBS duplex was forwardly and reversely engineered to modulate the dynamic range of translational activity. We further showed that Csy4 and its recognition site, together as a module, can also be replaced by orthogonal endoribonuclease-recognition site homologues. These modularly structured, high-performance translational activators would endow the programming of gene expression in the translation level with higher feasibility.



KEYWORDS: *translational activator, Csy4, CRISPR endoribonuclease, modularization, RBS calculator, orthogonal parts*

One important task of synthetic biology is to develop modular genetic parts that can be assembled into different biological circuits or pathways for desirable applications.[1−3] These genetic parts can function at the DNA, RNA, or protein levels. Previously, a handful of RNA-based functional parts have been engineered to control gene expression, including riboswitch,[4−6] small RNA based repressor or activator,[7,8] and ribozyme-based insulator.[9] These parts can potentially be used to modulate diverse biological processes by targeting different endogenous genes. The ability to reprogram sequence specificity through RNA base-paring gives such functional parts a great advantage in predictable manipulation of gene function.[10,11]

Previously an engineered translational activator adopting the regulation by the small RNA was reported by Isaacs et al.[12] They employed a cis-repressive RNA (crRNA) element that masks the ribosome binding site (RBS) of a reporter gene through base pairing. An ectopically expressed trans-activating RNA was then designed to compete the binding with crRNA and thus to unmask RBS and restore translation. However, when the crRNA is perfectly paired with RBS, the trans-activating RNA cannot compete the binding with crRNA,[12] limiting the application of such an activator. This issue was partially circumvented by a similar design in which a single strand RNA fragment (termed as "toehold") was included at the upstream of RBS to pair with sequence around the translation starting site and thus to suppress translation. A "trigger" sequence was used to base-pair with the toehold sequence for translational activation.[13] The toehold switch optimized the original riboregulator, but the sequence of each switch needs to be individually designed and may cause insertion of undesirable foreign sequences in the coding region of the target genes.

In addition to competitive RNAs, the RNA parts can also be controlled through programmable RNA cleavage. One of such systems is the clustered regularly interspaced short palindromic repeats (CRISPR) that mediate molecular immunity in many prokaryotes and archaea.[14−17] The CRISPR are transcribed as a long precursor containing various immune target sequences separated by repeat elements, and the precursor transcripts are subsequently processed into short CRISPR guide RNAs by Cas-family endoribonucleases that specifically recognize the repeat element.[18] One of Cas6 endoribonucleases, Csy4 from *Pseudomonas aeruginosa*, recognizes a specific 28-nt RNA and precisely cleaves between 20G and 21C,[19,20] making it possible to cleave an engineered Csy4 site in the coding region of a gene repression system.[21]

Received: July 19, 2015
Published: September 28, 2015

ACS Publications © 2015 American Chemical Society

DOI: 10.1021/acssynbio.5b00130
ACS Synth. Biol. 2016, 5, 74−80

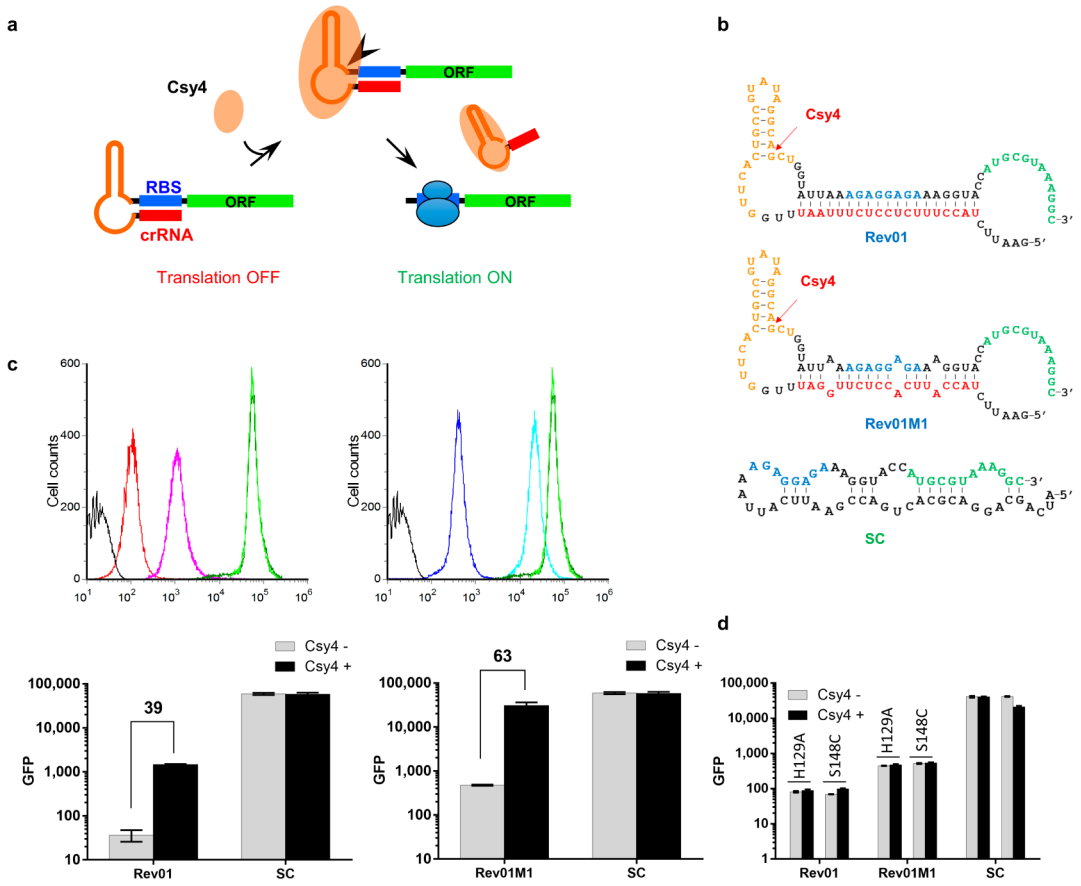


Figure 1. Csy4 cleavage of TrACR regulatory element activates translation of the reporter gene. (a) Schematic representation of translational activation by Csy4 cleavage of TrACR element. (b) Sequence and predicated structure of TrACR Rev01, Rev01M1 and SC. Red arrow indicates Csy4 cleavage site. RBS sequences are shown in blue. All crRNA sequences are shown in red. Csy4 recognition sequence is shown in yellow. The sfgfp coding sequences are shown in green. (c) Flow cytometric measurement of fluorescence of sfgfp. Histograms show distribution of fluorescence in samples with TrACR Rev01 (Red), Rev01&Csy4 (Purple), Rev01M1 (Blue), Rev0M1&Csy4 (Aqua), SC (Green) or SC&Csy4 (Lime). Cross-column number shows fold increase of sfgfp fluorescence. (d) Flow cytometric measurement of fluorescence of sfgfp in samples with mutated Csy4 H129A or S148C. Error bars show mean ± SD from three independent experiments.

In this study, we present the design and construction of a Cas-based translational activator. The translational control module was constructed by inserting a Csy4 recognition site between a RBS and cis-repressive RNA element, which can be specifically cleaved upon Csy4 expression. We optimized this system by replacing the RNA control module with several reversely and forwardly designed RBS-crRNA duplexes, and demonstrated that the RBS-crRNA duplexes can be modularly exchanged. We further determined how this translational activator can be affected by the length of the RBS-crRNA duplex, and examined the performance and orthogonality of different pairs of Csy4 analogues and their cognate recognition sites. This work represents a modular and tunable translational activator that could be integrated into a more complex system.

RESULTS AND DISCUSSION

Design and Performance of a Csy4-Based Translational Activator. We designed the translational activator with three modular parts: a crRNA to serve as translation suppressor by paring with RBS, a Csy4 site as linker between crRNA and RBS, and a CRISPR endoribonuclease Csy4 (Figure 1a). The expression of Csy4 will specifically recognize and cleave the RNA linker, releasing the crRNA to activate translation. We selected several crRNA sequences from known riboregulators to repress the translation of a reporter gene (sf-gfp) by masking the Shine-Dalgarno (SD) sequence of RBS,[12] resulting in a Translation Activator Controlled RBS (TrACR) regulatory element (Figure 1b).[12] In addition, a RiboJ insulator was inserted at the upstream of the TrACR sequence to buffer the synthetic circuits from genetic context of different promoters.[9] Upon recognition by Csy4, the RNA can be cleaved after a specific nucleotide (20G) within the Csy4 site (Figure 1a, Supplementary Figure S1), and the crRNA-Csy4 site piece of the TrACR element will be released from the masked SD sequence.

Interestingly, similar regulatory mode by RNA endoribonuclease may be responsible for translational activation in endogenous genes. For example, during lambda phage infection to *E. coli*, RNase III can recognize and cleave the RNase III-sensitive hairpin (RTS) in the N transcription antitermination complex, release the complex from the masked RBS sequence and restoring the translation of N protein.[22,23] Unfortunately, the exact recognition sequence of RNase III is still undetermined, making it unfeasible to engineer RNase III to become a translational activator. To obtain an endoribonuclease-based translational activator, a well-characterized recognition sequence and a specific cleaving site is required.

To test the translational activator, we coexpressed Csy4 with two versions of TrACR regulatory elements that control the expression of the reporter gene. One element, TrACR Rev01,

DOI: 10.1021/acssynbio.5b00130
ACS Synth. Biol. 2016, 5, 74−80

a

RBS	Sequence	Translation initiation rate (au)
Rev07	GATTTACTTTAAGGAGGTCAA	74748.6
Rev06	GCTACATAAGGAGGCCCTAA	25668.73
Rev05	TCTAGATTTAAGAAGGAGATATACAT	10974.7
Rev04	AAAATTAAGAGGTATATATTA	5678.7
Rev03	AAAAGATCTTTTAAGAAGGAGATATACAT	4265.22
Rev02	CCAAGACGGGGAGGTAATGAGCT	3024.24
Rev01	ATTAAAGAGGAGAAAGGTACC	1394.56

c

RBS	Ratio	Sequence	Translation initiation rate (au)
Rev01	1	ATTAAAGAGGAGAAAGGTACC	1394.56
Frw2	1/2	ATTAAAGAGGTCAGTATAACC	733.39
Frw4	1/4	ATTAAAGAGGCCACCCCTATT	341.24
Frw8	1/8	ATTAAAGAGGAGCTCCCCTAG	175.94
Frw32	1/32	ATTAAAGAGGCCCTATTCACC	47.11
Frw128	1/128	ATTAAAGAGGCGCGCCTTTCT	10.67
Frw1024	1/1024	ATTAAAGAGGAACAGCTCTTT	1.3

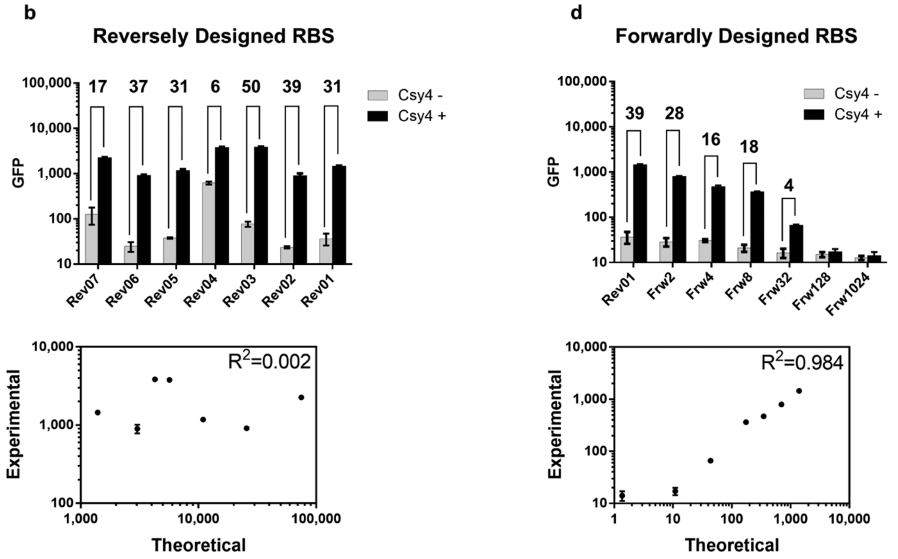


Figure 2. Modularization of RBS sequence in translational activator. (a) Sequence and calculated strength of reversely designed RBS. (b) Flow cytometric measurement of fluorescence of sfgfp in cells with each TrACR element containing reversely designed RBS (top). Linear regression of theoretical and experimental sfgfp fluorescence of Csy4 positive samples (bottom). (c) Sequence, designed ratio, and calculated strength of forwardly designed RBS. Underlined letters indicate mutated nucleotides in forwardly designed RBS. (d) Flow cytometry was performed to measure the sfgfp fluorescence of cells with each TrACR element containing forwardly designed RBS (top). Linear regression of theoretical and experimental sfgfp fluorescence of Csy4 positive samples (bottom). Error bars show mean ± SD from three independent experiments.

contains a crRNA perfectly matching RBS to achieve strong cis-repression, while the other TrACR element (Rev01M1) has three bulged mismatches within the crRNA-RBS duplex (Figure 1b, top and middle). We also used a control with scrambled crRNA sequence lacking Csy4 site (named SC), which allows a constitutive translation of the reporter gene (Figure 1b, bottom). Expression of the reporter gene was determined by measuring fluorescence intensity using flow cytometry, and we found that the coexpression of Csy4 produced a 39-fold increase in GFP fluorescence as compared to the control samples lacking Csy4 (Figure 1c). As a control, the GFP fluorescence in the SC sample remained constantly irresponsive to Cys4. Moreover, expression of two inactive Csy4 mutants failed to increase GFP fluorescence (Figure 1d). These data strongly support that the increased expression of the target gene in the Rev01 sample is indeed due to cleavage of Csy4 site that exposed the RBS to restore translation. To the best of our knowledge, this is the first demonstration of an endoribonuclease-based approach that activates translation of cis-repressed genes.

We noticed that Csy4 cleavage of TrACR Rev01 did not fully restore the GFP expression in comparison with the SC control (Figure 1c, top), probably because a fraction of mRNA still contains the RBS-crRNA duplex even after Csy4 cleavage. To address this possibility, we constructed a TrACR element, Rev01M1, with a weakened version of the crRNA-RBS duplex, which has three bulged nucleotides, to reduce the binding between RBS and crRNA (Figure 1b, middle). We found that, upon Csy4 induction, the new crRNA-RBS duplex can dramatically increase the GFP intensity, reaching the maximal level of GFP in SC control. As a result, the GFP fluorescence of Rev01M1 can be increased 63-fold by Csy4, indicating that adjusting the strength of the RBS-crRNA duplex is an effective route to optimize translation activation of cis-repressed sfgfp mRNA. Notably, the background expression of the reporter gene is also increased compared to that in samples with TrACR Rev01, presumably because the bulged nucleotides in RBS-crRNA duplex reduced the cis-repression.

Optimize TrACR System by Redesigning RBS. To further refine the translational activator, we seek to improve the crRNA-RBS duplex of TrACR element through redesigning the RBS. We obtained two sets of RBS sequences using either reverse or forward design strategy. The reversely designed sequences were collected by mining the database of known RBS, while the forwardly designed RBS sequences were generated by a thermodynamic simulation algorithm that predicts the strength of translation initiation (RBS calculator).[24,25] We first examined five reversely designed RBSs and the cognate crRNA-RBS duplex (Figure 2a), and found that the expression of the reporter gene was increased by 17- to 50-fold

DOI: 10.1021/acssynbio.5b00130
ACS Synth. Biol. 2016, 5, 74−80

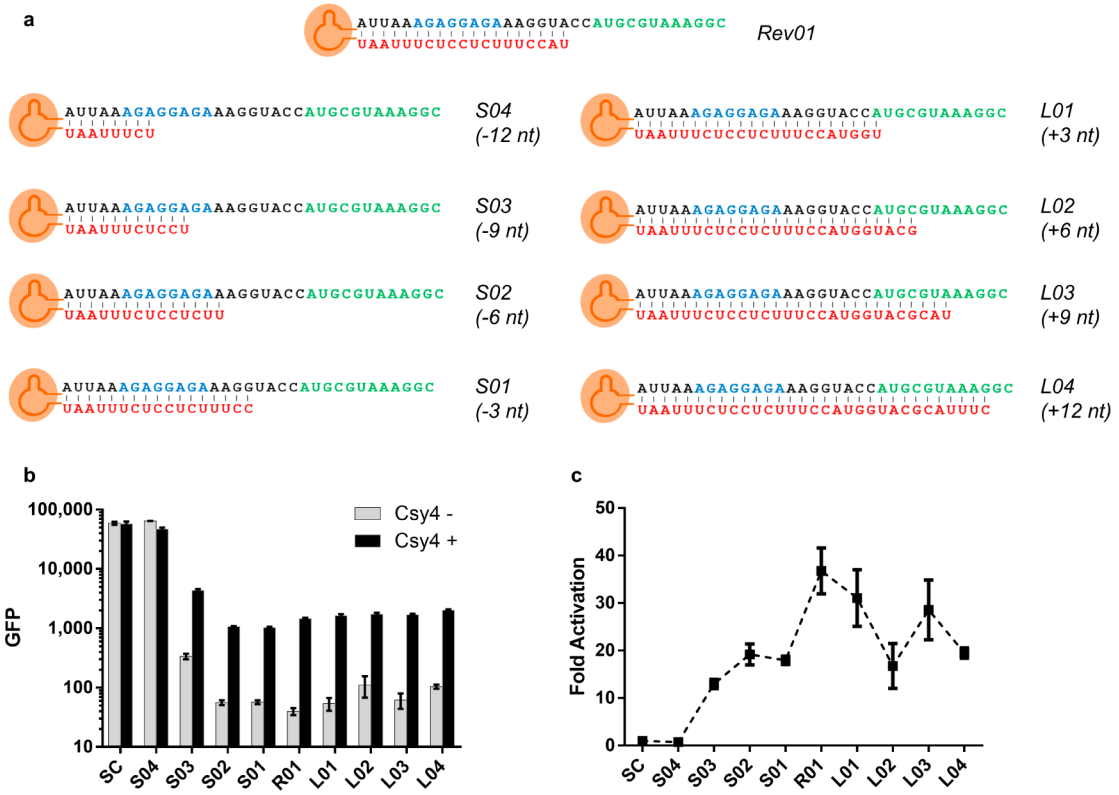


Figure 3. Length of RBS complementary crRNA sequence affects efficiency of translational activators. (a) Sequence and structure of TrACR S01-04 and L01-04. Orange circle represents the stem-loop structure containing Csy4 site. (b) Flow cytometric measurement of fluorescence of sfgfp in cells with indicated shortened or extended crRNA, respectively. (c) Fold activation for each TrACR element. Error bars show mean ± SD from three independent experiments.

upon expression of Csy4 (Figure 2b, Supplementary Figure S2), indicating that the endoribonuclease Csy4 can activate the duplex-repressed translation from various endogenous RBSs. The variation of their activation capacity seems mainly due to the differences in their basal repression. It is worth noting that the Rev06 duplex can be activated by 50-fold, a sizable improvement compared to that of the original Rev01 duplex.

Next, we constructed six crRNA-RBS duplexes using forward design strategy. We chose the new RBSs with translational activities are 1/2, 1/4, 1/8, 1/32, 1/128, or 1/1024 relative to that of original Rev01 (Figure 2c). As expected, the endoribonuclease Csy4 can activate TrACR-controlled translation in all the RBS sequences tested (Figure 2d, Supplementary Figure S3), suggesting this engineered system can function in a broad range of translation initiation activity. Importantly, we have demonstrated a strong correlation (R^2 = 0.984) between the theoretical and experimental level of Csy4-activated gene expression in the forward design strategy (Figure 2d). These results also provided an independent experimental validation of the prediction capacity for the "RBS calculator", enabling predictable tuning of translation initiation in the future engineering of gene regulators.

Optimize Translational Activator by Refining RBS-crRNA Duplex. In addition to the RBS sequence, the length of the RBS-crRNA duplex may also affect the performance of the translational activator. The TrACR regulatory element may be unable to cis-repress translation when the crRNA is too short, whereas extending the crRNA may abolish translational activation because of reinforced hydrogen bonds from the additional pairing of crRNA with target. Therefore, optimization of the crRNA length may potentially fine-tune the translational activator.

We systematically examined the effect of crRNA length by testing eight TrACRs with stepwise shortened crRNAs (S01 to S04) or extended crRNAs (L01 to L04) (Figure 3a). We found that the TrACR S01, S02, and S03 (with crRNA shortened by 3-, 6-, and 9-nt) maintain their translational switching ability (18-, 19-, and 13-fold upon Csy4 expression); whereas the switching ability is completely abolished in TrACR S04 with crRNA shortened by 12-nt to expose the Shine−Dalgarno (SD) sequence (Figure 3b). Notably, the crRNA in TrACR S03 partially exposed the SD sequence, causing increased expression of the reporter gene in both the absence and presence of Csy4 cleavage (Figure 3b, Supplementary Figure S4). This data suggests that the accessibility of the SD sequence is a key parameter in designing and refining the artificial translation switch.

Conversely, the longer crRNAs (L01-L04) with extended pairing between RBS and crRNA have a comparable performance as translational activators (Figure 3b, Supplementary Figure S5). These data suggested the increasing hydrogen bonds are less effective in disrupting the switching ability of the translational activator. However, we speculate that extending the length of crRNA could potentially reduce the expression leakage, which can be useful in manipulating genes with high background translation. However, further investigation may be needed to systematically investigate the impact of long base-pairs near translation initiation sites on design of translation regulators. Nevertheless, our data demonstrated the effect of the RBS-crRNA duplex length on the switch ability of the

DOI: 10.1021/acssynbio.5b00130
ACS Synth. Biol. 2016, 5, 74−80

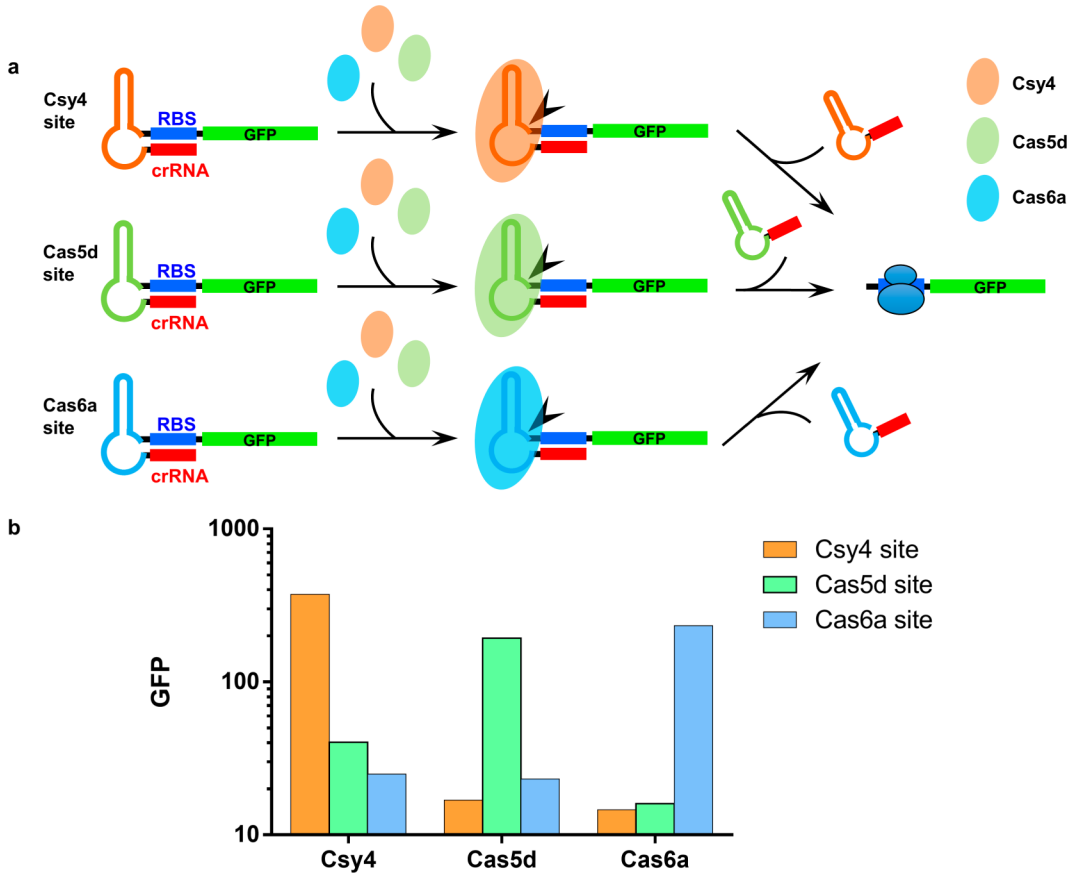


Figure 4. Orthogonal cleavage of the modularized endoribonuclease recognition site by various Csy4 analogues. (a) Schematic representation of cleavage orthogonality among Csy4 and its analogues Cas5d and Cas6a. (b) Flow cytometric measurement of fluorescence of sfgfp with each combination of indicated CRISPR endoribonucleases and corresponding recognition sites.

translational activator (Figure 3c), and suggested that adjusting the length of the crRNA is an effective strategy to tune the dynamic range of translation.

Taken together, with rational design and modularization of the RBS-crRNA duplex, we have achieved stepwise optimization of translational activators, providing a tunable module for a more complex genetic circuit.

Expanding the Translational Controller Tool Box with Diverse and Orthogonal Cas Endoribonucleases. To facilitate the application of TrACR in more complex circuits, we further expanded the specificity of this system with various members from CRISPR-Cas endoribonucleases and their cognate sites. The capability of recognizing and cleaving a highly specific RNA site is not limited to Csy4,[11,26,27] but has also been found in other CRISPR endoribonucleases that recognize different sequences.[28−32] We selected a diverse panel of Cas6 endoribonuclease analogues, including three from *Thermus thermophilus* H8, one from *Streptococcus pyogenes* MGAS5005, and five from *Sulfolobus solfataricus* P2. We seek to identify a set of Cas family members and their cognate sites that show minimal cross talk between each pair (i.e., strong orthogonality) and adequate translational switching ability.

We constructed new TrACR systems using all nine Cas-RNA pairs, and examined their ability to specifically control translation. Seven out of nine cas6 analogues showed notable translational activation activity (Supplementary Figure S5). We further tested the orthogonality (i.e., crosstalk between noncognate pairs) of these Cas enzymes, and identified three enzymes (Cas6a_TTHA0078, Cas5d_Spy1290, and Csy4) that can efficiently and specifically activate protein translation in the TrACR system with limited activity against noncognate recognition sites (Figure 4, Supplementary Figure S7).

Notably, in addition to the specific recognition between an endoribonuclease and its cognate site, the performance of a translational activator also may rely on other parameters (such as the length of RBS-crRNA duplex). Two Cas family members, Cas5d_TTP0133 and Cas6e_TTHB192 from *Thermus thermophilus* H8, show minimal translational activation activity, respectively, possibly due to low expression level or low enzymatic activity resulted by transferring gene from *Thermus thermophilus* to *E. coli*. This suggests that a broad range of cleavage activity can be generated by different Cas family enzymes, which could be exploited for distinct applications requiring various extent of translational activation. Furthermore, the dynamic range of a translational activator may be limited by background translation activity. Previously certain levels of cross reaction were proposed among the Cas6 endoribonucleases from *Sulfolobus solfataricus*,[31] and thus we examined all combinations of these cas6 endoribonucleases and their cognate site with the TrACR system. We found that while some combinations show translational activation, all have high background activity that significantly reduces the dynamic ranges of translational activation (Supplementary Figure S6). One possible reason is that these binding sites are linear rather than forming a stem-loop structure in TrACR,[31] and the linearized sequence might interfere with the adjacent base-pairing interactions between crRNA and RBS.

DOI: 10.1021/acssynbio.5b00130
ACS Synth. Biol. 2016, 5, 74−80

These findings expanded the diversity of the TrACR system with orthogonal CRISPR endoribonucleases, making it possible to modularly assemble TrACR into more complex circuits. The large number of endogenous CRISPR endoribonucleases from various archaea and bacteria provide a rich resource with a broad range of specificity and activity,[15] offering a great potential for future engineering of translational activators. The possibility of integrating multiple TrACRs in a genetic circuit will greatly expand the application of translational control in synthetic biology.

In conclusion, we have demonstrated the design of modular translational activators with CRISPR endoribonuclease Csy4. In our design, both the RBS-crRNA duplex and endoribonucleases recognized site can be modularized, offering diversity to future design and integration into complex genetic circuits. Additionally, fine-tuning of the translational activator can be achieved by introducing crRNA mutations, switching the RBS-crRNA duplex module, and adjusting the length of crRNA. The design flexibility would satisfy the various requirements of more complex genetic circuits.

MATERIALS AND METHODS

Plasmid Circuit Construction. Plasmid construction and DNA assembly procedures follow standard molecular biology techniques. The reporter plasmid pReporterA was assembled by inserting a constitutive BBa_J23119 promoter (http://parts.igem.org/), a LacZα expression module flanked by two BsaI sites, the RiboJ sequence,[9] the coding sequence of superfolder GFP (sfgfp), the synthetic terminator L3S2P22[33] and the double terminator BBa_B0015 (http://parts.igem.org/) between the XbaI and *Pst*I site of pSB4C5 plasmid,[34] (pSC101, Cmr) (Supplementary Figure S1). Then the sequence encoding each TrACR element was assembled between BsaI sites using the Golden Gate cloning method.[35] The Csy4 expression plasmid pNucleaseA-Csy4 was constructed by inserting Csy4 coding sequence into a custom built plasmid p15A origin, ampicillin resistance, Ptac promoter, and the RiboJ sequence (Supplementary Figure S1). The BBa_B0010 terminator (http://parts.igem.org/) was used to terminate expression of Csy4. A control plasmid pNucleaseA-Null was constructed by deleting the Csy4 expression system including the Ptac promoter, RBS, Csy4 coding sequence, and terminator from pNucleaseA-Csy4, and was utilized for samples without Csy4 expression. All Csy4 analogue genes were amplified from genomic DNA and inserted into pNucleaseA plasmid (Supplementary Table S1, Supplementary Figure S1). All RBS sequences used in this work were native *E. coli* or bacteriophage RBS.

Strains, Media, and Growth Condition. Plasmid construction works were all performed in *E. coli* DH5α strain. Cells were cultured in LB (Luria−Bertani broth) media (10 g/L peptone, 10 g/L NaCl, 5 g/L yeast extract) at 37 °C with shaking (225 rpm) for all plasmid circuit construction works. Cells for flow cytometric gene expression assay were cultured in 96 well plates (flat clear bottom) with M9 media (12.8 g/L $Na_2HPO{\cdot}4.7H_2O$, 3g/L KH_2PO_4, 0.5 g/L NaCl, 1.67g/L NH_4Cl, 1 mM thiamine hydrochloride, 0.4% glucose, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) at 37 °C with shaking (1000 rpm). Ampicillin, kanamycin, and chloramphenicol concentrations for all experiments were 100 μg/mL, 50 μg/mL, and 20 μg/mL, respectively.

Assay of Gene Expression by Flow Cytometry. Overnight cultured *E. coli* DH5α in LB was diluted 196 times into M9 medium with required antibiotics, followed by shaking at 37 °C for 3 h. IPTG (0.125 mM) induction was applied if necessary. Cells were then diluted 10 times into M9 medium with antibiotics and cultured at 37 °C with 0.125 mM IPTG induction. Cells were analyzed by BD LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA) after further dilution into PBS with kanamycin. The expression level of sfgfp was examined by measuring the fluorescence level of cells in each sample. The mean sfgfp fluorescence of each sample was calculated with Flowjo software (Treestar, Inc., San Carlos, CA, USA) and analyzed with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Data are shown as mean ± SD in all figures. Error bar represents three independent experiments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00130.

Supplementary figures and tables (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: louchunbo@im.ac.cn.

*E-mail: zefeng@med.unc.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Li Huang and Prof. Yong Tao for their material. This work was supported in part by the Ministry of Science and Technology of China (973 Grant No. 2013CB734001); and the National Natural Science Foundation of China (Grant No. 31470818).

REFERENCES

(1) Kemmer, C., Gitzinger, M., Daoud-El Baba, M., Djonov, V., Stelling, J., and Fussenegger, M. (2010) Self-sufficient control of urate homeostasis in mice by a synthetic circuit. *Nat. Biotechnol. 28*, 355−360.

(2) Weber, W., and Fussenegger, M. (2012) Emerging biomedical applications of synthetic biology. *Nat. Rev. Genet. 13*, 21−35.

(3) Juminaga, D., Baidoo, E. E., Redding-Johanson, A. M., Batth, T. S., Burd, H., Mukhopadhyay, A., Petzold, C. J., and Keasling, J. D. (2012) Modular engineering of L-tyrosine production in Escherichia coli. *Applied and environmental microbiology 78*, 89−98.

(4) Bauer, G., and Suess, B. (2006) Engineered riboswitches as novel tools in molecular biology. *J. Biotechnol. 124*, 4−11.

(5) Dixon, N., Duncan, J. N., Geerlings, T., Dunstan, M. S., McCarthy, J. E. G., Leys, D., and Micklefield, J. (2010) Reengineering orthogonally selective riboswitches. *Proc. Natl. Acad. Sci. U. S. A. 107*, 2830−2835.

(6) Verhounig, A., Karcher, D., and Bock, R. (2010) Inducible gene expression from the plastid genome by a synthetic riboswitch. *Proc. Natl. Acad. Sci. U. S. A. 107*, 6204−6209.

(7) Papenfort, K., Sun, Y., Miyakoshi, M., Vanderpool, C. K., and Vogel, J. (2013) Small RNA-Mediated Activation of Sugar Phosphatase mRNA Regulates Glucose Homeostasis. *Cell 153*, 426−437.

(8) De Lay, N., Schu, D. J., and Gottesman, S. (2013) Bacterial Small RNA-based Negative Regulation: Hfq and Its Accomplices. *J. Biol. Chem. 288*, 7996−8003.

(9) Lou, C., Stanton, B., Chen, Y. J., Munsky, B., and Voigt, C. A. (2012) Ribozyme-based insulator parts buffer synthetic circuits from genetic context. *Nat. Biotechnol. 30*, 1137−1142.

DOI: 10.1021/acssynbio.5b00130
ACS Synth. Biol. 2016, 5, 74−80

(10) Marraffini, L. A., and Sontheimer, E. J. (2010) CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat. Rev. Genet. 11*, 181−190.
(11) Childs, L. M., England, W. E., Young, M. J., Weitz, J. S., and Whitaker, R. J. (2014) CRISPR-Induced Distributed Immunity in Microbial Populations. *PLoS One 9*, e101710.
(12) Isaacs, F. J., Dwyer, D. J., Ding, C., Pervouchine, D. D., Cantor, C. R., and Collins, J. J. (2004) Engineered riboregulators enable post-transcriptional control of gene expression. *Nat. Biotechnol. 22*, 841−847.
(13) Green, A. A., Silver, P. A., Collins, J. J., and Yin, P. (2014) Toehold switches: de-novo-designed regulators of gene expression. *Cell 159*, 925−939.
(14) Grissa, I., Vergnaud, G., and Pourcel, C. (2007) The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinf. 8*, 172.
(15) Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J., Wolf, Y. I., Yakunin, A. F., van der Oost, J., and Koonin, E. V. (2011) Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol. 9*, 467−477.
(16) Chylinski, K., Makarova, K. S., Charpentier, E., and Koonin, E. V. (2014) Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Res. 42*, 6091−6105.
(17) Rousseau, C., Gonnet, M., Le Romancer, M., and Nicolas, J. (2009) CRISPI: a CRISPR interactive database. *Bioinformatics 25*, 3317−3318.
(18) Carte, J., Wang, R. Y., Li, H., Terns, R. M., and Terns, M. P. (2008) Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. *Genes Dev. 22*, 3489−3496.
(19) Haurwitz, R. E., Jinek, M., Wiedenheft, B., Zhou, K., and Doudna, J. A. (2010) Sequence- and structure-specific RNA processing by a CRISPR endonuclease. *Science 329*, 1355−1358.
(20) Haurwitz, R. E., Sternberg, S. H., and Doudna, J. A. (2012) Csy4 relies on an unusual catalytic dyad to position and cleave CRISPR RNA. *EMBO J. 31*, 2824−2832.
(21) Qi, L., Haurwitz, R. E., Shao, W., Doudna, J. A., and Arkin, A. P. (2012) RNA processing enables predictable programming of gene expression. *Nat. Biotechnol. 30*, 1002−1006.
(22) Wilson, H. R., Yu, D., Peters, H. K., 3rd, Zhou, J. G., and Court, D. L. (2002) The global regulator RNase III modulates translation repression by the transcription elongation factor N. *EMBO journal 21*, 4154−4161.
(23) Court, D. L., Gan, J., Liang, Y. H., Shaw, G. X., Tropea, J. E., Costantino, N., Waugh, D. S., and Ji, X. (2013) RNase III: Genetics and function; structure and mechanism. *Annu. Rev. Genet. 47*, 405−431.
(24) Salis, H. M. (2011) The Ribosome Binding Site Calculator. *Methods Enzymol. 498*, 19−42.
(25) Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009) Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol. 27*, 946−U112.
(26) Arslan, Z., Hermanns, V., Wurm, R., Wagner, R., and Pul, U. (2014) Detection and characterization of spacer integration intermediates in type I-E CRISPR-Cas system. *Nucleic Acids Res. 42*, 7884−7893.
(27) Haft, D. H., Selengut, J., Mongodin, E. F., and Nelson, K. E. (2005) A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS Comput. Biol. 1*, 474−483.
(28) Gesner, E. M., Schellenberg, M. J., Garside, E. L., George, M. M., and Macmillan, A. M. (2011) Recognition and maturation of effector RNAs in a CRISPR interference pathway. *Nat. Struct. Mol. Biol. 18*, 688−692.
(29) Niewoehner, O., Jinek, M., and Doudna, J. A. (2014) Evolution of CRISPR RNA recognition and processing by Cas6 endonucleases. *Nucleic Acids Res. 42*, 1341−1353.
(30) Koo, Y., Ka, D., Kim, E. J., Suh, N., and Bae, E. (2013) Conservation and variability in the structure and function of the Cas5d endoribonuclease in the CRISPR-mediated microbial immune system. *J. Mol. Biol. 425*, 3799−3810.
(31) Sokolowski, R. D., Graham, S., and White, M. F. (2014) Cas6 specificity and CRISPR RNA loading in a complex CRISPR-Cas system. *Nucleic Acids Res. 42*, 6532−6541.
(32) Garside, E. L., Schellenberg, M. J., Gesner, E. M., Bonanno, J. B., Sauder, J. M., Burley, S. K., Almo, S. C., Mehta, G., and Macmillan, A. M. (2012) Cas5d processes pre-crRNA and is a member of a larger family of CRISPR RNA endonucleases. *RNA 18*, 2020−2028.
(33) Chen, Y. J., Liu, P., Nielsen, A. A., Brophy, J. A., Clancy, K., Peterson, T., and Voigt, C. A. (2013) Characterization of 582 natural and synthetic terminators and quantification of their design constraints. *Nat. Methods 10*, 659−664.
(34) Shetty, R. P., Endy, D., and Knight, T. F., Jr. (2008) Engineering BioBrick vectors from BioBrick parts. *J. Biol. Eng. 2*, 5.
(35) Engler, C., Kandzia, R., and Marillonnet, S. (2008) A one pot, one step, precision cloning method with high throughput capability. *PLoS One 3*, e3647.

DOI: 10.1021/acssynbio.5b00130
ACS Synth. Biol. 2016, 5, 74−80