tRNA mimicking structures to control and monitor biological processes

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Chapter 2

Modular and versatile trans-encoded genetic switches

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2.1. INTRODUCTION

Synthetic non-coding RNAs with simple and limited conformational states have been deployed to build RNA switches to control bacterial translation\textsuperscript{1-3}. Many RNA switches suffer from low dynamic response and lack of versatility with respect to input signals that can be processed. Nonetheless, great progress involving sophisticated RNA switches has been made\textsuperscript{4-11}. These RNA switches act in \textit{cis} on the target mRNA (i.e., act on the same molecule, while \textit{trans}-acting switches act on a different molecule) to achieve fast and efficient functionality with high on/off-ratio. The “toehold switch” is a prime example of such an RNA device (Figure 1) with strong dynamic response, orthogonality, and possibility for logic-gate operations\textsuperscript{12}.

\textbf{Figure 1.} Schematic of the \textit{cis} encoded toehold switch. The Toehold switch represses bacterial translation through base pairs engineered before and after the start codon (AUG), leaving the RBS and the start codon unpaired. The translation is initiated when a trigger RNA, which binds to the ‘a’ and ‘b’ regions of the switch RNA, is expressed in cells. RNA-RNA interactions are initiated via linear-linear interaction domains called toeholds\textsuperscript{12}.

There are also ligand-dependent riboswitches that are \textit{cis}-acting with regard to the target mRNA\textsuperscript{13}. The incorporation of \textit{cis}-encoded switches at the leader mRNA may however interfere with folding of downstream mRNA\textsuperscript{14} and will require genetic modification upstream of the target gene. The importance of the leader mRNA sequence has been well documented for 5’-UTR-encoded regulators of pathogenic bacteria\textsuperscript{15}. \textit{Trans}-encoded switches would not present sequence constraints on the target mRNA. For example, a \textit{trans}-encoded switch based on the looped antisense oligonucleotide (LASO) can repress a downstream gene in presence of an input RNA\textsuperscript{16} but does not accept other signals such as
small molecules or proteins. Hence, limited by their design, current artificial switches lack modularity with respect to input signals.

To overcome these limitations, we present trans-encoded genetic switches based on a tRNA-mimicking structure (TMS; Figure 2). Bacterial tRNA is stable to RNase and allows stable expression of RNA constructs in the cells\textsuperscript{17,18}. Since the TMS switch is trans-encoded, it does not disturb the secondary structure of the target mRNA. The concept of TMS is based on metazoan mitochondrial tRNAs that display diverse sequences and structures\textsuperscript{19}. We hypothesized that sequence variation of different arms of the tRNA structure would allow easy incorporation of modules with different functions into the switch.

To achieve tight control over gene expression, we designed the switch to bind both flanking sites of the ribosome binding site (RBS) of the target mRNA, without disturbing the RBS and the start codon, to block ribosome entry and subsequent translation.

2.2. RESULTS AND DISCUSSION

2.2.1. Design and characterization of the TMS switch

We incorporated a repressor domain into the anticodon loop of the bacterial tRNA\textsuperscript{lys} that binds flanking sites of the RBS (Figure 2 and Supplementary Figure 2.1). To reverse the effect of the repressor, we designed an anti-repressor RNA that binds the TMS through loop–loop interactions and pulls off the repressor domain from the mRNA, thereby liberating the RBS for binding to the ribosome. To provide initiation sites for the binding between the TMS switch and the anti-repressor RNA, we incorporated two 9 nts initial binding elements (IBEs), one at each end of the repressor domain of the TMS switch. To make the design process simple, we consider the IBEs as a part of the repressor domain. We name this switch D$^\text{TMSIBE}$ (See appendix for designing the TMS switch using NUPACK software).
Figure 2. Concept to switch protein translation: Modification of the anticodon loop of a tRNA (1) blocks ribosome binding (2), which can be reversed by an anti-repressor RNA (3), thereby allowing GFP expression (4). A and B denote the two subdomains in the repressor domain. The initial binding element (IBE) hybridizes with the anti-repressor RNA.

For characterization of the switch, we used a two-plasmid system\textsuperscript{11} and co-expressed the anticodon-modified TMS switch and a GFP reporter from the two separate plasmids (Supplementary Figure 2.2) in *Escherichia coli* BL21(DE3) cells. We varied the length of regions A and B in the repressor domain and tested their ability to repress GFP by flow cytometry. Three out of eight D-TMS\textsuperscript{IBE} switches showed effective GFP repression (Supplementary Figure 2.3). We selected the D-TMS\textsuperscript{IBE} switch with the longest regions of A=10 nts and B=8 nts to obtain the best binding characteristics. We determined the minimum length of the stem in the repressor domain and the stem that connects the repressor domain with the tRNA structure (Supplementary Figure 2.4). The need for a minimum stem length is likely due to the stability provided by a tRNA structure in an RNase environment (see below). The D-TMS\textsuperscript{IBE} switch maintains its functionality even with a variable loop of 1 nt length.

2.2.2. In vivo stability of the D-TMS\textsuperscript{IBE} switch

Useful switches need to be stable to degradation by RNase enzymes. For example, a *trans*-encoded bacterial switch that binds the protein Hfq is stable to RNase\textsuperscript{20}. To investigate the stability of the D-TMS\textsuperscript{IBE} switch in RNase
environment, we compared repression of GFP expression by the $\text{D-TMS}^{\text{IBE}}$ switch with an RNA oligomer containing only the repressor sequence (Supplementary Figure 2.5). The $\text{D-TMS}^{\text{IBE}}$ switch repressed GFP expression 21-fold more effectively than the RNA oligomer, thus demonstrating that the $\text{D-TMS}^{\text{IBE}}$ provides stability to the RNA-based switch in an environment where RNase is present.

2.2.3. In vivo reversibility of the $\text{D-TMS}^{\text{IBE}}$ switch function

To reverse the repression of the GFP gene by the $\text{D-TMS}^{\text{IBE}}$ switch, we simultaneously expressed an anti-repressor RNA from the $\text{D-TMS}^{\text{IBE}}$ switch plasmid (Supplementary Figure 2.6). We again used a TMS structure for the anti-repressor RNA to provide stability. The anti-repressor RNA and the $\text{D-TMS}^{\text{IBE}}$ switch were expressed from similar promoters (T7 promoter) from two different regions of the plasmid. The anti-repressor RNA binds with the repressor domain of the $\text{D-TMS}^{\text{IBE}}$ switch and the RNA duplex structure gets detached from the target RNA, revealing the ribosome binding site for initiation of the translation process. Cells expressing both the anti-repressor RNA and the $\text{D-TMS}^{\text{IBE}}$ switch exhibited almost the same GFP intensity as in absence of both interacting TMSs (Figure 3 a). The ON/OFF ratio of the $\text{D-TMS}^{\text{IBE}}$ switch was around 200-fold. The ON state refers to the GFP fluorescence from cells expressing both the $\text{D-TMS}^{\text{IBE}}$ switch and its cognate anti-repressor RNA. On the other hand, the OFF state refers to the GFP fluorescence from cells expressing only the $\text{D-TMS}^{\text{IBE}}$ switch.

2.2.4. Orthogonality of the $\text{D-TMS}^{\text{IBE}}$ switch

We determined the orthogonality of six $\text{D-TMS}^{\text{IBE}}$ switches and their cognate anti-repressor RNAs (Figure 3 b). We varied the repressor domain sequence of the $\text{D-TMS}^{\text{IBE}}$ switches while retaining the ability to bind to the target mRNA by altering only the RBS flanking sites of the target mRNA so that each $\text{D-TMS}^{\text{IBE}}$ switch can bind with their corresponding target mRNA. Combination of each of the $\text{D-TMS}^{\text{IBE}}$ switches with each of the anti-repressor RNAs resulted in fold changes in GFP expression varying between 180 and 210 for each cognate pair and less than 2 for each non-cognate pair. Hence, our system is highly selective concerning the $\text{D-TMS}^{\text{IBE}}$ switch and cognate anti-repressor RNA.
Figure 3. a) GFP fluorescence measured by flow cytometry for the anticodon modified TMS switch in the ON and OFF states (presence and absence of the anti-repressor RNA). Negative control is without GFP induction, positive control is with GFP induction in the absence of TMS. b) Orthogonality of six different D-TMS\textsuperscript{IBE} switches and their cognate anti-repressor RNAs. The GFP fold change is the GFP fluorescence of the D-TMS\textsuperscript{IBE} switch ON divided by the OFF state without background correction.

2.2.5. Increasing versatility of the input signals of the RNA switch

To increase the versatility of input signals, we inserted an RNA aptamer as an additional module into the D-loop of the TMS switch. Previously, an aptamer was coupled to a self-cleaving ribozyme in order to create a ligand-dependent riboswitch\textsuperscript{21-23}, which was stabilized at its base-paired stem by binding its ligand. In our case, we hypothesize that such stabilization of a base-paired stem in the D-loop upon binding with its ligand would provide the energy to regain the tRNA structure with concomitant release of the TMS from the mRNA.

2.2.5.1. Increasing versatility of the input signals of the RNA switch: small molecule used as input signal

The well-studied hybridized structure of the neomycin B (NeoB) aptamer shows that it has increased stability upon neomycin B binding\textsuperscript{24, 25}. The aminoglycoside antibiotic is anchored within the RNA aptamer binding pockets through a network of intermolecular hydrogen bonds. These bonds are formed between the charged amine groups of the antibiotic and acceptor atoms on the base-pair edges and the backbone phosphates of RNA aptamer. To verify the TMS switch activity against a small molecule, we selected azide-modified neomycin B\textsuperscript{26} as input. We replaced the D-loop of the TMS switch with a neomycin B aptamer\textsuperscript{27} sequence (NeoB-TMS\textsuperscript{IBE}, Figure 4a and Supplementary Figure 2.7). The NeoB aptamer
exhibits a low micromolar binding affinity to azide-modified neomycin B (Supplementary Figure 2.8). Modifying the 2-deoxystreptamine ring of neomycin B with an azide reduces its antibacterial activity (Supplementary Figure 2.9 and Supplementary Figure 2.10) and allows its use as a non-bioactive input signal at lower concentrations. We removed the IBE elements at the repressor domain. With the incorporation of the neomycin B aptamer, the TMS switch is now comprised of a sensor (that recognizes a small-molecule input signal), an actuator (that controls the output signal), and a transmitter (that channels the signal from the sensor to the actuator; see Figure 4 a top left). This RNA architecture responds in a concentration-dependent manner to azide-modified neomycin B as input (Figure 4 b), thereby demonstrating that the NeoB-TMS⁻IBE structure can function as an analogue genetic switch against a small-molecule input signal²⁸.

**Figure 4.** a) Controlling gene expression by the NeoB-TMS⁻IBE and GFP-TMS⁻IBE switches (1). Binding of the switches prevents ribosome binding (2), which is reversed by binding of the corresponding aptamer ligand (3). The GFP-TMS⁻IBE
switch controls mCherry expression. b) Titration of azide-conjugated neomycin B leads to increased GFP production. c) Inducing GFP expression with anhydro-tetracycline leads to increasing mCherry fluorescence.

2.2.5.2. Increasing versatility of the input signals of the RNA switch: protein used as input signal

In addition to small molecules, the switch accepts GFP as an input signal with a GFP aptamer integrated into the D-loop of the TMS (GFP-TMS, Figure 4 a). We used mCherry as an output signal, controlled under an arabinose promoter, while expressing GFP from anhydrotetracycline inducible promoter. The behaviour of the GFP-TMS switch against a gradient GFP input signal was similar as that of the NeoB-TMS switch with the small-molecule input signal (Figure 4 c).

2.2.6. Construction of an OR logic gate using the TMS switch

Implementation of co-localized circuit elements in the same self-assembled molecular complex enhances signal propagation to the output gene and substantially decreases diffusion-mediated signal losses and metabolic cost. Apart from these, it also improves circuit reliability and enables one gate RNA to accomplish tasks that would otherwise require multiple independent RNAs. To demonstrate that our RNA switch can function as an OR logic gate in live cells, we programmed this gate by incorporating a repressor domain and the neomycin B aptamer together in a single TMS switch (NeoB-TMS, Figure 5). The logic gate gave the expected output signal: Cells only express GFP in the presence of the cognate anti-repressor RNA and/or azide-modified neomycin B (Figure 5), but cells do not express any GFP protein in absence of both inputs. This observation confirms that the NeoB-TMS switch can be implemented as an OR logic gate to perform complex computations in living cells.
Figure 5. Design of an OR logic gate with the NeoB-TMS\(^{+\text{IBE}}\) switch, responding to its cognate anti-represssor RNA and azide-modified neomycin B (100 µm). All experiments were performed in triplicate. Median fluorescence is reported. AR=Anti-repressor RNA; NeoB azide=azide-modified neomycin B.

2.2.7. Assessing the switch functionality based on affinity of ligand against the aptamer domain

To assess whether the ligand-mediated stabilization of the TMS switch solely depends on the interaction with the corresponding aptamer, we replaced the neomycin B aptamer of NeoB-TMS\(^{+\text{IBE}}\) with a kanamycin B aptamer\(^{30}\) (KanB-TMS\(^{+\text{IBE}}\)). Azide-modified neomycin B has a very low binding affinity for the kanamycin B aptamer (Supplementary Figure 2.11). We observed that due to the lower affinity of the kanamycin B aptamer for azide-modified neomycin B, KanB-TMS\(^{+\text{IBE}}\) is unable to control gene expression with azide-modified neomycin B (Supplementary Figure 2.12). This observation derives the conclusion that the ligand-mediated structural changes in the TMS switch depend on the affinity of the ligand for the aptamer component of the switch.

2.2.8. Implementation of the TMS switch to control gene expression in bacterial genome

2.2.8.1. Controlling the expression of the T7 RNA polymerase gene in bacterial genome

We next asked whether the TMS switches can be deployed to target the bacterial genome. A ϕ-TMS\(^{+\text{IBE}}\) switch was designed to target the T7 RNA polymerase gene
present in the genome of the E. coli BL21(DE3) cells (Figure 6a), binding from −30 to −1 bases upstream of the start codon in the corresponding mRNA. The D-TMS^{IBE} switch is under control of the strong constitutive lpp promoter. To induce T7 polymerase expression, we added 0.1 mm isopropyl β-d-1-thiogalactopyranoside (IPTG). As a marker, GFP expression from a plasmid controlled by a T7 promoter should be reduced when the D-TMS^{IBE} switch represses T7 RNA polymerase translation. Indeed, we observed that the presence of the D-TMS^{IBE} switch reduced GFP fluorescence (Figure 6b). Importantly, binding cognate anti-repressor RNA to the D-TMS^{IBE} switch showed full GFP fluorescence, thus showing that genomic expression can also be switched (Supplementary Figure 2.13). GFP expression is only marginally reduced further when a second D-TMS^{IBE} switch that targets the +11 to +30 bases upstream of the start codon of the T7 polymerase mRNA is employed simultaneously with the first D-TMS^{IBE}, thus indicating the excellent performance of the switches.

**Figure 6.** a) Switching genomic gene expression. The D-TMS^{IBE} switch prevents expression of T7 RNA polymerase, by binding the −30 to −1 bases of the T7
polymerase gene. GFP expression is recovered with the cognate anti repressor RNA. b) Corresponding flow cytometry data showing repression of expression with $D_{\text{TMS}^{\text{IBE}}}$ (OFF TMS switch), activation with the anti-repressor RNA (ON TMS switch), and controls as in Figure 3, but under a T7 promoter. c) Box plot showing that inhibiting a native $E.\ coli$ gene $ftsZ$ with two $D_{\text{TMS}^{\text{IBE}}}$ switches leads to filamentous growth compared to cells without any $D_{\text{TMS}^{\text{IBE}}}$ switch. Co-expression of the cognate anti-repressor RNAs leads to restoration of cell sizes similar to $D_{\text{TMS}^{\text{IBE}}}$-free cells. n=500 cells. d) Corresponding confocal microscopy brightfield images. Scale bar=7.6 $\mu$m. All experiments were performed in triplicate.

2.2.8.2. Controlling the expression of the $ftsZ$ gene in bacterial genome

A key advantage of a $trans$-encoded switch is that it allows genes in the genome to be targeted without sequence alteration of the genome. To demonstrate inhibition of native gene expression, we targeted the $D_{\text{TMS}^{\text{IBE}}}$ switch versus the $ftsZ$ gene in the genome, which encodes for the FtsZ protein (Figure 6d). FtsZ is an essential protein for cell division that forms a contractile ring structure ($Z$ ring) at the future cell-division site$^{31}$. One of the functions of the FtsZ ring is to recruit other cell division proteins to the septum to produce a new cell wall between the dividing cells$^{32}$. Inhibiting the FtsZ production would hamper the cell-division process, leading to filamentous growth of $E.\ coli$ cells$^{33,34}$. Unlike the T7 polymerase gene in the bacterial genome, the $ftsZ$ gene is controlled by a constitutive promoter. Therefore, we decided to express two $D_{\text{TMS}^{\text{IBE}}}$ switches simultaneously to target the $ftsZ$ gene in order to achieve tight control of FtsZ expression. One $D_{\text{TMS}^{\text{IBE}}}$ switch binds to the $-19$ to $+11$ nucleotides region and the other binds to the $+32$ to $+61$ nucleotides region from the start codon of the $ftsZ$ gene. Transcription of both $D_{\text{TMS}^{\text{IBE}}}$ switches led to filamentous cells and a larger spread of cell lengths in comparison with cells without the switches (Figure 6c; Supplementary Figure 2.14). Both switches accept inputs from their respective cognate anti-repressor RNAs, also expressed simultaneously, giving cells with the same average length and distribution as untreated cells. Hence, we can repress translation of mRNAs transcribed from both plasmid and genome without alteration of the target mRNA sequence, and subsequently turn on gene expression with input signals.

2.3. CONCLUSION

In summary, we present powerful new RNA-based switches that provide unparalleled versatility in controlling bacterial gene expression. Unlike other $cis$-
acting genetic switches, the TMS switches do not pose sequence constrains on the target mRNA. By designing RNA switches that accept versatile inputs (RNA, small molecules, and proteins), we have been able to compensate for the low chemical diversity of input signals of the current RNA switches compared to their protein counterparts. A remarkable feature of the TMS switches is that the simple replacement of modules enables sensing of new input signals, and even addition of a second sensing module does not compromise performance of the RNA device as realized by the logic-gate design. The switches that we developed here display a strong dynamic range, likely due to the stability of the TMS structure tuned by the unique design of the repressor domain. With their unique combination of signal processing of desired inputs and targeting any desired mRNA, the TMS switches will play an essential role in novel genetic circuitry to allow advanced information processing in synthetic biology.

2.4. EXPERIMENTAL SECTION

2.4.1. Plasmids construction

All the DNA fragments were purchased from Integrated DNA Technologies and the primers were acquired from Sigma-Aldrich. The fragments were inserted into the vector backbone through conventional cloning (Supplementary Ref. 1). The pBluescript vector backbone contained the DNA sequences of the TMS and anti-repressor switches. The reporter plasmid was based on the pZE21 vector backbone. The pBluescript vector encoded the pUC origin of replication (Supplementary Ref. 2) and the pZE21 vector contained p15A origin of replication (Supplementary Ref. 3). The pBluescript and the pZE21 plasmids contained ampicillin and chloramphenicol resistance genes, respectively. All constructs were transformed in *Escherichia coli* (E. coli) DH5α cells and were sequenced.

2.4.2. Growth of the *E. coli* cells and transcription of the TMS switches

To verify the function of the TMS switches, we used *E. coli* BL21(DE3) cells. Two separate tubes of the BL21(DE3) cells were used to execute the transformation process. In the first tube of BL21(DE3) cells, the TMS switch plasmid and the reporter plasmid were co-transformed. The anti-repressor RNA plasmid, which contains both the gene for the anti-repressor and TMS switch, was co-transformed with the reporter plasmid in the second tube of the cells. The transformation was carried out by electroporation (using MicroPulser Electroporator from BIORAD with 2.5 kV for 5 milliseconds). We spread the
transformed cells on antibiotic plates, single colonies were picked and grown in LB media shaking at 200 rpm overnight at 37°C in the presence of antibiotics (ampicillin 50 μg/ml and chloramphenicol 25 μg/ml). The starter culture was then diluted by 200 times in LB medium containing antibiotics and four separate cultures were prepared – the first culture was to express the reporter gene only, the second culture was to express the switch and the reporter gene, the third culture was to express the switch, the reporter and the anti-repressor RNA and the fourth culture was used as a negative control. All the samples were grown at 37°C with 200 rpm to OD600 0.4 – 0.6, after which 0.1% arabinose (w/v) was added into the first culture to induce the expression of the reporter gene only. Into the second culture, 1mM IPTG and 0.1 % arabinose (w/v) were added to induce the switch and the reporter. Into the third culture, 1mM IPTG and 0.1 % arabinose (w/v) were added to induce the switch, the antirepressor and the reporter. In the fourth culture, no inducer was added. After inducing each sample at the log phase, we incubated for six hours and then the output signal from each sample was measured by flow cytometry. To control gene expression by the TMS switch with protein as an input, we used mcherry as an output signal by replacing the GFP in the reporter plasmid with mcherry protein. The mcherry protein was placed under the control of an arabinose promoter. Here the GFP was used as an input signal to control the expression of the mcherry by the TMS switch. The GFP was cloned in the same reporter plasmid under a tet promoter. To study the gene expression with the TMS switch and protein as input, all the experimental procedure was same as mentioned above except the third culture was further divided into five separate subcultures. At the log phase 1 mM IPTG, 0.1% (w/v) arabinose were added into each five sub-cultures. Along with IPTG and arabinose, anhydrotetracycline was also added into those cultures with different concentrations (0.0125-0.2 μM). The flow cytometry measurement was taken after six hours of incubation.

2.4.3. Switching genomic FtsZ expression in E. coli

E. coli BL21(DE3) competent cells were either transformed with the TMS_FtsZ plasmid, or with both the TMS_FtsZ and TMS-AR_FtsZ plasmids: The TMS_FtsZ encoded the TMS switch that targets mRNA from the FtsZ gene, and the TMS-AR_FtsZ plasmid produced the corresponding anti-repressor switch. Expression of the switches was achieved under constitutive lpp promoters. Single colonies were grown in LB media shaking at 200 rpm at 37°C in the presence of antibiotic (ampicillin 50 μg/ml). The bacteria were analysed by fluorescence
confocal microscopy upon reaching log phase (Leica TCS SP8, 63×water objective). Five μl cells were added on a glass slide and a cover slide was put on top. The images of the cells were analysed by ImageJ and the Feret diameter of each cell was taken as cell length. As control, BL21(DE3) cells without plasmid were grown, measured and analyzed in the same manner.

2.4.4. Flow cytometry measurements

Flow cytometry measurements were performed on a BD FACS Canto flow cytometer. The BD FACS Canto was calibrated with CST beads from BD Biosciences (Cat No: 655051). Samples were washed with 1X PBS and were diluted by 50-fold into 1X PBS buffer prior measurement. For GFP fluorescence measurement, 488 nm excitation filter (optical power 20mW) and 515-545 nm emission filter were used. For mcherry fluorescence measurement, 561 nm excitation filter (optical power 40mW) and 615-620 nm emission filter were used. For each sample, 50,000 events were recorded. All the samples were measured with low sample flow rate (approximately 12 μl/min). Cells were gated based on the positive fluorescence level. The flow cytometry analysis was performed using FlowJo software (version 10).

2.4.5. Isothermal Titration Calorimetry (ITC) measurement

ITC experiments were executed using the ultrasensitive ITC 200 calorimeter (MicroCal) at 25°C. First, we degassed all solutions for 15 min using a vacuum pump in order to prevent the formation of bubbles in the sample cell during the experiment. We filled the reference cell with degassed distilled water and rinsed the sample cell with the buffer two times. We filled the sample cell with 7 μM aptamer solution (prepared in phosphate buffer solution with pH 6.8) and calorimeter syringe with 70 μM target ligand solution (prepared in phosphate buffer solution with pH 6.8). A purge-refill cycle was performed during filling up the syringe to avert the formation of air bubbles inside the syringe. To determine the binding constant, the ligand solution was added to the cell containing aptamer solution in a stepwise manner. The instruction of the instrument was set up in such a way that 20 injections of 2 μl volume from the syringe were added into the aptamer solution with intervals of 60 or 120 seconds between each injection. Control experiments were done by titrating the phosphate buffer without ligand into the aptamer solution. Data were analysed by using the nonlinear curve-fitting functions for one binding site provided by the ORIGIN software of MicoCal.
2.4.6. Minimum Inhibitory Concentration (MIC) assay

*E. coli* BL21(DE3) cells were grown in LB medium at 37 °C with 200 rpm until an OD600 of 0.6, after which they were diluted to OD600 0.1 with fresh LB medium. NeomycinB-azide was serial diluted in 200 μL LB medium from 25.6 mM to 50 μM in 1 mL Deepwell 96-well plates (Eppendorf, Germany). 200 μL of the diluted *E. coli* culture was added to each well, diluting the NeomycinB-azide further from 12.8 mM to 25 μM. After 18 h incubation at 37 °C with 200 rpm, 200 μL of the culture was transferred to clear 96-well microtiter plates (Brand, Germany), and the turbidity was measured at 600 nm using a SpectraMax M3 platereader (Molecular Devices, USA). To conduct the MIC test for pristine NeomycinB, same procedure was adopted but the range of the serial dilution for NeomycinB was from 100 μM to 0.78 μM.

2.4.7. Secondary structure prediction of RNA

All secondary structure predictions of RNA were performed using the mfold Web Server (Supplementary Ref. 4).

2.4.8. Statistical analyses

All statistical analyses were performed using GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com).

Author contribution

Andreas Herrmann and Avishek Paul conceived the research. Avishek Paul carried out most of the experiments. Eliza M. Warszawik synthesized the antibiotic derivative. Mark Loznik performed the MIC test of the modified antibiotic. Arnold J. Boersma designed and assisted microscopy experiments. Andreas Herrmann, Arnold J. Boersma and Avishek Paul wrote the manuscript.

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Appendix

Designing the TMS switch with region A = 10 nts and region B = 8 nts

To design the repressor domain of the TMS switch, we first determined the secondary structure of the target region of the mRNA molecule. A region of 47 nts length of the target mRNA molecule has been chosen to make the design process straightforward. To design the A and B regions of the repressor domain, we took the reverse complementary sequences of the flanking regions of the Ribosome Binding Site (RBS) in the target mRNA. For region A, 10 nts reverse complementary region was chosen and for region B, 8 nts reverse complementary region was selected. An ideal repressor domain should not impose any sequence constrains on the anti-repressor RNA and therefore we did not include the complementary sequence of the 6 nts RBS in the repressor domain. Instead, we incorporated random nucleotides in between the A and B regions of the repressor domain.

Next, we decided to include two 9 nts sequences of Initial Binding Elements (IBE) into the repressor domain, to provide initial binding sites between the repressor domain and the anti-repressor RNA. To attach the IBE to the repressor sequence, a stem of 8 nts length was incorporated into the structure. Finally, a stem of 4 nts length was chosen to attach the repressor domain to the respective tRNA structure.
After setting up these initial parameters, we used NUPACK software (Supplementary Ref. 5) to design the whole repressor domain and the stem that connects the repressor domain with the tRNA structure. In order to design these parts of the switch, the following algorithm was used:

```plaintext
#
# design material, temperature (C)
#
material = rna
temperature = 37.0
#
# target structures
#
structure hairpin1 = D4 (U9 D8 (U24) U9)
#
# sequence domains
#
domain a = N4
domain b = N9
domain c = N8
domain d = GAUUUCAUN6AGCAAUUUAA
domain e = N9
#
# thread sequence domains onto target structures
#
hairpin1.seq = a b c d c* e a*
#
# stop conditions for normalized ensemble defect
```
In this algorithm, we specified the sequence of the A and B regions of the repressor domain and 6 random nts in “domain d”. For the design algorithm, 1.0 M Na\(^+\) and 0 M Mg\(^{2+}\) were selected as buffer condition. After executing the algorithm, the NUPACK software determined the sequences of the IBE domains, the stem that connects the repressor sequence to the IBE and the stem that connects the repressor domain to the tRNA structure.

The sequence of the IBE domains was the following:

5’ AAAAUAAAGA 3’

5’ GAAGCCAGA 3’

The sequence of the stem that connects the IBE to the repressor sequence was the following:

5’ UCGAGUCG 3’

AGCUCAGC

The sequence of the stem that connects the repressor domain to the tRNA structure was the following:

5’ GCGG 3’

CGCC

We also determined the free energy of the structural complex formed by the binding of the repressor domain and the target mRNA by executing the following algorithm in the NUPACK software,

```plaintext
# default: 1.0 (percent) for each target structure

# In this algorithm, we specified the sequence of the A and B regions of the repressor domain and 6 random nts in “domain d”. For the design algorithm, 1.0 M Na\(^+\) and 0 M Mg\(^{2+}\) were selected as buffer condition. After executing the algorithm, the NUPACK software determined the sequences of the IBE domains, the stem that connects the repressor sequence to the IBE and the stem that connects the repressor domain to the tRNA structure.

The sequence of the IBE domains was the following:

5’ AAAAUAAAGA 3’

5’ GAAGCCAGA 3’

The sequence of the stem that connects the IBE to the repressor sequence was the following:

5’ UCGAGUCG 3’

AGCUCAGC

The sequence of the stem that connects the repressor domain to the tRNA structure was the following:

5’ GCGG 3’

CGCC

We also determined the free energy of the structural complex formed by the binding of the repressor domain and the target mRNA by executing the following algorithm in the NUPACK software,

```
The free energy of the structural complex was calculated to be -22.18 kcal/mol. The software also allowed to obtain the average number of nucleotides (2.9 nts) in the complex that could be incorrectly paired at the equilibrium relative to the specified secondary structure (the number was evaluated over the Boltzman-
weighted ensemble of secondary structures). The normalized ensemble defect was 4.4%.

We also used NUPACK software to design the different repressor domains used in the orthogonality test. To conduct the orthogonality test, we designed six different TMS switches with the same stem sequence that connects the repressor domains to the tRNA structure. The TMS switches differ in the sequences of IBE domains and the stem that connects the IBE to the repressor sequence. They also contain different sequences in the A and B regions of the repressor domain. After setting up these parameters we executed the following algorithm:

```python
# # design material, temperature (C)
#
material = rna
temperature = 37.0
#
# target structures
#
structure hairpin1 = D4 (U9 D8 (U24) U9)
#
# sequence domains
#
domain a = GCGG
domain b = N9
domain c = N8
domain d = N24
domain e = N9
#
# thread sequence domains onto target structures
After executing the algorithm, the NUPACK software provided different sequences of the TMS switches where each TMS switch contained a specific repressor domain sequence.
Supplementary Figure 2.1. The secondary structure of the TMS switch containing the repressor domain at the anti-codon arm. The nucleotides which are modified during tRNA processing are denoted by red circles. Target sequence of the GFP mRNA is 5’TTAAATTGCTAAGGAGATGAAATC3’. The secondary structure of the TMS switch was determined by mfold web server. Red colour represents the binding domains of the TMS switch.
Supplementary Figure 2.2. The plasmid maps of the TMS switch and GFP.
Supplementary Figure 2.3. Repression of the GFP fluorescence by the candidate TMS switches. We designed eight different TMS switches, where each TMS switch contains a unique length of A and B regions (the two subdomains located in the repressor domain). We designed TMS1 switch in such a way that it contains region A of 10 nts and region B of 8 nts. We constructed the subsequent TMS switches by reducing the length of the A and B regions stepwise by 1 nt. We noticed that TMS switch number 1, 2 and 3 displayed GFP repression capability after 6 hours of incubation. The experiment was performed in triplicate (sequences of the eight different TMS switches are available in supplementary sequences 2). GFP fluorescence on Y axis represents the GFP fluorescence values in log scale.
Supplementary Figure 2.4. Determination of the minimum length of the stem in the repressor domain, the stem that attaches the repressor domain with the tRNA structure and the variable loop required to maintain the functionality of the TMS switch. We designed five different TMS switches with different length of the stem in the repressor domain (from 1 nt to 8 nts) and measured the median GFP for each candidate TMS switch against five corresponding reverse complementary target mRNA sequences. Similarly, we changed the length of the stem connecting the repressor with the tRNA structure in the five TMS switches and measured the median GFP intensity against the same five different target sequences that were used previously to characterize the stem length of the repressor domain. In the same way, we changed the variable loop length of the five TMS switches and determined the median GFP intensity with the same five target sequences (the
sequences of the five TMS switches and their target sequences are available in supplementary sequences 2).

**Supplementary Figure 2.5.** Stability of the TMS switch in RNase environment. GFP positive cells contain only the GFP reporter plasmid which was induced by arabinose and GFP negative cells contain GFP reporter plasmid but without any inducer. GFP repression by the pristine repressor refers to the repression of the GFP with only the repressor sequence without any tRNA scaffold. GFP repression by the TMS switch refers to the repression of the GFP with the repressor sequence incorporated into the tRNA scaffold.
Supplementary Figure 2.6. The plasmid map of the Antirepressor.
Supplementary Figure 2.7. Secondary structures of the TMS switch and the NeomycinB aptamer. The aptamer was incorporated into the D-loop of the TMS structure without any sequence modification of the NeomycinB aptamer.
**Supplementary Figure 2.8.** Isothermal titration calorimetry (ITC) test to measure the binding affinity between the NeomycinB aptamer and NeomycinB-azide. Analysis of the ITC binding curve showed a binding affinity of 28 μM. The sequence of the NeomycinB aptamer: 5’GGACTGGCGAGAAGTTTAGTCC3’.
Supplementary Figure 2.9. MIC test with NeomycinB-azide to determine its working concentration. We noticed from the MIC test that concentration higher than 100 μM of NeomycinB-azide disturbs bacterial growth. Therefore, we picked 100 μM NeomycinB-azide concentration to verify the TMS switch activity against the NeomycinB-azide.
Supplementary Figure 2.10. MIC test with pristine NeomycinB. We noticed from the MIC test that concentrations higher than 3.12 μM of NeomycinB can disturb bacterial growth.
**Supplementary Figure 2.11.** Isothermal titration calorimetry (ITC) test to measure the binding affinity between the KanamycinB aptamer and NeomycinB-azide. Analysis of the ITC binding curve showed a binding affinity of 2 mM. The sequence of the KanamycinB aptamer: 5’GGGAGCUCGUACCGAAUUCUC3’.
**Supplementary Figure 2.12.** Study of TMS switch functionality in presence of NeomycinB and KanamycinB aptamers. Two separate TMS switch were prepared by replacing the D-loop of the tRNA with the respective aptamers. To check the functionality of each switches, NeomycinB-azide (100 μM) was used as target ligand.
Supplementary Figure 2.13. Control expression of the T7 RNA polymerase gene by the TMS switch. TMS switch was designed to target the mRNA of the T7 RNA polymerase gene and thus affects the expression of a GFP protein, which was under the control of a T7 promoter. (1) Negative control (without addition of IPTG); (2) Positive control (with addition of IPTG); (3) Single TMS switch targeting the mRNA of the T7 RNA polymerase gene and thereby reducing the GFP expression from the T7 promoter; (4) Dual TMS switch targeting the mRNA of the T7 RNA polymerase gene; (5) Simultaneous expression of the TMS switch and its cognate anti-repressor RNA.
Supplementary Figure 2.14. Histograms of controlling FtsZ translation by the TMS switch. (a) Cells expressing only the TMS switches; (b) Cells expressing both TMS switches and their cognate antirepressor RNAs; (c) Cells that neither express TMS switches nor antirepressor RNAs.
**Supplementary Sequences 1.** Sequences of the different switches, origin of replications and the target GFP

| Supplementary Sequences 1 | 5’TTATGACA AAC TTGACGGCTACATCATCATTTTCTTCAACAACCGG |
|---------------------------|----------------------------------------------------|
| **A-RBS-binding region**  | CACGGAACTC GCTCGGCTGCGCCGCCGTTGCAATTTTTATAATACCCGC |
| **B-GFP**                 | GAGAAATAGA GTTGTACG TCAAACCAACAT TCGACCGAC CGGTTG |
|                           | CGATAGGCA TCCGCGGCTGCTCAGTTAACGCTCTAGTGAATACTT |
|                           | CTGACAAGCCT CGGTACCTCGCATACCGGATATCCCCGTGCTGATGTA |
|                           | CGTTAACGCT CAGTGATTTTCTTCAACGTAAGTACCCGAGGAGAT |
|                           | ATCCGCGAG CAGCTCGAATAAGCGGCGGCTTCCAATCCGCGCAGG |
|                           | GATTTGCGCA AACAGCTCGTGAATCGGCTGCGCTTGCTCACTCCG |
|                           | GCCGAAGAAC CGCCGATTGCGAATATGACGCGCCGCAGGCAAGC |
|                           | TTCATGCCAGT GAGCCGCGGACGAAAGTAAACCCACTGGTACAC |
|                           | CATTCCGCGA GCTCCGGAATGAGCGGCAATGCTACGCTGCAGG |
|                           | CGGGGAACAG CAAATATCACCACGGGCTGCAAAACACCACAAAT |
|                           | GATTTTCGCA ACCACCCCTGACCGCAGATGTTGAGATGAGAATAA |
|                           | CTTTCCATTCCCAGCGTCCGTCGATAAAAATCGAGATAACCGTT |
|                           | GGCCTCAAATCGCGTTAAACCGCCACCAGATGGGCAAT TAAACGAGT |
|                           | ATCCGGCA GCAGG GATCATTTTGCGCTTGCTAGCCATACTTTT |
|                           | CTCCC GCA CTCCAGAGAAGAAAAACGC ATTGTCCATA TTGCAATCAGACA |
|                           | TTGCGTC ACTGCGCTTTTACTGGCTTTCGCTAAACAAACCGGT |
|                           | AACCCGCTT ATTAAAGACAT TCGTAAACACAGGGACCAAAG |
|                           | ATGACAAAAACGCGTAAACAAAGTGTCTATAATCAGCGCAAAAG |
| Sequence | T7P-TMS-GFP Apt-TMS-binding region A-TMS-binding region B-T7 TT | T7P-Anti repressor RNA-T7 TT | Ptet/pLtet-GFP-tetR-araC-pBAD-binding region A-RBS-binding region B-mcherry |
|----------|---------------------------------------------------------------|--------------------------------|---------------------------------------------------------------|
| GGTCAGGGTTCAAGTCCCTGTTCGGGCGCCTAGCATACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGGA3’ | 5’TAATACGACTCACTATAGGGCCCGGATAGGACAGATCTGGGAGCA CGATGGCGTGCGAATTGGGTGGGGAAAGTCCTTAAAAGAGGGCCA CCACAGAAGCAATGGGCTTCTGGACTCGGTAGATCTGTCCTGAGTC GGGTTTCACCTGCAATCCTTGGTTGCGACTCGAGGGTCCAGGGTTTTTGGA3’ | 5’TAATACGACTCACTATAGGGCCCGGATAGGACAGATCTGGGAGCA CGATGGCGTGCGAATTGGGTGGGGAAAGTCCTTAAAAGAGGGCCA CCACAGAAGCAATGGGCTTCTGGACTCGGTAGATCTGTCCTGAGTC GGGTTTCACCTGCAATCCTTGGTTGCGACTCGAGGGTCCAGGGTTTTTGGA3’ |
| 5’CTCGAGTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATA GAGATCTCAGCACTAGCAGCAGGACGCACTGACCGAATTCATTAAA TTTAACTTTAAGAAGGAGATATACATATGCGTAAAGGCGAAGAGCT GGGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGGAAGGTGACGCAACT AATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGGAAGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAA GGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGC GATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAAGA AGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCC ACAATGTTTACATCACCAGCGGATAAACAAAAATGGCATTAAAGC |
GAATTTAAAAATTGCCACACGTTGAGGATGCCAGCTGCTGGAGCTGGC
TGATCAGTACCAGCAAACACTCCTATCGTGTGATGGGTTCCTGGTTCTGC
TGCCAGAAATACACTATCTCAGACGGCAGCTGCTGGAGCTTTAGATGAT
CCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGC
AGCGGGCATCAGCATGATGTTAGGATGAAACTGCTACATGTAACAAAT
GATGATGGCATGCCAGTTCTAGCTAACCCTAATGAGTGAGCTAACTTCA
TTAATTGCGTTGCGCCTTAATTACGGAAGGCTCCTCAAGCACAATATAAT
GACCCCTTTGATAAACCAGAGAGGCTATCTTAAAATATTGCTGGGCTG
TACCACAGCTAACAACACGTCGTCCTCATTCTGTGCGCTCCTAGGTC
AGTGGGTTGCTGGATAACTTACAGGGCAGCTGCTGAGCTAGGCT
ATATTCAGGGGACCCAAACGTTTTCCCTCTACAAATAATTTTTGGTTT
AATTTGAAATAAGGAGGTAATACAAATGTCCTGTTAGATAAAAGGT
AAAGTGATTAACACGCATTAGAGCTGCTTAATGAGGTCGGAATCG
AAGGTTTAAACAAACCGTAAAACCTCGCCCAGAAGCTTTAGGGAGCA
GCCTACATTGTATTTGCCGCATTAAAATAAAGCAGGCTTGGGCTCGAC
CCTAGCCATTGAGATGTAGATTAGGACCACCATCTCATTGCTCCCTT
TAGAAGGGAAAGCTGGAAGATTTTTACGTGTAATAACGCTAAAAG
TTTGTAGTGTCCTTACTAAGTCATCGCAGTGGAGCGAAAAGTACTATT
TAGTGACTACGGCCTACAGAAAACAGTTAGGAAACTCTGAAAATCA
ATTAGGCTTTTTATCAGCCAACAGGTTTTTCATAGAGAATGCATTATA
TGCACTAGCAGCTGTGGGCGATTATTACTTTAGGTTGCCGATTGGAAG
ATCAAGAGCATCAAGTCGCTAAAGAAGAAAGGGAAACACCTACTAC
TGATAGTATGCGCCATTATTAGCACAAGCTACGTAATTGATGTC
ACCAAGGTGAGCCAGCCTTTCTATTCCGCGCTTTAAATTTATGCTCA
TGCGGATTAGAAAAACACTTAAATGTGAAAGTGGGTCTTAATTATG
ACAACTTGACGGCTACATTGACCTTTTTCTTCTACGCAACGACCGG
AATCAGCTCGGCTGGCCCGGCTGAGTTCCTTTAATACCCGCGAGAA
| GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC | pUC |
|-----------------------------------------------|-----|
| TTCGCCTGGGACATCCTGTCCCCCTCAGTTTCTAGTGCTGCTCT | CCGTAGAAAAAGATCAAAGGATCTTTTCTTGAGATCTCTTTTTTTCTGC | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| CTACGTGAAGCACCCCCGCCGACATCCCCGACTACTTTGAGCTGCTGCTCT | CCGTAGAAAAAGATCAAAGGATCTTTTCTTGAGATCTCTTTTTCTGC | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| TCCCCGAGGCGTTCAAGTGGAAGCGGCGTGATGAACTTCTGAGGACGG | TCCCCGAGGCGTTCAAGTGGAAGCGGCGTGATGAACTTCTGAGGACGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| CGGCGTGGTGAGCAGCAGACCAGACTCTCCTCCCTGAGGACGG | CGGCGTGGTGAGCAGCAGACCAGACTCTCCTCCCTGAGGACGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| TCATCTACAAGGAGTGTAAGCTGCGCGCGACAAACTTCCTCCCCCTCAGGACGG | TCATCTACAAGGAGTGTAAGCTGCGCGCGACAAACTTCCTCCCCCTCAGGACGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| CCGTAATGCGAGAAGACACCATTGGGGCGAGGCTGCTCTCTCTCCAGGACGG | CCGTAATGCGAGAAGACACCATTGGGGCGAGGCTGCTCTCTCTCCAGGACGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| GGATGTACCACGGAGGACGCGGCCTGAGAAGGGCGAGATCGAGGACGG | GGATGTACCACGGAGGACGCGGCCTGAGAAGGGCGAGATCGAGGACGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| GCTGAAAGCTGAAGGACGG | GCTGAAAGCTGAAGGACGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| ACCTACAAGGCAAGAAAGCCTCCGTGACGCTGCCGGGCGCCTACACAGG | ACCTACAAGGCAAGAAAGCCTCCGTGACGCTGCCGGGCGCCTACACAGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| TCAACATCAAGTTGCGACATCCTCCCCCAACAGAGACTACAACCACTC | TCAACATCAAGTTGCGACATCCTCCCCCAACAGAGACTACAACCACTC | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| GTGGAAACAGTACGAAAGCGCGCGAGGCGAGCAGGACGCTCAGG | GTGGAAACAGTACGAAAGCGCGCGAGGCGAGCAGGACGCTCAGG | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |
| TGGACGAGCTGTAACAGTAG3’ | TGGACGAGCTGTAACAGTAG3’ | GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCCCGCCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCC |

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### Supplementary Sequences 2.

Sequences of eight different TMS switches with different length of A and B sub-domains against the target GFP sequence 5’TTAAATTGCTAAGGAGATGAAATC3’

| TMS switch | sequence |
|------------|----------|
| 1          | 5’GGGCCCCCCGATAGCTCAGTCGGTAGAGCAGCGGAAAATAAGATCGAGTGGATTCTGTCTGACAACCTCTGAGCAAGGACATGACGCACTACGAGGACTATAAAGATACAG |
|            | ACTTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGGCAAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| 2          | 5’GGGCCCGGATAGCTCAGTCGGTAGAGCAGCGGAAAATAAGATCGAGTCGGATTCACTGCAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
|            | TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |

p15A

| sequence |
|----------|
| GGGAAACGCTGATCCTGTCTGTCGGGTTTCGCCACCTCTG
| ACTTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |
| TTTTCCATAGGCTCCGCCCTCTGAACAAGGACATCGAAGCAATTTAACGACTCGAGAAGCCAGACCGCGGGTCCAGGGTTCAAGTCCCTGTCGGGCGCC |

GATTACGCGCAGACCAAAACGATCTCAAGA
Supplementary Sequences 3. Five sequences of the loop domain of the TMS switch and the reverse complementary sequence of the target RBS region in the GFP mRNA. These were tested with different stem lengths in Figure S3.

| Sequence No | Sequence of the target region of the GFP | Sequence of the repressor domain of the TMS switch |
|-------------|------------------------------------------|-----------------------------------------------|
| 1           | 5’GTTGGTTCCTAAGGAGCAGACTTTTG3'          | 5’CAAAAGTGGTGCAATAGGAAACCAAC3'                |
| 2           | 5’TAAATTGCTAAGGAGATGAAATC3’             | 5’GATTTCACTCTGCAAAGCAATTTAA3’                 |
| 3           | 5’TGCTGTAGGCAAGGAGATGAAATC3’            | 5’AAAGACTATCTCCCGCCCTACAGCA3’                 |
| 4           | 5’ATTACGAAATAAGGAGAGCTTAGT3’            | 5’ACTAAGCTCTGGGTATTTCCATAT3’                  |
| 5           | 5’AGTCAGAGTAAAGGAGGAATAGA3’             | 5’TTCATATTCCAGCAATTACTCTGACT3’                |
**Supplementary Sequences 4.** Sequence of the construct to express pristine repressor RNA.

| T7P-Repressor sequence-T7 TT | 5’TAATACGACCTCTATAGGGTTTTTCAGTCACTTCCAAATCCTTGGTTGCTAGCATAACCCCTTGGGGCTCTAAACGGGTCTTGAGGGGTTTTTTGGA3’ |

**Supplementary Sequences 5.** Sequences used in the orthogonality test, corresponding to Figure 1C.

| Index No | Target region in the GFP mRNA | Repressor loop in TMS switch | Reverse complementary sequence in the loop of the anti-repressor |
|----------|-------------------------------|----------------------------|---------------------------------------------------------------|
| 1        | 5’ATTGATTTGTAAAGGAGGGCCTTTA3’ | 5’GCGGGGACACATCGAGGCTGACAATCACGACTCCACTTACCCAGACCCGACG3’ | 5’GCGGTCTTTAAAGTAGGGCGTGTATTGATTTGAGGGTCGGTTTTAACACGCCTCGATGTCGCCG3’ |
| 2        | 5’CTCTCTGTGTAAGGAGGCTCCTCTACACGAGAAAAGACACGCTACTTTAAGGACCCG3’ | 5’GCGGCTTTAAAGTAGGGCGTGTCTTCTGTGTAAGGAGGCTCCTCTACACGAGAAAAGACACGCTACTTTAAGGACCCG3’ | 5’GCGGTCTTTAAAGTAGGGCGTGTCTTCTGTGTAAGGAGGCTCCTCTACACGAGAAAAGACACGCTACTTTAAGGACCCG3’ |
| 3        | 5’TGTATTTTTGTAAGGAGGCTCCTCTACACGAGAAAAGACACGCTACTTTAAGGACCCG3’ | 5’GCGGCTTTAAAGTAGGGCGTGTCTTCTGTGTAAGGAGGCTCCTCTACACGAGAAAAGACACGCTACTTTAAGGACCCG3’ | 5’GCGGTCTTTAAAGTAGGGCGTGTCTTCTGTGTAAGGAGGCTCCTCTACACGAGAAAAGACACGCTACTTTAAGGACCCG3’ |
| 4        | 5’ATTGATTTGTAAAGGAGGGCCTTTA3’ | 5’GCGGGGACACATCGAGGCTGACAATCACGACTCCACTTACCCAGACCCGACG3’ | 5’GCGGTCTTTAAAGTAGGGCGTGTATTGATTTGAGGGTCGGTTTTAACACGCCTCGATGTCGCCG3’ |
### Colour code:

- **Sequence of the stem connecting the repressor domain to**
- **Sequence of the IBE**
- **Sequence of the stem connecting IBE to the repressor**
- **Sequences of the repressors**
- **Sequence of the ribosome in GFP target region**

### Supplementary Sequences 6. RNA sequences to target FtsZ and T7 polymerase genes

| FtsZ | 5’CACAAATCGGAGAGAAACTATGTTTGAACC3’ |
|------|-------------------------------------|
| T7 polymerase | 5’TCCGGATTTACTAAGAGAGACTGACTAA3’ |
Supplementary Reference

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