De novo engineering of *trans*-activating riboswitches in *E. coli*

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By

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Satya Prakash

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Abbreviation

aTc	Anhydrotetracycline
asRNA	Antisense RNA
ATP	Adenosine Triphosphate
Cis11	CisRNARAJ11 or 5UTRRAJ 11
Coop1	Cooperative RNA1
Coop2	Cooperative RNA2
CoopU	5'UTR (cooperative 5'UTR)
DMSO	Dimethyl sulfoxide
dsDNA	Double stranded DNA
DTT	Dithiothreitol
2D-PAGE	2-Dimensional polyacrylamide gel electrophoresis
EB	Elution buffer
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
HDV	Hepatitis D virus
HHR	Hammerhead ribozyme
HR	Homologous recombination
IPTG	β-D-1-thiogalactopyranoside
MFE / mfe	Minimum free energy
miRNA	Micro RNA
MiR11	MIRRAJ11or aniRAJ11
mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
PACE	Phage assisted continuous evolution
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
Riboregulation	RNA based regulation
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNase	Ribonuclease
RT	Reverse transcription / Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
TTCA	Translation-transcription coupling adaptor
SELEX	Systematic evolution of ligands by exponential enrichment
SfGFP	Superfolder green fluorescent protein
sRNA	Small RNA
ssDNA	Single stranded DNA
ssRNA	Single stranded RNA
taRNA	Trans-activating RNA
TE buffer	Tris EDTA buffer
tracrRNA	Trans activating crRNA
tRNA	Transfer RNA
TTP, dTTP	Thymidine triphosphate, deoxy-TTP
UTR	Untranslated region (OF mRNA)
WT	Wild type

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Summary of work.

RNA molecules play a major role in cellular processes such as replication, transcription, and translation. As a result, RNA-based engineering methods have emerged as important tools in biotechnology. However, the structure and function of RNA depends on global interactions, which often prevents the use of a modular design strategy, particularly with allosteric conformations. Using RNA secondary structure prediction tools, computational methods can successfully design RNA switches that work in *E. coli*. The overarching aim of my research is to develop synthetic RNA switches that could be used for regulation and sensing of molecules in living cells.

We have engineered RNA based synthetic signal transduction cascade consisting of a single RNA molecule (regazyme, an RNA chimera of an aptazyme with a riboregulator) that upon sensing a ligand (theophylline) self-cleaves and releases a riboregulating small RNA. This small RNA binds to a cis-repressed mRNA allowing translation of a reporter protein. This system can be adapted to be induced by other ligands and can be used as a biosensor. I have also integrated a riboregulated RNA switch into the *E. coli* genome to study its behaviour at single-cell level. This reduces the transcriptional and translational noise in data collection to inform more accurate computational design of RNA regulatory units. We used computational design to engineer higher-order RNA-triggered riboregulators organized as a hierarchical toehold activation cascade. This has been studied in a single cell as well as in a population of *E. coli* cells. These RNA riboregulators can be used for construction of new, complex and portable synthetic gene circuits.

In addition, I have engineered sense and antisense riboregulators consisting of the small RNA reverse complement of a known riboregulator. This riboregulator can transcribe two small RNAs from the same DNA template depending on the direction of transcription. These two small RNAs independently *trans*-activate translation of their cognate target genes and

both RNAs also silence each other by antisense interaction. We have also engineered an RNA-based tunable antiterminator, a TNA-derived adaptor that acts as a signal converter in a genetic circuit, converting a translation signal to a transcription signal (unpublished). I have engineered a minimum alphabet riboregulator that has only three nucleotides (GCU) that currently validating (unpublished).

In order to explore the use of directed evolution for the engineering of RNA switches, I am developing an evolution-based system for generation and selection of new biomolecules. These evolved new biomolecules could be used in future medical applications such as molecular sensing. I have been using T7 and P2 bacteriophages as the basis for this evolutionary system. I have engineered the genome of the T7 phage with (regazyme, Riboswitch and riboregulators) using homologous recombination with marker-based selection. These engineered phages can be used to evolve new biomolecules such as other regazymes, riboswitches and riboregulators.

0.0 General Introduction

Synthetic biology is an emerging interdisciplinary area which applies engineering principles towards design and construction of biological parts, devices and systems for useful biotechnological applications (Endy 2005; Khalil & Collins 2010; Qi & Arkin 2014). In past decades, a number of promoters (Alper et al. 2005), scaffolds (Dueber et al. 2009) proteins and RNAs (Pfleger et al. 2006); (Win et al, 2008) have been designed and characterized. There are currently a number of complex circuits including oscillators (Elowitz et al. 2000; (Stricker et al. 2008); (Danino et al., 2010), toggle switches (Gardner et al., 2000; Atkinson et al., 2003), riboregulators (Isaacs et al., 2004; Rodrigo et al., 2012; Na et al., 2013), riboswitches (Tucker et al., 2005; Blount et al., 2006) and biological logic gates (Tamsir et al., 2011; Moon et al., 2012) engineered into a wide range of organisms.

The goal of synthetic biology opens a new door to the engineers and biologists for the designing of a novel biomolecules, reprogram the cell, evolve the new medically applicable biomolecules (Khalil & Collins 2010). These engineered organisms could be useful for diagnostic, targeted therapy, such as cancer and to kill superbug. This reprogrammed cell would have many applications such as dynamic control on gene expression sensing of molecule. The cell regulators such as DNA, protein, RNA and DNA modifying the regulators control gene expression (Nielsen & Voigt 2014). The reprograming cell or engineering genetic circuits are challenging because, genetic circuits should be robust, cell regulator should not be toxic. Cell regulators should also have fine tuning. The regulator should not interact with in cell and no cross talk with in cell and host cell. That's we need to engineer novel regulatory circuits, and RNA switches to reprogram cell.

Currently, the development of synthetic riboswitch, genetic circuits and related technologies is a high priority for the synthetic biology community because it allows programmable control over the cell. However, RNA synthetic biology is in its infancy, there will be more research to be done in near future in order to understand the function and mechanism of RNA

switches within the cellular systems. In the past decades, RNA was known as an information carrier molecule, carrying genetic information from DNA to protein. Recently, RNA has emerged as a key regulatory molecule and is now known as a highly versatile molecule with regulatory, enzymatic and structural roles in the cell. RNA can regulate major cellular functions such as replication, transcription, and translation.

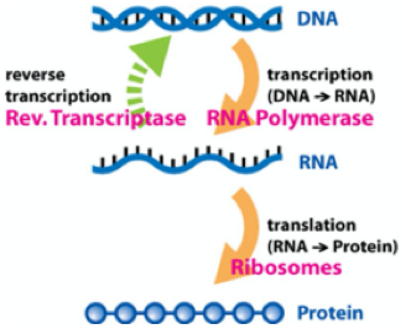


Figure 0.1 Explains Mechanism of central dogma (figure credit to wiki)

It has important roles in post-transcriptional regulation, including gene repression and activation by using synthetic riboregulators, which can be explored for constructing novel regulatory circuits (Isaacs et al., 2004; Rodrigo et al., 2012; Na et al., 2013). To develop RNA switches RNA based circuits, different aspects of RNA function used that are described below.

0.01 Base pairing functionality

The base-pairing or antisense RNA regulation is primarily involved in the regulating by small non-coding RNAs present in bacteria (sRNA) and eukaryotes (microRNA) (Wassarman & Storz 2011).This RNA performs their function by hybridizing to target RNA by the base-pairing interactions. In eukaryotes, microRNAs are short RNAs (19-22bp) that bind 3'UTR region of mRNAs and regulate the transcript stability or translation efficiency (Lim et al., 2007). In prokaryotes, small RNAs (sRNAs) show promising role in the gene regulation for controlling of cellular physiology and growth. Recently, (Rodrigo et al. 2016) have engineered an antisense sRNA that could function in *E. coli* and one template could transcribe two small RNA that could activate two gene independently. Thus, base pairing has proved to be an essential element for gene regulation by sRNAs.

There are number of advantages by using sRNA, the specificity of RNA base-pairing being an important one, such that a single nucleotide mutation in riboregulator can change specificity and function (Davidson and Ellington 2007). The advantages of RNA molecules allow them to adapt to form a regulatory sub-circuit that in turn could be linked together in *cis*, in *trans* and in multiple layers to create different synthetic biological circuits. These properties allow us to engineer RNA based circuits in *E. coli.* Some of these sRNAs use the RNA chaperone Hfq, which facilitates the binding of sRNA to target mRNA at faster rate than the ribosome and regulates the gene function (Sakai et al. 2014).

0.2 Binding functionality

In addition to hybridizing with DNA and RNA in a sequence-specific manner, RNA molecule has high specificity of binding to proteins and ligands. This binding property of RNA can control cellular functions like transcription, processing, and translation. Researchers have designed a library of synthetic RNA aptamers and selected by SELEX (Systematic Evolution of Ligands by Exponential Enrichment). These synthetic aptamers can control the signal processing in cell, protein function, and gene regulation (Shen et al., 2015). These synthetic or natural aptamer can be specifically bound to their target (protein, RNA or ligand) and changed the conformation of RNA to activate or repress the gene expression. This allows us to use aptamers for medical and therapeutics applications. We have engineered a switchable ribozyme that emits an sRNA upon binding of theophylline which is called as regazyme. The emitted sRNA then specifically to the 5' UTR of its target mRNA and changes the conformation of mRNA to open the RBS (Shen, et al. 2015). This exposure of the RBS allows landing of the ribosome and start the translation process.

0.3 Catalytic functionality

RNAs catalytic activity was discovered in the year 1982 that catalyse the biochemical reactions which is known as ribozyme (Kelly et al. 1982). Natural and synthetic ribozymes can both catalysed the biochemical reactions.

Ribozyme catalyses the reaction in two ways the first is self-cleavage and second is trans-cleavage. Ribozymes also catalyse the RNA splicing, where they break a phosphodiester bond and rearrange it by reformation or ligation. Recent studies suggested that RNA splicing also present in prokaryotes. The circular RNA splicing in prokaryotes removes the intron and re-ligates the exons (Jeck et al. 2013) (Rostain et al. in revision) and this mechanism may protect against the RNA degradation (Arraiano et al. 2010). The hammerhead ribozyme is the smallest natural ribozyme regulating rolling circle replication in viroid's by self-cleavage mechanism (Ellington et al. 2006). These regulatory and enzymatic properties of sRNA attract the biologist to design new riboregulator, RNA switch, riboswitch and new ribozyme by computational and rational design with predictive behaviours.

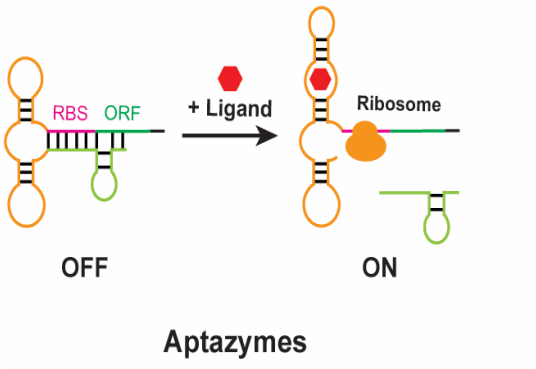


Figure 0.3 explains mechanism of aptazyme (adapted Kushwaha et.al., ACS synthetic biology 2016)

0.4 Riboregulators (trans-activating riboswitches)

In1986 Kozak discovered first riboregulator and first synthetic riboregulator has been built by Isaacs et al (2004) that allows us to design a novel sRNA-based circuits with predictive functions. Riboregulator is a small regulatory RNA that responds to a signal nucleic molecule by Watson and Crick base pairing. The base pairing of riboregulator with RNA molecule changes the conformation and structure of target RNA and activate or repress the cellular reaction such as replication, transcription and translation. The sRNA base pairing with cis-repress mRNA (5'UTR) changes the conformation and opens the RBS that binds ribosome to translate protein (Isaacs et al. 2004; Rodrigo et al. 2012) or repression, and also DNA replication (origin of replication

repression), base pairing with complementary or antisense RNA repress the translation(Rodrigo et al. 2016.)

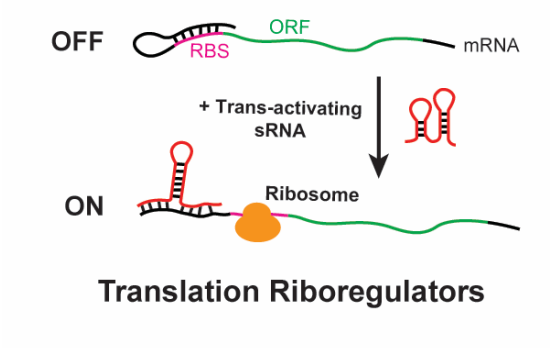


Figure o.4 Explains the Mechanism of *trans*-activating riboswitch(riboregulator) adapted Kushwaha et.al., ACS synthetic biology 2016

The translational riboregulator regulates translation by changing the conformation and make RBS accessible to ribosome to start the translation. Ribozyme based riboregulators regulate the ability of a catalytic RNA molecule to cleave a target nucleic acid sequence. A hammerhead ribozyme (Wieland et.al., 2008) RNA molecule is activated or inactivated depending on the change of the secondary structure that is induced by hybridizing a signal molecule such as a cognate DNA or RNA sequence.

0.5 Riboswitches

Riboswitches are the versatile RNA molecule typically found in the 5' UTR regions of mRNA, where they control the gene expression by binding of ligand (Tucker & Breaker 2005). Riboswitch is also known as extracellular sensor of vitamins and derivatives (Sedlyarova et al. 2017). The riboswitches consist of two domains, an aptamer domain and an expression platform. The aptamer domain is a highly specific structure that selectively binds to the target ligand or metabolite and change the conformation.

The expression platform change conformation after binding metabolite activated or repressed gene expression. Riboswitch have a wide a range of small metabolite, ligand and they regulate transcription, translation, splicing and RNA stability. Currently, the riboswitch is used for developing new antibiotics, designing new molecular sensors and engineering synthetic circuits.

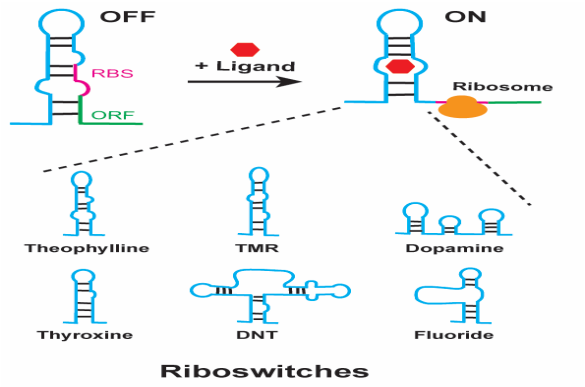


Figure 0.4 Explains the Mechanism of ligand activating riboswitch adapted Kushwaha et.al., ACS synthetic biology 2016

My overall goals of PhD study to use computational and rational design to engineer *novel trans*-activating riboswitches (RNA switches**)** in *E. coli.* The ccomputational methodologies principally rely on the Watson-Crick interactions, whereas this is not adapted to *in vivo* operations where RNAs are unstable. Recently, Jaramillo's Lab has reported a fully automated design methodology and experimentally validated synthetic RNA interaction circuits working in *E. coli*. Starting with well-defined structures of all single species of RNA, our aim to minimize the free energy and the activation energy of complex formations by proposing an objective function, using a Monte Carlo simulated annealing algorithm has been used. It has been validated by engineering a synthetic riboregulator which is able to *trans*-activate the translation of a *cis*-repressed gene. To this end, we have successfully engineered *novel trans-activating* riboswitches in *E. coli* at multiple layers such as single component riboswitches and multi-components riboswitches that function independently and also in combination with transcription regulation to create a complex logic gate circuit.

To This end for thesis 1st chapter, we have been engineered RNA based a tuneable antiterminator system. The sRNA responds to a signal directly regulates the control of structure and conformation of cis-repress mRNA and RBS. The RBS regulates the translational control of transcription and termination.

In the first chapter of thesis, we have engineered sRNAs (act as a signal) that behave as a translation-transcription coupling adapter (TTCA element). Here, the synthetic TNA derived adaptor acts as a signal converter in a

genetic circuit, converting a translation signal to a transcription signal by closely interfacing the ribosome and the RNA polymerase(Liu et al. 2012)

Transcriptional terminators in the prokaryotes are of two types, Rho dependent and rho independent. Rho independent termination is responsible for 80% termination events in bacteria. A terminator is AT rich DNA sequence in bacteria. The RNA Polymerase (RNAP) has two regions to pause at terminator region. First mRNA transcript of stem loop region of terminator makes hairpin loop in bacteria and the RNAP unable to scan hairpin and slow down as well as ultimately pause. The second terminator region has a poly(U) repeat sequence, at which the RNAP back tracks. This hairpin structure and poly(U) repeat sequence destabilizes the transcription elongation complex and RNAP drops off from the DNA sequence to finally terminate the transcription.

In electrical engineering, digital circuits are organized into Boolean gates that establish the basic logical operations. In the second parts of thesis we have built single component a *trans*-activating riboswitch in *E. coli.* For reprogramming cell, we need more complex and robust system. However, in previous implementations of cellular logic, complex gates such as XOR gate (ambisense riboregulator) have required the layering of multiple genetic circuits, thus necessitating substantial efforts in circuit construction and tuning. We have taken advantage of our computational *de novo* design of the sRNA based riboregulators to design ambisense riboregulator with two sRNA signals. This was attempted through the base pairing between two complementary riboregulators, leading to an output of 0 when all inputs are either 1 or 0 (Rodrigo et al. 2016). This could be used for the conditional activation or repression of targeted gene. In this part we are able to transcribe two RNA signal (two riboregulator) depends on polarity of DNA and promoter direction. These two small RNA signals able to activate two different gene independently. Aim of to engineer this type of system is to reduce metabolic load on the cell. This end we are also able to engineer trans-activator riboswitch that having three nucleotides only.

The above properties allow us to engineer genetic circuits that are useful in synthetic biology. In third part of thesis, we have engineered a trans-acting

multicomponent riboswitches (cooperative) that used two sRNAs signal. The sRNA based cooperative riboregulator that performs an AND gate operation on the input signals: that returns a 1 only when all inputs are 1. A pLtetO1 promoter that controlled riboregulator which can be exposed the 5'-UTR *cis*-repressed RBS of the mRNA to allow the translation of the reporter protein GFP. These switches regulate the mRNA translation by controlling access of ribosomal machinery to the mRNA. The most common point of control is translation initiation, when the 16S rRNA anti-SD interacts with the RBS to form the translation initiation complex. Cooperative sRNA-mediated activation is also possible in mRNAs where a structured 5′UTR occludes the RBS in the OFF state, and only allows RBS access upon the conformational change which is induced by the two sRNAs signal binding cooperatively (Rodrigo et al. 2017). The cooperative regulation is common in proteins to control many biochemical reactions. We have designed cooperative regulation in bacteria using synthetic sRNA signal, which to our knowledge has not been previously designed and tested in *E. coli*. However, in eukaryotes cooperative microRNA based regulation has been previously reported (Schmitz et al. 2014).

In this section additionally, we have explained the orthogonality. The orthogonality results explain robust system and does not have cross talk with other RNA and host cell. To this end, we have studied the orthogonality of our RNA triggered riboregulators and riboregulators engineered in host lab recently. (Rodrigo et al. 2017., Rodrigo et al.2012). Our data explain that our system is robust and no crosstalk with other RNA and host cell. Previously, we have engineered and characterized a new RNA molecule which is regazyme (Shen et al., 2015). We have fused riboregulator RAJ11 with theophylline aptamer, this riboregulator is active in presence of theophylline. In the presence of theophylline, it can change the conformation and get cleave and release riboregulator. This riboregulator binds to cis-repress mRNA change the conformation that allows RBS to land ribosome and obtained expression of GFP.

In order to have consistency results and less metabolic burden on host cell, we have integrated riboregulator (RAJ11) into genome of *E. coli* by the site-

specific recombination (Kuhlman & Cox 2010) and studied at single copy(Lee et al. 2016).

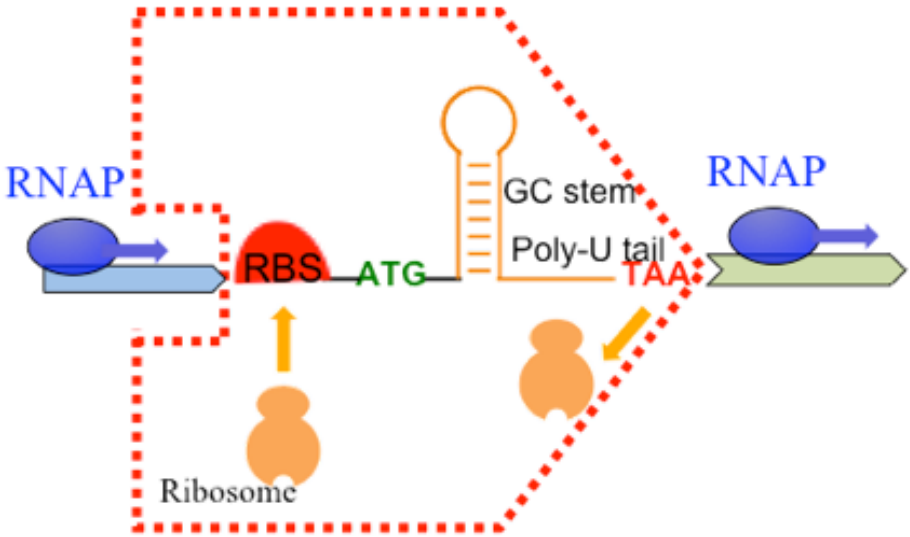
Currently, directed evolution is a fast and robust approach for evolving biomolecules in changing environmental conditions and allow us to fit in that conditions(Esvelt et al. 2011). We have used interactive cycles that we could find best fitted molecules with higher stability. We have used T7 bacteriophage as a tool for directed evolution of RNA molecules, RNA circuits, riboswitch and protein molecules. We have designed and characterized three recombinant T7 bacteriophages including (T7riboswitch, T7 regazyme and T7 sRNARAJ11 bacterteriophage). This has a number of future applications for controlling of multidrug resistant bacteria, gene therapy and diagnostic.

AIM of work

- **Engineering of a tunelable antiterminator riboswitches**
- **Engineering single component advanced riboswitches**
- **Engineering multi-component advanced riboswitches**

Chapter 1

Engineering of tuneable antiterminator riboswitches



Schematic of tuneable antiterminator system

1.1 Abstract. In prokaryotes, transcription and translation both reactions proceed simultaneously. We exploit this property of *E. coli* to engineer a system that converts translational signal into transcriptional signal. Here, we report a trans-activating small RNA (sRNA) that binds to cis-repress mRNA and opens the RBS that allows access of ribosome. This landing of the ribosome at the RBS initiates the translation and initiation of translation prevents the hairpin formation of newly transcript mRNA at termination region and also prevents back tracking of RNA polymerase. This allows transcription is continuing on terminator region and reporter protein GFP is expressed. The efficiency of converting translational signal to transcription is tightly regulated by strength of RBS. This type regulation will open new way of gene regulation and could be important in synthetic biology.

1.2 Introduction

The central dogma is one of the key components of cellular systems. Gene regulation at the transcriptional and posttranscriptional level play a vital role in synthetic biology. Recently, the synthetic biologists are more interested in riboswitches and small non-coding RNA due to easy to design, use and characterize in living cells. The transcription process is the key regulatory step in gene expression where RNA Polymerase binds to a promoter sequence and start the transcription at the transcription start site (TSS). In the *E. coli* transcription and translation processes are simultaneously started. Once the ribosome binds on the ribosome binding sites and it starts the protein synthesis. Transcription termination has important role in the cellular mechanism as follows-

It prevents un-useful transcription of downstream gene and interference of antisense RNA (Ray-Soni et al. 2016).

1. The recycling of RNAP is free for efficient gene expression.
2. The termination is also prevents collisions with replication machinery. This collision can cause double strand break in the DNA (Ray-Soni et al. 2016).
3. Transcription termination also specify structure and polarity of 3' of RNA.

Bacteria have three type mechanisms of transcription termination

1. Intrinsic termination is regulated by hairpin structure and RNAP back tracking.
2. Rho dependent (homohexameric protein) protein binds to transcribe RNA and move along the RNA and hydrolyses ATP to drive RNA translocation and RNAP-RNA-DNA complex dissociate finally release RNA (Ray-Soni et al. 2016)
3. Mfd protein an ATP dependent DNA translocase that binds to double strand DNA (rewinded DNA after transcription) just behind transcription bubble. This Mfd inhibits NTP polymerisation and nascent RNA chain dissociate (Park et.al., PNAS 2006).

A termination signal induces a rapid and irreversible dissociation of the nascent RNA from RNAP and DNA. In *E. coli* rho independent terminator structure composed of GC rich (5-9) nucleotide and 3-5 nucleotide stem loop followed by 7-9 poly(U) tail. The Rho independent transcription termination involves the formation of an RNA hairpin in the nascent RNA (Park et.al., 2006**).** They reported that palindromic sequence should be sufficiently long to form an RNA hairpin to destabilise the RNA-DNA hybridisation. If the palindromic sequence is not sufficiently long then a hairpin would not form and transcription will go on continuously on termination (Li et.al., 2015). The hairpin formation of mRNA transcript may disrupt the RNA-DNA hybrid.

There are two key factors responsible for transcription termination at nascent mRNA in *E. coli*. The first key factor is terminator sequence forms a stem loop sequence and newly transcribed mRNA that make hairpin loop and regulate the RNAP. The second factor is terminator region that contains a poly(U) sequence, and RNAP read this sequence as an error that allows start back tracking as well as transcriptional elongation complex dissociate; resulting in the eventual dislocation of the RNAP from DNA.

In our *de-novo* design of a trans-activating riboswitch, small RNA acts an external signal regulated by PLlcacO1 promoter and the 2nd Part is the cis-repress mRNA controlled by constitutive promoter BBa_J23119. The

external signal sRNA bind to expression region of the part of cis-repressed mRNA of riboswitch which opens the conformation and activates it.

In our newly created *de-novo* tuneable antiterminator system, we have designed a 5'UTR that contains a *cis*-repress RBS and the start codon ATG just before terminator sequence that is regulated by constitutive promoter BBa_J23119. This *cis*-repress RBS is tuned by small RNA signal (Rodirgo et. al., 2012) regulated by PLlacO1. Normally, the constitutive promoter (BBa_BBa_J23119) always expresses 5'UTR but it forms secondary structure and sequester the RBS that will not allow for translation. However, in the presence of inducer (IPTG) the PLlacO1 promoter expresses the sRNA signal that hybridizes with cis-repress 5'UTR and changes conformation and opens the RBS to bind ribosome. In bacterial systems, the transcription and translation are proceed simultaneously. The landing of ribosome at the RBS and initiation of translation prevents hairpin formation at the stem loop sequence. This prevents hairpin formation and if RNA polymerase has ribosome and translation just behind poly(U) sequence then RNAP does not read as an error. It prevents backtracking and transcription is continued on terminator sequence and GFP of ORF2 is expressed.

We have engineered sRNA signal system that works as a translation-transcription coupling adapter (Liu et al. 2012). In our system, sRNA binds and *cis*-repressed 5'UTR to open the RBS for ribosome landing. The landing of ribosome on RBS initiates translation. This initiation of translation, continues transcription elongation at the terminator region by preventing backtracking of RNA polymerase. This preventing of backtracking by RNAP prevents hairpin formation of transcript at terminator region (Cambray et al. 2013; Liu et al. 2012). This continued transcription of the terminator region allows transcription of ORF2 and the subsequent translation of reporter gene (super folder green fluorescent protein with degradation tag sfGFP-LAA). The transcription elongation rate is tightly regulated (Proskin *et al.*, 2010) by the rate of translation initiation. Increasing and decreasing the rate of translation initiation (ribosome binding on RBS or RBS strength) directly

affects elongation of transcription (RNAP speed).

We have also engineered a library of this novel RNA signal-based transcriptional attenuation regulator based on the rho-independent terminators in bacteria. The TTCA constructions contain two separate parts that is expressed from a single plasmid. Firstly, an sRNA signal (RAJ11) is placed under the control of a PLlacO-1 promoter. Secondly, a cisRAJ11-Terminator-RBS-sfGFPLAA mRNA is transcribed under the control of a constitutive promoter (BBa__J23119). The inducible sRNA RAJ11 activates the expression of GFP from the mRNA by binding to its cisRAJ11 region. The terminator region in mRNA must be flanked by 5' ATG codon and a 3' TAA codon, however the structural region of the terminator is variable (i.e. we have used any of the terminators B1002, tonB, rrnB, T21, Tfur, rev-Tfur, T500 and synthetic terminator). We designed the library of different terminators and characterized in different riboregulators 12 (Isaac et al., 2007), Collins toehold switch (Green et al., 2014) and Gallivan riboswitch (Lynch et.al., 2007). Downstream of the terminator region is another RBS, a start codon and the reporter gene (sfGFP with the LAA degradation tag showing in Figure 1.1). When translation is activated by the sRNA, transcriptional termination at the terminator poly(U) is prevented by the ribosomes that block the backtracking of the RNA polymerase.

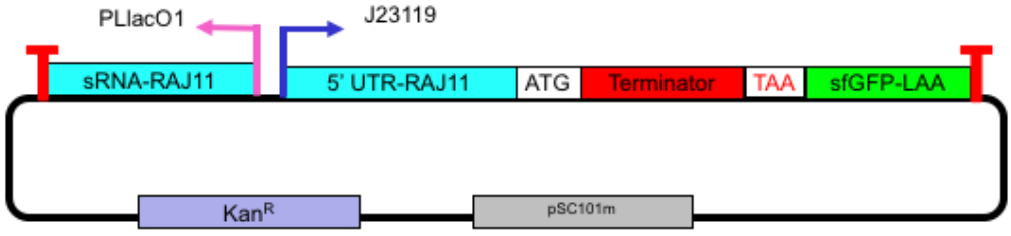


Figure1.1 Design of antiterminator RNA switch Plasmid

Figure 1.1. Showing the design of a tuneable antiterminator plasmid. The promoter PLlacO1 (IPTG inducible) controlling sRNARAJ11 and withT500 the terminator. The constitutive promoter BBa_J23119 controls cis-repress 5' UTR-RBS and ATG. I fused terminator with stop codon and then RBS2 and reporter protein sfGFP-LAA.

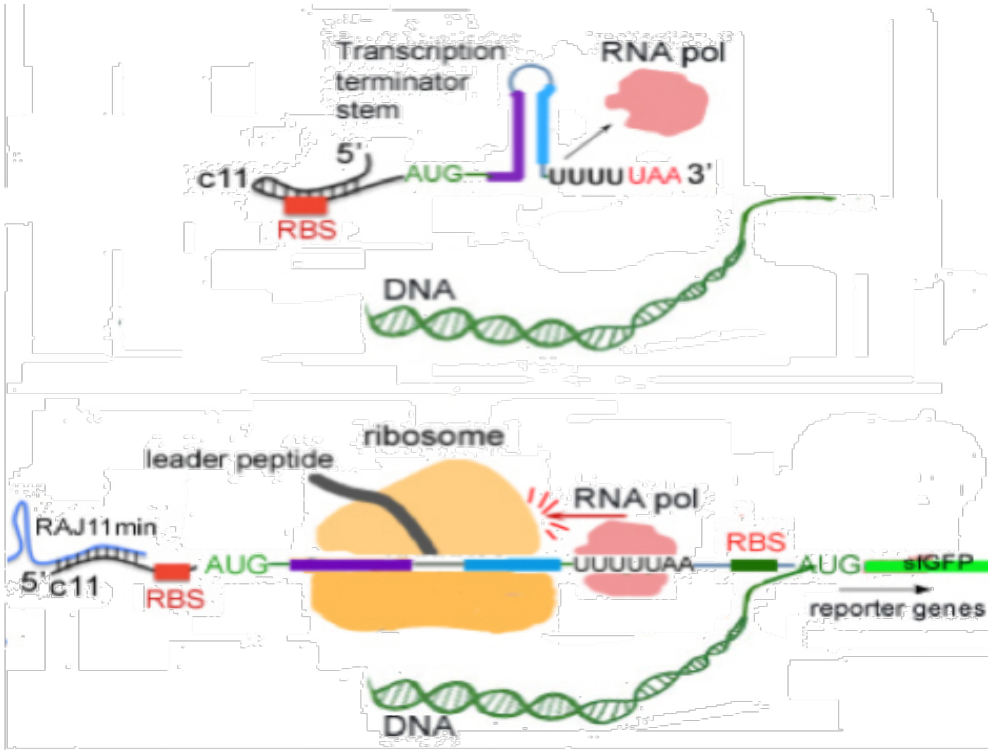


Figure1.2 Schamatic TNA system

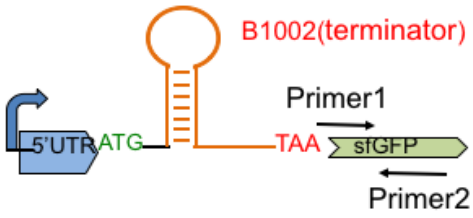
Figure1.2**.** The mechanism shown in figure, In absence of IPTG, mRNA is transcribe but the RBS is not exposed and the mRNA makes a hairpin loop at the terminator region. The RNA polymerase unable to read and pause. In the presence of IPTG, PLlacO1 promoter gets activated and sRNA is transcribed. The sRNA binds to cis-repress mRNA and change the conformation to expose RBS. The Ribosome land on RBS and prevent backtracking of RNA polymerase and transcription continue at terminator sequence resulting reporter protein is expressed.

1.3 Results and discussion

We have characterized our anti-terminator by population level by Fluorescence plate reader, RT-PCR, Flow-cytometry and microfluidics analysis to study single cell dynamics. These results are showing in the below.

1.3.1 Gel assay.

Electrophoretic analysis of RT-PCR showing GFP expression with induction of IPTG and without induction of IPTG. As showing in figure1.3A primer of GFP region and figure 1.3B Showing, no GFP expression without induction.



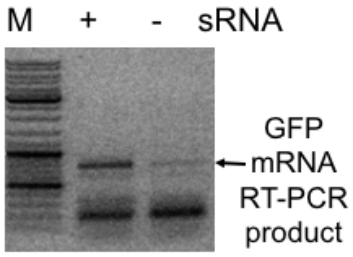


Figure 1.3A design of plasmid **Figure 1.3 B results of RT-PCR**

Figure1.3 Design and RT-PCR gel results of antiterminator RNA switch

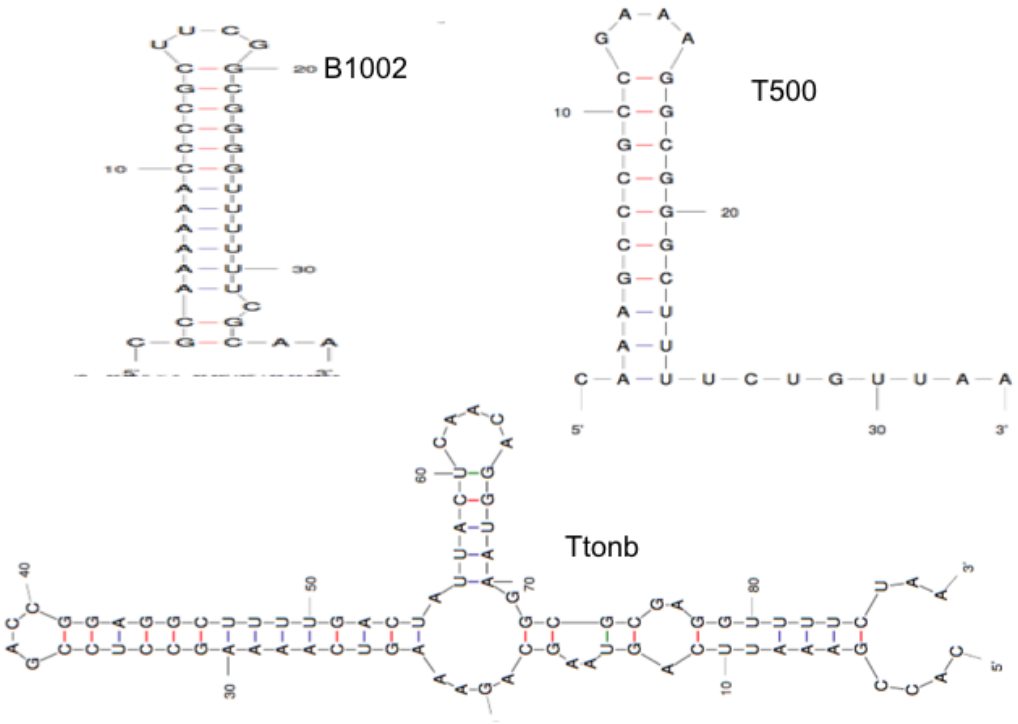
Figure1.3A Schematic of B1002 terminator and sfGFP and primers1 and primer2. Figure3.3B showing RT PCR results in gel. The Gel band showing in presence of sRNA signal gel band is sharp. This shows in presence IPTG promoter PLlacO1 is activated and sRNA transcribed. This sRNA activates cis-repress mRNA and and transcription continue on terminator reason and reporter protein is expressed. We isolate total RNA in two conditions (none and IPTG).

We made cDNA and amplified with sfGFP primer1 and primer2. The PCR product was run on a 1% agarose gel with 100bp Plus ladder (Thermo-fisher). We have sharp band in presence of sRNA (IPTG) and light band in absence of IPTG. Ideally in the absence of IPTG there should not be an expressed band, but the system is leaky as such.

In the down table1 showing the list of terminators with their sequence and free energy of activation. We also explains the RNA secondary structure of terminators used in our research. The mRNA fold online free software used to predict secondary structure. We used library of terminators, the figures showing structure and sequence

Terminator name	Sequence	=ΔG
B1002	CGCAAAAAACCCCGCTTCGGCGGGGTTTTTTCGCAA	-25.30 kcal/mol
Ttonb	CACCGAAATTCAGTAAGCAGAAAGTCAAAAGCCTCCGACCGGAG GCTTTTGACTATTACTCAACAGGTAAGGCGCGAGGTTTTCTAA	= -30.70 kcal/mol
TT21	TTGAGCAAGTGGCAACACTATTCGCATAAGCTGCCGTTAGTGACTC TTAAGTTGCAACGGTGGCTTTTTTTATTTGGGTCAGTCGTATAAAG GTCATTACGG	-28.20 kcal/mol,
rev-Tfur	TGCATAAAAAAGCCAACCCGCAGGTTGGCTTTTCTCGTTTAA	-15.30 kcal/mol,
Tfur	AACGAGAAAAGCCAACCTGCGGGTTGGCTTTTTTATGCATAA	-17.90 kcal/mol
T500	CAAAGCCCGCCGAAAGGCGGGCTTTTCTGTTAA	-22.50

Table1: Sequence and ΔG of different terminator



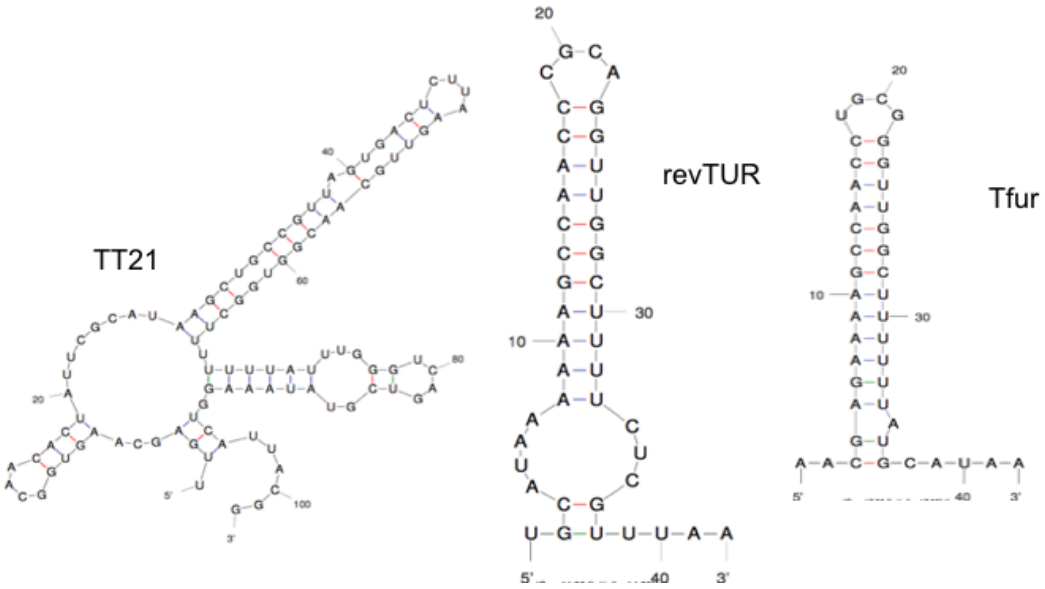
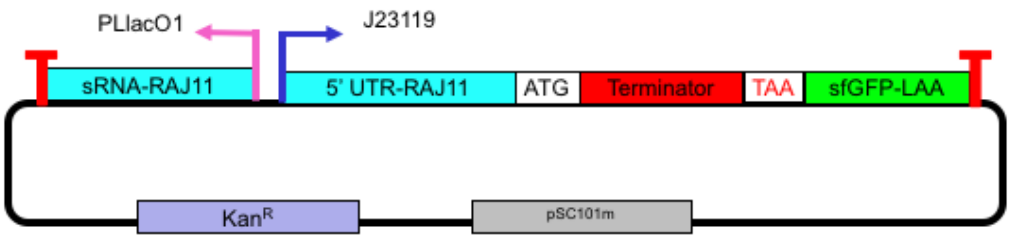


Figure 1.4A showing structure of hairpin and stem loop.

1.3.2 Fluorimeter characterization results

We have characterized the antiterminator system MG16555Z1 and also cross-checked results in Dh5alphaproZ1 *E. coli* strains. We have used a library of different terminators (B1002, Ttonb, TT21, T500, Rev-TFUR and Tfur. We have also engineered a library of same terminators based on template of previously published (Isaacs et al., 2007) and toehold Collins Toehold RNA switch. Additionally, we have engineered TNA circuits TNA31 (a trans-activating riboswitch-based cascade), additionally also tested our system in riboswitch (Arkin TNA system). Double riboswitches (theophylline and adenine) based feed forward (cascade) also designed and constructed and characterized in *E. coli*., to make TNA based cascade system.



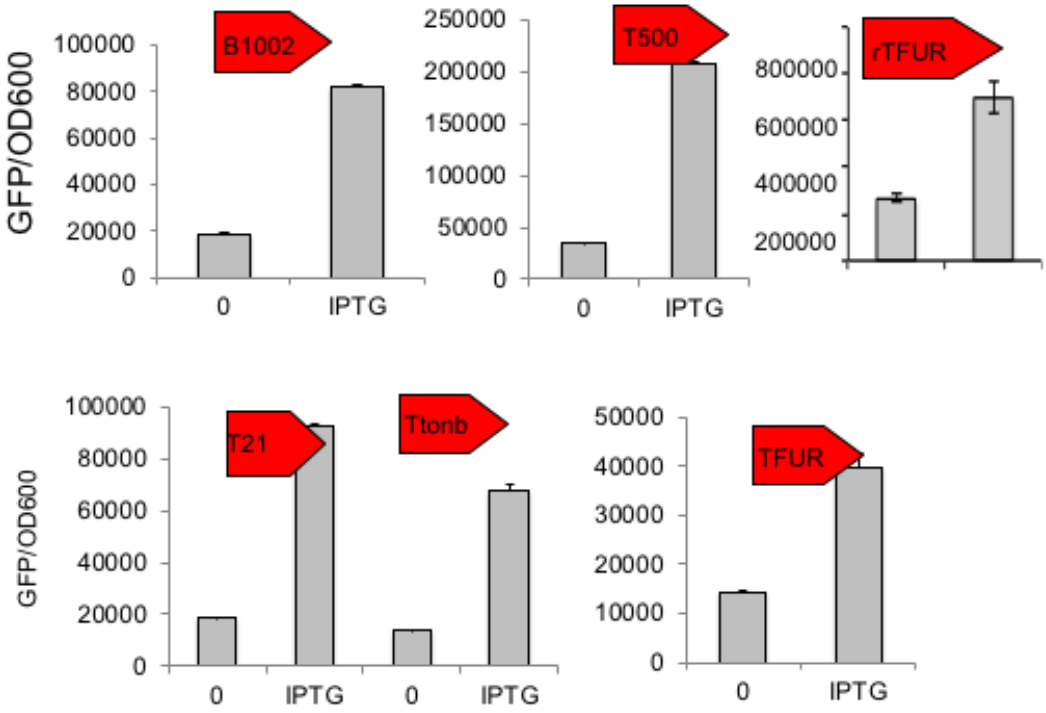


Figure1.4 B Design and Results of different terminator

Figure 1.5 Above figure showing results of antiterminator. The results are in two conditions (0 and IPTG.) In the absence of IPTG PLlacO1 promoter is not active and no small RNA transcribed finally no GFP expressed. I have characterized library of terminator.

Results of RAJ11 based TNA system. As shown in the figure 1.4B we have characterized different terminator (B1002, T500, rev-Tfur, T21, Ttonb, and Tfur. We have characterized in two conditions (0 and IPTG). As shown in the figure we get the expression in presence of IPTG. In the Presence of IPTG PLlacO1 gets activates and sRNA is expressed. This sRNA binds cis-repressed mRNA and change conformation and makes activates. We have characterized library terminators all have different activation fold because all having different stem loop and hairpin structure.

1.3.3 Single cell dynamics of TNA B1002 terminator

To further explore a tunable anti-terminator behavior of the system at the single-cell level, we have performed a time-dependent characterization of system TNA B1002 and TNA B1002 (Theophylline riboswitch) using the microfluidic lab-on- chip devices (Figure 1.5). This allowed us to monitor GFP expression in individual cells under a varying concentration of inducers IPTG.

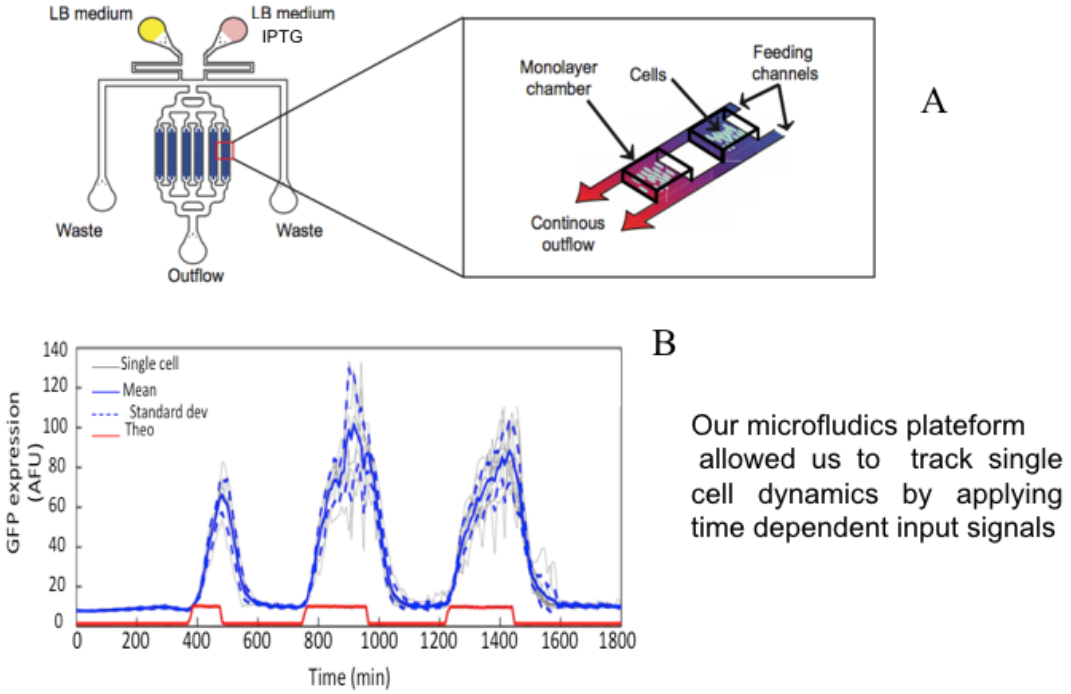


Figure1.5 Sigle cell dynamics of B1002terminator

Figure1.5Figure showing single cell dynamics of B1002 terminator in presence of inducer IPTG and absence of IPTG. Single cell tracking of fluorescence GFP expression in arbitrary fluorescence units (AFU)] in one micro chamber of the microfluidics device under time-dependent induction with IPTG (1mM) for system B1002 in Dh5alphaproZ1. A square wave of IPTG inducers with period 8 h (i.e., 4 h induction and 4 h relaxation) was applied. The solid and dashed lines (in blue) correspond to the mean and plus/minus standard deviation over the cell population. Sulforhodamine B was used to monitor the inducer time-dependent profile (in red)

1.3.4 FACS data for TNA system

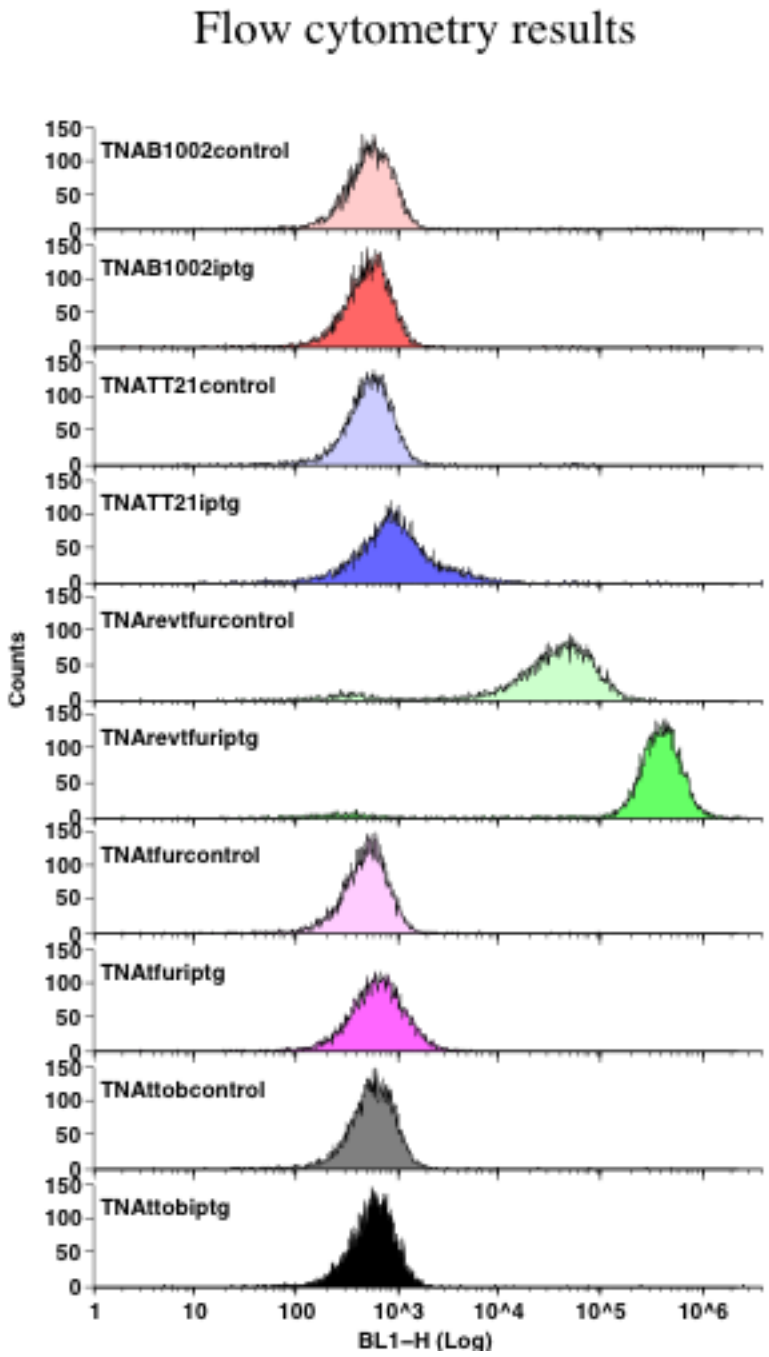


Figure1.6 FACS results of TNA system

Figure1.6. The Flow cytometry characterization results of different terminator. The left side showing scatter plot and right-side value showing value.

1.3.5 Tunability with RBS

The transcriptional control by transcription tune with RBS. We have generated an RBS knock out. We made construct with weak RBS deleted RBS. These controls were characterized in MG1655Z1 cells in two conditions (0 and IPTG). The results show that weak RBS gave a less GFP expression. The construct deleted RBS gave, no expression in both conditions (0 and IPTG). These results suggest that landing of ribosome prevents the hairpin formation and backtracking of RNAP. The weak RBS, ribosome unable to land on all transcripts and less transcriptional signal converted to translation. In the deleted RBS, there are no ribosome landing and no expression.

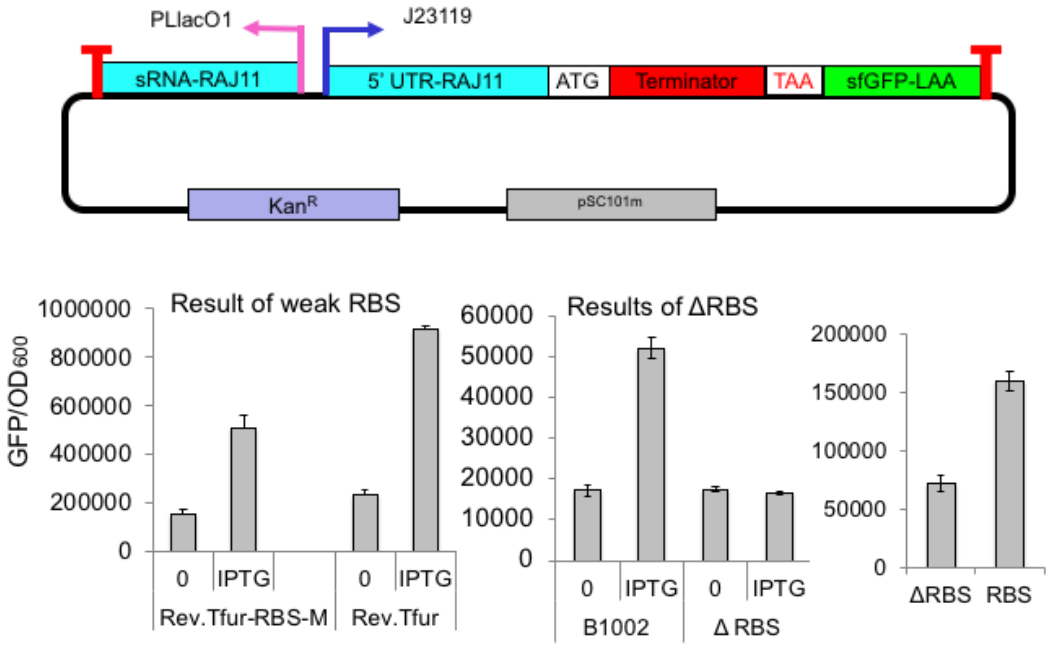


Figure 1.7 TNA results tuning with RBS strength

Figure 1.7 The above figure showing antiterminator results of RBS tuning. The weak RBS work as a weak antiterminator. The RBS deleted system having no expression in presence of RBS and absence of RBS. These results explaing that initiation of translation prevents hairpin fromation and backtracking of RNAP.

1.3.6 Results of weak terminator (spacer)

As shown in figure 1.8, we have mutated the terminator and kept the same amino acids. The ideally, we want test weak poly(U) (as reported in literature mutation in poly(U) decrease terminator efficiency (Sedlyarova & Nudler et.

al., 2016) but in our case same amino acids also synthesized. Our 2nd control, we have completely disrupted the hairpin and poly(U) that synthesized different protein as well. We have characterized both control with ΔT(terminator deleted) *E. coli* MG1655Z1 cells in both possible condition (0 and IPTG). As the results shown in both condition system gave the expression but the system poly(U) and hairpin and ΔT was showed very high expression, even in absence of IPTG.

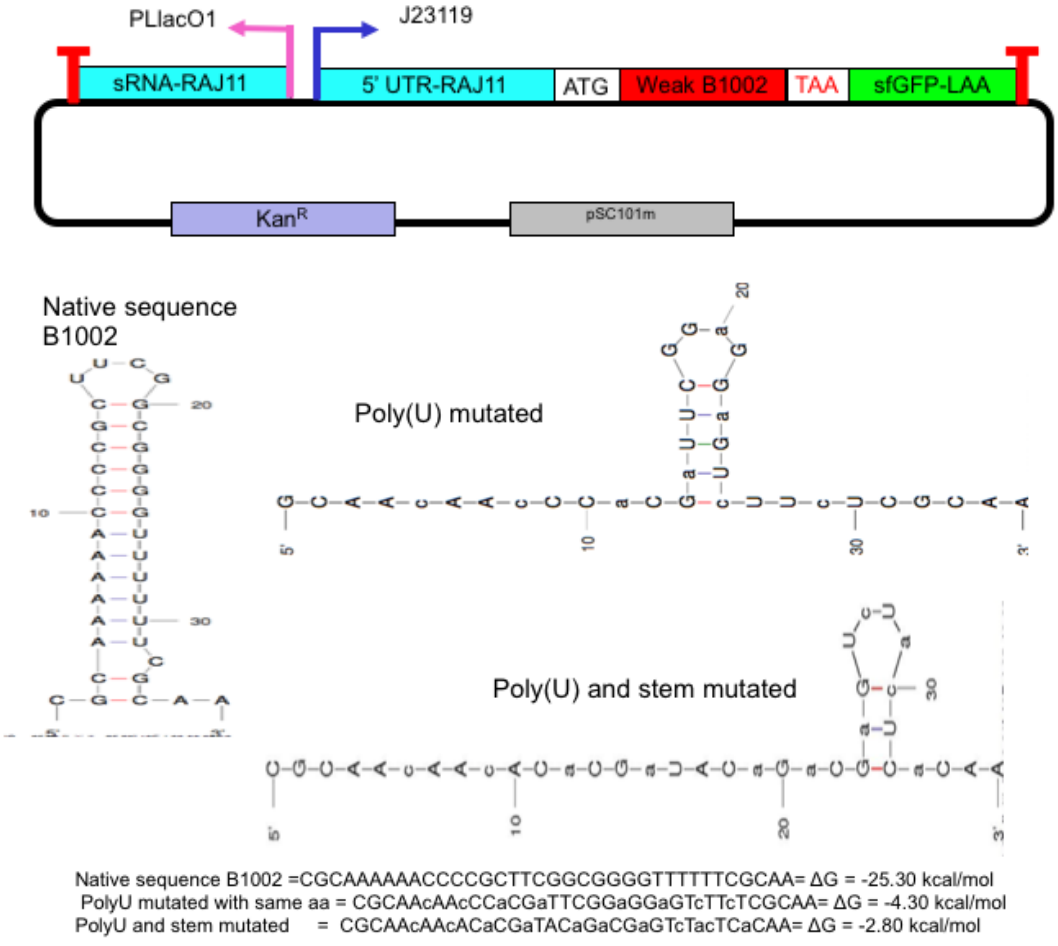


Figure1.8 Sequence and structure of Poly(U) mutate terminator

figure 1.8 (A**)** A poly(U) mutated but stem loop is same, but gene expressing same protein. The figure 1.B poly(U) and stem loop both are mutated. Terminator also encoding different protein. The total GFP expression is increase, because terminator not inhibiting. The 3rd control terminator is removed this is also giving total more GFP.

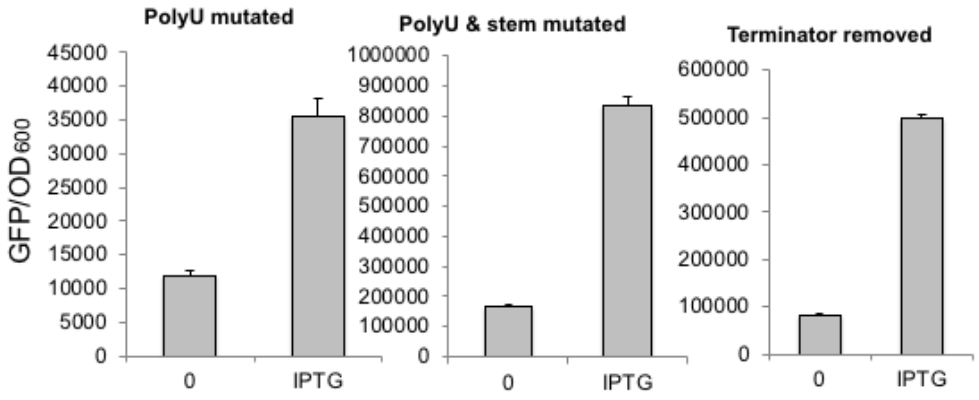


Figure1.8B Results of B1002 terminator mutation

1.3.7 ATG and stop codon knock out

We have made knock out of ATG and stop codon. We have inserted the AAG in place of ATG and stop codon (TAA) in place of ATG as shown in figure1.9. These knock out construct were characterized in *E. coli* MG1655Z1 in both condition (0 and IPTG). The results in the figure shown that total expression is increased in ATG Knockout but observed the similar activation fold. The argument is ATG and terminator is very closed. Once ribosome is land on RBS and translational initiation complex cover the region of terminator and it prevents hairpin formation back tracking of RNAP and transcription continue on terminator sequence. The 2nd argument is that newly transcribed mRNA is too small in size and it is unable to form hairpin formation. We need a minimum length of nascent mRNA to form proper hairpin and termination of transcription.

We have constructed more control in order to support this argument. We have inserted some spacer (30 and 45 base pair) between (ATG and terminator) in both condition ATG and ΔATG construct as shown in the figure 1.9. We transformed these constructs into *E. coli* MG1655Z1 strain and characterized in both possible condition (0 and IPTG). The results shown in figure 1.9, the ATG knock out have no expression when we inserted the spacer. But wild type (without knockout) express in presence of IPTG.

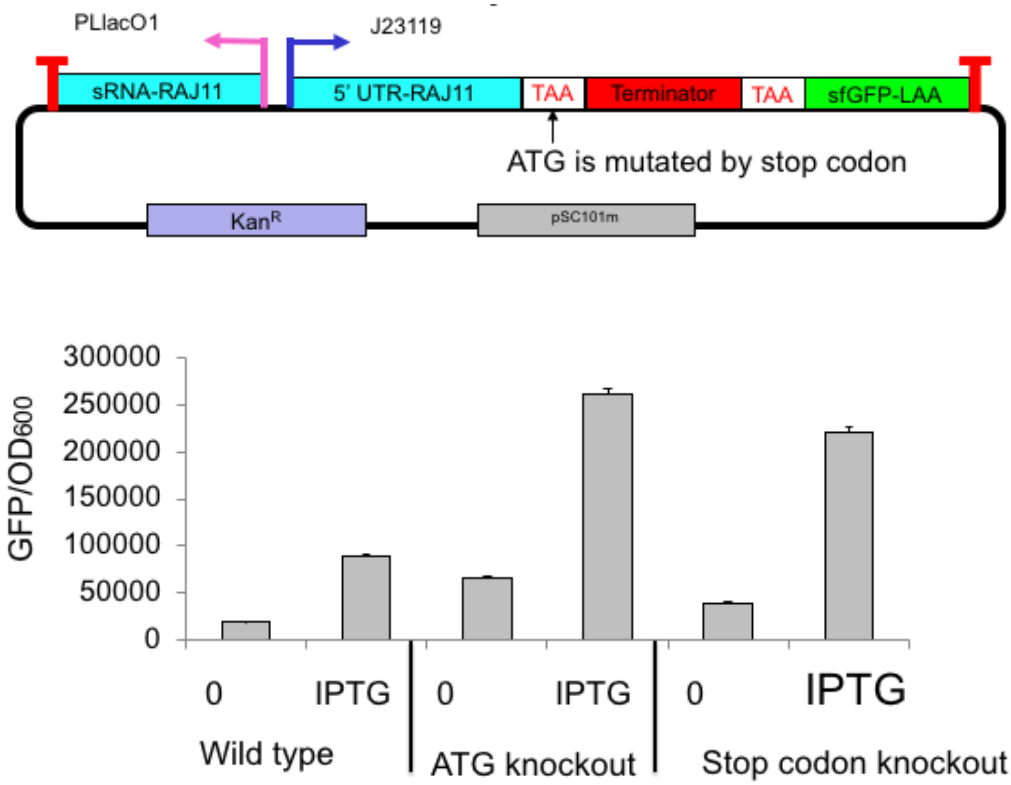
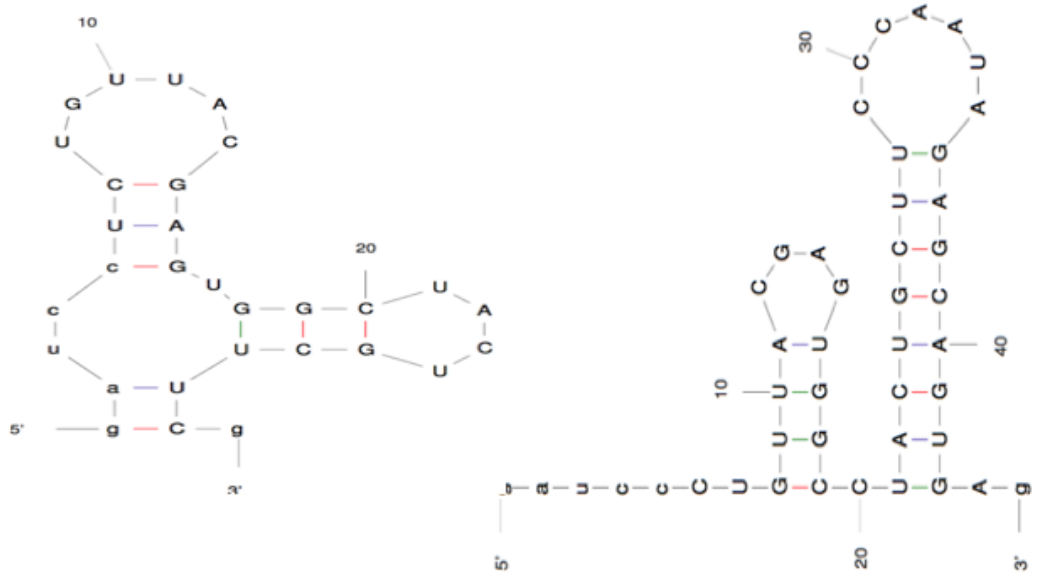


Figure1.9 Results of ATG and ATG/stop codon knock out

Figure.1.9 Above results showed ATG knockout and stop codon knock out, not change the results. The terminator and ATG is to close and Translation initiation complex prevent hairpin formation and backtracking and transcription continue on terminator region.



Spacer 30 = gatccTCTGTTACGAGTGGCTACTGCTTCg= ΔG = -2.40 kcal/mo
Spacer 45 =gatccCTGTTACGAGTGGCCTACTGCTTCCCAATAGAGCAGTGAg= ΔG = -9.50 kcal/mo

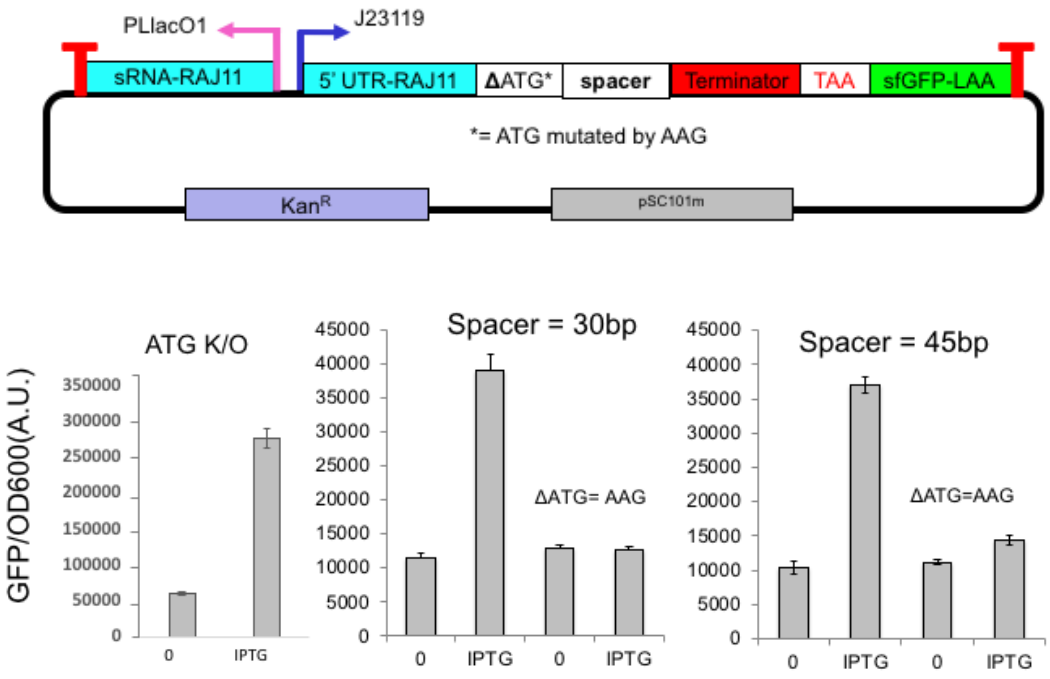


Figure1.10 Results of ΔATG/spacer

Figure 1.10 The resuts showed that after insertion of 30 bp and 45 bp spacer, no GFP expression in presence and absence of IPTG. These results explained that after ATG knockout (ATG mutated by AAG) not giving any expression.

1.3.8 Synthetic translational to transcription control by Isaac riboregulator

In order to have more consistency in results, we additionally design a tuneable antiterminator system based on Isaac riboregulator (Isaacs and Collins 2006). This design is the similar as our RAJ11 based design. In this design small RNA (RR12) controlled by PLlacO1 promoter. The Promoter BBa_J32119 control 5'UTR -RR12 that is cis-repressed. We fused ATG and Terminator just after 5'UTR and then

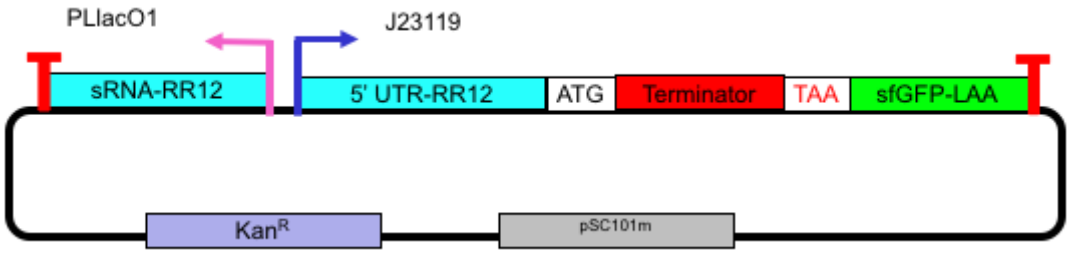


Figure 1.11 showing design of antitermination system based on Isaac riboregulator. We constructed this system in B1002, Ttonb, rev-Tfur and Tfur.

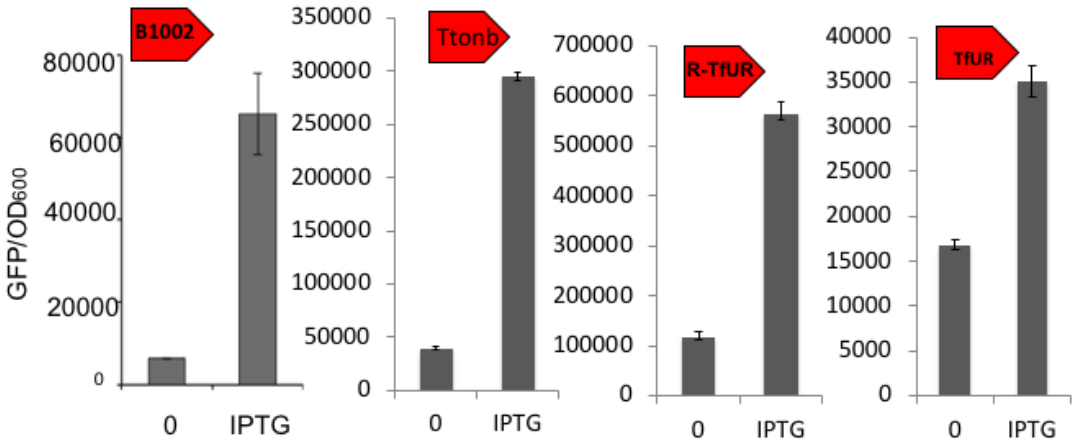


Figure1.12 results of Isaacs riboregulator and riboswitch

Figure1.12 Reporter protein sfGFP-LAA. We characterized these systems in MG1655Z1 in two conditions (none and IPTG). Our explain that in presence of IPTG small RNA bind to cis-repressed 5'UTR change conformation and open RBS and start expression of GFP.

The above results explaining that our a tuneable antiterminator system also working Isaac's riboregulator system. My lab colleague Dr. Michel also tested this system in toehold switch developed by (Green and Collins 2014).

1.3.9 Synthetic translational to transcription control by RNA cascade

Our goal to engineer more robust and complex system that we engineered a cascade where one RNA signal regulates multiple RNA. We engineered a more complex system, where small RNA RAJ11 activates small RNA RAJ31. This small RNA 31 activates cis-repressed mRNA and expressed GFP. The design is shown in the figure. The small RNA-RAJ11 is regulated by PLtetO1 promoter. This promoter is activated by inducer aTc (100ng/ml). After induction of aTc Promoter gets activated and sRNARAJ11 synthesized. This small RNA-RAJ11 binds to cis-repressed RAJ11 and, regulated by constitutive promoter BBa_J23119. This cis-sRNA31 get activated after binding sRNARAJ11. This activated sRANA-RAJ31 and it bind cis-repressed mRNA31 and make it activate to express GFP reporter protein.

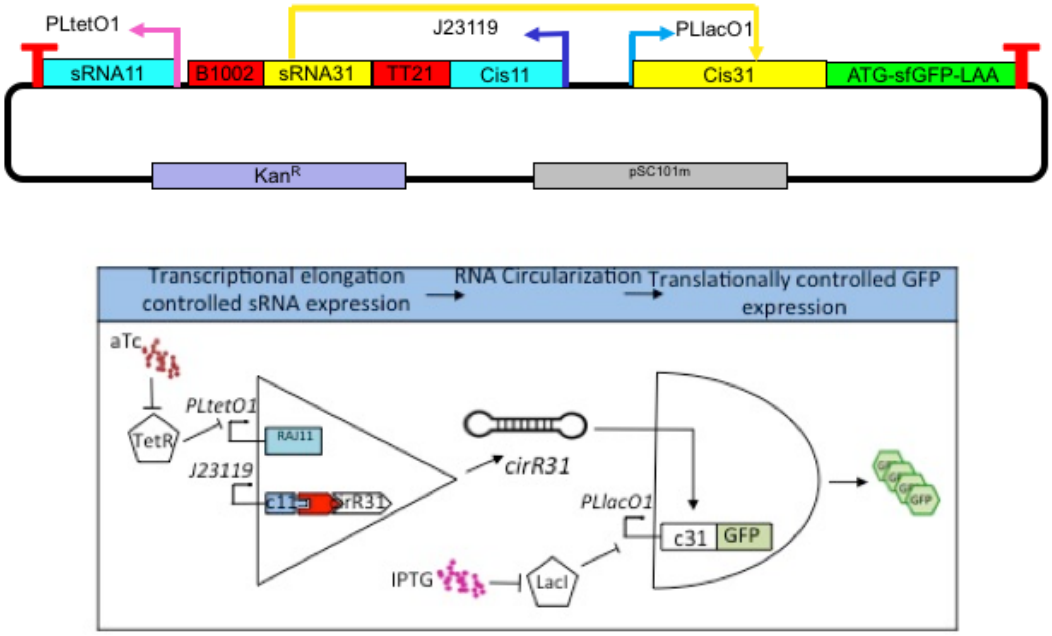


Figure 1.13 Trans-activating TNA 31 RNA cascade

Figure1.13 The above figure showed the schematics of design. After induction of aTc PLtetO1 promoter and get activated and sRNA-RAJ11 transcribed. The lower left figure showing expression of GFP in four conditions (0, aTc, IPTG and aTC+IPTG). The maximum GFP is expressed in Presence of aTc+ IPTG. The system is also leaky that GFP is expressed in IPTG induction.

1.1.10 FACS results of TNA 31 circuits

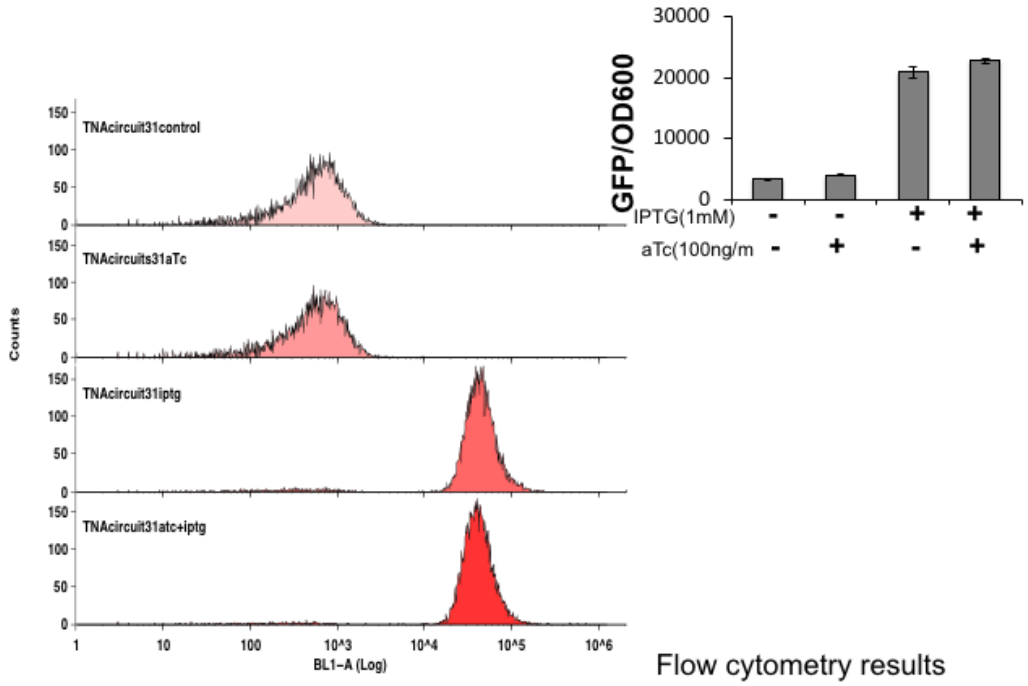


Figure 1.14 Results of TNA 31 cascade.

Figure 1.14 showing FACS results in all four condition (0, aTc, IPTG and aTc+IPTG). The GFP value also maximum in presence of aTc+IPTG both inducer present.

We characterized TNA 21 cascade in MG1655Z1 strain in all foour conditions (0, aTc, IPTG and aTc+IPTG). The floeimeter and FACS both results explaing, no activation in (none) and aTc induction. We got actation in IPTG and aTc+ IPTG. We should not have activation of reporter protein in presence of IPTG, but our system is leakey.

The TNA cascade system was leakey that we designed spacer to reduce the translational rate. We have designed 4 spacers (6bp, 9bp, 12bp and 15bp) which is inserted using the golden gate cloning. We have transformed into *E. coli* MGZ1655Z1 strain and charecterized using the 4 conditions (0 , aTc, IPTG and aTc+IPTG).

Spacing before ATG reduce translational rate

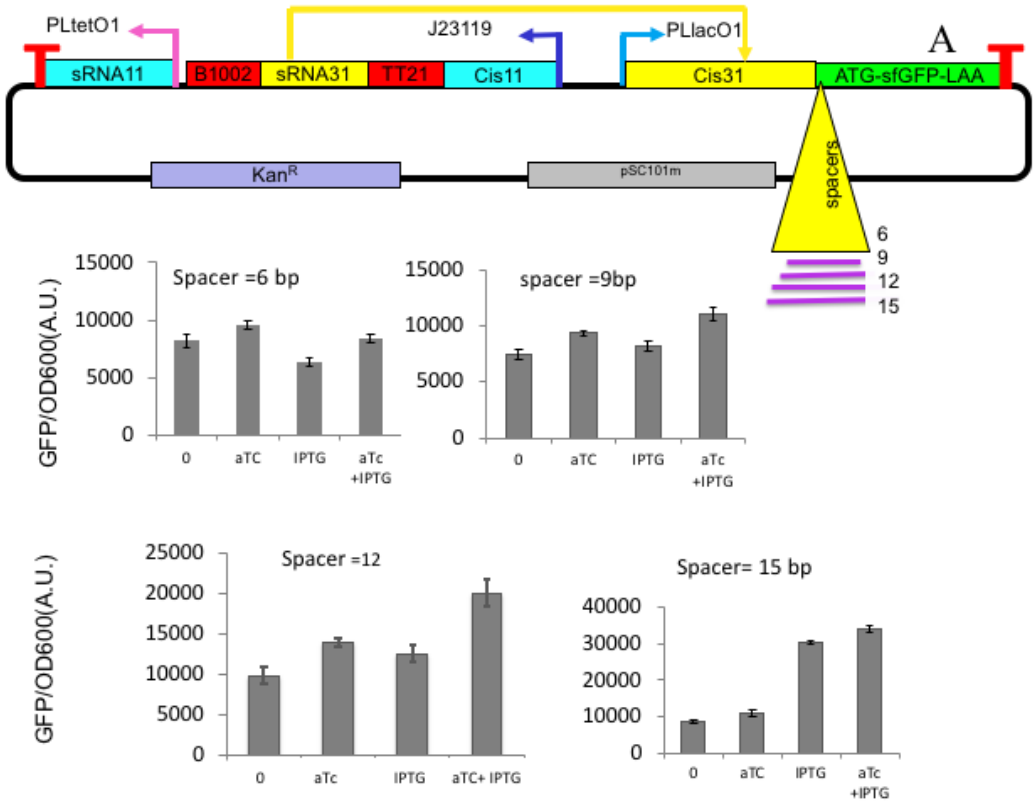
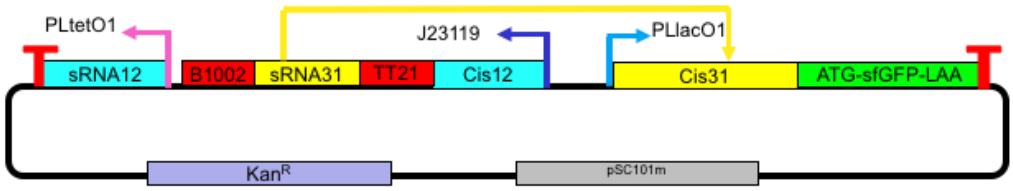


Figure 1.15 Results of spacer 6, 9, 12, 15 TNA 31 cascade

Figure1.15 The above is characterization results of TNA31 circuits in MG1655Z1. The leakiness of system is reduced and system giving better activation fold.

To explore more possibility, we have designed more cascade based on the Isaacs RNA-RR12. Designed is shown in figure**.** The design is the similar as TNA31 cascade.



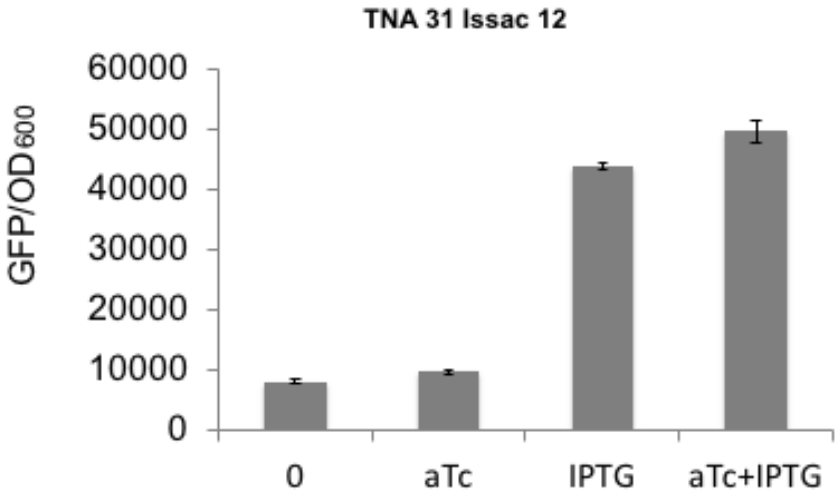


Figure1.16 Design and results of RNA cascade of RR12 and sRNARAJ31

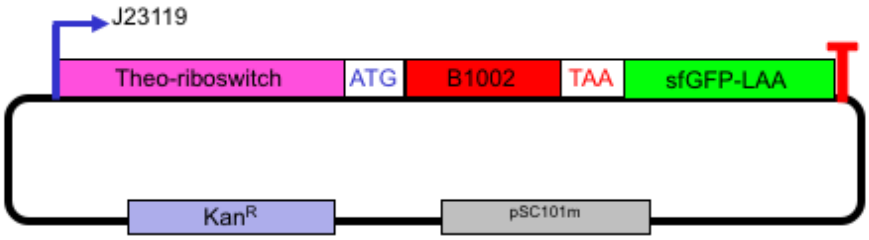
We characterized TNA-RR12 cascade in MG1655Z1 in all four conditions (0, aTc, IPTG and aTc+IPTG). This system also not having any activation in 0 and aTc but having with IPTG. The system having maximum activation in presence of both inducers. But IPTG also having so this system is also leaky.

1.1.11 TNA system based on riboswitch and cascade

In our above antiterminator riboswitch, the external signal is the small RNA that is present in trans. The trans activating small RNA signal activates cis-repressed mRNA by binding of sRNA. In this design external signal is theophylline and bind to aptamer domain of mRNA and change the conformation open RBS and make it active. In this design riboswitch is activates with external signal theophylline. We used theophylline riboswitch (Lynch and Gallivan et.al.,2007). Theophylline bind to aptamer change the conformation and get activate.

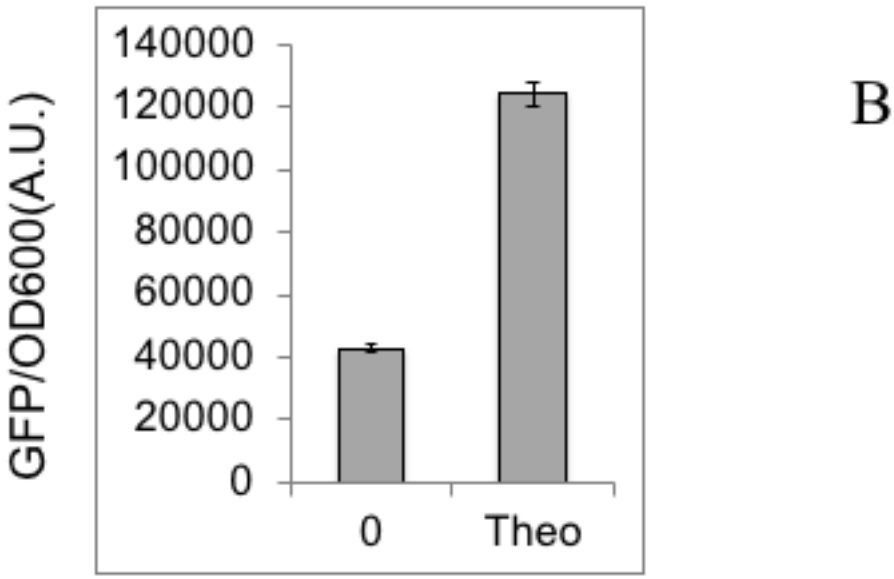
The TNA system we also tested in other riboregulator such as Isaac (RR12), theophylline riboswitch, and our recently published regazyme (Shen et al., 2015). The results are shown below. This system we obtained very high activation fold in Gallivan riboswitch.

Antiterminator cascade based on theophylline riboswitch



A

Figure1.17A Design of Theophylline based antiterminator



B

Figure1.17A Results of Theophylline based antiterminator

1.1.12 Single cell dynamic of Theophylline riboswitch TNA system

The theophylline riboswitch (Lynch et al.,2007) based antiterminator system was characterized in microfluidics to study single cell dynamics. The system is studied in two conditions (none and theophylline. In presence of theophylline system activate GFP reporter protein.

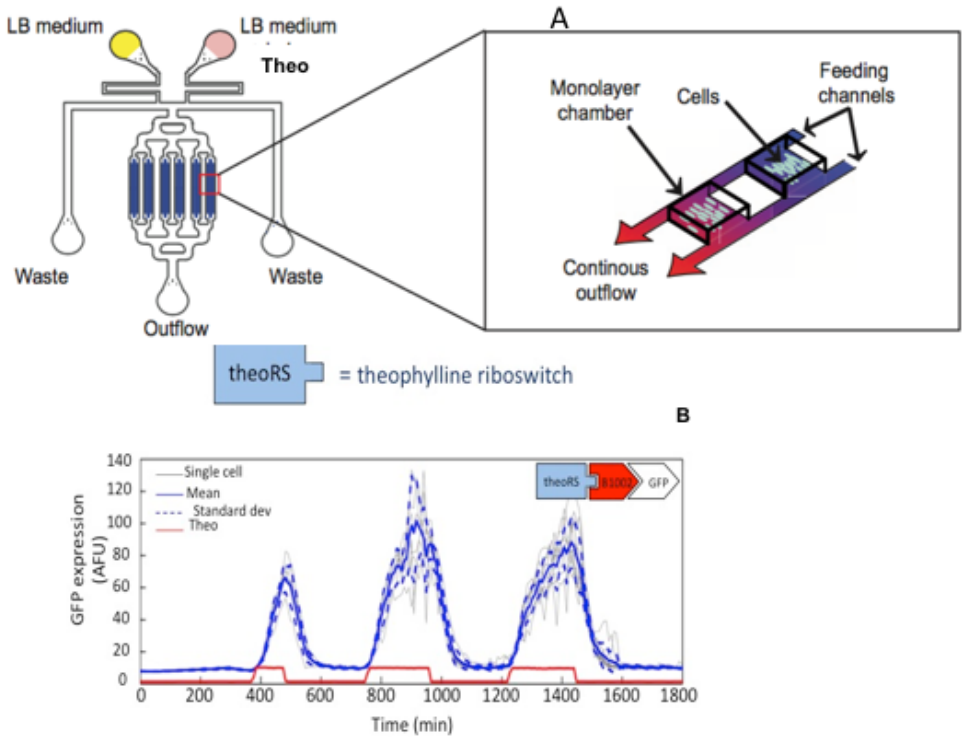


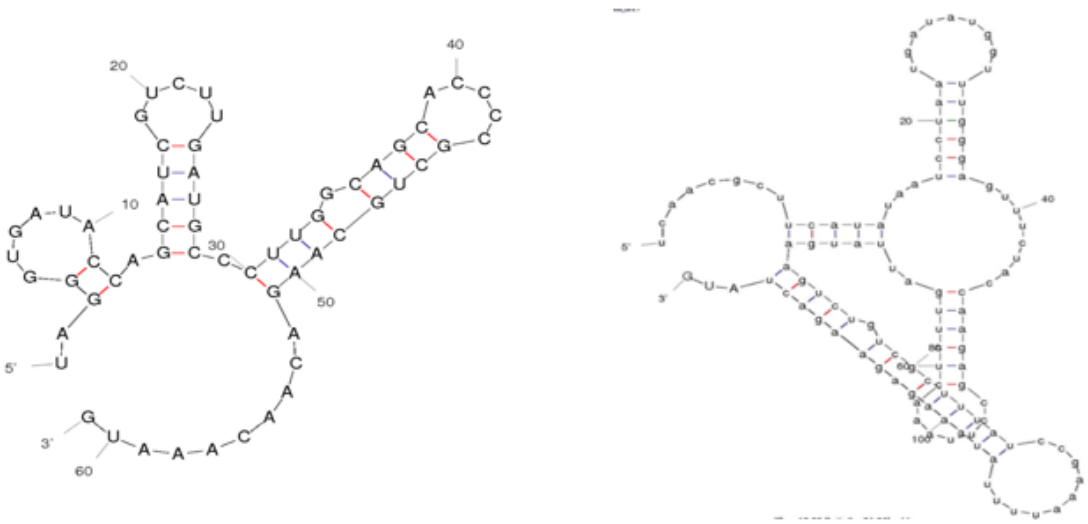
Figure1.17C Single cell dynamics of theophylline riboswitch based antiterminator RNA switch

Figure1.17C showing single cell dynamics of B1002 terminator in presence of inducer theophylline and absence of theophylline. Single cell tracking of fluorescence GFP expression in arbitrary fluorescence units (AFU)] in one micro chamber of the microfluidics device under time-dependent induction with theophylline (6 mM) for system TNA B1002 Galivan riboswitch in MG1655Z1. A square wave of theophylline inducers with period 8 h (i.e., 4 h induction and 4 h relaxation) was applied. The solid and dashed lines (in blue) correspond to the mean and plus/minus standard deviation over the cell population. Sulforhodamine B was used to monitor the inducer time-dependent profile (in red)

We additionally study single cell dynamics of Gallivan riboswitch based antiterminator.

Antiterminator cascade based on double riboswitch

Synthetic translational to transcriptional control by double riboswitch cascade



Theophyllineriboswitch=TAGGGTGATACCAGCATCGTCTTGATGCCCTTGGCAGCACCCCGCTG CAAGACAACAAATG= ΔG = -17.30 kcal/mol

addAriboswitch=TCAACGCTTCATATAATCCTAATGATATGGTTTGGGAGTTTCTACCAAGAGC CTTAAACTCTTGATTATGAAGTCTGTCGCTTTATCCGAAATTTTATAAAGAGAAGACTATG =ΔG = -21.90 kcal/mol

Figure1.8A Structure and sequence of Theophylline and addA riboswitch

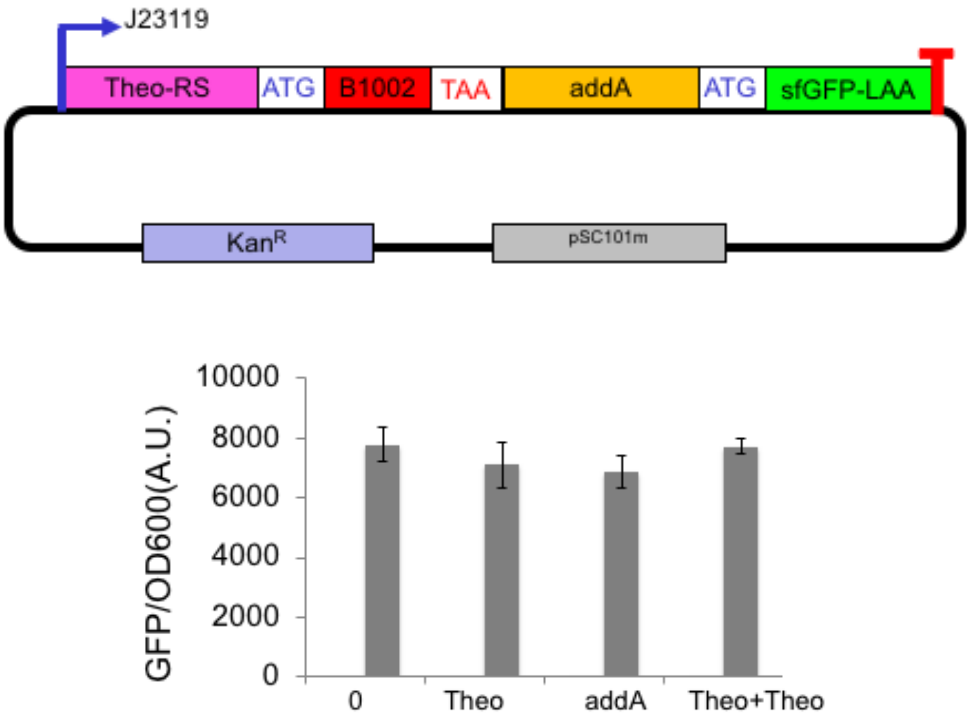
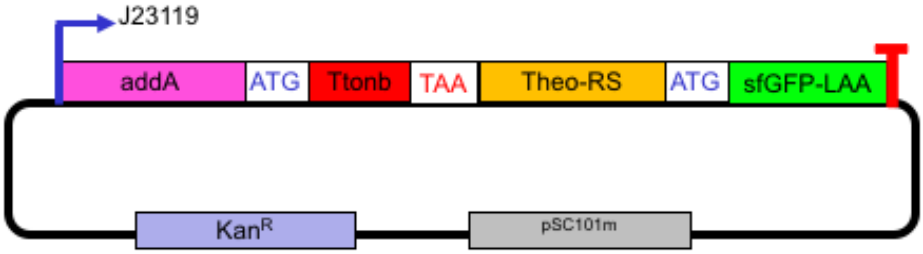


Figure1.18 Design & results of theophylline and addA based antiterminator switch



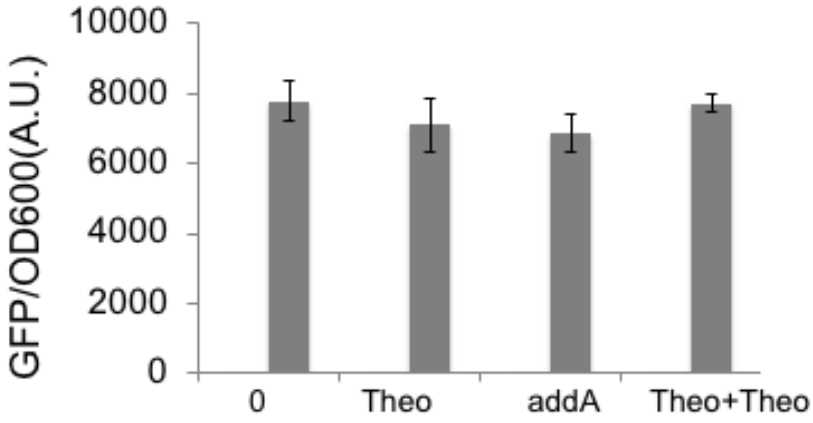
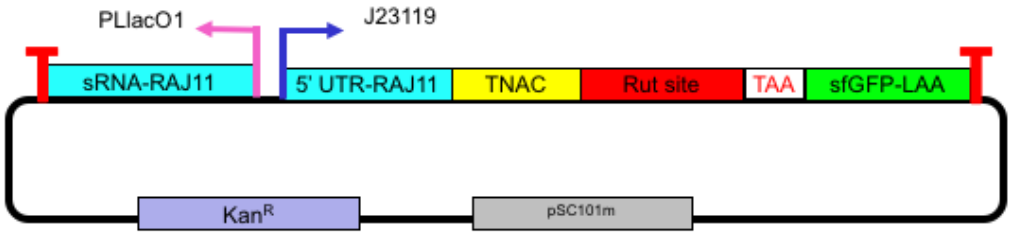


Figure1.19 Design & results of addA and of theophylline based antiterminator switch



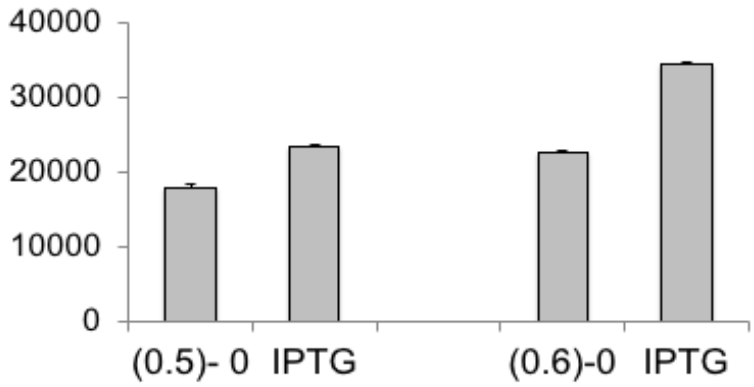


Figure1.20 design and results of Arkin's terminator system

Conclusion. As our experimental results, we conclude that the stimulating effect of a ribosome on transcription elongation occurs predominantly as a result of its anti-backtracking mechanism. Also, strong termination hairpins do not pause RNAP. Instead, it is a poly-T stretch of the terminator that induces backtracking type pauses at the site of termination. They also say that hairpins do not affect the rate of elongation.

Materials and methods

Common materials and methods

RNA sequence design. We applied a Monte Carlo Simulated Annealing optimization algorithm to design cascades of regulatory RNAs to finally control gene expression. The system was composed of three different RNA species: two small RNAs (sRNAs) and one 5' untranslated region (UTR). To implement this algorithm, we constructed a physicochemical model based on free energies and RNA structures, which involved the energies of activation and hybridization corresponding to the interaction between the two sRNAs and the energies of activation and hybridization corresponding to the interaction between the sRNA complex and the 5' UTR (see more details in Supplementary Information). The model also accounted for the degree of repression and exposure of the RBS within the 5' UTR intramolecular and intermolecular structures. Rounds of random mutations were applied and selected with the energy-based objective function (figure 3.4). For that, we extended a previously reported algorithm for RNA design. We used the Vienna RNA package for energy and structure calculation. The sequences of riboregulators engineered in this work, as well as their cognate 5' UTRs.

Plasmid construction

The plasmids were constructed with standard cloning methods (Sambrook and Russel, 2000). For ease of cloning, EcoRI and SpeI sites, allowing Bio Brick compatibility, flanked each system. The sRNA systems (sRNA – in reverse orientation controlled by Inducible promoter PLlacO1, fused – to the 5' UTR with ATG and then terminator and reporter protein (GFP) of sTB1002, were synthesized (Integrated DNA technologies, USA). The synthesized DNA was used for spinning to assure all DNA in bottom. The total DNA dissolved in 20 µL in MQ water (DNAase and RNAase free) vortexed then incubated at 50^0C for 5 minutes.

Restriction digestion of plasmid and insert

Type II restriction endonucleases were used to digest double-stranded DNA. The 10μL dissolved DNA was used for digestion with EcoRI and SpeI (fast digest enzyme from Thermo fisher).

Insert DNA	10μl
10X reaction buffer	2μl
Fast digest EcoR1	2μl
Fast digest SpeI	2μl
Water	2μl
Total	20μl

Total reaction volume is 20μl and incubate 37^0C for 35 minutes.

Purification of digested DNA

We have used QIAGEN PCR purification kit for purification of insert for further processing. We have followed the manufacturer instructions.

Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA fragments on the basis of their size. The larger DNA will be migrated slowly and smaller migrated faster. The length of DNA fragments compares with marker and isolated the desire DNA fragments. The 1% (w/v) agarose dissolve in 1X TAE buffer were used and melted. We added 0.5μg/ml ethidium bromide or gel red (10000X dilution) and casted in casting tray. The polymerized agarose gel was placed in gel running tank in 1X TAE buffer. The digested plasmid was loaded in the gel along with marker and control. The gel was run nearly 85V and cut the gel in blue light trans-illuminator.

DNA recovery from agarose gel

The vector DNA (pSTC1) fragment and insert were cut out with razor blade under blue light transilluminator. The gel slice dissolved in 500 μl QG buffer (3X volume of gel) incubated at 50^0C for 10 minutes. The dissolved gel

transferred in 2 ml Qiagen gel purification column. The column was centrifuged at 13000rpm for 1minute. Discard the flow-through and place the column back in the same tube. The add 750μl PE wash buffer to the column and spin for 1-minute same speed. Discard flow-through and placed the column back the same tube. The centrifuge the column one additional time to assure to remove residual ethanol and wash buffer were performed. The pre-incubated (450C) 35 μl elution buffer adds in column and incubated 42^0C for 2 minutes. Centrifuged for 90 second at 13000 rpm. We have measured the concentration of insert and vector using the Nano-drop and ligated as below.

Dephosphorization of linearized Vector DNA

In order to prevent self-ligation of vector DNA, we have used dephosphorization of vector with calf intestine alkaline phosphatase (CIP) enzyme. The alkaline phosphatase catalyses the hydrolysis of 5'phosphate from 5' end and 3' phosphate groups from DNA.

Dephosphorization reaction

Plasmid vector	33μl
10X reaction buffer for AP used in reaction	4μl
Fast AP Alkaline phosphatase	1μl
Water (Nuclease free)	2μl
Total	40μl

After adding all mix gently, we incubated the reaction mixture at 37^0C for 25 minutes. The reaction was stopped by heat inactivation 75^0 C for 5 minutes.

Ligation of plasmid vector

Plasmid vector having either sticky ends, blunt ends or circularization of vector can be ligated using the T4 DNA ligase.

Plasmid vector	5.50μl (10- 100 ng)
Insert	2.5 μl (3:1 molar ratio with vector
10X ligation mixture	2μl (Thermo Fisher)
Fast T4 DNA ligase	1μl (Thermo Fisher)
Water	9 μl
Total	20μl

The total reaction volume is 20μl and incubate at 220C for 35 minutes in PCR.

Heat shock Transformation of ligated vector in E. coli

We have used E. coli TOP10 for routine cloning experiments. The ligated 5μl final vector mixed into 50μl thaw chemical *E. coli* competent cells that were incubated for 30 minutes on ice. Subsequently, we gave heat shock 42^0 C for 90 seconds. Immediately placed the cells on ice for 10 minutes and added 900 μl LB media. The 100μl-200μl culture were taken and spread on LB agar with appropriate antibiotics and kept for overnight (16 to 20h) at 37^0 C.

Primer ligation method to construct control plasmid

All Control plasmids such as spacer plasmid, ATG knockout, synthetic terminator plasmid, leader less plasmid controls were constructed using oligos ligation method. We have ordered forward and reverse primers with specific restriction site (AvrII and BamH1) and BamH1 and Spe1. The forward and reverse primers were phosphorylated in a solution (NEB, and Thermos fisher kit and protocol).

The plasmid vector was also digested with same restriction enzyme, gel purify and dephosphorylated. Spacer and terminator were ligated with digested and dephosphorylated plasmid backbone. Plasmid backbone of ≈ 50ng and 1μl of anneal primer with 0.1μm cone with 1μl of T4 DNA ligase (Thermo fisher) and 1μl T4 DNA ligase buffer at 22^0 C for 45 minutes. The ligated vector was transformed in 50μl Top10 competent cells thawed on ice. The transformed cells were spread on LB/agar that contains kanamycin (50μg\ml). The plate was incubated 37 ^{0}C. The single colony was picked from plate and screen by colony PCR for further verification (VF2 5-tgccacctgacgtctaagaa-3 and VR 5-ttcttagacgtcaggtggca-3. The positive PCR colonies were screened and confirmed by sequencing.

PCR based site directed mutagenesis to construct control

Site directed mutagenesis is one of the most important modern biology techniques to introduce mutation at specific site of DNA. We have constructed some control plasmids (ATG knock out, RBS partially knock out, and RBS deletion knockout) by PCR based Site-Directed Mutagenesis method. We have designed the forward and reverse primer with specific mutation / deletion on target gene. The both forward and reverse primers amplified vector with high-fidelity DNA polymerase (NEB). The primer generated mutation/ deletion in specific nucleotide of gene. The PCR reaction treated with Dpn1 enzyme. The Dpn1 is susceptible to parental vector DNA. It digested only parental DNA because parental E. coli is dam methylated and it digest the parental vector.

Golden gate cloning

Golden gate cloning (Engler et al., 2008) was carried out using 1µL BsaI (New England Bio labs, Ipswich, USA), 1 µL T4 Ligase (NEB), 2 µL T4 ligase buffer and 100 fmol of each DNA fragment used (PCR product, Gblock, plasmid or annealed oligo's), topped up to 20 µL. Reaction was then cycled in a thermocycler. 30 cycles: 37°C, 2 minutes; 16°C, 5 minutes then 10 minutes, 50°C; 10 minutes, 80°C; 4°, hold. Oligos for goldengate were annealed by diluting them to 0.1 µM in EB, heating them to 96°C for 5 minutes in a heat block, switching it off and letting them cool to room temperature. 3µL of golden gate reaction were electroporated. Four standard bio brick-compatible golden gate cloning plasmids were built and used for generic golden gate cloning.

Gibson Assembly

1.5X Gibson assembly master mix was prepared with 320 µL 5X ISO buffer, 0.64 µL T5 exonuclease, 20 µL Phusion polymerase, 160 µL Taq ligase (all enzymes from NEB), and 600 µL H_2O and kept at -20°C until use. 5X ISO buffer preparation: 3 mL Tris-HCl pH 7.5 (1M); 150 µL $MgCl_2$ (2M); 240 µL dNTP mix (25mM each dGTP, dCTP, dATP, dTTP) ; 300 µL DTT (1M) ; 1.5 g

PEG-8000; 300 µL NAD (100 mM) and 2 mL H_2O. Assembly was carried out with 100 fmol of each fragment (5 µL added in 15 µL master-mix) and incubated at 50°C for 1 hour. 2 µL of reaction were electroporated.

Bacterial culture and reagents

E. coli strain DH5α (Invitrogen) was used for plasmid construction purposes as described in the manual. All the characterization experiments were performed in E. coli DH5α-Z1 cells (Clontech) or in *E. coli K-12 MG1655-Z1* cells (both lacI+ tetR+) for control over the promoters PLlacO1 and PLtetO1. For characterization in a fluorometer (TECAN) or in a flow cytometer, plasmids carrying systems coop11, coop1 and coop2 were transformed into DH5α-Z1 cells, whilst plasmids carrying systems coop31 and coop32 into MG1655-Z1. Moreover, plasmid carrying system trigR2 was transformed into MG1655-Z1 cells for characterization in a microfluidic device.

A single colony were grown in LB medium or in M9 minimal medium, prepared with M9 salts (Sigma-Aldrich), glycerol (0.8%, vol/vol) as only carbon source, CaCl2 (100 µM), MgSO4 (2 mM), and FeSO4 (100 µM). Kanamycin concentration was 50 µg/mL. Cultures were grown overnight at 37 ^{0}C and at 225 rpm from single-colony isolates before being diluted for *in vivo* characterization. One mM IPTG (Thermo Scientific) was used for full activation of promoter PLlacO1 when needed, and 100 ng/mL aTc (Sigma-Aldrich) was used for full activation of promoter PLtetO1. For microfluidic cultures, cells were grown in fresh LB and in LB supplemented with 0.05% sulforhodamine B (Sigma-Aldrich), and IPTG + aTc (i.e., we used sulforhodamine B to monitor the presence of inducers in the chamber).

Gel assay (RNA-RNA inter-action)

To perform the *in vitro* transcription, 3µg of each pUC18-derived plasmid was digested with Eco31I and purified with silica- based columns (Zymo). We used approximately 1 µg of digested plasmid in the reaction. This was in 20 µL: 10 µL of plasmid, 2 µL buffer 10x (Roche), 0.4 µL DTT 10 mM, 1 µL NTPs 10 mM (Thermo-Scientific), 0.5 µLRibolock (~40 U/µL, Thermo Scientific), 1 µL inorganic pyro phosphatase (0.1 U/µL, Thermo Scientific), 1

μL T7 RNA polymerase (50 U/μL, Epicentre), and 4.1 μL H2O. We incubated the mix for 1 h at 37 oC, and then added 20 μL of loading buffer with formamide. The samples were heated at 95^0 C for 1.5 min, then cooled on ice, and then separated by PAGE in a 10% polyacrylamide gel, containing 8 M urea, TBE (1x), (200 V, 2.5 h). We cut the bands corresponding to the full-length RNAs for purification. The presence of RNA was confirmed by loading a small part of the purified preparations in another polyacrylamide gel.

For the reaction of RNA-RNA interaction, we used approximately 20 ng of RNA for each of the transcripts. The buffer of the reaction was 50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 20 mM NaCl. The mix (20 μL) was denatured (1.5 min at 95^0 C) and slowly cooled (15 min at room temperature) We then added 1.5μL glycerol (87%) and 0.2μL bromophenol blue-xylene cyanol (100x) to load the gel (15% polyacrylamide, buffer TAE, 1 mm thick), which was run for 2 h at 75 mA at 4^0 C. The gel was stained first with ethidium bromide and then with silver. We used the DNA molecular weight marker XIII (50 bp ladder, Roche).

Fluorescence quantification

Single colony was inoculated and grown overnight (approximately 16 hours) in LB medium with antibiotics (spectinomycine 35ng\μL and kanamycin 35ng\μL. The overnight cultures were then refreshed by diluting 1:200 in M9 medium with inducer (none, aTc, IPTG, and aTc+ IPTG) and antibiotics. They were grown for additional 2 h to then load 200 μL in each well of the plate with three technical and biological replicates (Custom Corning Costar). The plate was incubated in an Infinite F500 multi-well fluorimeter (TECAN) at 37 °C with shaking. It was assayed with an automatic repeating protocol of absorbance measurements (600 nm absorbance filter) and fluorescence measurements (480/20 nm excitation filter - 530/25 nm emission filter for sfGFP) every 15 min. All samples were present in triplicate on the plate.

Normalized fluorescence was obtained by subtracting the background values corresponding to M9 medium (in both fluorescence and absorbance values) and then dividing fluorescence by ^{absorbance} at OD600 ≈ 0.5. Corrected normalized fluorescence was obtained by subtracting the fluorescence of

plain cells (auto fluorescence). In systems coop1 and coop11, the cis-repression is very efficient and then the autofluorescence is even higher than the normalized fluorescence of transformed cells (assumed to be 0).

Single-cell microfluidic analysis

Preparation of PDS chip

The design of our microfluidic device was performed in AUTOCAD (AUTODESK) and was already applied to study a synthetic genetic oscillator. All images were acquired using Zeiss Axio Observer Z1 microscopy (Zeiss). The microscope resolution was 0.24 μm with Optovariation 1.6X, resulting total magnification 1600X for both bright field and fluorescent images. Images were analyzed with MATLAB (MathWorks). Cells were tracked by defining a cell-to-cell distance matrix and the cell lineages were reconstructed. Finally, the fluorescence level of each cell in each fluorescence frame was extracted.

Flow cytometry analysis

Single colony was inoculated and grown overnight (approximately 16 hours) in LB medium with antibiotics (spectinomycin 35ng\ μL and kanamycin 35ng\ μL at 200 rpm. The overnight cultures were then refreshed by diluting 1:200 in LB medium with inducer (none, aTc, IPTG, and aTc+ IPTG) and antibiotics. The refreshed culture was grown 2-3 hour to reach an OD600 of 0.2-0.4. Afterwards, cells were centrifuge 3000 rpm for 5minuts. The supernatant discarded and pellet was diluted again in 1 mL PBS. All expression data were analyzed using a Becton-Dickinson FACScan flow cytometer with 488 nm argon laser for excitation and a 530/30 nm emission filter (GFP). Gene expression of each sample was obtained by measuring the fluorescence intensity of thousands of cells. Data were analyzed using the Cytobank webserver by gating the events using scatter ranges, and then fluorescence histograms (subtracting auto fluorescence) plotted with MATLAB.

CHAPTER 2

Engineering single component riboswitches

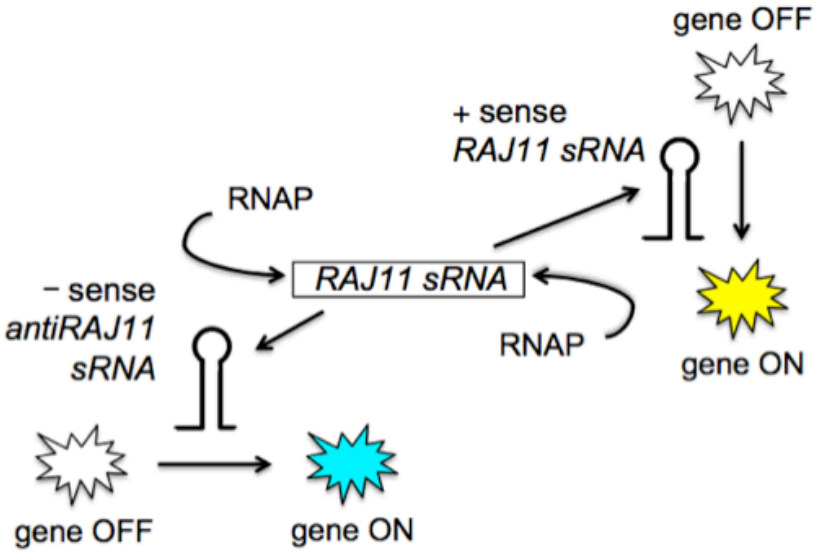


Figure 2.1 Schematic of ambisense riboregulator

2.1 Abstract

The coding sequence of transcripts could theoretically encode a different protein in each reading frame and some viruses use this mechanism. Non-coding sequences could still theoretically encode a different RNA molecule in each sense of transcription, but it has not been seen in nature aside from in the antisense. We hypothesized that it should be possible to engineer a synthetic sequence where it would produce a different riboregulator when transcribed in each sense. For this, we considered the sequence of a known riboregulator and created a 5' untranslated region (5' UTR) sequence that would interact with the molecule corresponding to the reverse-complement of the riboregulator.

Using computational design, we engineered a cis-repressed 5' UTR that can be activated by this new regulator. As a result, both sequences can independently activate the translation of their cognate targets. As expected, the two riboregulators can also silence one another by antisense interaction. We show that it is possible to engineer regulatory elements that can regulate different genes depending on the direction of transcription, and that they can be interfaced with other circuits used in synthetic biology.

2. 2 Introduction

Four combinatorial bases offer the possibility of encoding vast information, and even the same sequence can provide, in some cases, multiple functions (Johnson and Chisholm 2004). The question we asked was whether a single piece of DNA of a given size could encode multiple different functional elements for the cell (Kozak 1986). In theory, as the same DNA sequence could be transcribed in two ways depending on the localization of the promoter, two anti-parallel RNA molecules could be synthesized.

RNA is a key regulatory molecule as it regulates major cellular reactions such as DNA replication, transcription, and translation. There is a growing interest for using non-coding RNAs rather than proteins for a number of reasons. There are easy tools available to predict secondary structure (predictability of folding), and calculation of free energy (ΔG), (Rodrigo et al., 2013). The ambisense riboregulatory system would be the new way of

synthetic gene regulation in *E. coli.* This could allow the programmable regulation of a gene, depending on the direction of transcription. This work focused on bacterial riboregulators (trans-activating riboswitch) which are small RNAs (sRNAs) able to induce a conformational change in a specific cis-repressed 5' untranslated region (5' UTR) of an mRNA (messenger RNA) to modulate gene expression. The 5' UTR contains a functionally repressed ribosome-binding site (RBS). When the sRNA signal binds to the cis-repressed mRNA, the RBS is uncovered into a fully functional motif and the riboswitch gets activated.

Previously our lab has engineered a small RNA riboregulator (Rodrigo et.al 2012), sRNARAJ11, that performs a multiplication of the input signals: activating gene expression only when all inputs are high. A *PLtetO1* promoter controlled the sRNA RAJ11 which interacted with a cognate 5' UTR containing *cis*-repressed RBS to initiate the translation of a reporter protein (GFP) (Rodrigo et al. 2012). The engineering of an ambisense riboregulator often requires the layering of multiple genetic circuits, thus necessitating substantial efforts in circuit construction and tuning. Here we have taken advantage of our computational *de novo* design of riboregulators to develop two riboregulators that interact with each other through the base pairing between the two complementary sequences (Figure 2.1)

2.3 Design and results

2. 3.1 Design of ambisense riboregulator

Computational sequence design

The sequence of the RAJ11 sRNA was used as a template. The sequence of the antiRAJ11 sRNA was obtained as the negative-sense strand (reverse complement) of the RAJ11 sRNA (excluding the transcription terminator). We used our original sequence; the RAJ11 sRNA had the B0015 terminator, and the antiRAJ11 sRNA the T500 terminator. In order to design the sequence of the 5' UTR regulated by the antiRAJ11 sRNA, we used the web-based

software Ribomaker (collaboration with Dr. Guillermo Rodrigo, ICPM Spain) to automatically design regulatory RNAs that exploit conformational changes to control gene expression. It uses a Monte Carlo simulated annealing optimization algorithm and the Vienna RNA package to calculate secondary structures of different species in the system. Here, the system comprised three RNA molecules: the small RNA (sRNA), the messenger RNA (mRNA, with the 5' UTR), and the interacting complex. To implement this algorithm, we constructed a physicochemical model based on free energies and RNA structures that included the energies of activation and hybridization corresponding to the interaction between the antiRAJ11 sRNA and its cognate 5' UTR. The model also accounted for the degree of repression and release of the ribosomal binding site (RBS) within the 5' UTR intramolecular and intermolecular structures. Successive rounds of random mutations over the 5' UTR sequence were applied and selected with an energy-based objective function (Fig. S1). Designed sequences are shown in Fig. S2.

Ribomaker was run several times to design the 5' UTR responding to the antiRAJ11 sRNA. The heuristic algorithm performs multiple cycles of random mutations and selection, and each run produces a different sequence. The objective function accounts for on one side, the *cis*-repression of the 5' UTR, and on the other, the interaction ability with the sRNA. Ribomaker outputs the values of effective free energies (to be minimized) corresponding to all partial objectives; and the global objective function is a sum of these free energies. From all sequences designed, we selected the one with the minimal value of objective function.

To engineer an ambisense riboregulator, we have designed a DNA sequence that produces transcripts that are not translated but are able to perform function in the cell by activating gene (i.e., regulatory RNAs). These transcripts can encode two different target genes depend on the direction of the transcript. In our *de novo* design in *E. coli*, of the two small RNA transcripts one is the reverse complement of the other. We used *in-silico* evolution methods to design our sequence of a new riboregulator (antiRAJ11).

By using computational design, we have engineered a new riboregulator antiRAJ11 (sRNA is the reverse complement of sRNARAJ11 but has the same transcriptional terminator) containing an sRNA antiRAJ11 controlled by the PLtetO1 promoter which can bind to cis-repressed 5' UTR mRNA and hence lead to the exposure of the RBS of the targeted mRNA and translation of the reporter protein Figure 2.2. The sRNARAJ11 and sRNAantiRAJ11 are two complementary sequences that can hybridize with each other when co-expressed in cells (Rodrigo et al. 2016). This leads to the mutual repression of the two riboregulators and hence inhibition of their abilities to activate downstream translation of their corresponding targets mRNA figure 2.2. To implement this firstly, we constructed and characterized each of the two riboregulators in two different vectors to demonstrate their ability to activate the translation of their target mRNA. Both riboregulators (sRNA) were under control of the promoter PLtetO1 (Lutz & Bujard 1997), while the *cis*-repressed mRNA was under control of the promoter PLlacO1 (Lutz & Bujard 1997). The binding to the 5' UTR of the target mRNA was initiated by the seeding site in the 5' terminal of the riboregulators and hence opening the *cis*-repressed RBSs in the 5' UTR, leading to the binding of the ribosome on the RBS and the translation of the reporter protein (GFP). The two constructs were transformed into MG1655 Z1 (expressing lacIQ and TetR (Lutz & Bujard 1997) repressors in genome) and the activation of GFP was characterized using a fluorescence plate reader Figure 2.2. The two riboregulators activate their target mRNAs, as shown in the results.

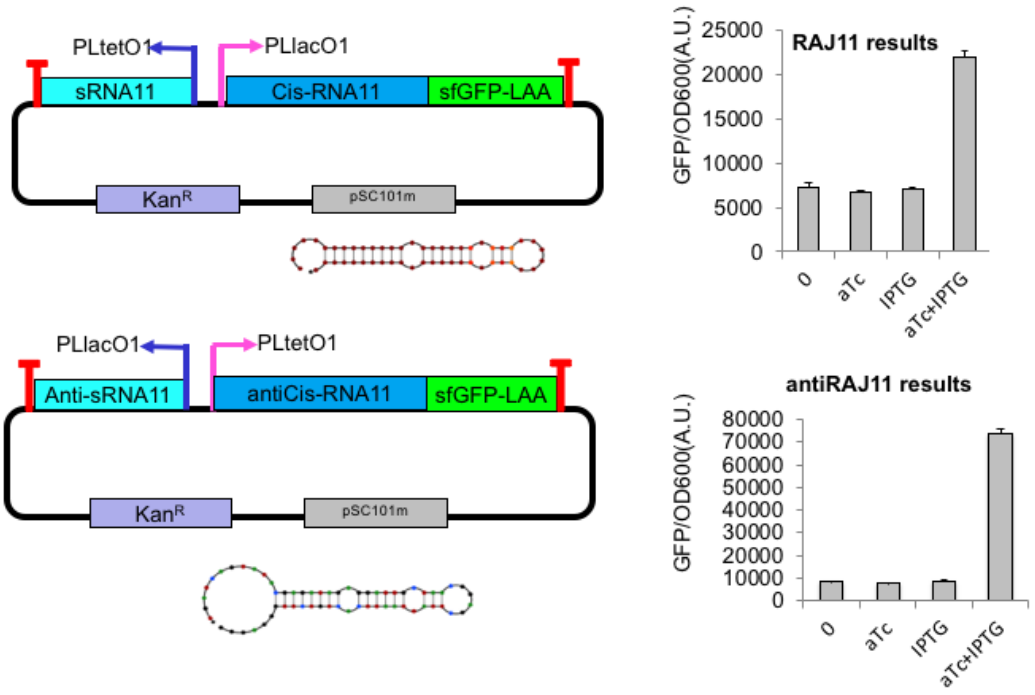


Figure 2.2 Design and results of RAJ11 and antiRAJ11

Figure 2.2 Design and characterization of the two riboregulators RAJ11 and antiRAJ11. As shown in the map, PLlacO1 promoters' control both riboregulating small RNAs are controlled by PLtetO1 promoter and mRNA expression. The GFP is downstream of the 5' UTR. The GFP-mut3 in case of the RAJ11 system and superfoder GFP with degradation tag in the antiRAJ11 system. Both plasmids were transformed separately into MG1655 Z1 and characterized under all possible inducer combinations (none, aTc, IPTG and aTc+IPTG).

Figure 2.2 is comprised of 2 sections. The upper half shows the design of the riboregulator pRAJ11, the sRNARAJ11 activates the target cis-repressed 5' UTR mRNA-GFP (Rodrigo et. al, 2012). The Tecan (fluorometer) characterization results of GFP in four conditions (none, 100ng/mL aTc, 1mM IPTG and aTc+ IPTG) in MG1655Z1 *E. coli* cells and secondary structure mRNA. The lower figure shows the design of the antisense riboregulator (antiRAJ11) and characterization results in all four conditions and secondary structure of mRNA.

2.2.2 Design of a compact ambisense riboregulator

The characterization results of the RAJ11 (Rodrigo et al. 2012) and antiRAJ11 riboregulator (Rodrigo et.al 2016) have shown the successful

activation of the *cis*-repressed mRNAs. After these experimental results, we have designed a compact ambisense riboregulator into a single plasmid. As shown in Figure 2.3A**,** small RNA RAJ11 was controlled by a promoter *PLlacO1* in the forward orientation and is controlled by the promoter *PLtetO1* in the reverse orientation that will drive transcription of sRNAantiRAJ11 (complementary to RAJ11). With this design we can transcribe two complementary small RNAs on the same DNA template, depending on orientation of the promoter and polarity of the DNA template. In this work, we focused on DNA sequences that can produce two small RNA transcripts that are not translated but are able to activate two cis-repressed mRNAs independently. In this design I have integrated two cistronically fused *cis*-repressed 5' UTR-mRNA (RAJ11 and antiRAJ11) to the sfGFP-LAA. This design principally should be able to express sRNA-RAJ11 on induction by IPTG and activate cis-repressed 5' UTR to express the reporter protein sfGFP-LAA. The system can also express sRNA-antiRAJ11 on induction by aTc and activate, cis-repressed 5' UTR antiRAJ11 GFP. The co-expression of both riboregulators (combination induction of aTc and IPTG) would lead to hybridization between the two riboregulators as mentioned before, thus returning 0 output of GFP. This system output resembles the electronic circuit XOR gate truth table.

We have characterized the ambisense riboregulator in the *E. coli* strain MG1655Z1 and DH5alpha Z1 ($lacI^R$, $tetR^+$ and $spect^R$) strains. The fluorescence of the reporter GFP was measured in plate reader upon different treatment (none, aTc, IPTG and aTc+IPTG) as indicated in Figure 2.3D. As shown in the results, IPTG treatment activated the expression of the GFP as indicated by the increase of the fluorescence. However, no activation was found in the aTc treatment, nor inhibition upon combination of aTc and IPTG.

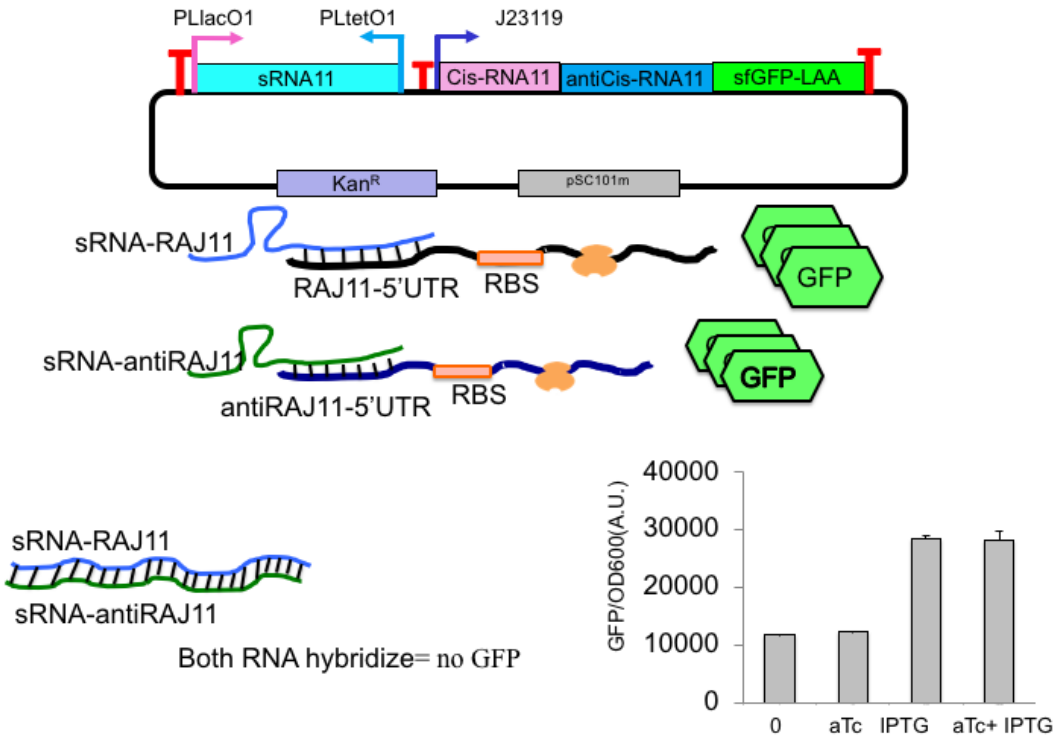


Figure 2.3 Design and results of compact abmisense riboregulator

Figure 2.3 **(**A) Design of compact ambisense riboregulator. Two promoters PLlacO1 and PLtetO1 depending on its polarity regulate the sRNARAJ11. The constitutive promoter BBa_J23119 controls both 5' UTRs (binding sites for RAJ11 and antiRAJ11) placed upstream of the superfolder GFP. The plasmid transformed MG1655Z1 was characterized with all four conditions (0, aTc, IPTG and aTc+ IPTG). The promoter PLlacO1 controls the expression of riboregulator sRNARAJ11, while PLtetO1 controls the expression of riboregulator sRNA-antiRAJ11 (reverse complemental to sRNARAJ11), and the GFP mRNA was under control of the constitutive promoter BBa_J23119.

(B). The induction of riboregulator with IPTG; PLlacO1 promoter will activate and transcribe sRNARAJ11, this will activate cis-repression mRNA-RBS of RAJ11 and GFP will be expressed.

(C). Induction of riboregulator with aTc, PLtetO1 promoter will activate and transcribe sRNAantiRAJ11. This will activate cis-repression mRNA-RBS of antiRAJ11 and exposed RBS leading to expression of GFP.

(D). Induction of riboregulator with inducer aTc and IPTG would express sRNARAJ11 and sRNAantiRAJ11, respectively. Both sRNAs (RAJ11 and antiRAJ11) are complementary, so they hybridize and no GFP is produced.

The construction (ambisense riboregulator plasmid) shown in figure 2.3A was transformed into MG1655Z1 cells and the fluorescence was characterized with plate reader (Tecan 500) under different treatment of

inducers as indicated in the Figure 2.3E.

The failure of the output 0 in the combination of the two small RNAs could be due these possibilities:

1. **Promoter Competition**: Face-to-face promoters controlling the reverse complementary riboregulators could lead to competition for RNA polymerase between the two promoters. In our case, it seems that PLlacO1 promoter dominates the expression, leading to no transcription of the antiRAJ11 which is under control of PLtetO1 (Brophy & Voigt 2016).
2. **Occlusion**: RNAP binds to the strong promoter, in this case PLlacO1, and RNAP elongates over the other promoter (PLtetO1) during transcription and transiently blocks the other (Brophy & Voigt 2016).
3. **Collision**: Two actively transcribing RNAPs collide and the stronger wins.
4. The fused 5' UTR cis-regulatory regions in our case can interfere each other. The riboregulator antiRAJ11, ever if it was expressed from the PLtetO1 promoter, could not bind to the 5' UTR (antiRAJ11) if the first 5' UTR (RAJ11) was not opened. This may explain why there is no activation of fluorescence upon treatment of aTc inducer.
5. The two-different small RNA have different stabilities.
6. The PLacO1 promoter is leaky and it always express sRNARAJ11 and inhibits sRNAantiRAJ11.

To further understand my design and in the end successfully construct an ambisense riboregulator, I have co-transformed this system together with another high copy number plasmid expressing the antiRAJ11 small RNA riboregulator under control of PLtetO1 (Figure 2.4). We have used the same strain MG1655Z1 to characterize the fluorescence of the GFP upon treatment of aTc or IPTG, or both. As shown in Figure 2.4, co-expression of another plasmid, which could normally express the riboregulator sRNAantiRAJ11 upon treatment of aTc, has led to the return 0 in the combination of the aTc and IPTG. In other words, sRNAantiRAJ11 could inhibit the sRNARAJ11 to activate the 5' UTR cis-repressed GFP expression. This result indicated that our complementary riboregulators could

successfully generate an ambisense riboregulator gate in a new manner that does not exist so far.

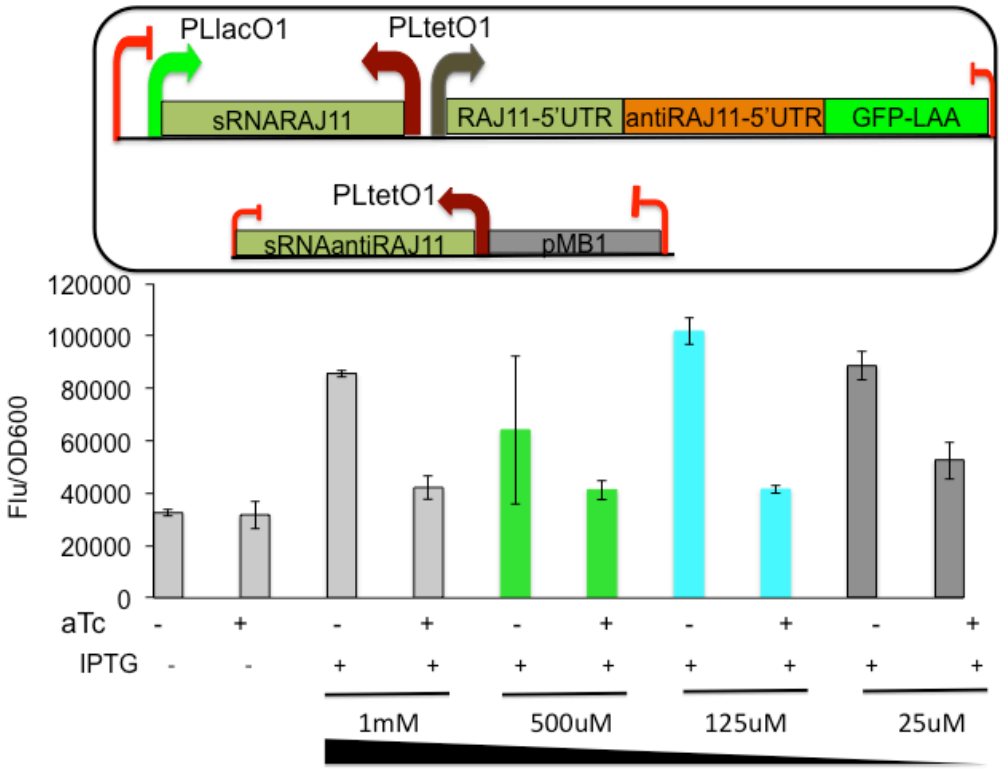


Figure 2.4 Results of co-transformed sRNA

Figure 2.4. A Co-transformation of another plasmid expressing riboregulator sRNA antiRAJ11 with the previous design; (b) characterization of the fluorescence of the GFP. Although there was still no activation of the fluorescence upon treatment with aTc, the combination of aTc and IPTG could lead to inhibition of the fluorescence indicating the functional RNA-based XOR gate (0 output with two inputs). The Different concentration of IPTG values demonstrated that there were no significant changes in the inhibition of the GFP.

We have also treated the cells co-transformed with the two plasmids with different concentration of IPTG. However, we didn't see any significant change in the 0 output upon treatment of both inducers. These results underscored that our previous design was problematic. On the one hand, the face-to-face promoters could not work properly since there was no transcription of sRNA antiRAJ11 upon treatment of aTc. On the other hand, the cistronically fused 5' UTR was non-functional for the second 5' UTR. These two important factors led to the hypothesis that:

1. Two promoters could interfere if they are too close two each other.

2. Two cistronically fused RBSs could interfere each other and may be making a pseudoknot.

2.2.3 Design of an ambisense riboregulator using ribozymes

My above design (compact ambisence riboregulator) did not work properly, so I designed an ambisense riboregulator based on ribozyme. Ribozymes (ribonucleic acid enzymes) are RNA molecules that are capable of catalyzing specific biochemical reactions, similar to the action of protein enzymes. Many ribozymes cleave RNAs in *trans*, such as RNase P which processes transfer RNAs (tRNAs), or self-cleave, such as the Hammerhead or Hepatitis D virus (HDV) ribozymes (Serganov and Patel, 2007). We used hammerhead ribozyme, which is a small catalytic RNA motif made up of three base-paired stems and a core of highly conserved, non-complementary nucleotides essential for catalysis. Similarly, to what occurs in some RNA organisms such as viroids, we can use ribozymes (Markus Wieland and Jörg S. Hartig 2008), (Yangbin Gao and Yunde Zhao 2014) in each sense (or polarity, as it is called in viruses) that will cleave the transcript at appropriate positions to remove undesired sequences. I took advantage of the self-cleavage activity of ribozyme, hypothesizing the following: that PLlacO1 promoter controlled HHR1 ribozyme and terminator sequence and after induction of IPTG the system transcribed small RNA-RAJ11 and HHR1 ribozyme. The ribozyme HHR1 would then self-cleave and release small RNA. This small RNA would activate 5' UTR RAJ11and express RFP. The same mechanism would occur for opposite polarity but in the reverse orientation using the PLteO1 promoter, the antisense strand of sRNARAJ11 (antiRAJ11) and HHR2 ribozyme and terminator. After induction of aTc, the system would transcribe in reverse orientation with antiRAJ11 and HHR2. The antiRAJ11-HHR2 complex would then cleave and release sRNAantiRAJ11. This antiRAJ11 would trans-activate 5' UTR antiRAJ11 and express GFP.

Each sense transcribes two different sRNAs targeting two different 5' UTRs where they activate their respective genes. The Raj11sRNA, transcribed by

the PLtetO12 promoter in the forward direction, binds to the CisRaj11 5' UTR to activate the mRFP reporter protein. The antiRaj11sRNA (the reverse complementary of Raj11sRNA), transcribed by the PLlacO1 promoter in the reverse direction, binds to the cis_antiRaj11 5' UTR to activate the sfGFP.

Our goal was to transcribe two small RNA transcripts with the same DNA template depending on polarity of DNA sequence and has these transcripts activate two separate genes independently. The first compact ambisense riboregulator design we made was unable to transcribe two sRNAs from the same DNA template. That is why we designed a new ribozyme based ambisense riboregulator as shown in the Figure 2.5A

1. In this design, the PLlacO1 promoter controls HHR1 ribozyme sRNARAJ11and transcriptional terminator. The inducer IPTG will activate the promoter PLlacO1 and this will trigger the transcription of ribozyme-sRNARAJ11. The ribozyme will self-cleave and free the sRNARAJ11. This sRNARAJ11 will hybridize to 5' UTR-RAJ11 and activate the expression of the RFP reporter protein.
2. The aTc inducer will also activate the promoter PLtetO1 in the reverse direction and transcribe sRNA antiRAJ11ribozyme2. The ribozyme will self-cleave and free the sRNAantiRAJ11. This sRNAantiRAJ11 would hybridize antiRAJ11 5' UTR and open the RBS and the GFP will be expressed.
3. Upon induction by both inducers IPTG and aTc, IPTG activates PLlacO1 promoter and transcribes sRNARAJ11 and aTc will activate the PLteto1 Promoter in reverse direction and transcribe sRNAantiRAJ11. Both sRNAs are reverse complements of each other and would hybridize and repress each other. Ultimately there would be no activation of either cis-repressed 5' UTR and so no expression of the reporter protein.

We characterized our ribozyme based ambisense system in four different conditions (none, aTc, IPTG, aTc+IPTG). As shown in the figure 2.5B, no activation was seen in any conditions. Possible reasons for failure are:

1. The terminator could be working bidirectionally so it may terminate in

reverse direction as well, such that no sRNA is transcribed.

2. The ribozyme may be unable to cleave, such that sRNA is not free to activate cis-repressd 5' UTR, ultimately leading to no expression of the reporter protein.

3. The promoter is too close so that they interfere each other and ultimately no expression of reporter protein occurs.

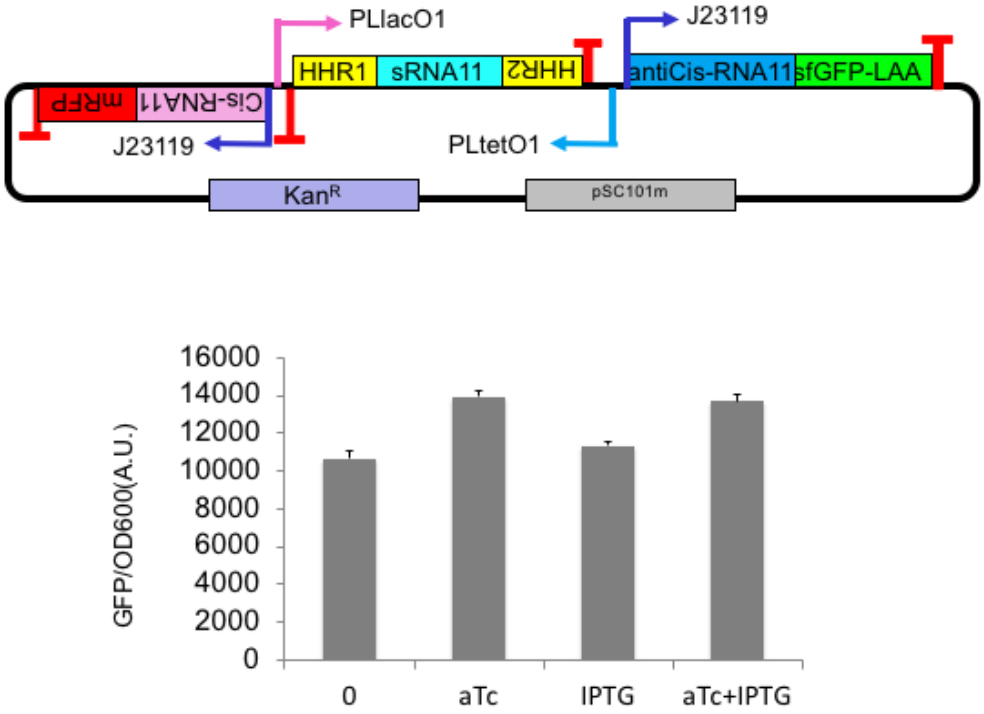


Figure 2.5 Design and results of ribozyme based on ambisense riboregulator

Figure 5.A Design of the ribozyme-based ambisense riboregulator. The promoter PLlacO1 controls ribozyme HHR1 sRNARAJ11 with reverse direction ribozyme and terminator (Tt1). The IPTG induction will activate PLlacO1 promoter and sRNA RAJ11 will transcribe and cleave and release sRNARAJ11. This sRNA would bind cis-repressed 5' UTR-RAJ11 and will activate reporter protein RFP. The inverse promoter PLtetO1 controls expression of sRNAantiRAJ11 ribozyme HHR2 and its terminator (tim liu term). The aTc induction would activate promoter PLtetO1 and transcribe sRNAantiRAJ11. The sRNAantiRAJ11 would bind 5' UTR-antiRAJ11 and activate the reporter protein. The induction of aTc and IPTG would trigger the expression of both complementary RNAs. They are reverse complement of each other and they would hybridize resulting in no expression of reporter protein.

Design of RNA based on orthogonal ambisense riboregulator

To solve our problem with the activation of PLtetO1 promoter, we have designed and synthesized two orthogonal plasmids (pMR01 and pMR02 as

shown in the Figure 2.6). These are orthogonal rioboregulators. This new design enabled us to test the orthogonality between the two riboregulators and their corresponding 5' UTR. Meanwhile, it also finally allowed us to construct an ambisense riboregulator.

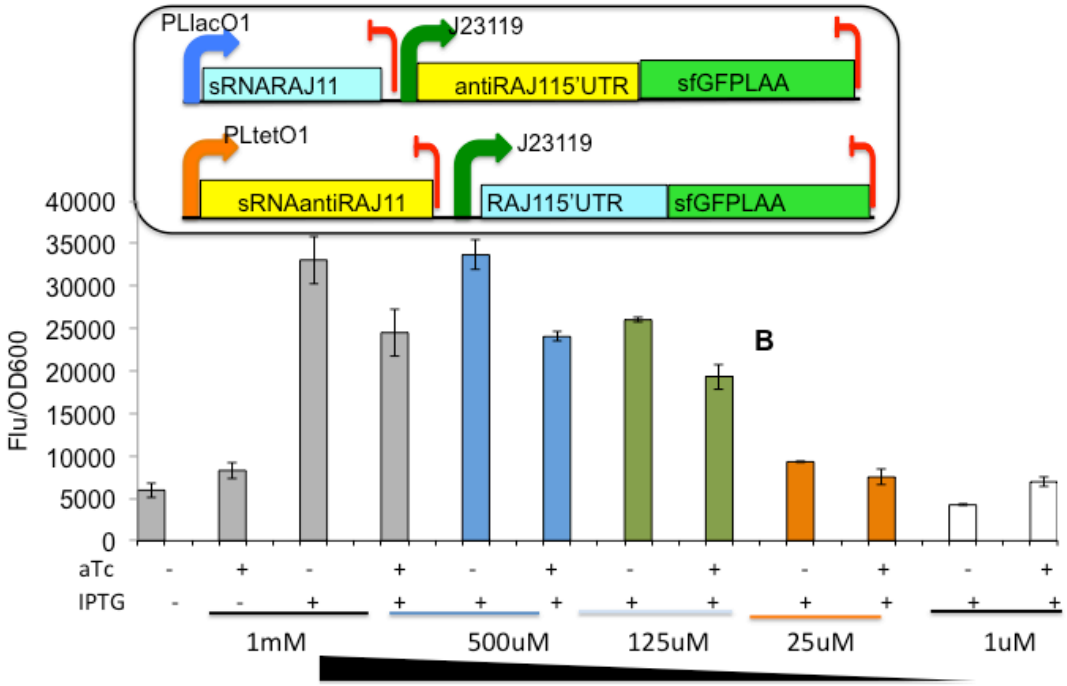


Figure 2.6 Design and results of orthogonal ambisense riboregulator

Figure 2.6. New design of the ambisense orthogonal riboregulator and its characterization. Two orthogonal riboregulators were designed and constructed. These orthogonal riboregulators were co-transformed in MG1655Z1 strains. The inducer IPTG activates PLlacO1 promoter and transcribes sRNARAJ11. This sRNARAJ11 activates cis-repress mRNARAJ11 of another plasmid and expresses GFP. The induction with aTc would activate promoter PLtetO1 and transcribe sRNAantiRAJ11, expressing GFP of another plasmid. These sRNARAJ11 and sRNAantiRAJ11 are both complementary to each other and would hybridiz if co-expressed with induction aTc+ IPTG.

The characterization results showed:

1. The induction of the system with inducer aTc triggers the transcription of sRNAantiRAJ11. This sRNAantiRAJ11 binds to cross system and activates cis-repressed mRNA of antiRAJ11 and GFP is expressed.
2. The induction of the system with inducer IPTG triggers the

transcription of sRNARAJ11. This sRNARAJ11 binds to the cross system and activates cis-repressed mRNA and GFP express. The figure shows huge expression of GFP.

3. The induction of the system with both inducers aTc and IPTG triggers the transcription of both sRNARAJ11 and sRNAantiRAJ11. According to the hypothesis, both sRNA (RAJ11 and antiRAJ11) are reverse complementary of each other and so they should hybridize. The results in figure show that GFP expression is reduced.
4. This suggests that both small RNA (sRNARAJ11 and antiRAJ11) are transcribed with the induction of inducer IPTG or aTc and are able to activate their respective cis-repress mRNA. The induction of the system with both inducers (aTc and IPTG) results in the transcription of both sRNAs and they are able to hybridize and repress GFP expression.

   The two plasmids are of different copy number that they transcribe different amounts of sRNA. They cannot mutually hybridize completely and therefore the system cannot repress completely. Both the systems have autofluorescence and system could not show good activation. The *E. coli* has RNAse III that could degrade some amount of sRNA.

   To balance both system sRNA, I tuned the system with different concentrations of IPTG and characterized and analyzed the results.

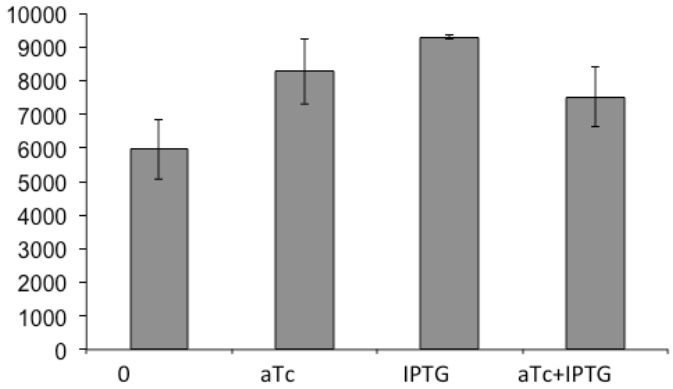


Figure 2.7 Results at 25 ng/ml cone. and 1mM IPTG

Figure 2.7 the characterization of orthogonal ambisense riboregulator at aTc (25ng/ml) and IPTG 1mM concentration. The results show activation upon induction with aTc and IPTG independently.

The induction with both inducers (aTc+IPTG) represses the system, but both activation and repression are very much reduced. Here we have success at some level of engineering an ambisense riboregulator. The system has auto-fluorescence and PLlacO1 has leakiness so at some level it hybridizes sRNAantiRAJ11 such that activation and repression is not significant.

Engineering of minimum alphabet (riboregulator with three nucleotides

The RNA-world hypothesis suggest that's life is based on RNA, before arrival of DNA and protein (Roger and Joyce 1999). The RNA had a role both genetic information and catalytic function. Protein have much diversity (20 a.a) as compared of RNA (4 nucleotide only). We proposed a hypothesis and experimentally validated that original genetic material contains fewer nucleotides than four different have now. It is possible that three nucleotides of RNA are sufficient for maintaining secondary structure and activation. Therefore, the engineering of RNA with a minimal alphabet is proposed.

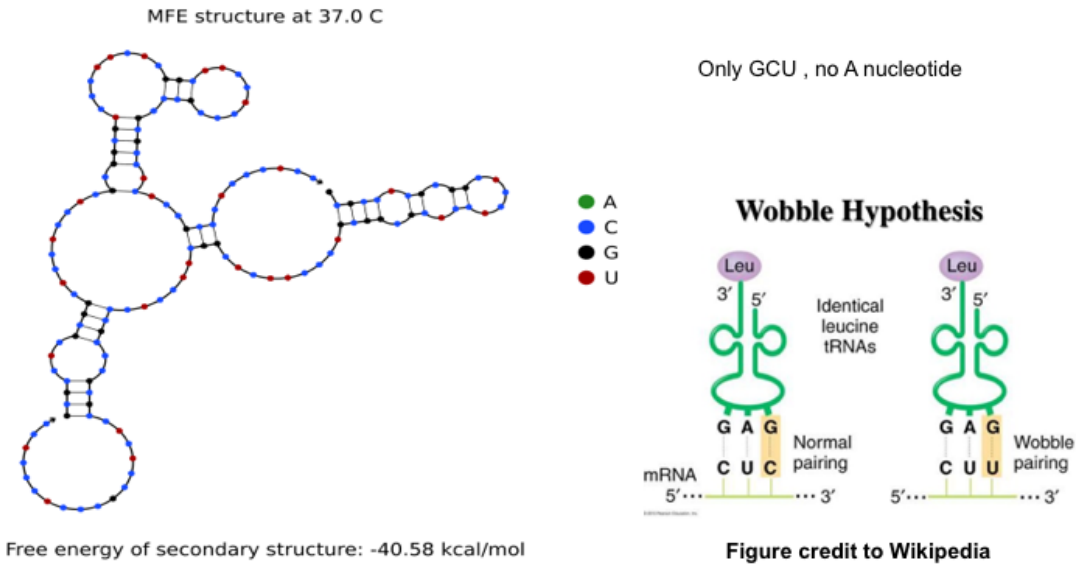


Figure 2.7A RNA switch with three alpha-bate GCU only

We engineered new RNA switch that has three nucleotides only. We engineered without adenine nucleotide in the sequence. The idea is based, on Watson-crick base pairing theory, where Guanine base pair with cytosine and it has strongest interaction. Although Guanine will also pair with uracil.

The base pair between guanine and cytosine is the strongest of the Watson-Crick base pairs as they form 3 hydrogen bonds. Whereas the guanine and uracil wobble pair only have 2 hydrogen bonds, which means they have a weaker interaction (Szatylowicz and Sadlej-Sosnowska 2010). Therefore, sequences that can only form G-C Watson-Crick pairs and G-U wobble pairs should be sufficient to produce complex secondary structures.

We successfully engineer small RNA that have three nucleotide and this small RNA activate cis-repressed mRNA that also has three nucleotides. The three nucleotides (GCU) riboregulator bind with cis-repressed mRNA.

This is possible by *In-silco* evolution of Guanine beginning with mutating the adenine bases of a synthetic RNA into guanines. Mutating adenines into guanines was the starting point as guanines are able to interact with cytosine, following the Watson-Crick model, as well as being able to form wobble pairs with uracil. Therefore, there would be more opportunities for the guanine to form a base pair when the sequence is folded into its secondary structure compared to if the adenines were initially mutated to a cytosine or uracil.

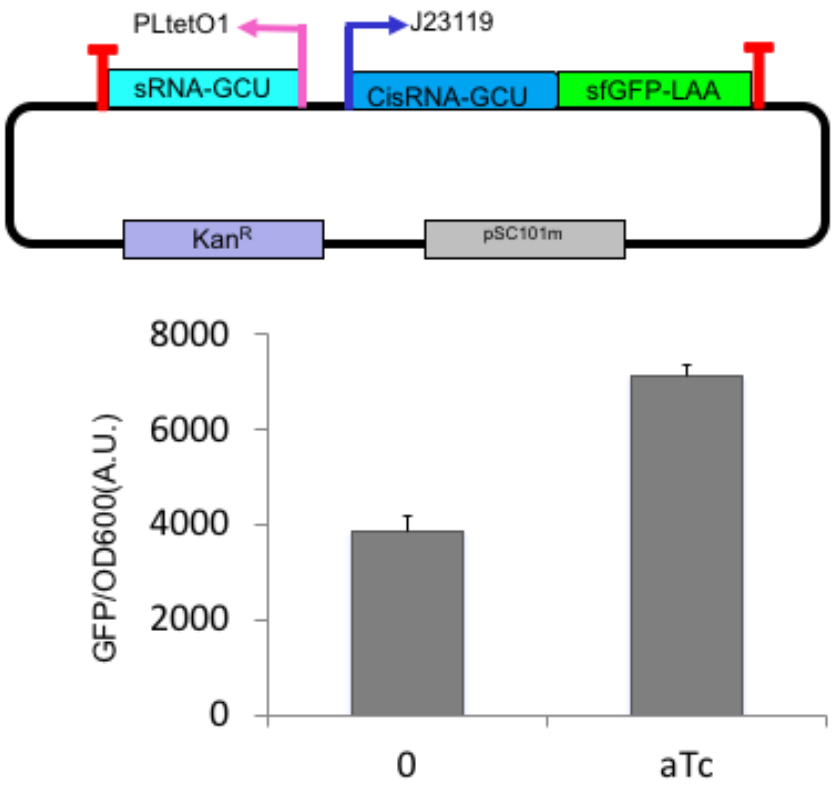


Figure2.7B Design & results of GCU (minimal alpha bates) RNA switch

Study of orthogonality of riboregulator.

In order to engineer a robust system, we studied orthogonality of different small RNAs to each other and the host cell, the orthogonality of cooperative riboregulators and additionally the orthogonality of riboregulators that have been engineered in our laboratory (Rodrigo et al. 2012). The results explain that neither small RNAs have crosstalk between their small RNA and host cell. Additional tested orthogonality of previously engineered riboregulators in our lab (Rodrigo et.al. 2012). The all riboregulator tested. I made two combinations riboregulator RAJ12 tested with all small RNA (sRNARAJ11, sRNARAJ12, sRNARaJ21, sRNA RAJ23 and sRNA31). The 2nd combinations were RAJ31 with all small RNA (sRNARAJ11, sRNARAJ12, sRNARaJ21, sRNA RAJ23 and sRNA31).

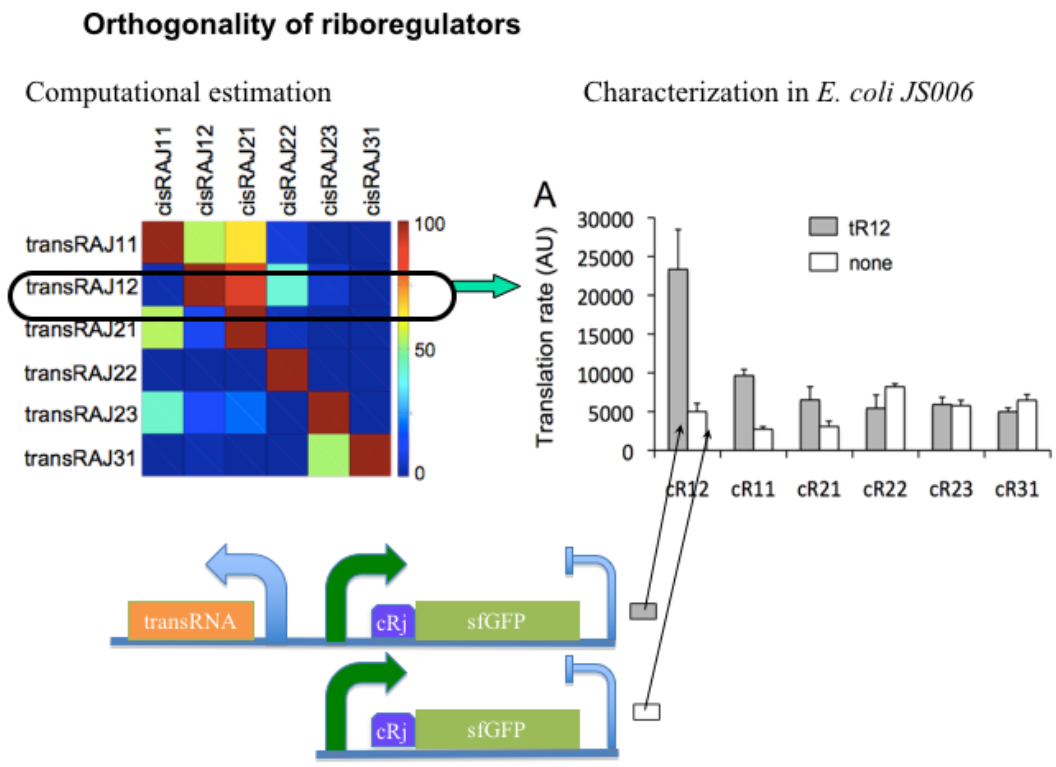


Figure 2. 7 C orthogonality of single component RNA switch12

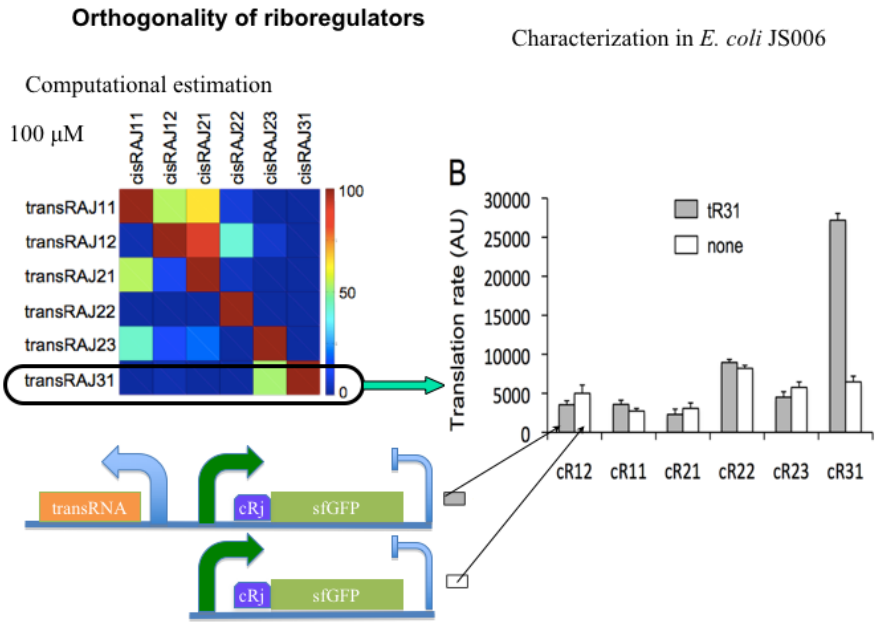


Figure 2. 7 D orthogonality of single component RNA switch 31

Constructing single copy RNA switches in *E. coli*

2.7.1 Abstract

Researchers are becoming more interested in the ability to engineer genetic circuits. These genetic circuits are engineered in multi copy plasmid. This multi copy plasmid is unstable in the cell and increases metabolic burden. The multi copy plasmid has different rates of growth which is dependent on strain-to-strain. We have engineered our genetic circuits in this plasmid and studied the gene regulation at the post-transcriptional level. These plasmids have variable copy numbers which can differ between cells. This fluctuation in the growth rate causes a hindrance in data analysis and the resulting outcomes are not constant between strains. This causes the gene expression analysis to be noisier. Hence, there is a need to develop a system for making genetic circuits in a single copy that will reduce the metabolic burden and variability of data. This could be possible by integration of the genetic circuits into the chromosome of the *E. coli* genome. These circuits, once integrated in the chromosome would have minimum metabolic burden, consistent results and the circuits would be stable in the chromosome without antibiotics.

In this study, we have integrated in house constructed RNA circuits RAJ11 (Rodrigo et al. 2012) into the *E. coli K12* (MG1655Z1) strain. These RNA circuits had studied in multi copy plasmid pSTC1 (Rodrigo et al. 2012) pSC101mutated medium high copy plasmid. The integration of large size circuits in the *E. coli* is challenging and limited methods are available:

1) Lambda based recombineering
2) Phage derived method
3) Cas9 based system.

In the following study we used the Lambda based recombineering method based on the Thomas and Khulman 2010 NAR paper. Chromosomal integration of DNA is thus critical in synthetic biology, biotechnology, and metabolic engineering.

2.7.2 Introduction

Chromosomal engineering allows us to study gene regulation at the single copy level *in vivo* and is therefore an essential application of synthetic biology. Previously developed methods include a two-step recombineering methodology for the integration of large fragments of DNA into the *E. coli* chromosome (Thomas and Cox et al., 2010; François et al.,2013; Pengfei et al., 2015). This integration system includes three plasmids (figure 2.7.1) pTKRED plasmid, which carries λ-RED recombineering enzyme control by LacI regulated promoter and induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG). The plasmid also has I-SecI endonuclease excision enzyme control by pBAD promoter and induced by arabinose and, constitutively expressed Rec-A protein. The endonuclease I-SecI makes double stranded breaks in the *E. coli* chromosome and λ-RED promote Recombineering. The second plasmid (pTK/Cs), which carries 1.3kb size Landing pad tetracycline resistance gene (tetA) flanking with I-secI restriction site. This landing pad was designed to be unique to the *E. coli* chromosome and provide platform for the integration of large fragments of DNA into the chromosome. *YgcE-YgcE* location in chromosome as mentioned by Thomas and Cox (Cox and khulman 2010) was used.

- Site-specific chromosomal integration of large synthetic constructs. Kuhlman, Thomas E and Cox, Edward C

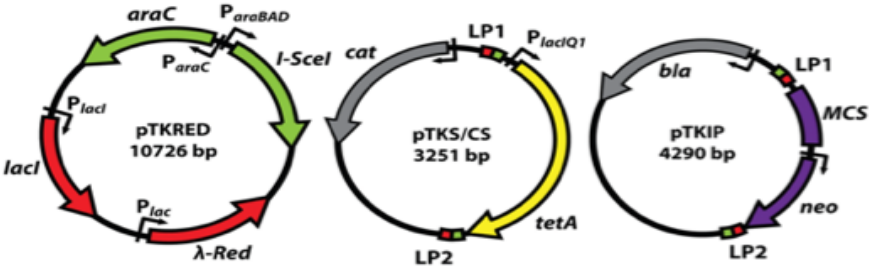


Figure 2.7.1 Plasmid used for chromosome integration (adapted Kuhlman and Cox 2010 NAR)

pTKRED: λred system under the pLac promoter, I-SceI endonuclease under the Para BAD promoter.

pTCS/CS: landing pad, recombination sites and I-SceI restriction sites

pTKIP: MCS where to put the gene of interest, antibiotic resistance

A third donor plasmid (pTKIP), has multiple cloning site. We constructed our house engineered riboregulator RAJ11 (Rodirgo 2012 et, al.,) in donor plasmid pTKIP- RAJ11. This pTKIP-RAJ11 ready to harbors the riboregulator and integrated into the chromosome. In RAJ11 we have previously developed a small RNA riboregulator based gene expression regulatory system in vivo, in which a small trans-RNA hybridizes with the cis-repress 5' UTR of the targeted mRNA to expose the ribosome binding site (RBS) to launch the protein translation. In the following study, we have integrated small RNA regulatory (RAJ11) system into the chromosome of E. coli to study gene regulation at single copy in the chromosomal level. This would enable a better understanding of the regulation mechanism and parameters of the small RNA based gene circuits. In summary, here we have established previously discovered recombineering system, which is a simple technique used to integrate large fragments of DNA into any desired location in the chromosome.

2.7.3 Material and method

2.7.3.1A-Landing pad integration-Competent cell preparation

Plasmids (pTKRED, pTK/CS and pTKIP) were a kind donation from Thomas and Khulman, USA (Department of Molecular Biology, Princeton University, Washington Road, Princeton USA). Electro-competent cells of MG1655Z1 (a modified strain with *E. coli* K12, having laciQ and tetR gene in the chromosome and spectinomycine antibiotic resistance) were cultured following a standard protocol. pTKRED plasmid was transformed in MG1655Z1 electro-competent cells and grown in 30°C temperature. The plasmid pTKRED is a temperature sensitive plasmid and cannot survive at 37°C. The transformation of pTKRED was confirmed by PCR since plasmid and cells were resistant to the same antibiotic. Electro-competent pTKRED-transformed cells were developed. The single colony was inoculated in 2 mL LB media with spectinomycin antibiotics and overnight grown for nearly 16 hours at 30°C. The overnight culture was subcultured in 100 mL LB media in a 1L conical flask with 100-fold dilution. the culture was supplemented with 0.5% w/v glucose and 100 mg/ml spectinomycin and 2mM IPTG after 2

hours of growth. This enabled production of the Lambda red enzyme which increases the efficiency of recombineering. When the optical density (OD) (λ = 600 nm) of the culture reached 0.5–0.6, the cells were placed on ice and washed three times with ice-cold 10% v/v glycerol.

2.7.3.2 Landing pad integration

Plasmid pTKS/CS was used as a PCR template to amplify landing pad fragments using the landing pad regions as standardized priming sites. The forward primer was **ygcE/ygcF S/CS F**, with the following sequence:

5'-
TCAACAGCGTATAGAGGCGGTTATGTAAAACCACTCATTAGCCTCAAAACTACGGCCCC
AAGGTCCAAACGGTGA-3'

The reverse primer was **ygcE/ygcF S/CS R**, with the following sequence:

5'-
GATACAAAAAATTAAATTTAATCAAAGTGTTATTTGTATGATTCTTAAATTTGGCTTCAGG
GATGAGGCGCCATC-3'.

The primers included 50-bp sequence homology for the desired insertion location in the chromosome (Thomas and Khulman 2010). NEB Phusion polymerase (HF) DNA polymerase PCR conditions were as follows: 98°C for 60 seconds, followed by 30 cycles of: 98°C for 35 seconds, 55°C for 45 seconds and 72°C for 45 seconds. The resulting PCR reactions was digested with 1μ DpnI for at least 2 hours at 37°C. The PCR product was run on a 1% agarose gel and purified using a Qiaquick spin column. The 100 μ plasmid pTKED containing competent cells and 300 ng of purified landing pad PCR fragment was electroporated. Additionally, 1μl pTKRED plasmid was added for safe site and increasing recombineering. Mechanism of integration of large fragment.

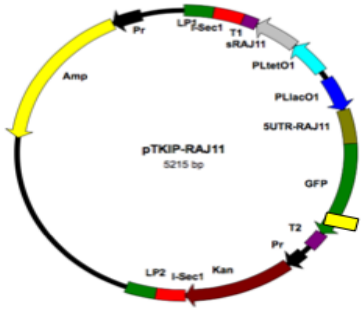


Figure 2.7.2 . pTKIP-RAJ11 plasmid constructed by cloning of RAJ 11 in the pTKIP plasmid.

Mechanism of integration of large fragment.

Following the electroporation, 1 ml SOC/LB medium was added with 2 mM IPTG. After recovery for at least 2 hours, antibiotics were added and the cultures were grown at 30°C for 6 hours. After 6 hours of recovery, 500µl of the culture was plated on LB agar plates containing 10 mg/ml tetracycline, 100 mg/ml spectinomycin and 0.5% glucose and grown overnight at 30°C. The cells were grown overnight at 30°C so that the pTKRED plasmid did not die. The colonies were collected after 24 hours, three colonies were inoculated and grown in LB with specific antibiotics at 30°C.

Following the protocol detailed in the thermo-fisher bacterial genomic DNA isolation kit the genomic DNA of *E. coli* of culture was isolated.

The overnight 1ml bacterial culture was centrifuged in a 1.5ml micro-centrifuge for 10 min at 6000 x g. The bacterial cells were re-suspended in 200µl of TE buffer. 200µl of sample was mixed with 400 µl of lysis solution and incubated at 65°C for 5 min. After incubation, 600 µl of chloroform was added and gently emulsified by inversion (3-5 times) and the sample was centrifuged at 10,000 rpm for 2 min. Precipitation solutions were prepared using 80 µl of supplied 10X concentrated precipitation solution mix (provided in the kit) and mixing 720 µl of sterile deionized water. The upper aqueous phase containing DNA was transferred to a new tube and 800 µl of freshly prepared precipitation solution mix was added. The tube was gently mixed at room temperature for 1-2 min and centrifuge at 10,000 rpm (~9400 x g) for 2 min. The supernatant was removed completely and the dissolved DNA pellet was resuspended in 100 µl of NaCl solution by gentle vortexing. To the tube,

300 µl of cold ethanol was added and the DNA was precipitated (10 min at -20°C) and spun down at 10,000 rpm (~9400 x g), 3-4 min. The ethanol was removed and the pellet was washed once with 70% cold ethanol. The precipitated DNA was resuspended in 100 µl of sterile deionized water by gentle vortexing. (Thermo-fisher genomic DNA isolation kit (K0512)).

The genomic DNA was amplified by PCR with specific verification primers. These primers were nearly 100 bp outward of insertion site in order to ensure the right location of the insertion of the landing pad. The verification primers were:

ygcE/ygcF ver F (Sequence: 5'- CGCTGATGCAGGGAACATAATAAAAAC-3')

ygcE/ygcF ver R (Sequence: 5'-CCCCGCGCCAGCGGGGATAAACCA-3')

Thermo-fisher dream-taq master mix DNA polymerase was used and the PCR conditions were as follows: 95°C for 60 seconds, followed by 30 cycles of: 94°C for 35 seconds, 55°C for 45 seconds and 72°C for 90 seconds. Three colonies were amplified and one of MG1655Z1 genomic DNA was used as a negative control. 10 µl of PCR product was checked on 1% agarose. The positive PCR were purified (Qiagen purification kit) and sent for sequencing for conformation.

2.7.3.3 Construction of the RNA circuit donor plasmid pTKIP-RAJ11

The pTKIP-RAJ11 plasmid was constructed with standard cloning method. The pTKIP plasmid was digested with the enzymes EcoR1 and Spe1. The sRNA cassette in RAJ11 plasmid also has same restriction site. The digested backbone and RNA cassette were both gel purified. The pTKIP backbone was given dephosphorization treatment to prevent self-ligation. The backbone and RNA cassette were ligated with standard method used thermos-fisher kit. The positive clone was confirming by colony PCR and sequencing.

2.7.3.4 Integration of RNA cassette in bacterial genome

Competent cell preparation of landing pad integrated *E. coli* strain

Competent cells were prepared which had the landing pad. Landing pad-integrated clone was grown overnight in the antibiotics spectinomycin and tetracycline. The overnight culture was sub-cultured in 1:100 dilutions and supplemented with 0.5% w/v glucose and 100 mg/ml Spectinomycin and tetracycline. To the culture, 2mM IPTG was added after 2 hours for the synthesis of the lambda red enzyme. Once the OD600 of the culture reached 0.5 the cells were made electro-competent. I PCR amplified an RNA cassette with an additional Landing pad and I-SecI restriction site from pTKIP-RAJ11 Plasmid. One hundred fifty ng of purified PCR product and 1μl pTKED (for safe guard of integration) was transformed in 1ml SOC media with supplemented 2mM IPTG (for synthesis of Lambda RED) and 0.2% W/V L-arabinose. The culture was incubated for 1 hour in a shaking water bath at 37°C and Spectinomycin was then added to the culture tube which was transferred to a 30°C bath. The remaining 500 ml was allowed to recover overnight and plated the next day. Potential integrants were picked onto LB plates supplemented with 34 mg/ml chloramphenicol to screen against colonies which had been transformed with undigested pTKS/CS plasmid. The high level of expression from the PlacIQ1 promoter combined with the significantly decreased growth rate of cells expressing tetA (24) made it easy to distinguish landing pad integrants from higher copy number pTKS/CS transformants, and, consequently, all potential integrants passed this screen. Chromosomal landing pad integrants cannot grow on low nutrient LB agar plates supplemented with fusaric acid (25). Samples were verified by colony PCR across the desired insertion junctions and confirmed by sequencing.

2.7.3.5 Insertion of Raj11 in genome

Plasmid pTKIP has multiple cloning sites, therefore we digested pTKIP and Raj11 with EcoR1 and Spe1. Raj11 ligated into the backbone of pTKIP. The

pTKIP plasmid was then transformed into the landing pad in MG1655Z1 cells. This integration was confirmed by sequencing.

2.7.3.6 Tecan characterization

Before characterization experiments, cells were grown in LB overnight in order to reach the stationary phase. Overnight cells were diluted 200 times and induced with ATC and IPTG inducer. When cells reached an OD between 0.30 to 0.40 they were diluted 200 times in 200 mL of M9 within each well of the plate (Custom Corning Costar 96 well microplate, black transparent bottom with lid). The plate was incubated in an Infinite F500 multi-well fluorometer (TECAN) at 37 °C with shaking (orbital mode, frequency of 33 rpm, 2 mm of amplitude) and assayed with an automatically repeating protocol of absorbance measurements (600 nm absorbance filter) and fluorescence measurements (480/20 nm excitation filter - 530/25 nm emission filter for GFP. The ime between repeated measurements was 15 minutes. All samples were present in triplicate on the plate. Each measurement was repeated 2-3 times on independent days to verify reproducibility in the results. All data were analyzed by MATLAB.

Landing Pad integration

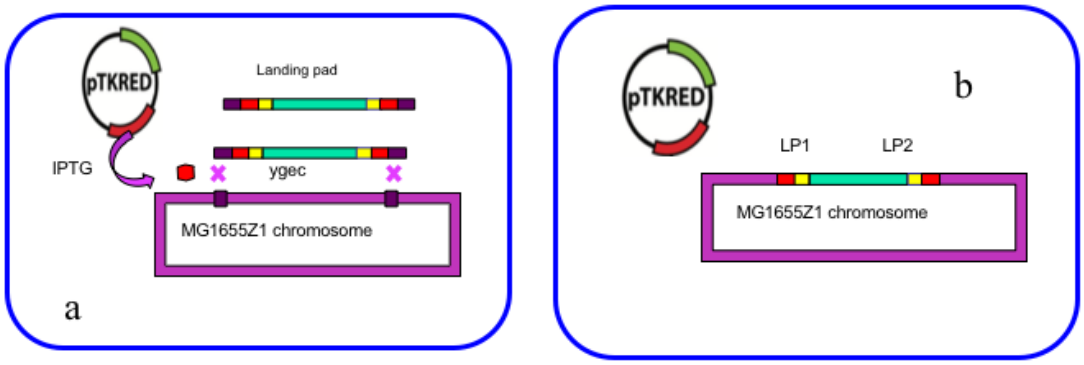


Figure 2.7.3 mechanism of Integration of landing pad in the *E. coli* chromosome (a&b),

(a) mechanism used to integration of landing pad in *E. coli*. The PCR product of Landing pad is transformed in pTKRED bearing *E. coli MG1655Z1* . Small green boxes are I-SceI restriction sites; landing pad regions 1 and 2 are small red boxes labeled LP1 and LP2 respectively.
(b) Annotated sequence of the pTKIP MCS showing LP1, available restriction sites, and the first four bases of the adjacent FRT site.
(c) Strategy for large construct chromosomal integration.
Step 1: the host strain is transformed with the helper plasmid pTKRED,
bearing I-SceI endonuclease (green) and -Red (red). Linear landing pad fragments (yellow) are integrated into the chromosome at the desired location (black squares) when -Red expression is induced by IPTG. Landing pad integration confirmed by PCR and sequencing

Screening of positive landing pad insert

The figure 2.7.5 showing position of ygcE-ygcE in chromosome of *E. coli.* The Gel showing the PCR confirmation. Lane1 – 100 bp Marker. Lane-2,3&4 three clone showing positive band. I used ygcE-ver FW and ygcE ver RW prime nearly outside of ygcE origin in chromosomal showing right position of integration.Lane 5. Negative control (MG1655Z1 cells without landing pad).

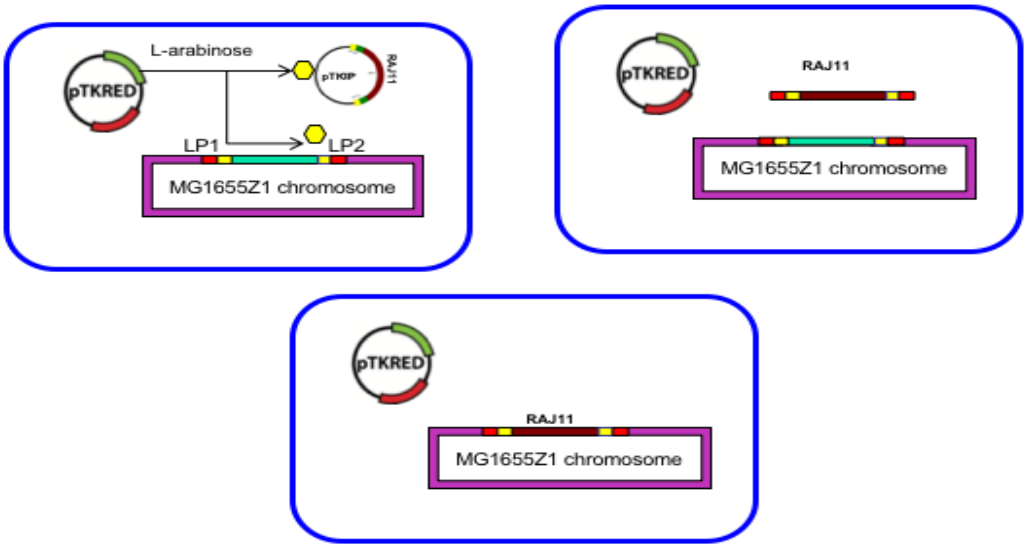


Figure 2.7.4 integration of RNA circuit is *E. coli* Chromosome.
Step 2: The landing pad congaing the host strain is transformed with pTKIP having RAJ11 riboregulator (purple) to be inserted into the *E. coli* genome. Enzyme I-SceI expression is induced via the suppliment of L-arabinose, and the I-SceI recognition sites (green)n the donor plasmid pTKIP-RAJ11 and landing pad containg *E. coli* chromosome are cleaved. The RAJ11 riboregulator also Integration of the fragment is facilitated by IPTG-induced lambda-red expression. I additionally able to transformed PCR product of RAJ11 with I-SceI site . PCR product integrated plasmid does not required curing of plasmid pTKIP- RAJ11. The RAJ11 I
Step 3: pTKRED is cured by growth at 42 C and serial dilution method . Finaly I screen by spectinomycin resistance and confirmed by PCR.

- Site-Specific Chromosomal Integration

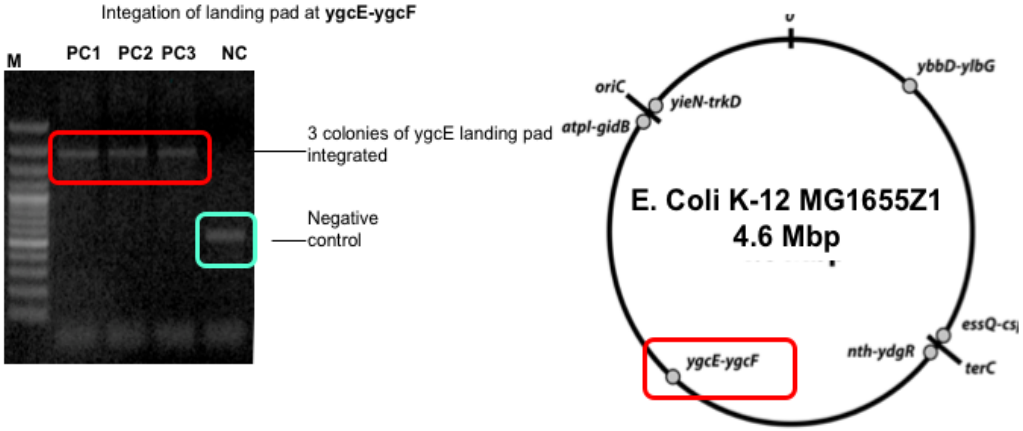
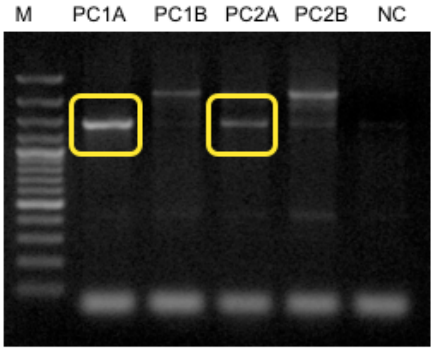


Figure 2.7.5 Landing pad integration in *E. coli* chromosome landing pad by 1% agarose gel.
Landing pad verification primer (ygcE Forward primer and ygcE reverse primer) to confirm actual location of landing pad. I PCR amplified landing pad chromosome and run on 1% agarose gel. I used M marker and three positive clone (PC1, PC2 and PC3) and negative control (*E. Coli* chromosome with out landing pad)
All three positive sample showing integration of landing pad integration positive PCR and negative showing very small band.
This PCR product sequenced and confirmed .

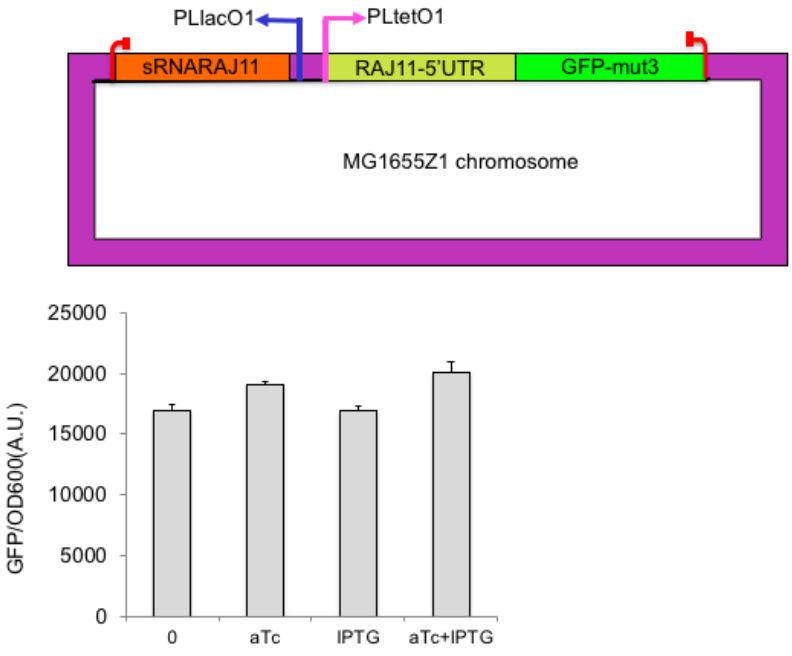
Integration of Raj11 in the *E. coli* chromosome

Integration of RAJ11 in the chromosome of MG1655Z1



M = 100 bp marker
PC1A= positive control with middle primer of clone 1
PC1B= positive control with outward primer of clone 2
PC2A= positive control with middle primer of clone 2
PC2B= positive control with outward primer of clone 2
NC = negative control pcr MG1655Z1

Figure 2.7.6 Verification of RNA circuits integration in chromosome by 1% agarose gel.
The PC1A and PC2A are two clone with two middle primer and PC1B and PC2B are amplified with outward primer chromosome.



Results of RAJ11 chromosome of *E.coli.* MG1655Z

Rodrigo, Major and Prakash et. al. Biophysical J. 2015

2.7.7Results and discussion. The figure 2.7.7 showing fluorimeter characterization results in four conditions (none, aTc, IPTG and aTc+IPTG).

The activation of GFP is not shown. The conclusion is that RNA has a very sort life period. The single copy RNA is degraded before reaching to the target mRNA.

The characterization data of these results, I collaborate this data with Dr. Rodrigo (ICMP, Valencia Spain.) and he develop a biophysical model and we publish in Biophysical journal 2015.

Adapted from JMB paper.

Functionalization of antisense RNA.

Antisense RNA has a potential role in posttranscriptional regulation of gene. The antisense oligo was used as to control growth of bacteria. The antisense technology (RNA interference) is most common to regulate gene expression in eukaryotes. In our system antisense regulation depends on polarity of DNA sequence. Our system able to transcribe two sRNA from same DNA template depends on polarity of of DNA template and direction of promoter. We engineer a system that working as an antisense and we validate in *In-vivo* as well *In-vitro*. To this end, we focused on DNA sequences that produce transcripts that are not translated but are able to perform a function in the cell (i.e., regulatory RNAs), (Storz et al., 2009). In particular, we considered a scenario of an antisense small RNA as pre-existing gene, a transcript able to sequester a functional sRNA in *trans*. This antisense regulatory mechanism has already been described in prokaryotes (Georg and Hess 2011) and eukaryotes (Ebert et al., 2007) and this work will show that an antisense small RNA may acquire an additional ability to directly control gene expression. We present a synthetic case in *Escherichia coli*, where the two-transcribed small RNAs, one the reverse complement of the other, can independently activate gene expression and repress each other when both are present.

The sRNA antiRAJ11 was designed as the reverse complement of the sRNA RAJ11 (without accounting for transcription terminators). Then, using RiboMaker (Rodirgo and Jaramillo 2014), a computational approach following certain energetic and structural criteria we designed a 5' UTR

sequence that is able to *cis*-repress the RBS (and block translation) and to be *trans*-activated by the new riboregulator antiRAJ11. The RNA sequences are shown in table1. The heuristic algorithm performs multiple cycles of random mutations and selection, and each run produces a different sequence. Hence, we ran the RiboMaker software multiple times and selected the best sequence according to the objective function. Note that it is not possible to construct the 5' UTR antiRAJ11 as the reverse complement of the 5' UTR RAJ11 because it would lack a suitable RBS sequence. Moreover, riboregulatory systems require a precise secondary structure, followed by toehold formation, which is not maintained after a reverse complementation operation due to the existence of GU wobble pairs.

We used inducible promoters (Lutz and Bujard 1997) to dynamically control the expression of our system with isopropyl-β-D-1-thiogalactopyranoside (IPTG) and anhydrotetracycline (aTc). The design of the full expression cassette (including the promoters and terminators of the sRNA and mRNA) was performed according to the protocol previously published (Rostain et al., 2015) The characterization of the system *in vivo* at the population level revealed a high dynamic range Figure. 2.2 antiraj11 results. We then tested the ability of the riboregulator RAJ11 to inhibit the action of the riboregulator antiRAJ11, as they hybridize perfectly with each other. When co-expressing both riboregulators, we observed a remarkable decrease in GFP expression Figure. 2.8. We also tested the ability of the riboregulator antiRAJ11 to inhibit the action of the riboregulator RAJ11, obtaining a reduction in GFP expression but less substantial than in the previous case Figure. 2.9.

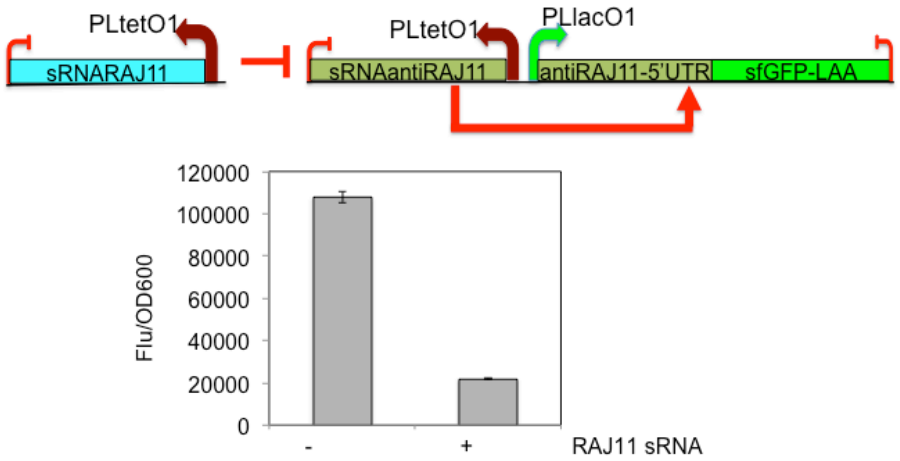


Figure 2.8 activation of antisense riboregulator(-sense strand) and repression with sRNARAJ11

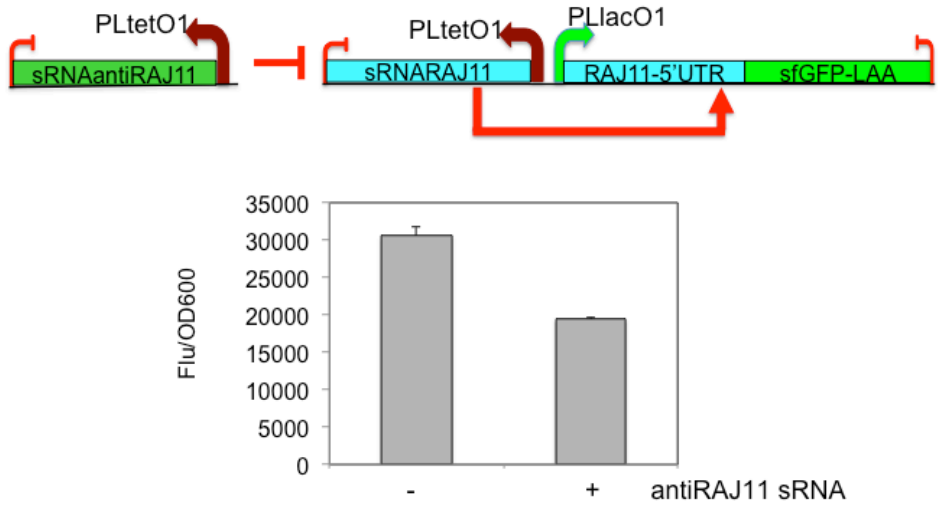


Figure 2.9 activation of sense riboregulator and repression with sRNAantiRAJ11

Using *in vitro* translation, where the complementary DNAs (cDNAs) corresponding to the RNA species were first transcribed *in vitro*, we also proved the dynamic behavior of the new system antiRAJ11, and that the sRNA RAJ11 can inhibit it Figure. 2.10. Consequently, it turns out that the appropriate activation of the target genes (genes regulated either by the riboregulators RAJ11 or antiRAJ11) could require non-simultaneous expression regimes of the riboregulators.

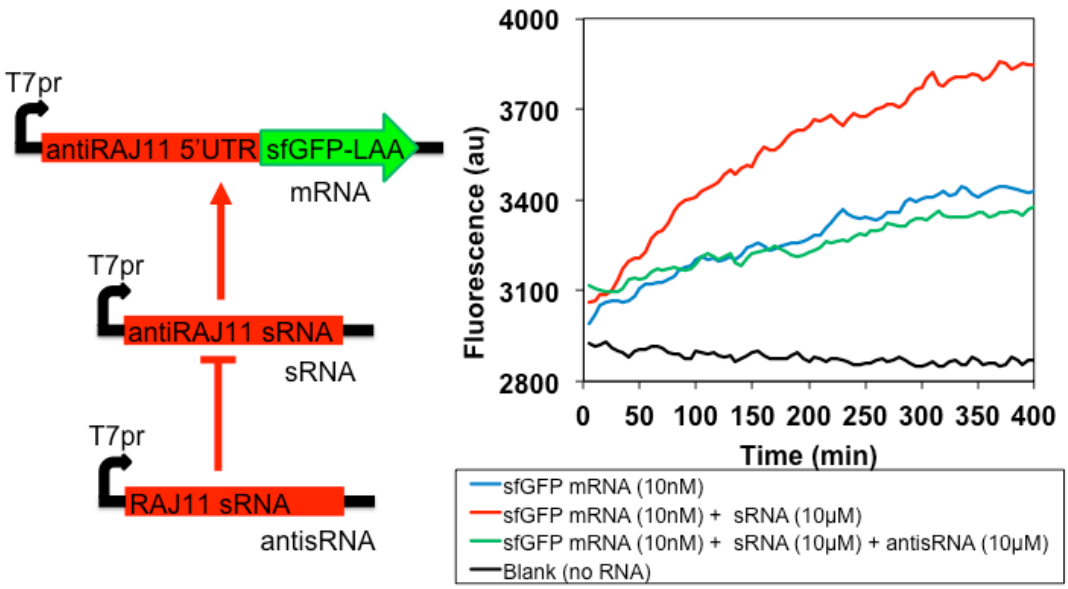


Figure 2.10 sRNA-mediated activation and deactivation of mRNA translation *in vitro*

(Note figure 2.10 adapted from Guillermo and Prakash JMB 2016)

In addition, we studied to what extent one riboregulator can affect the targets of the other (i.e., if they are orthogonal). For this, we measured the change in GFP expression from the mRNA controlled by the non-cognate 5' UTR in presence or absence of the sRNA (i.e., crossed systems). We found that the riboregulator antiRAJ11 has no significant impact on the GFP controlled by the 5' UTR RAJ11 and the same applies for the riboregulator RAJ11 on the GFP controlled by the 5' UTR antiRAJ11. We also found that in both cases the 5' UTRs are very efficient at repressing translation. Simulations with an RNA physicochemical model (Hofacker et al.,) revealed no significant free energy of hybridization between the non-cognate sRNAs and mRNAs, supporting the orthogonal behavior. Finally, and to get mechanistic insights, we performed a native polyacrylamide gel electrophoresis (PAGE; details in material and methods), where the cDNAs were again first transcribed *in vitro*. We mixed two species per lane, with the amount of each

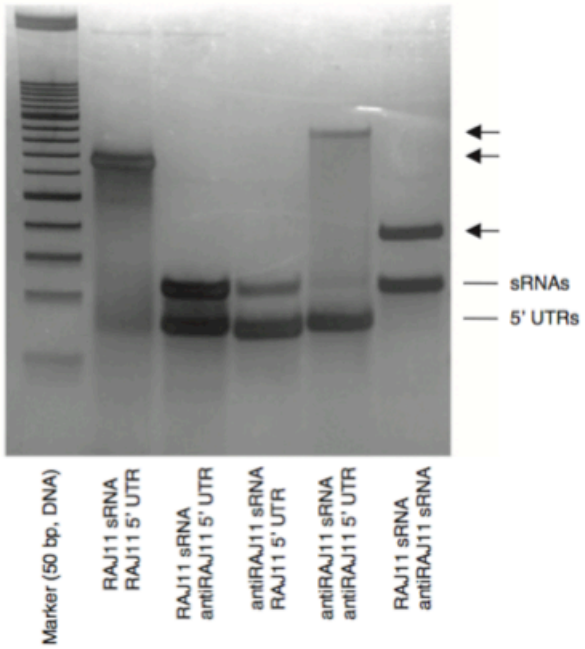


Figure 2.11 In-vitro RNA-RNA interaction PAGE gel

RNA adjusted to introduce the same. The gel revealed the intermolecular interactions between the sRNA and 5' UTR of systems RAJ11 and antiRAJ11, as well as the interaction between the two riboregulators. No interaction was detected for the non-cognate pairs.

Functionalization of an antisense small RNA

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Évry, France.
 # Equal contribution.

Materials and Methods

Computational sequence design

The sequence of the RAJ11 sRNA was used as a template [1]. The sequence of the antiRAJ11 sRNA was obtained as the negative-sense strand (reverse complement) of the RAJ11 sRNA (excluding the transcription terminator). In our original designs, the RAJ11 sRNA has the B0015 terminator, and the antiRAJ11 sRNA the T500 terminator (see ref. [2] for details). In order to design the sequence of the 5' UTR regulated by the antiRAJ11 sRNA, we used the web-based software RiboMaker [3] to automatically design regulatory RNAs that exploit conformational changes to control gene expression. It uses a Monte Carlo simulated annealing optimization algorithm [4] and the ViennaRNA package [5] to compute the free energies and secondary structures of different species in the system. Here, the system comprised three RNA molecules: the small RNA (sRNA), the messenger RNA (mRNA, with the 5' UTR), and the interacting complex. To implement this algorithm, we constructed a physicochemical model based on free energies and RNA structures that included the energies of activation and hybridization corresponding to the interaction between the antiRAJ11 sRNA and its cognate 5' UTR. The model also accounted for the degree of repression and release of the ribosomal binding site (RBS) within the 5' UTR intramolecular and intermolecular structures. Successive rounds of random mutations over the 5' UTR sequence were applied and selected with an energy-based objective function (Fig. S1). Designed sequences are shown in Fig. S2.

Ribomaker was run several times to design the 5' UTR responding to the antiRAJ11 sRNA. The heuristic algorithm performs multiple cycles of random mutations and selection, and each run produces a different sequence. The objective function accounts, on one side, on the *cis*-repression of the 5' UTR, and, on the other, on the interaction ability with the sRNA. Ribomaker outputs the values of effective free energies (to be minimized) corresponding to all partial objectives; and the global objective function is a sum of these free energies. From all sequences designed, we selected the one with minimal value of objective function.

In vitro RNA–RNA interaction

We first constructed the complementary DNAs (cDNAs) of the different RNA species of the designed system to then perform in vitro transcription. We analyzed the systems RAJ11 and antiRAJ11. We considered the sRNAs without transcription terminators and the 5' UTR until the start codon. Amplification by polymerase chain reaction (PCR) using Phusion DNA polymerase (Thermo Scientific) was done over the template plasmids. The PCR products were cloned into the plasmid pUC18, where the restriction site Eco31I was previously removed. The resulting plasmids with inserts were selected by DNA cleavage with appropriate restriction enzymes. Sequences were also verified by sequencing.

In order to perform *in vitro* transcription, 3 µg of each pUC18-derived plasmid was digested with Eco31I and purified with silica-based columns (Zymo). We used approximately 1 µg of digested plasmid in the reaction. The reaction mixture (20 µL) contained 10 µL of plasmid, 2 µL of 10x buffer (Roche), 0.4 µL 10 mM DTT, 1 µL 10 mM NTPs (Thermo Scientific), 0.5 µL Ribolock (~40 U/µL, Thermo Scientific), 1 µL inorganic pyrophosphatase (0.1 U/µL, Thermo Scientific), 1 µL T7 RNA polymerase (50 U/µL, Epicentre), and 4.1 µL H_2O. We incubated the mix for 1 h at 37 ºC and then added 20 µL of loading buffer with formamide. The samples were heated at 95 ºC for 1.5 min, cooled on ice, and subsequently separated by polyacrylamide gel electrophoresis (PAGE) in a 10% polyacrylamide gel, containing 8 M urea and 1x TBE (200 V, 2.5 h). The bands corresponding to the full-length RNAs were cut for purification. The presence of RNA was confirmed by loading a small part of the purified preparations in another polyacrylamide gel.

For the RNA–RNA interactions, we used approximately the same amount of RNA for each of the transcripts (50 ng). The buffer of the reaction was 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 20 mM NaCl. The mix (20 µL) was denatured (1.5 min at 95 ºC) and slowly cooled (15 min at room temperature). We then added 1.5 µL glycerol (87%) and 0.2 µL bromophenol blue–xylene cyanol (100x) to load the gel (15% polyacrylamide, TAE (tris, acetate, and ethylenediaminetetraacetic acid) buffer, 1-mm thick), which was run for 2 h at 75 mA at 4 ºC. The gel was stained first with ethidium bromide

and then with silver.

In vitro translation

PCR was used to prepare DNA for *in vitro* transcription. T7 promoter was added upstream of the expression cassettes consisting of the small RNAs (RAJ11 and antiRAJ11 trans elements), or the 5' UTRs (RAJ11 and antiRAJ11 cis elements) and fluorescent gene (sfGFP). The primers and plasmid templates used are listed below:

	PCR product	Primer (forward)	Primer (reverse)	Template DNA	Amplicon size
1	RAJ11 sRNA	T7RAJ11_FW 5'-TAATACGACTCACTATA GGGAGGGTTGATTGTGTGAG-3'	T7RAJ11_RW 5'-GCCTCGCATAAATCTGTCA CAG3'	pMIR05 (this study)	88 bp
2	antiRAJ11 sRNA	T7antiRAJ11_FW 5'-TAATACGACTCACTATAGG GCCTCGCATAAATCTGTCACAG-3'	T7antiRAJ11_RW 5'-GGGAGGGTTGATTGTGTA AGTC-3'	pMIR03 (this study)	90 bp
3	antiRAJ11 mRNA	T7antiRAJ11GFP_FW 5'-TAATACGACTCACTATAGGGA GAGGGAGGGTTCTGTCACT TAATTATTCG-	GFP_RW 5'-AGCGTAATAACTGCAGGAGT CACTAA-3'	pMIR03 (this study)	856 bp

RNA was prepared by transcription using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific #K0441) and template DNA was subsequently removed by DNase I digestion according to the manufacturer's protocol. RNA was purified by extraction once with acid phenol:chloroform (1:1) and twice with chloroform:isoamyl alcohol (24:1). The purified RNA was used for in vitro translation using the PURExpress In Vitro Protein Synthesis Kit (New England Biolabs #E6800S). Each mRNA and sRNA was used at end concentration of 10 nM and 10 μM, respectively. The reaction volumes were scaled down to 10 μL each and prepared at 4 °C. The reactions were incubated at 37 °C, and green fluorescence was monitored using the Mx3005P qPCR System (Agilent Technologies) with filter set FAM/SYBR Green I (492 nm - 516 nm) (Fig. S8).

Supplementary Figures

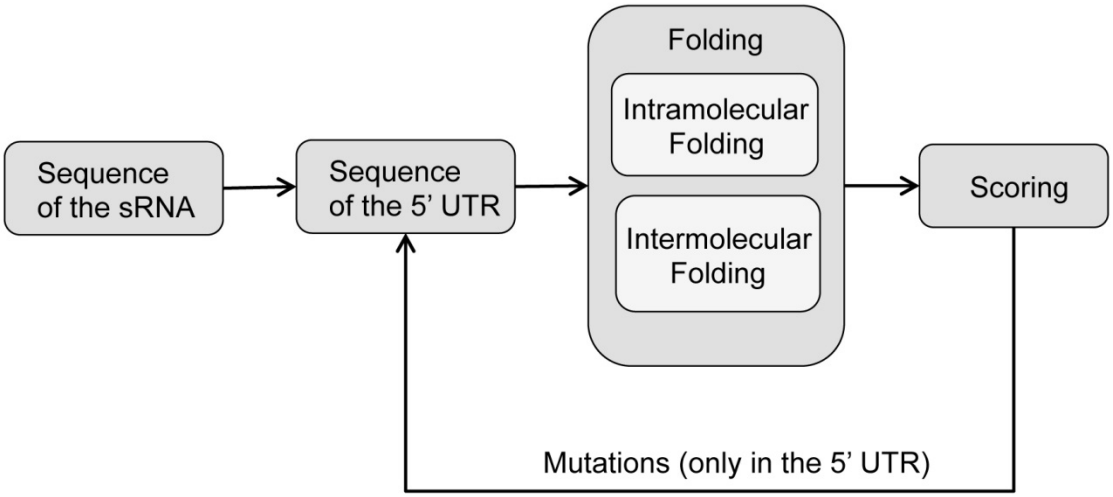

Figure S1: Scheme of the optimization loop to obtain a 5' UTR sequence able to interact with the antiRAJ11 sRNA, whose sequence is kept fixed.

```
RAJ11 sRNA
GGGAGGGTTGATTGTGTGAGTCTGTCACAGTTCAGCGGAAACGTTGATGCTGTGACAGATTTATGCGAGGC

RAJ11 5'UTR
CCTCGCATAATTTCACTTCTTCAATCCTCCCGTTAAAGAGGAGAAATTATGAATG

antiRAJ11 sRNA
GCCTCGCATAAATCTGTCACAGCATCAACGTTTCCGCTGAACTGTGACAGACTCACACAATCAACCCTCCC

antiRAJ11 5'UTR
GGGAGGGTTCTGTCACTTAATTATTCGAGGCAATCGTTAGGAGATAGAGCATATG
```

Figure S2: Sequences of the RNA species.

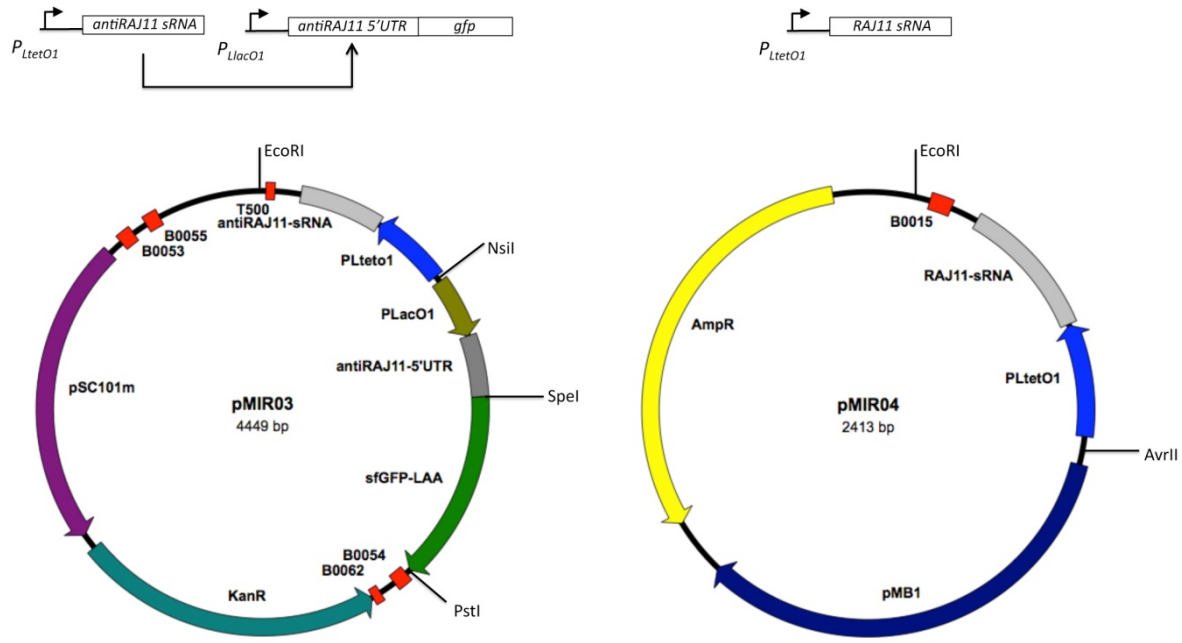

Figure S3: Maps of the plasmids used in this work for expressing the designed sRNA

systems.

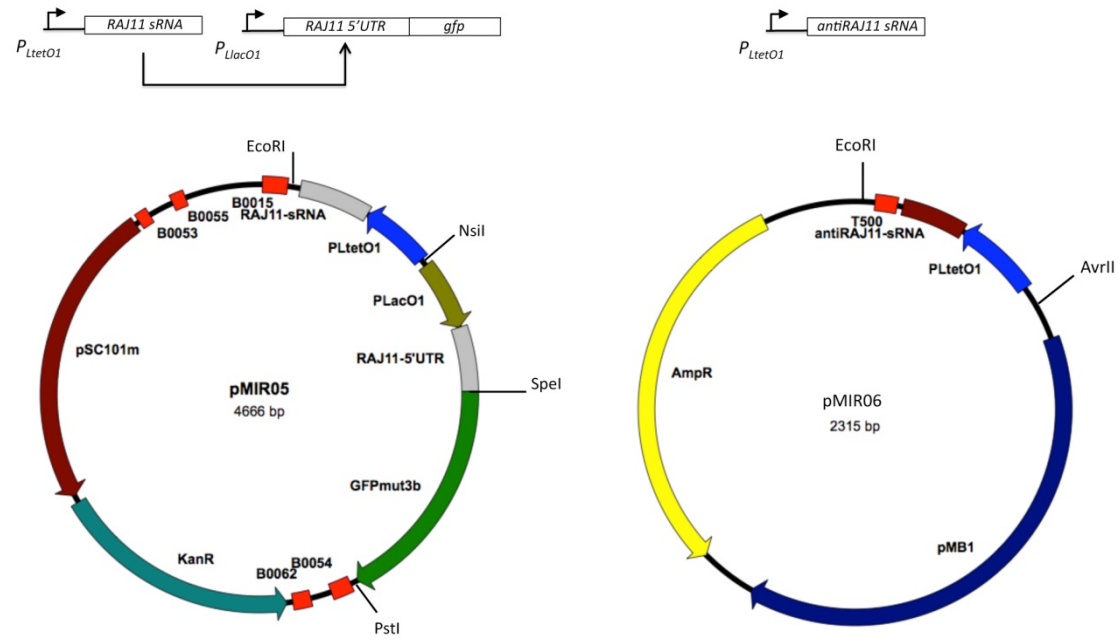

Figure S4: Maps of the plasmids used in this work for expressing the designed sRNA

systems.

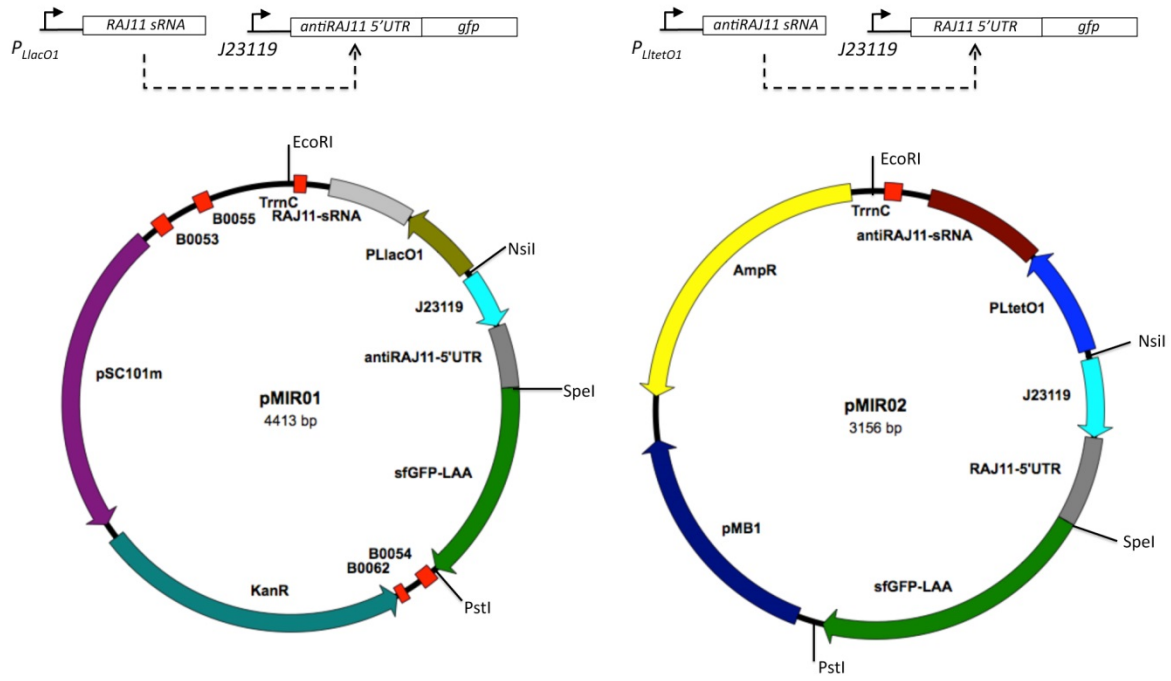

Figure S5: Maps of additional plasmids used in this work for studying the orthogonality of the designed sRNA systems.

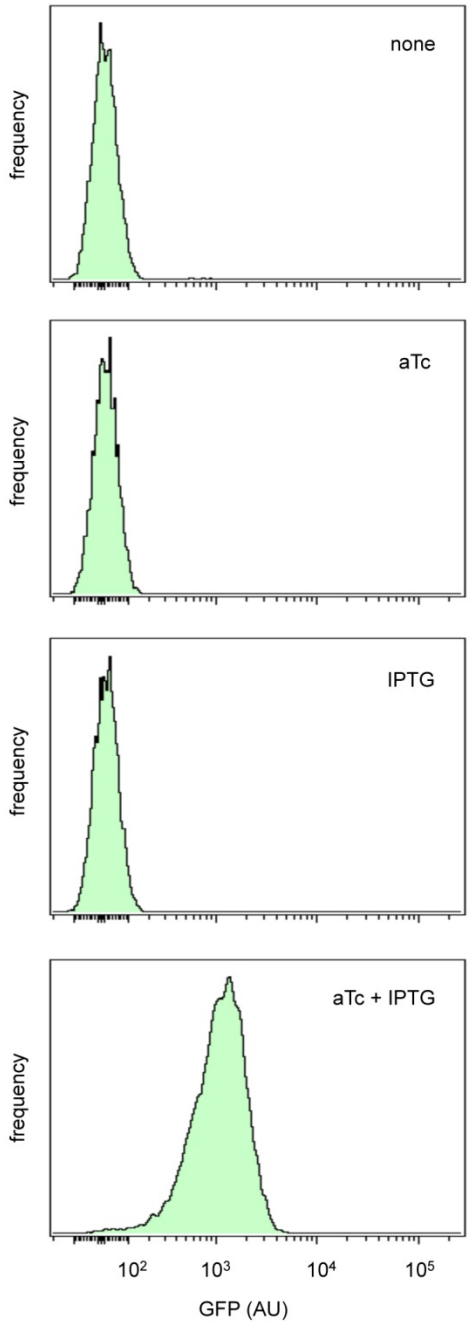

Figure S6: Characterization result of antiRAJ11 system by flow cytometry which shows the behavior at the single-cell level.

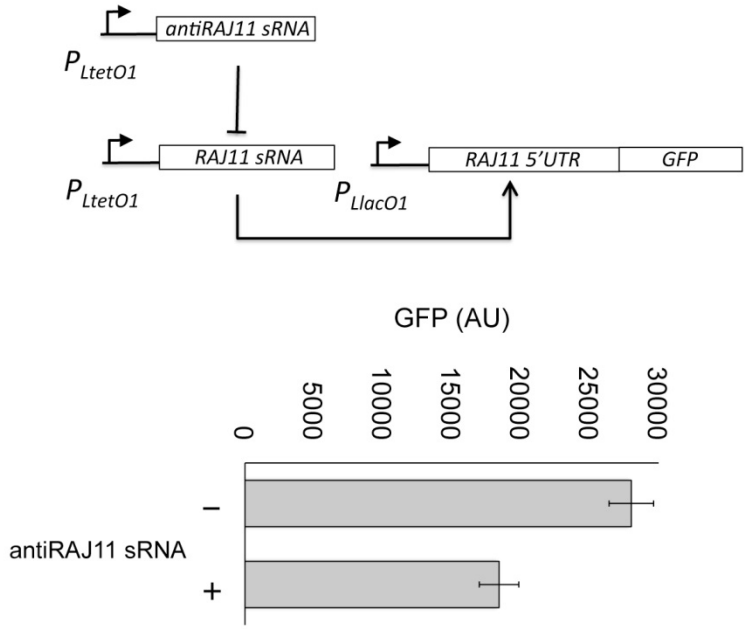

Figure S7: Scheme of the regulatory circuit and characterization result of antiRAJ11 sRNA inhibition of the action of RAJ11 sRNA. Here, + (or −) means that the antiRAJ11 sRNA is (or is not) present (introduced with a different plasmid). Assays performed in JS006 cells.

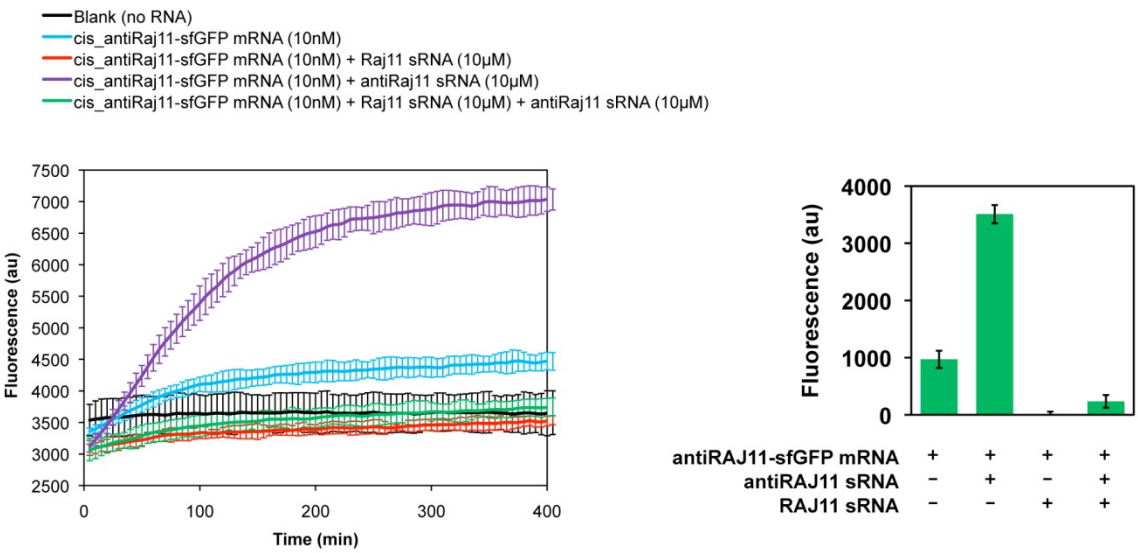

Figure S8: On the left, dynamics of in vitro translation (three replicates) for different combinations of RNAs (pre-transcribed). On the right, stationary behavior (background subtraction).

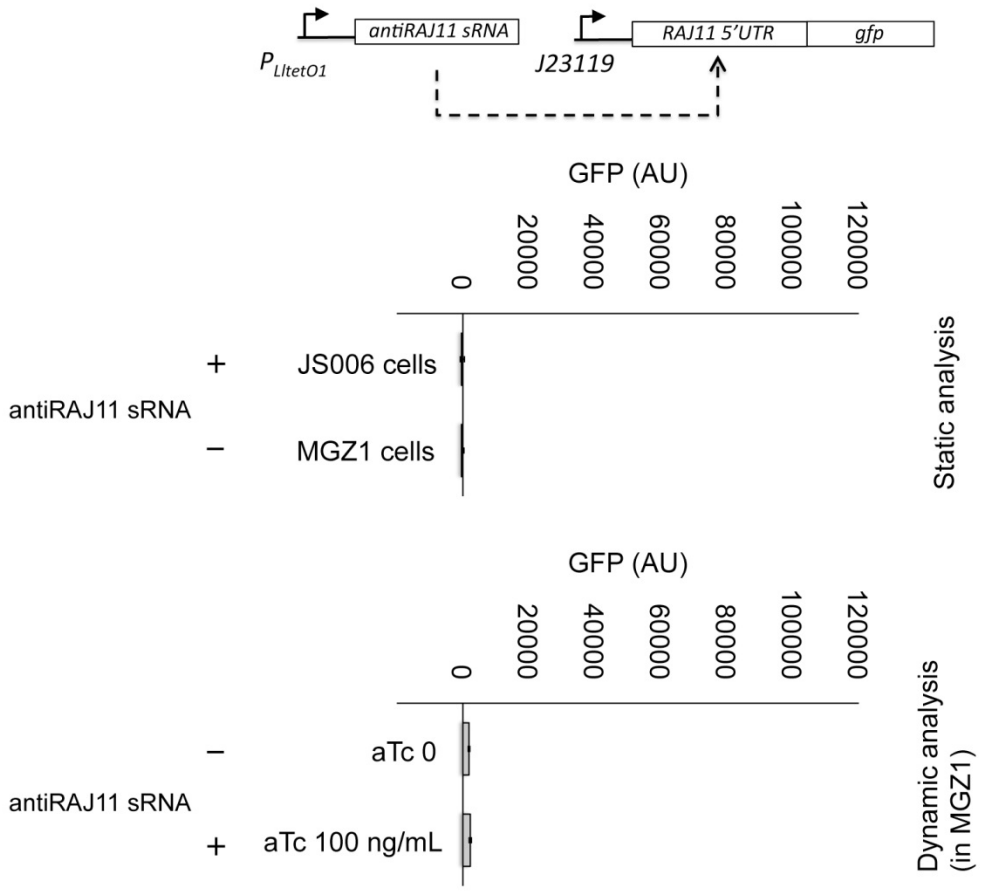

Figure S9: Scheme of the regulatory circuit and characterization result of antiRAJ11 sRNA transactivation of cis-repressed GFP by RAJ11 5' UTR. + stands for an assay where the sRNA is expressed (performed in JS006 cells or with aTc in MGZ1 cells), while – for an assay where the sRNA is highly repressed (performed in MGZ1 cells).

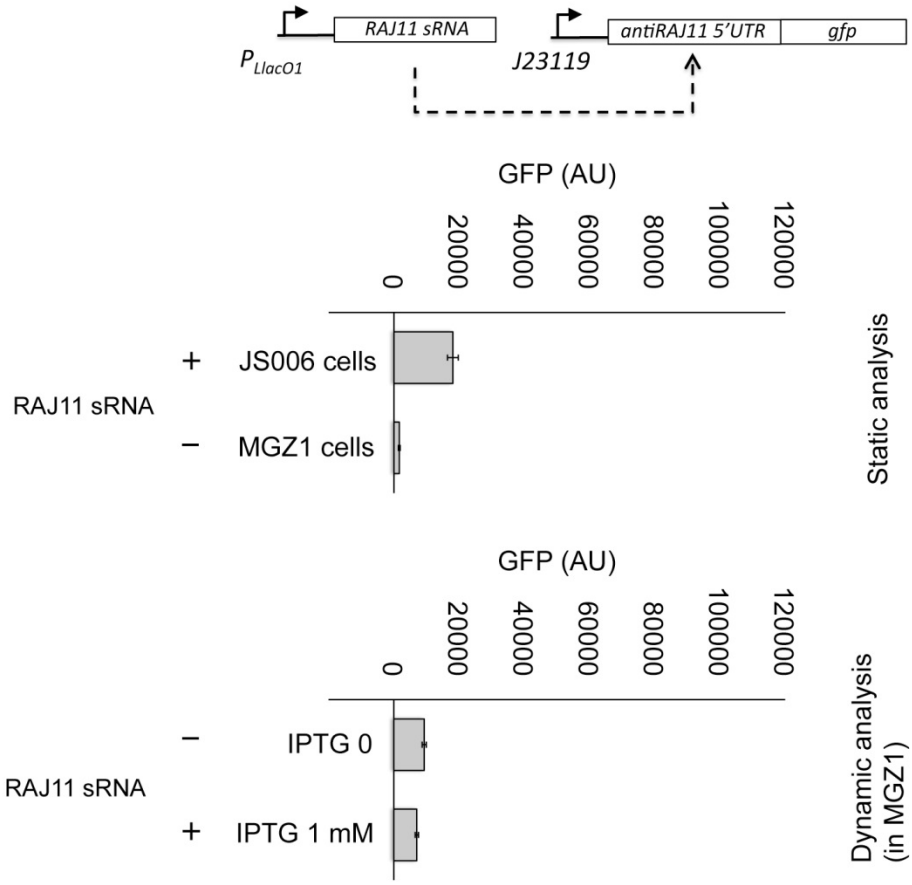

Figure S10: Scheme of the regulatory circuit and characterization result of RAJ11 sRNA transactivation of cis-repressed GFP by antiRAJ11 5' UTR. + stands for an assay where the sRNA is expressed (performed in JS006 cells or with IPTG in MGZ1 cells), whilst – for an assay where the sRNA is highly repressed (performed in MGZ1 cells).

PCR was used to prepare DNA for *in vitro* transcription. T7 promoter was added upstream of the expression cassettes consisting of the small RNAs (RAJ11 and antiRAJ11 trans elements), or the 5' UTRs (RAJ11 and antiRAJ11 cis elements) and fluorescent gene (sfGFP). The primers and plasmid templates used are listed below:

	PCR product	Primer (forward)	Primer (reverse)	Template DNA	Amplicon size
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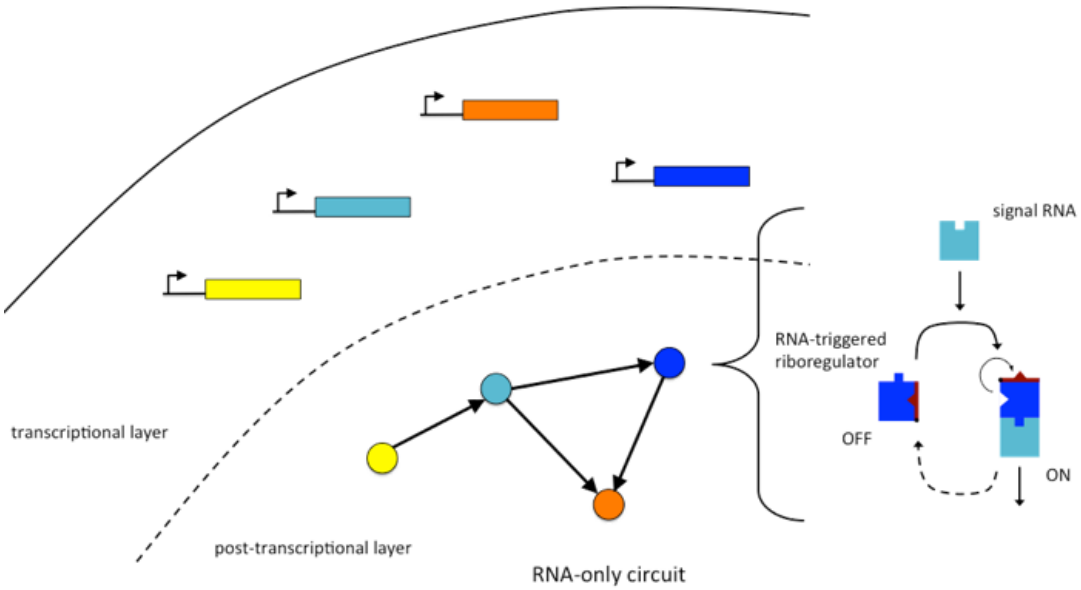
2	antiRAJ11 sRNA	T7antiRAJ11_FW 5'-TAATACGACTCACTATAGG GCCTCGCATAAATCTGTCACAG-3'	T7antiRAJ11_RW 5'-GGGAGGGTTGATTGTGTA AGTC- 3'	pMIR03 (this study)	90 bp
3	antiRAJ11 mRNA	T7antiRAJ11GFP_FW 5'-TAATACGACTCACTATAGGGA GAGGGAGGGTTCTGTCACT TAATTATTCG- 3'	GFP_RW 5'-AGCGTAATAACTGCAGGAGT CACTAA-3'	pMIR03 (this study)	856 bp

RNA was prepared by transcription using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific #K0441) and template DNA was subsequently removed by DNase I digestion according to the manufacturer's protocol. RNA was purified by extraction once with acid phenol: chloroform (1:1) and twice with chloroform: isoamyl alcohol (24:1). The purified RNA was used for in vitro translation using the PURExpress In Vitro Protein Synthesis Kit (New England Biolabs #E6800S). Each mRNA and sRNA were used at end concentration of 10 nM and 10 μM, respectively. The reaction volumes were scaled down to 10 μL each and prepared at 4 °C. The reactions were incubated at 37 °C, and green fluorescence was monitored using the Mx3005P qPCR System (Agilent Technologies) with filter set FAM/SYBR Green I (492 nm - 516 nm) (Fig. S8).

Chapter 3

Engineering *trans*-activating multi-component riboswitches



Schematic of Multicomponent RNA switch

3.1 Abstract

Living cells rely on small non-coding RNAs (sRNAs) to regulate gene expression at the post-transcriptional level. Contrary to most protein-based activators of transcription, the riboregulators, known till date, do not exploit cooperative binding mechanism to activate gene expression. We conceived a general strategy to design trans-activating multicomponent riboswitches by programming a hierarchical toehold activation cascade, which we implemented to develop a *de novo* sequence design algorithm. We engineered different trans-activating multicomponent riboswitches systems relying on the conditional formation of a heterotrimeric species. We characterized the specificity of each RNA-RNA interaction *in vitro* and the cooperative activation of gene expression in *Escherichia coli*. As we only rely on a biochemical model to compute allosteric regulation, our strategy could be applied to reach more complex RNA-based nanostructures regulating gene expression for synthetic biology applications.

3.2 Introduction

Trans-activating multicomponent riboswitches (Cooperative riboswitches) interactions at the molecular level are crucial factors for the living systems in order to sustain complex behaviors (Strogatz 2001). Indeed, the control mechanisms of gene expression found in higher organisms (e.g., mammals) present many cellular factors at play and are highly combinatorial (Ravasi et al. 2010), but most of these are limited to protein interactions. As nucleic acids are molecules with more programmable interactions than proteins, they prove to be better material for nanotechnology to engineer complex nanostructures, conceived by designing interactions using toehold-mediated strand-displacement reactions (Yin et al. 2008, Afonin et al 2010). However, RNAs have not yet been engineered to control gene expression in a sophisticated manner in living cells.

In this study, I considered RNA as a substrate to design and characterize cooperative riboregulators of gene expression, exploiting a physicochemical model involving free energies and secondary structures. Previous work in our lab (Jaramillo lab) on the *de novo* design of synthetic regulatory RNAs has

allowed the engineering of new systems to control protein expression in living cells (Isaacs et al., 2004; Tucker and Breaker, 2005; Blount and Breaker, 2006; Rodrigo et al., 2012; Na et al., 2013). Here, I went beyond by designing obligate heterodimers of sRNAs activating the initiation of translation. There are one reports (Green and Collins 2014) till date that reports natural or synthetic riboregulators to be able to regulate gene expression in microbes, cooperatively. Although, there are a few studies on higher organisms, regarding the synergistic repression of microRNAs with target sites optimally separated (between 8 and 40 nucleotides) (Grimson et al., 2007). The present piece of work, in addition illustrates the designability of synthetic Trans-activating multi-component riboswitches, provides experimental evidence for combinatorial mechanisms employed by microbial riboregulome.

To design Trans-activating multi-component riboswitches, we exploited the facts that *i*) RNA-RNA interactions are kinetically dominated by a toehold-mediated mechanism (i.e., interacting regions remain unpaired within the intramolecular structures of the species), *ii*) the ribosome-binding site (RBS) of a given mRNA can be considered as a toehold mediating the its interaction with the ribosome, and *iii*) activating toeholds in cascades implement a hierarchical assembly of higher-order complexes (Rodrigo et al., 2012). We propose programming RNA species holding hidden/inactive toehold domains that become active after a conformational change following a binary interaction. Once the toehold is active, the complex is able to interact with a third RNA specie. The energy landscape associated with our hypothesized hierarchical interaction mechanism is shown in Figure 3.2.

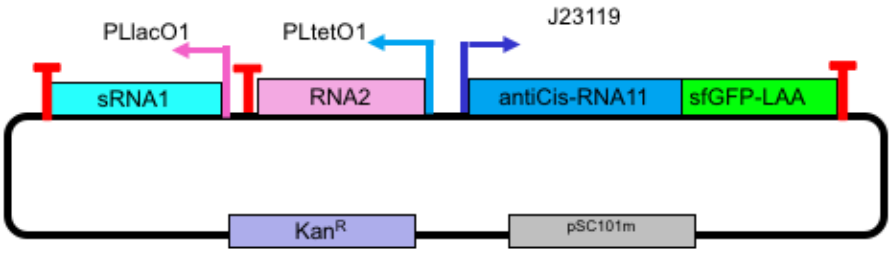


Figure 3.1 Design of a trans-activating multicomponent riboswitches plasmid. The sRNA1 (coop A) is controlled by PLlacO1 promoter and sRNA2 (coopB) is regulated by PLtetO1 promoter. The Cis-repress 5'UTR (coopU) is under the control of a constitutive promoter, BBa_J23119. The super-folder GFP with degradation tag fused with the 5' UTR.

To control the gene expression, we used a *trans*-acting riboswitch, able to activate a *cis*-repressed 5'UTR of a given messenger RNA (mRNA), via allosteric regulation (Isaacs et al., 2004; Rodrigo et al., 2012; Na et al., 2013). We identified different conformational states and their free energy levels, which could be predicted with a physicochemical RNA model (Hofacker et al., 1994; Mathews et al., 2004; Wright et al., 2014). The reaction coordinate was defined as the number of intermolecular hydrogen bonds (or base pairs), on one side, between the two sRNAs (sRNA1-coopA and sRNA2-coopB, hereafter) and, on the other side, between the resulting sRNA complex and the 5' UTR of the target mRNA (coopU, hereafter). On the energy landscape, two barriers (free energies of activation; $\Delta G_1^{\#}$and $\Delta G_2^{\#}$) impinge the progression of the reaction, one for each intermolecular interaction that defines the reaction coordinate. These free energies of activation are associated with the degree of exposition of the toeholds to the solvent, and shall be low (i.e., three or more nucleotides shall be unpaired in the toehold (Rodrigo et al., 2013;) to permit the initiation of the reaction (kinetic aspect). In addition, for an efficient reaction, the free energies of hybridization (ΔG_1 and ΔG_2) have to be as low as possible (at least, lower than -15 Kcal/mol (Rodrigo et al., 2012;) to ensure irreversibility in the intermolecular interactions (thermodynamic aspect) (Laidler et.al., 1983).

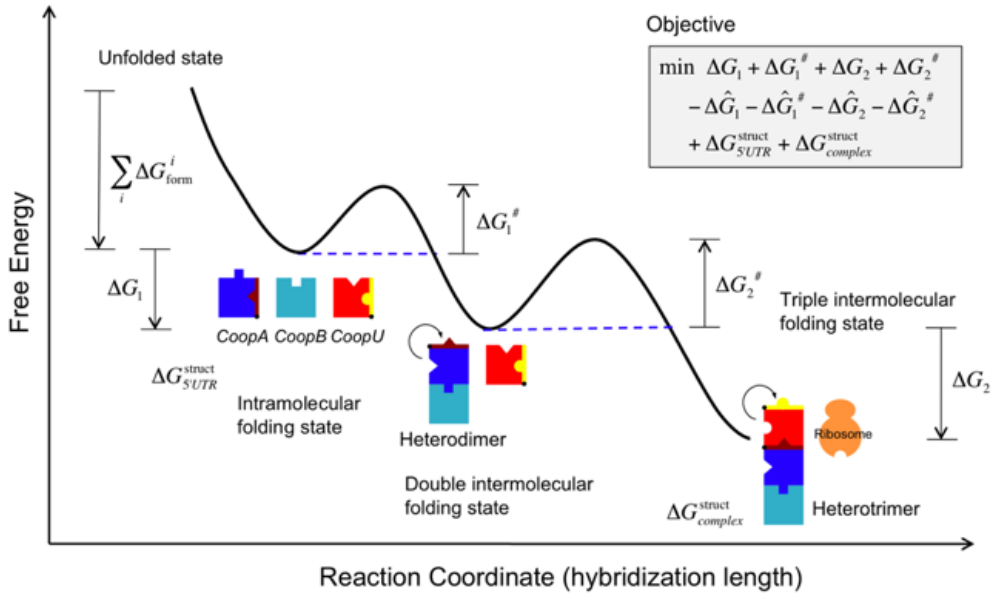


Figure 3.2 Adapted from Rodirgo & Prakash et. al., NAR 2017

Figure 3.2 Energy landscape of Trans-activating multi-component riboswitches (cooperative riboswitches) of gene expression in bacterial cells. On left top, the objective function to be minimized is explicated. The terms ΔG1, ΔG1#, ΔG2, and ΔG2# correspond to the free energies of hybridization and activation, respectively, between the two sRNAs (subscript 1) and between the sRNA complex (heterodimer) and the 5' UTR (subscript 2). Note that the free energy of hybridization is a negative magnitude, whereas the free energy of activation is a positive magnitude. The terms ΔGˆi and ΔGˆi# correspond to the free energies of hybridization and activation, respectively, between the sRNA i and the 5' UTR (subscript i = 1 or 2, for coopA or coop B). They have to be maximized in order to be maintained in negative magnitude. Finally, the terms ΔG 5'UTR complexstruct and ΔG 5' UTRstruct are the works required to fold 5' UTR complex the 5' UTR (coopU) and the heterotrimer according to the structure specifications (in this case, RBS is paired in the 5' UTR and unpaired in the complex). On right top, the scheme of the optimization loop is depicted, where three RNA sequences (coopA, coopB, and coopU) are iteratively mutated and evaluated according to the objective function. The energy landscape shows the different conformational states (intra- and intermolecular), together with the free energy terms of the objective function, as a function of a reaction coordinate (number of

intermolecular base pairs). The trajectory illustrates the interaction between two sRNAs to form a heterodimer that is able to subsequently interact with the 5' UTR to release the RBS and then allow translation.

To show that the proposed hierarchical toehold activation mechanism (based on the kinetic aspect) is a sufficient criterion to provide arbitrary cooperative riboregulation, we developed a computational algorithm (with collaboration Dr. Rodirgo, Spain) that addressed the *de novo* sequence design (iterative process of random mutations and selection according to an energy-based objective function) Figure3.2 (Rodrigo et al., 2012, Rodrigo et al., 2013). The objective function was calculated with a nucleotide-level energy model considering all conformational states of the system's species (coopA, coopB, coopU, all possible heterodimers, and the heterotrimer), following a combined strategy of positive and negative design (Koga et al., 2012). On one hand, as positive objectives (to be minimized), we considered the free energies of activation and hybridization corresponding to the interactions between the two sRNAs and between the resulting sRNA complex and the 5' UTR. We also considered the intramolecular structure of the 5' UTR (to have the RBS paired), and the intermolecular structure of the 5' UTR with the sRNA complex (to have the RBS unpaired). On the other hand, as negative objectives (to be maximized), we took the free energies of activation and hybridization corresponding to the interactions between each sRNA and the 5' UTR.

We computationally designed two systems: (coop1 and coop2) (Table 1). System coop1 was obtained by specifying the intramolecular structures of the two sRNAs, whereas for system coop2, the specification was of the intermolecular structure of the sRNA complex (introduced as soft constraints in both cases). These specifications, although were not required functionally, were introduced to prevent premature degradation of unstructured sRNAs. For these systems, the toehold that nucleates the interaction between coop1: coop2 and coopU is hidden within the intramolecular structure of coop1 (or coop2). However, the toehold that nucleates the interaction between coop1 and coop2 (or coopA2 and coopB2) is unpaired (active) within their intramolecular structures, so that the hybridization reaction between both

sRNAs becomes feasible. As a result, within the intermolecular structure of the resulting sRNA complex, the toehold that nucleates the interaction with coopU (or coopU2) becomes active. The structures of system coop2 are shown in Figure 3.2 explaing the hierarchical activation of toeholds.

However, even if a toehold is not hidden within the corresponding intramolecular structure sRNA1 (of coopA), it may still remain inactive. In this case, the hybridization free energy would not be sufficient to ensure irreversible interaction (with coopU), and an additional species sRNA2 (coopB) would be required for the reaction. The free energy of hybridization between coopA: coopB and coopU would then be sufficient to form the triple intermolecular folding state with a three-way junction. To explore this possibility (based on the thermodynamic aspect), we considered a design based on a three-way junction. Indeed, naturally occurring three-way junctions have already been exploited to engineer RNA-based structure.

We took advantage of our previously published riboregulatory systems, RAJ11 and RAJ12 (Rodrigo et al., 2012). We split the sRNA into two halves, coopA11 and coopB11 hereafter, and considered the cognate 5' UTR, which we named here as coopU11 (figure3.2). The free energies of hybridization were automatically fair due to the three- way junction formed within the intermolecular conformation between the sRNA and 5' UTR in the native system, RAJ11. While constructing coopA11 and coopB11, we found that both sRNAs had an active toehold that allowed them to interact. The heterodimer coopA11: coopB11 has another active toehold that nucleates it's binding to coopU11 by forming a heterotrimer with the three-way junction. This resulting structure activates the RBS for recognition by the ribosome and then initiates translation. The coop12 system did not work after splitting into two parts of small RNA.

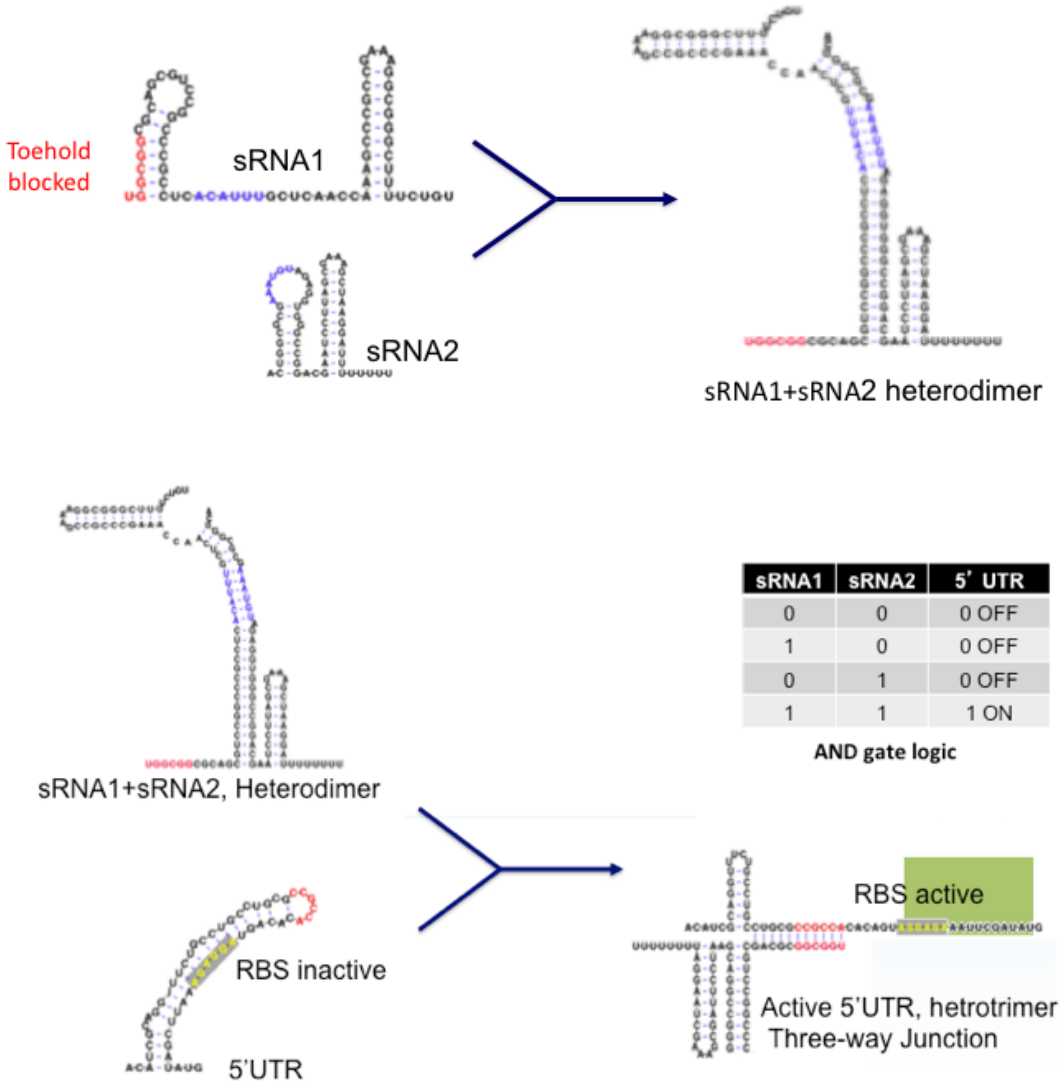


Figure 3.3 showing Sequences and structures of the species of the designed sRNA system coop2. The toehold for the interaction between the two sRNAs is shown in blue. The toehold for the interaction between the heterodimer (sRNA complex) and the 5' UTR is shown in red. In the 5' UTR (cCoopU2), the RBS is shown in yellow. The transcription terminators T500 and B1002 were used in coopA2 and coopB2, respectively. As shown in the figure, toehold is inactive in sRNA1 (coopA). The sRNA2 (coopB) is also inactive. The two small RNAs hybridize and make heterodimer and toehold is free. This heterodimer hybridizes to cis-repress. 5'UTR (coopU) change the conformation and expose RBS and make hetrotrimer and a three-way junction. This system also explains the AND logic circuits.

We performed in a PAGE gel the molecular characterization of the higher-order RNA-RNA interactions by gel analysis (Shu et al., 2011). The different RNA species were first transcribed *in vitro* (for the sRNA species without transcription terminators). We prepared, in each lane, the three-individual

species: coopU, coopA, and coopB. Then, we prepared the three possible combinations of two species and finally, the three species together. The gel finely shown for system coop11, the intermolecular interactions *i*) between the two sRNAs, and *ii*) between the resulting sRNA complex and the 5' UTR (Figure 3.4). It also revealed mild intermolecular interaction between one sRNA and the 5' UTR (Figure3.4). We also confirmed the intermolecular interactions between the sRNA complex and the 5' UTR for system Coop11 by PAGE. Taken together, these results validated our description of the energy landscape.

The designed RNA systems were implemented as separate operons (with their respective transcriptional terminators) in plasmids, which were transformed in *E. coli* strains, expressing the transcriptional repressors LacI and TetR (illustrates the engineered sRNA circuit). The use of P_L-based inducible promoters allowed controlling the expression of the two sRNAs with the external inducers, isopropyl-β-D-thiogalactopyranoside (IPTG) and anhydrotetracycline (aTc) (Lutz and Bujard 1997) Figure 3.5 shows the dynamic ranges (characterized by fluorimeter) of our engineered systems, probing the regulation of gene expression in living cells with two cooperative sRNAs (at the population level). To assess that the combined action of the two sRNAs was enhanced over the expected action from their independent contributions, we compared the increase in gene expression with both inducers with respect to the additive increase with IPTG, on one side, and aTc, on the other (Welch *t*-test, $P < 0.02$ for systems, coop11 and coop2; and $P = 0.06$ for system, coop1). We also observed that *cis*-repression is much stronger in systems coop11 and coop1 than in system coop2. Figure3.3 demonstrated a graded response with both the inducers. To further explore the cooperative behavior at single cell level, we performed a time- dependent characterization of system coop11 (in Dh5alphaproZ1 *E. coli* strain) and coop2 (MG1655Z1) using microfluidic devices Figure 3.8 (Bennett and Hasty 2009). This allowed us to monitor GFP expression in single cells under a varying concentration of both the inducers Figure 3,8 B. These results showed that each individual cell responded to the inducers, and that the system, as expected, is reversible *in vivo*. Flow cytometry experiments

also revealed significant population shift in response to both the inducers Figure 3.7.

In conclusion, we programmed with RNA, a hierarchical activation of hidden toeholds after intermolecular interactions (systems coop1 and coop2; sequences obtained by computational design). Cooperative behavior can also be programmed without relying on the activation of a hidden toehold when forming the obligate heterodimer if a thermodynamically stable three-way junction is used (system coop11). This coupling between hierarchical assemblies of RNA structures with gene expression could allow the application of known nano- structures (Bhadra and Ellington 2014) to engineer the higher-order regulation of gene expression in living cells, and potentially novel trans-activating multicomponent riboswitches with sophisticated functionalities.

Engineering of New RNA molecule (regazyme)

In our lab we engineer synthetic transduction system. (Shen et.al.,2015) We developed small RNA based sensing. In this work I fused small RNA-RAJ11 with theophylline aptamer to engineer the novel sensing system. This aptazyme acting as a moleculer sensing elements (regazyme). In the presence of theophylline, aptazyme cleave and release riboregulator (small RNA). This sRNA binds to cis-regulated mRNA and expressed GFP

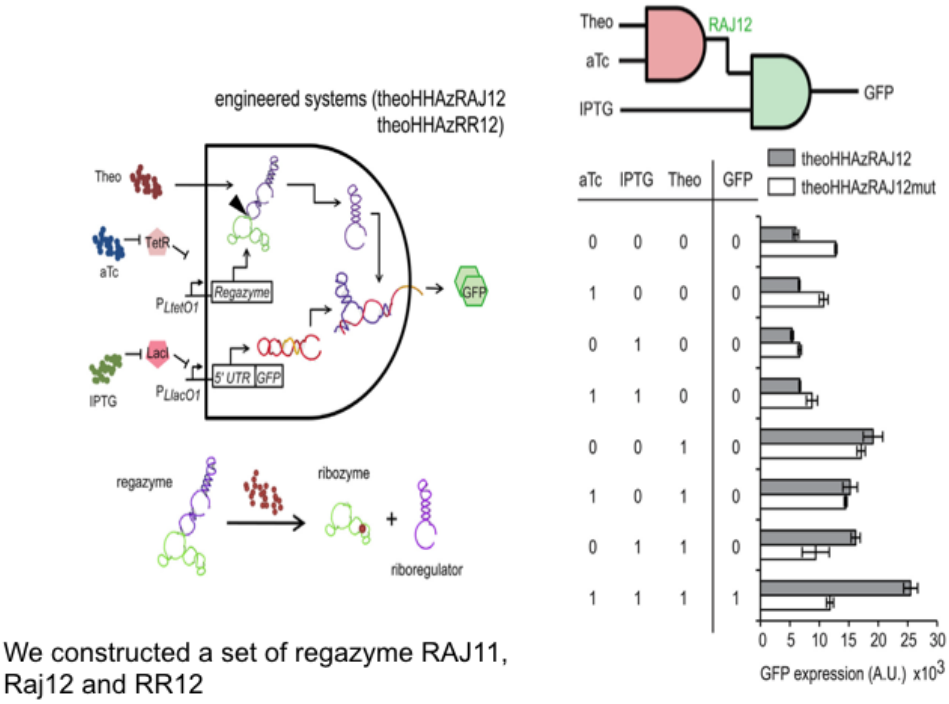


Figure 3.3 B Functional characterization of small-molecule-sensing regazyme

Figure 3.3B Schemes of the engineered RNA-based circuit to sense a small molecule and its corresponding control. (Digital scheme, associated Truth table, and fluorescence results of regazyme theoHHAzRAJ12.

The figure Adapted Shen, Rodirgo and Prakash et al. NAR 2015

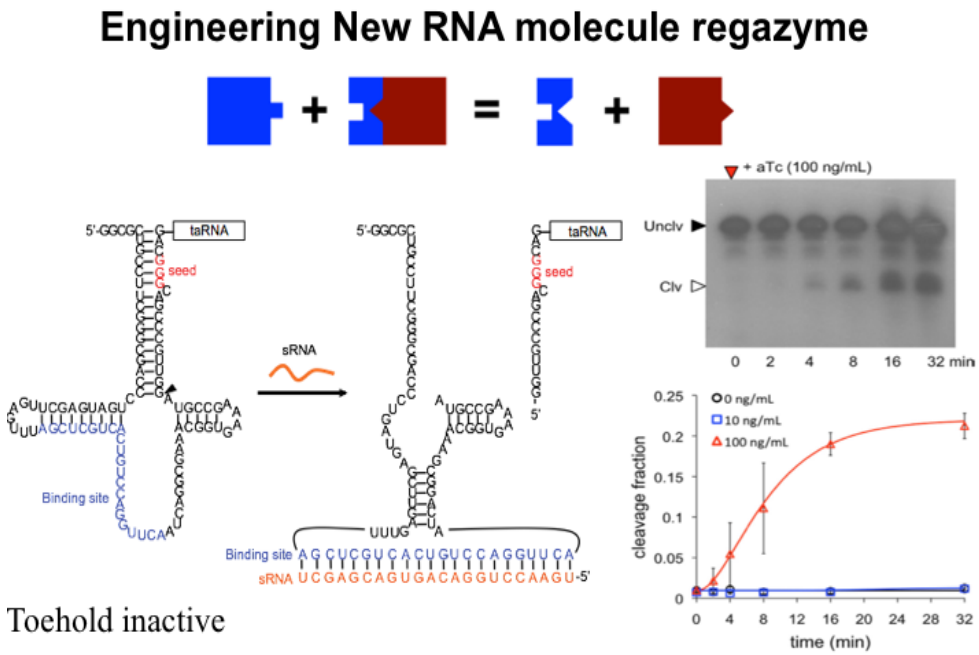


Figure 3.3 C Molecular characterization of small-molecule-sensing regazyme.

Figure 3.5 SequenceandstructureoftheregazymetheoHHAzRAJ12.Asmallmolecule (Theo) binds to the regazyme to reconstitute the active conformation of the ribozyme and then produce the cleavage. An arrow marks the cleavage site, between the transducer module and the ribozyme core. The seed of the riboregulator is paired in the uncleaved state. (B) Time-dependent electrophoretic analysis of cellular RNA extracts taken at different time points; gel shown for 4 mM Theo. Quantification of dynamic RNA processing for different concentrations of the signal molecule (Theo). Data fitted with a generalized exponential decay model with production, where the temporal factor is $(1 - \exp(-\lambda t))^m$, with $m \approx 1$. Error bars represent standard deviations over replicates.

The figure Adapted Shen, Rodirgo and Prakash et al. NAR 2015

3. 3 Results discussion

The thermodynamic model to create trans-activating multicomponent riboswitches (cooperative riboswitches)

We engineered a cascade of three RNA molecules (Fig3.3). The small RNA1 (coop1) is able to interact with sRNA2 (coop2). The coop2 is initially in 'off' state because toehold hidden and inactive. The binding of coop1 to coop2 changes the conformation and thus activates the toehold. This coop1: coop2 heterodimer interacts with the inactive 5'UTR (coopU) and forms a heterotrimer. This heterodimer binding to 5'UTR changes the conformation and exposes the RBS, which then allows the landing of ribosome and thus translation initiation factors start translation. This strategy leads to the formation of larger cascades at post- transcriptional level. The energy landscape associated to our hypothesized hierarchical interaction mechanism is depicted in Figure 3.2

RNA-RNA interaction in-vitro (PAGE)

To get mechanistic insight about the regulatory behavior, we characterized the higher- order RNA-RNA interactions by polyacrylamide gel electrophoresis (PAGE). The complementary DNAs (cDNAs) corresponding to the RNA species were first transcribed in vitro (for the sRNA species without transcription terminators), then purified and quantified. We

mixed the three-individual species (coopU, coopA, and coopB), and all combinations of two of these species. These mixes, along with the individual RNAs as controls, were loaded in polyacrylamide gels and separated electrophoretically. The PAGE gel shown for coop system

Figure 3.4 Electrophoretic analysis of system Coop11. The different lanes correspond to all combinations of species. The arrow marks the complex of the three RNAs. PAGE gel showing RNA –RNA interaction between coop11A, coop11B and coop11U.

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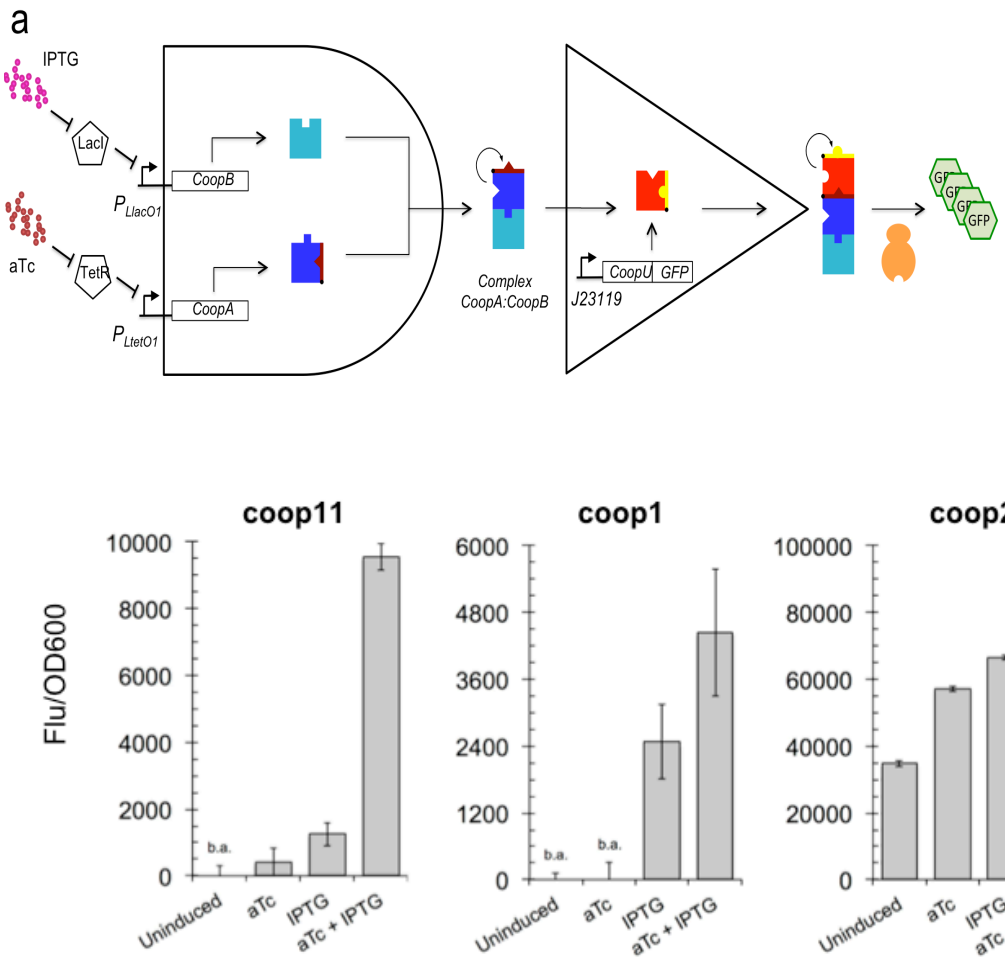
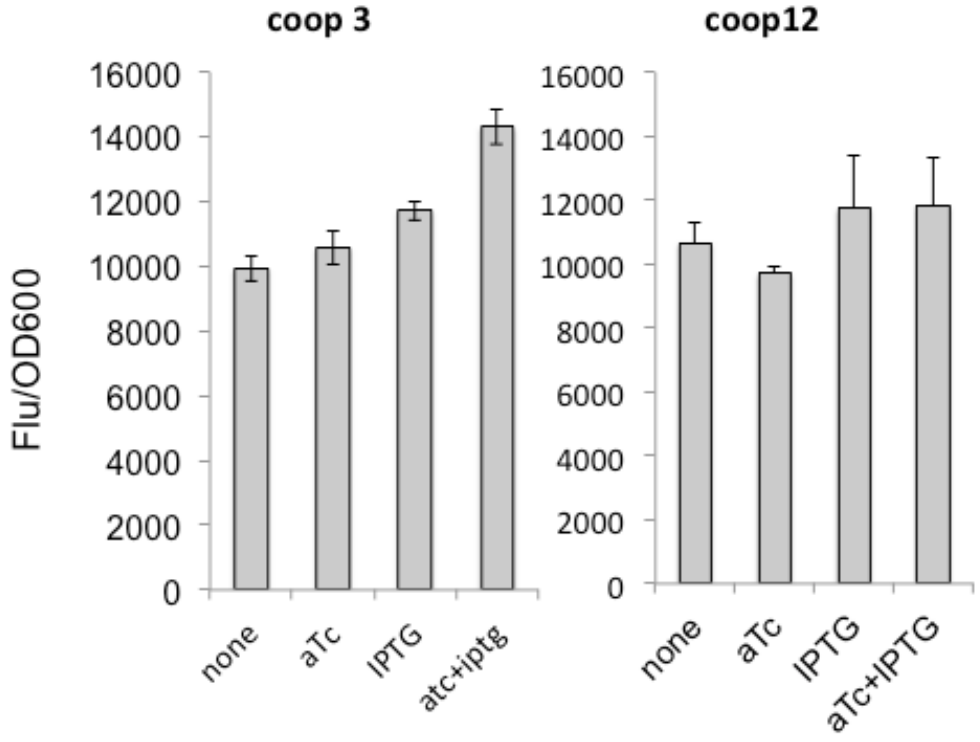
Characterization of *trans-activating multicomponent riboswitches*



b.a. = Below Auto fluorescence


coop 3
coop12
Flu/OD600
16000
14000
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8000
6000
4000
2000
0
none
aTc
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atc+iptg
aTc+IPTG

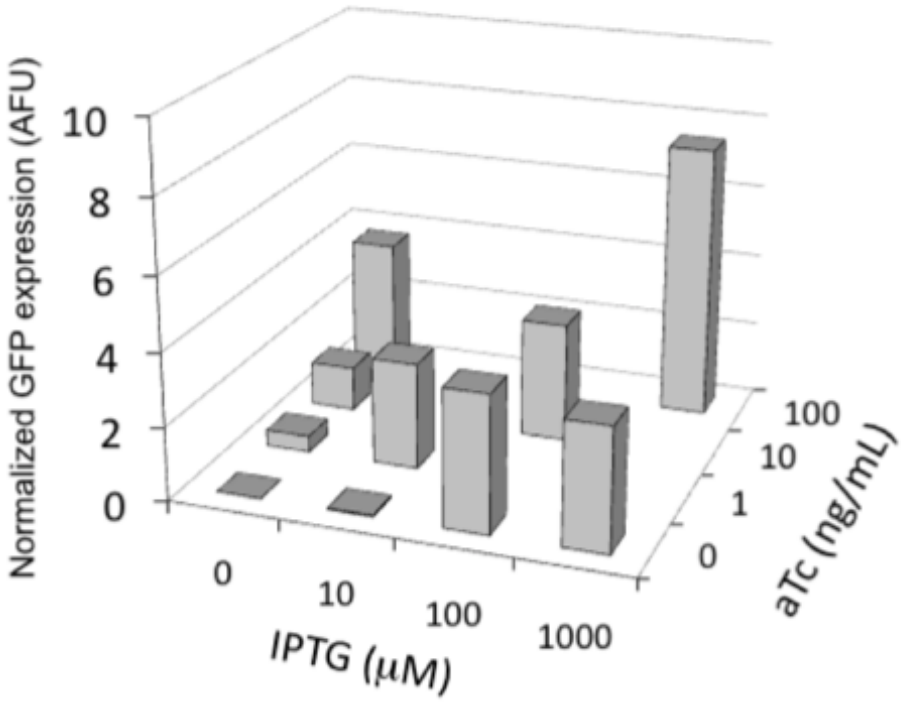
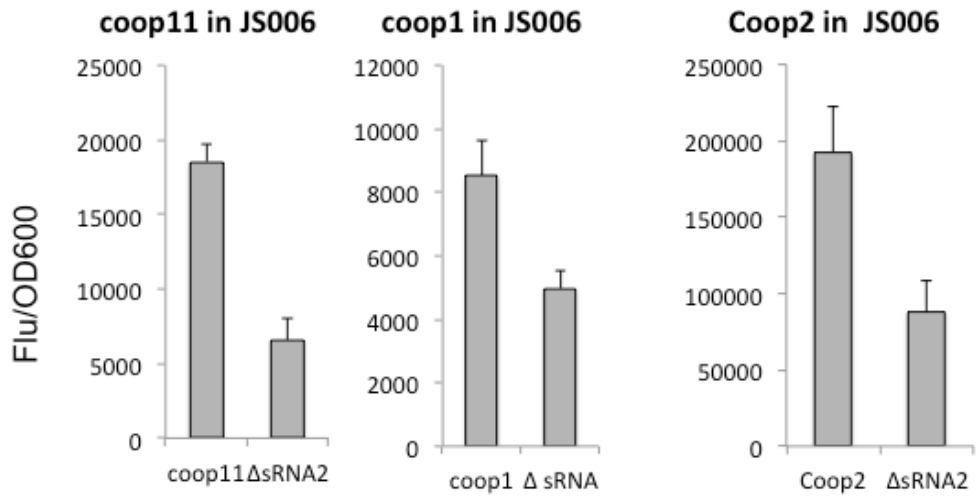

Normalized GFP expression (AFU)
10
8
6
4
2
0
0
10
100
1000
IPTG (μM)
100
10
1
0
aTc (ng/mL)

Figure 3.6 Functional characterization of trans-activating multicomponent riboswitches (cooperative riboswitches) systems in bacterial cells. (a) Digital-like scheme of the engineered sRNA circuit. Promoters P_{LlacO1} and P_{LtetO1} control the expression of the two sRNAs, which can be tuned with external inducers IPTG and aTc, whereas the mRNA is constitutively expressed from promoter BBa_J23119.

To test variability and reproducibility, I characterized cooperative riboregulators in different *E. coli* strain (MG1655Z1, Dh5alphaproZ1 and JS006). I characterized in technical and biological replicate and in different days. As Shown in figure1.5. Fluorescence results of the designed systems Coop11, Coop1, Coop2, coop3 and coop 12. The characterization in all four possible combinations of inducers (none, aTc, IPTG, aTc+IPTG) Error bars represents standard deviations over replicates. As results showing coop1 (sRNA1) hybridized to coop2 (sRNA2). This heterodimer bind to coopU (cis-repress 5'UTR) and change the conformation open RBS and express reporter protein. As results showing some riboregulators giving very good expression and some giving poor expression.

To assess that the combined action of the two sRNAs was enhanced over the expected action from their independent contributions, we compared the increase in gene expression with both inducers with respect to the additive increase with IPTG, on one side, and aTc, on the other (Welch *t*-test, $P < 0.02$ for systems Coop11 and Coop2; and $P = 0.06$ for system Coop1). We also observed that *cis*-repression is much stronger in systems Coop11 and Coop1 than in system Coop2. Figure 3c demonstrated a graded response with both inducers Figure 3.7 showing dynamic range of both inducer aTc and IPTG.

Functional characterization of trans-activating multicomponent riboswitches in JS006 *E. coli* Figure 3.8. The JS006 *E. coli* strain have no is the lacQ, $^{-}$ tet^{R-}. This strain always expresses both coop RNA that I deleted sRNA2 (coop2). Results showing that in presence of both coopA and coopB, the coopU is active and GFP are express. In the absence of sRNA2 (coopB), toehold is inactive and cis –repress 5' UTR inactive and no GFP exprees.

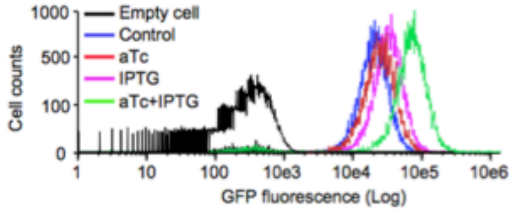


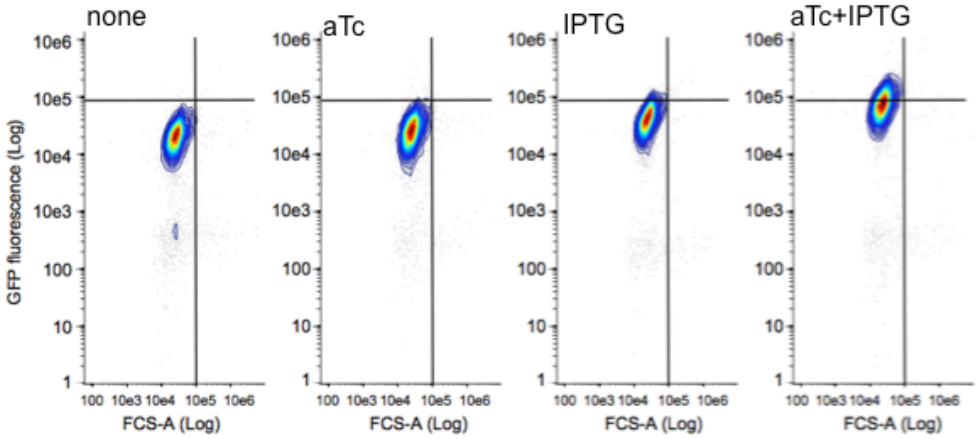
Orthogonality of cooperative riboregulator. I also cheked the interaction of RNA with in circuits and crosstalk with in the cis and transRNA.

Flow-cytometry characterization *trans*-activating multicomponent riboswitches.

Flow cytometry experiments revealed significant bacterial population shift in response to both inducers (Figure 3.9A. results of trans-activating multicomponent riboswitches). The reported dynamic ranges at the single-cell level are similar to those measured for the whole population.

These results showed that every individual cell responds to the inducers, and that the system, as expected, is reversible in vivo. Flow-cytometric results for system Coop2 for all possible combinations of inducers (none, aTc (100ng/ml), IPTG (1mM) and (aTc+IPTG) in MG1655Z1 E. coli strain. The black curve corresponds to plain cells.





Study of single cell dynamics of *trans*-activating multicomponent riboswitches.

To further explore the cooperative riboregulatory behavior at the single-cell level, we performed a time-dependent characterization of system coop11 and coop2 using microfluidic lab-on- chip devices Figure 3.10). This allowed us to monitor GFP expression in individual cells under a varying concentration of both inducers. Single cell tracking of fluorescence [GFP expression in arbitrary fluorescence units (AFU)] in one micro-chamber of the microfluidics device under time-dependent induction with IPTG (1 mM) and aTc (100 ng/mL) for system Coop2 in MG1655Z1 and coop11 in Dh5alphaproZ1. A square wave of both inducers with period 8 h (i.e., 4 h induction and 4 h relaxation) was applied. The solid and dashed lines (in blue) correspond to the mean and plus/minus standard deviation over the cell population. Sulforhodamine B was used to monitor the inducer time-dependent profile (in red). The (Figure 3.10a) showing chamber of PDMS CHIP.

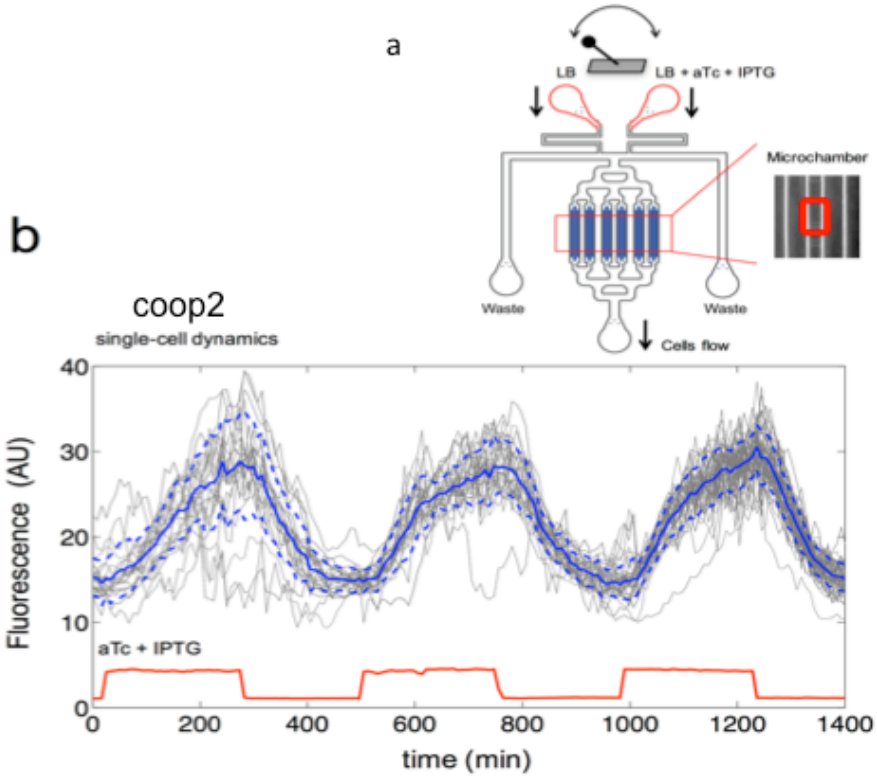


Fig 8. A Single cell dynamics of coop2 in MG1655Z1 (adapted from the manuscript un-publish)

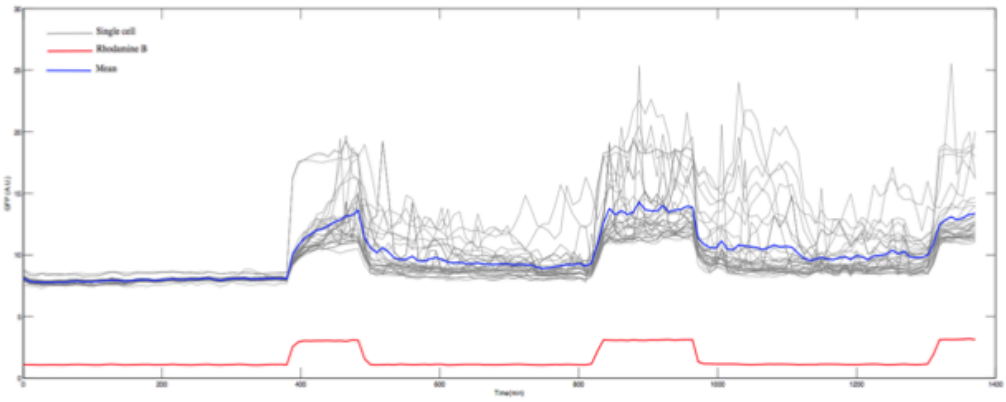


Fig 8. B Coop11 Single cell dynamics in Dh5alphaProz1

(Figure 3.10 B) showing single cell tracking of GFP of trans-activating multicomponent riboswitches Coop11 in Dh5alpharoZ1.

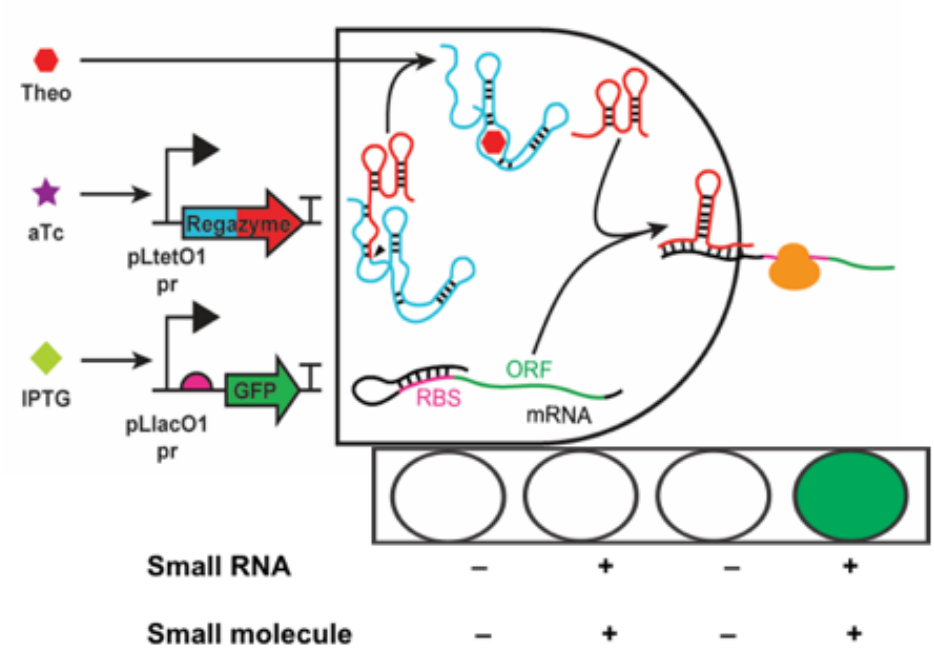


Figure 3.11 We have engineered a new type of RNA molecule (regazyme) that does not exist in nature, and experimentally validated in lab This "regazyme" molecule combines a riboregulator and a ribozyme, and gets activated in the presence of a small molecule. The system produces green fluorescence when all three molecules (ligand, small RNA and mRNA-GFP) are present. (Shen et.al 2015)

Chapter 4

Discussion and conclusion.

Nucleic acids are molecules with a much higher interaction programmability than proteins. The overall aim of my research is to engineer trans-activated RNA switch that would be useful for reprogram cell, RNA sensing, reduce the metabolic load, to evolve future medically applicable new biomolecule and devolve anti-terminator system trans-activated RNA switches cascade-based feedforward loop. In order to do this, we engineered a trans-activated tuneable antiterminator RNA switch. The engineering of **the** trans-activating RNA switches was done in *E. coli.* RNA-switches i.e., riboregulators that are ON/OFF in terms of activity according to the binding with another RNA. These are hence new elements offering new possibilities for engineering functional, synthetic gene circuits in living cells. In addition, as these circuits would be mainly based on physical interactions between RNAs, their adaptation to other organisms than *E. coli* (including eukaryotic hosts) might be favored by only re-designing the interface with the output-protein-expression machinery (either at transcriptional or post-transcriptional level).

To design our RNA switches we used a computational and rational design. For the trans-activating RNA switches, we used a small RNA that works as an antiterminator element. The small RNA acts as an adapter converting a translational signal into a transcriptional one. The small RNA hybridizes with a cis-repressed mRNA, this interaction free up an RBS, allowing the ribosome to bind and initiate translation (Figure 1.2). We have successfully been able to engineer an antiterminator RNA switch. We tested a library of terminators (table 1.1) and shown that they are working well (figure 1.4). To further justify our hypothesis, we tuned our system with different RBS strengths (from weak RBS to strong and including a no RBS control). Our results in figure 1.7 show that translation-transcription coupling is directly controlled by the RBS. Strong RBS's convert more signal and weaker RBS's convert less, and our no RBS no signal is converted to translation to

transcription. In order to explain our hypothesis, I made a knockout of the 'ATG' start codon, replaced with 'AAG'. The idea was that if ATG is deleted then translation would does not start even ribosome is land on RBS for translation. But we do not get any difference in the GFP expression in the Wild type and ATG knockout. We hypothesized that the start codon and the terminator are very close, and that even after the deletion of 'ATG' nascent mRNA RNA is very small and unable to make a hairpin stem loop. This allow translation continue on terminator sequence and GFP expressed. To combat this, we inserted a 30 to 45 bp spacers between the 'ATG' and terminator (figure 1.10). After insertion of spacer the 'ATG' knockouts show no expression in no, but wild type strain shows GFP expression with IPTG induction. Alongside this we made the control system of a constitutive RBS and spacer, and our results show that if the RBS is present then the system expresses GFP. We also tested our antiterminator system with Isaac's riboregulators RR12 (Issacs and Collins 2006) (figure1.12). The system does not work well. We further engineered a complex feedforward loop system, the TNA31cascade. The results are not as we hypotheses. This could be possibly two RNA interfere each-other, they may have cross talk or the system could be leaky.

To further explore the possibility to construct antiterminator riboswitch. Theophylline riboswitch (Lynch et. al 2007) based system was design and validates and (figure 1.7 A &B and 1.8). The Fluorimeter characterization and single cell dynamics results explains system working well. After experimentally validating theophylline riboswitch based antiterminator. I designed and characterized double riboswitch based cascade (feedforward loop based). The results explain (figure1.8 and figure 1.9). but system does not work. Possibly two riboswitches could interfere each other or two inducers may be toxic to cell or interfering each other. We need to engineer much robust system (riboswitch) to engineer feedforward and feedback loop. For this end we are developing directed evolution system. The system will be based on PACE phage assistance evolution. In our case T7 bacteriophage using as an evolution tool. Bacteriophages are viruses of bacteria that infect and often kill them. Viruses have faster mutation and

adaptation rates for new environment than the cells they infect. Therefore, we use T7 bacteriophage as a tool for evolution to make novel biomolecule such as better riboswitch or new riboswitch. We have integrated our riboswitch for evolution in the T7 bacteriophage genome. We design the system in a way that riboswitch function directly controls the rate of bacteriophage propagation. This results in the cargo molecule being evolved to become better in its function. We engineered T7 riboswitch recombinant bacteriophage engineered.

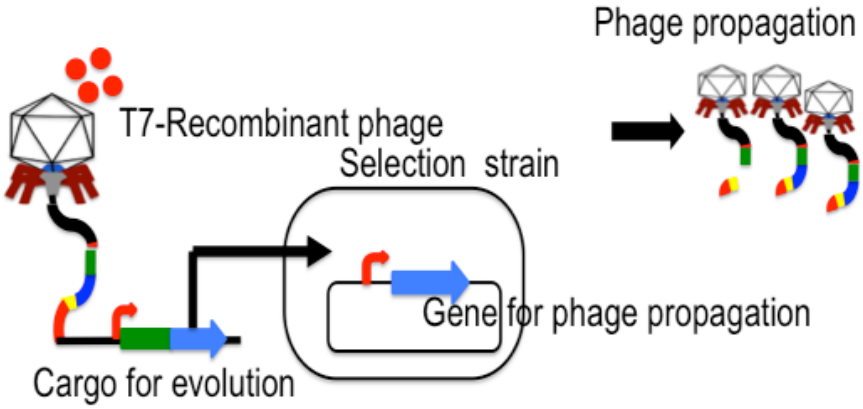


Figure 4. 1Design of T7 recombinant phage with riboswitch.

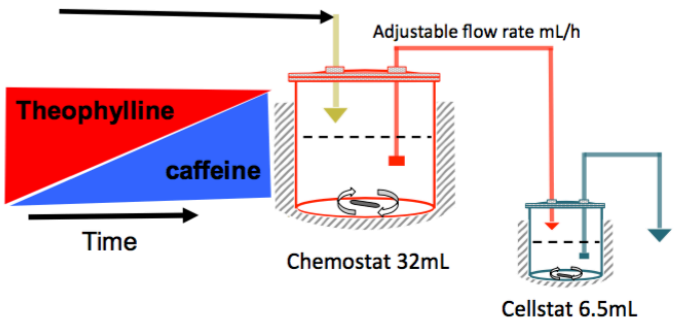


Figure 4.2 Design of bioreactor to evolve new riboswitch/ better riboswitch.

In the 2nd part of my research we engineered a trans-activated single component RNA switch (Rodirgo et.al 2016). Here, we hypothesized that it

should be possible to engineer a synthetic sequence, where it would be possible to produce a different riboregulator when transcribed in each sense. We are able to engineer a reverse complement of known a riboregulator. We used computational design to create a cis-repressed 5'UTR. In our design one DNA template is able to transcribe two small RNA depends on the direction of transcription. These are activated by two genes independently. In conclusion this overlapping reading frames can have transcribed two transcript and reduce the information encoded in the genome and metabolic load on cell.

Our results also explain (figure 2.3), if two promoter face to face each other than transcription of both transcript sRNA-RAJ11 and sRNAanti-RJA11 is not possible at the same time on the same DNA template. This is due to both promoter's competition, only the stronger could win. The two promoters also collide finally the canceled to each other or incomplete transcription. We also hypothesized ambisense based on ribozyme figure 2.5. According to results figure 2.5, the ribozyme ambisense RNA switches are leaky, it is likely both sRNAs are not transcribed properly. Finally, we have demonstrated that it is possible to use an antisense sRNA as a new regulatory agent in the cell. This was accomplished by designing an appropriate 5′ UTR. These results largely indicate the development of sophisticated RNA-only circuits where sRNAs interact with each other to form arbitrary regulatory architectures. We also hypothesize that antisense transcripts could be exploited in synthetic biology as overlapping reading frames of transcription (i.e., ambisense) for engineering bi-functional systems with minimal genetic material, as well as non-linear behavior with RNA, as convergent transcription has been shown to confer a bistability. We are able to transcribe two RNA switch that activate two gene independently and that also cancel each other if both are present.

In order to engineer minimum genetic material for reduction of the metabolic load on cells we constructed a single component RNA switch with reduced nucleotide usage. This switch created with three nucleotides only (G, C and U) (unpublished results). This RNA switch without adenine.

In order to engineer, a robust system to reduce metabolic load on the host

cell RNA switch does not having crosstalk with host cell. To This end I tested orthogonality of our RNA switch. Our results in figure 2.30 explains that our RNA switch does not having crosstalk to other RNA and within the host cell.

In 3rd chapter of my thesis we engineered a multi-input trans activating RNA switch. We programmed RNA with a hierarchical activation of hidden toeholds, after intermolecular interactions (Coop1 and Coop2; sequences obtained by computational design) Cooperative behavior of RNA switch can also be programmed without relying on the activation of a hidden toeholds when forming the obligate heterodimer (Rodirgo et.al.2017) if a thermodynamically stable three-way junction can be used (system Coop11) (figure 3.2). This coupling between hierarchical assembly of RNA structures with gene expression could allow the application of known nanotechnology structures to engineer higher-order regulation of gene expression in living cells. This will contribute to have building blocks with increased nonlinearity, and potentially to create novel RNA circuits with sophisticated functionalities. We characterized multicomponent RNA switch in different dynamic range. We study reporter protein expression at both the single cell and population level. Additionally, we studied possible molecular interaction between different component of RNA with PAGE figure 3.4. We also study single cell dynamics of our system in MG1655Z1 and Dh5alphaproZ1 strain.

We have designed and experimentally validated new type of RNA molecule(regazyme). This is the chimera of an aptazyme with a riboregulator. The regazyme sensing a ligand (theophylline) self-cleaves and releases a riboregulating small RNA (Shen et al., 2105). This small RNA binds to a cis-repressed mRNA allowing translation of a reporter protein. The regazyme able to transduce signals in a modular way in live cells, introducing the one-to-two-component signal transduction paradigm. The design strategy relies on the hierarchical activation/inactivation of RNA elements with specific function. In our case we developed theophylline-based signal transduction system. The ligand should be different and we can develop more regazyme with different ligand or environment signal.

We have engineered RNA based synthetic signal transduction cascade consisting of a single RNA molecule (Regazyme, an RNA chimera of an aptazyme with a riboregulator) that upon sensing a ligand (theophylline) self-cleaves and releases a riboregulating small RNA (Shen et al., 2105). The regazyme able to transduce signals in a modular way in live cells, introducing the one-to-two-component signal transduction paradigm. The design strategy relies on the hierarchical activation/inactivation of RNA elements with specific function. In our case we developed theophylline-based signal transduction system. This system can be adapted to be induced by other ligands and can be used as a biosensor.

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List of Published papers

Apendix1

Sequence used in this work

Plasmid sequenced of chapter1

Sequence and plasmid map. This is the sequence of functional part. The plasmid backbone, reporter protein, antibiotics marker is same as pSTC1 in chapter1. The sequence having colour code accordind to their order.

1. EcoR1+ T500+ (sRNARAJ11) REV +(Promoter PLacO1) Rev + PromoterJ23119

+ 5'UTR-RAJ11 +Terminator B1002+ B0034 RBS

gaattcAAAAAAAATCCTTAGCTTTCGCTAAGGATGCCTCGCATAATCA CACAATCAACCCTCCCGTGCTCAGTATCTTGTTATCCGCTCACAAT GTCAATGTTATCCGCTCACATTTtctagaTTGACAGCTAGCTCAGTCCT AGGTATAATGCTAGCCCTCGCATAATTTCACTTCTTCAATCCTCCCG TTAAAGAGGAGAAATTATGAATGggatccCGCAAAAAACCCCGCTTCG GCGGGGTTTTTTCGCAATAAATTAAAGAGGAGAAAGGTACCATG+S pe1 + SfGFP

TNA_T500 (terminator)

CAAAGCCCGCCGAAAGGCGGGCTTTTCTGT

TNA_B1002 (terminator)

CGCAAAAAACCCCGCTTCGGCGGGGTTTTTTCGC

>TilvGEDA (terminator, very good according to Endy et al.)

TAGAGATCAAGCCTTAACGAACTAAGACCCCCGCACCGAAAGGTCCGG
GGGTTTTTTTTGACCTTAAAAACATAACCGAGGAGCAGACA

>mutated version of TilvGEDA (to remove stop codon)

E. coli DH5□-Z1	Commercial (DH5□□ *lacI*Q, PN25-*tetR*, SpR)	Clontech
E. coli MG1655-Z1	*lacI*Q, PN25-*tetR*, SpR	Gifted by M.B. Elowitz
P-TNA B1002	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNAttonb	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA-TFUR	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA RefTFUR	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA-21	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA -T500	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA-TNA31	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA TNA 12	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA double riboswitch	pSC101m ori, kanR, sfGFP-LAA	This work
P-TNA FFL	pSC101m ori, kanR, sfGFP-LAA	This work

Plasmid sequence of Chapter2

1. **Design of compact ambisense Riboregulators**

>E + rev(TrrnC) + PLlacO1 + transRAJ11 + rev(PLtetO12) + T500 + X + J23119 + cisRAJ11 + B + cRMIR11 + S

gaattcAAAAAAAATCCTTAGCTTTCGCTAAGGATAATTGTGAGCGGATAAC
AATTGACATTGTGAGCGGATAACAAGATACTGAGCACGGGAGGGTTGA
TTGTGTGAGTCTGTCACAGTTCAGCGGAAACGTTGATGCTGTGACAGAT
TTATGCGAGGCGTGCTCAGTATCTCTATCACTGATAGGGATGTCAAACT
CTATCAATGATAGAGTgagcacCAAAGCCCGCCGAAAGGCGGGCTTTTCT
GTtctagaTTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCCCTCGCAT
AATTTCACTTCTTCAATCCTCCCGTTAAAGAGGAGAAATTATGAATGggat
ccGGGAGGGTTCTGTCACTTAATTATTCGAGGCAATCGTTAGGAGATAG
AGCTATGactagt

Design of orthogonal riboregulators

pMIR1.

XOR11: C+E+rrnc (rev)+transRaj11 (rev)+ PLlacO12(rev)+ spacer+J23119 +cisMI11+S+Nc

atcgatgaattcAAAAAAAATCCTTAGCTTTCGCTAAGGATGCCTCGCATAAAT
CTGTCACAGCATCAACGTTTCCGCTGAACTGTGACAGACTCACACAATC
AACCCTCCCGTGCTCAGTATCTTGTTATCCGCTCACAATGTCAATGTTAT
CCGCTCACATTTATGCTAGCCAATTGATGCATTTGACAGCTAGCTCAGT
CCTAGGTATAATGCTAGCGGGAGGGTTCTGTCACTTAATTATTCGAGGC
AATCGTTAGGAGATAGAGCATATGactagtccatgg

pMIR

C+E +

rrnc(rev)+transMiraj11(rev)+PLtetO12(rev)+spacer+J23119+cisRaj11+S+Nc

atcgatgaattcAAAAAAAATCCTTAGCTTTCGCTAAGGATGGGAGGGTTGAT
TGTGTGAGTCTGTCACAGTTCAGCGGAAACGTTGATGCTGTGACAGATT
TATGCGAGGCGTGCTCAGTATCTCTATCACTGATAGGGATGTCAAACTC
TATCAATGATAGAGTGCTAGCCAATTGATGCATTTGACAGCTAGCTCAG
TCCTAGGTATAATGCTAGCCCTCGCATAATTTCACTTCTTCAATCCTCCC
GTTAAAG**AGGAGA**AATTATGAATGactagtccatgg

Design sequence of Plasmid antiRAJ11(MIRAJ11)

E-X-revcom(PLtetO1-tRMIR11-T500)-N-M-Ns-PLlacO1-cRMIR11-S-
gaattcgcggccgcttctagagACAGAAAAGCCCGCCTTTCGGCGGGCTTTGGGG
AGGGTTGATTGTGTAAGTCTGTCACAGTTCAGCGGAAACGTTGATGCTG
TGACAGATTTATGCGAGGCGTGCTCAGTATCTCTATCACTGATAGGGAT
GTCAATCTCTATCACTGATAGGGAgctagccaattgatgcatATAAATGTGAGCG
GATAACATTGACATTGTGAGCGGATAACAAGATACTGAGCACGGGAGG
GTTCTGTCACTTAATTATTCGAGGCAATCGTTAGGAGATAGAGCATATGa
ctagt

Schematic of the final of ribozyme based ambisense riboregulator

EcoRI +Rev(Ttonb term) + Rev(LAA) + Rev(mRFP)+ LacO + XbaI +
Rev(cisRaj11)+Rev(J233119)+ **NsiI** + PLtetO12 +Rev (rnpB
term)+Raj11sRNA+rrnC term + Rev (PLlacO1)+J23100+ cis_antiRaj11
+**SpeI** +sfGFP + LAA + rrfB term+ PstI

Sequence of the final of ribozyme based ambisense riboregulator

5'-
gaattcGAAAACCTCGCGCCTTACCTGTTGAGTAATAGTCAAAAGCCTCCG
GTCGGAGGCTTTTGACTTTCTGCTTACTGAATTTCGGTGTTATCAAGCG
GCCAGGGCGTAGTTTTCATCGTTGGCAGCGCTGGTagcaccggtggagtgacg
accttcagcacgttcgtactgttcaacgatggtgtagtcttcgttgtgggaggtgatgtccagtttgatgtcggtt
ttgtaagcacccggcagctgaaccggtttttttagccatgtaggtggttttaacttcagcgtcgtagtgaccacc
gtctttcagtttcagacgcattttgatttcacctttcagagcaccgtcttccgggtacatacgttcggtggaagct
tcccaacccatggtttttttctgcataaccggaccgtcggacgggaagttggtaccacgcagtttaactttgta
gatgaactcaccgtcttgcagggaggagtcctgggtaacggtaacaacaccaccgtcttcgaagttcata
acacgttcccatttgaaaccttccgggaaggacagtttcaggtagtccgggatgtcagccgggtgtttaacg
taagctttggaaccgtactggaactgcggggacaggatgtcccaagcgaacggcagcggaccacctttg

gtaactttcagtttagcggtctgggtaccttcgtacggacgaccttcaccttcaccttcgatttcgaactcgtga
ccgttaacggaaccttccatacgaactttgaaacgcatgaactctttgataacgtcttcggaggaagcATT
GTGAGCGGATAACAAtctagaCATTCATAATTTCTCCTCTTTAACGGGAGG
ATTGAAGAAGTGAAATTATGCGAGGGCTAGCATTATACCTAGGACTGAG
CTAGCTGTCAA**atgcat**ACTCTATCATTGATAGAGTTTGACATCCCTATCA
GTGATAGAGATACTGAGCACAAAAGCAAAAACCCGCCGAAGCGGGGGGG
AGGGTTGATTGTGTGAGTCTGTCACAGTTCAGCGGAAACGTTGATGCTG
TGACAGATTTATGCGAGGCATCCTTAGCGAAAGCTAAGGATTTTTTTTGT
GCTCAGTATCTTGTTATCCGCTCACAATGTCAATGTTATCCGCTCACATT
TATtctagaTTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGCGGGAGG
GTTCTGTCACTTAATTATTCGAGGCAATCGTTAGGAGATAGAGCATATG**a
ctagt**cgtaaaggcgaagagctgttcactggtgtcgtccctattctggtggaactggatggtgatgtcaacg
gtcataagttttccgtgcgtggcgagggtgaaggtgacgcaactaatggtaaactgacgctgaagttcatct
gtactactggtaaactgccggtaccttggccgactctggtaacgacgctgacttatggtgttcagtgctttgct
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gcacgatttcctttaaggatgacggcacgtacaaaacgcgtgcggaagtgaaatttgaaggcgataccct
ggtaaaccgcattgagctgaaaggcattgactttaaagaagacggcaatatcctgggccataagctgga
atacaattttaacagccacaatgtttacatcaccgccgataaacaaaaaaatggcattaaagcgaatttta
aaattcgccacaacgtggaggatggcagcgtgcagctggctgatcactaccagcaaaacactccaatc
ggtgatggtcctgttctgctgccagacaatcactatctgagcacgcaaagcgttctgtctaaagatccgaac
gagaaacgcgatcatatggttctgctggagttcgtaaccgcagcgggcatcacgcatggtatggatgaac
tgtacaaaGCTAGCGCAGCGAACGACGAAAATTACGCCCTGGCAGCGTAA
TAAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGA
CTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTC
TCCTGctgcag-3’

Sequence and plasmid list of chapter 3

Tabe of content and plasmid sequences

Table 1: Sequences of the cooperative sRNA systems designed in this work. Dot- bracket structures are also shown. The seed region for the interaction between the two sRNAs (CoopA and CoopB) is shown in cyan. The seed region for the interaction between the sRNA complex and the 5’ UTR (CoopU) is shown in red. In CoopU, the RBS is shown in yellow and the start

codon in green. The transcription terminator T500 (efficiency > 90%) was used in CoopA, shown in dark red, and the terminator TrrnC (efficiency > 90%) or B1002 (efficiency about 90%) in CoopB,..

Sequence of coop11. (Rev coop11A with T500)+(PLlacO1)+ (Rev coop11 B with Trrnc + PLtetO1 rev) + PrJ23119+ coop11U+ sfGFPLAA +kanR +pSC101 mutated

gaattcAAAAAAAATCCTTAGCTTTCGCTAAGGATGCCTCGCATAAATCTGT
CACAGCATCAACGTTGTGCTCAGTATCTTGTTATCCGCTCACAATGTCA
ATTGTTATCCGCTCACAATTcaattgACAGAAAAGCCCGCCTTTCGGCGGG
CTTTGTCCGCTGAACTGTGACAGACTCACACAATCAACCCTCCCGTGCT
CAGTATCTCTATCACTGATAGGGATGTCAAACTCTATCAATGATAGAGTa
tgcattctagaTTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCCCTCGCA
TAATTTCACTTCTTCAATCCTCCCGTTAAAGAGGAGAAATTATGAATGaC
TAGTCGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTG
GAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAG
GGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTA
CTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGA
CTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCA
TGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCAC
GATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAA
ATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGAC
TTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTA
ACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAAATGGCATTAA
AGCGAATTTTAAAATTCGCCACAACGTGGAGGATGGCAGCGTGCAGCT
GGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTG
CTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATC
CGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAG
CGGGCATCACGCATGGTATGGATGAACTGTACAAAGCTAGCGCAGCGA
ACGACGAAAATTACGCCCTTGCAGCGTAATAACTGCAggagtcactaagggtta
gttagttagattagcagaaagtcaaaagcctccgaccggaggcttttgactaaaacttcccttggggttatc
attggggctcactcaaaggcggtaatcagataaaaaaaatccttagctttcgctaaggatgatttctgctagt
attattagaaaaactcatcgagcatcaaatgaaactgcaatttattcatatcaggattatcaataccatattttt
gaaaaagccgtttctgtaatgaaggagaaaactcaccgaggcagttccaaagaatggcaaggtcctggt

aacggtctgcgattccgacccgtccaacatcaatacaacctattaatttcccctcgtcaaaaataaggttatc
aagtgagaaatcaccatgagtgacgactgaatccggtgagaatggcaagagcttgtgcatttctttccaga
cttgttcaacaggccagccattacgctcgtcatcaaaatcactcgcatcaaccaaaccgttattcatgcgtg
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accggcgcaggaacacggccagcgcatcaacaatattttcacctgaatcaggatattcttctaatacctgg
aaggctgttttcccaggaatcgcggtggtgagtaaccacgcatcatcaggagtacggataaaatgcttgat
ggtcgggagaggcataaactccgtcagccagttgagacggaccatctcatctgtaacatcattggcaacg
ctacctttgccatgtttcagaaacaactctggcgcatcgggcttcccatacaagcgatagattgtcgcacctg
attgcccgacattatcgcgagcccatttatacccatataaatcagcgtccatgttggagtttaagcgcggac
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attgttcatgatgatatattttatcttgtgcaatgtaacatcagagattttgagacacaacgtggctttgttgaat
aaatcgaacttttgctgagttgaaggatcagctctagtagttacattgtcgatctgttcatggtgaacagcttta
aatgcaccaaaaactcgtaaaagctctgatgtatctatctttttacaccgttttcatctgtgcatatggacagttt
tccctttgatatctaacggtgaacagttgttctacttttgtttgttagtcttgatgcttcactgatagatacaagagc
cataagaacctcagatccttccgtatttagccagtatgttctctagtgtggttcgttgtttttgcgtgagccatgag
aacgaaccattgagatcatgcttactttgcatgtcactcaaaaattttgcctcaaaactggtgagctgaatttttt
gcagttaaagcatcgtgtagtgtttttcttagtccgttacgtaggtaggaatctgatgtaatggttgttggtattttg
tcaccattcatttttatctggttgttctcaagttcggttacgagatccatttgtctatctagttcaacttggaaaatca
acgtatcagtcgggcggcctcgcttatcaaccaccaatttcatattgctgtaagtgtttaaatctttacttattggt
ttcaaaacccattggttaagccttttaaactcatggtagttatttcaagcattaacatgaacttaaattcatcaa
ggctaatctctatatttgccttgtgagttttcttttgtgttagttcttttaataaccactcataaatcctcatagagtatt
tgttttcaaaagacttaacatgttccagattatattttatgaatttttttaactggaaaagataaggcaatatctctt
cactaaaaactaattctaatttttcgcttgagaacttggcatagtttgtccactggaaaatctcaaagcctttaa
ccaaaggattcctgatttccacagttctcgtcatcagctctctggttgctttagctaatacaccataagcattttc
cctactgatgttcatcatctgagcgtattggttataagtgaacgataccgtccgttctttccttgtagggttttcaat
cgtggggttgagtagtgccacacagcataaaattagcttggtttcatgctccgttaagtcatagcgactaatc
gctagttcatttgctttgaaaacaactaattcagacatacatctcaattggtctaggtgattttaatcactatacc
aattgagatgggctagtcaatgataattacatgtccttttcctttgagttgtgggtatctgtaaattctgctagacc
tttgctggaaaacttgtaaattctgctagaccctctgtaaattccgctagacctttgtgtgttttttttgtttatattca
agtggttataatttatagaataaagaaagaataaaaaaagataaaaagaatagatcccagccctgtgtat
aactcactactttagtcagttccgcagtattacaaaaggatgtcgcaaacgctgtttgctcctctacaaaaca
gaccttaaaaccctaaaggcttaagtagcaccctcgcaagctcgggcaaatcgctgaatattccttttgtctc
cgaccatcaggcacctgagtcgctgtctttttcgtgacattcagttcgctgcgctcacggctctggcagtgaat

gggggtaaatggcactacaggcgccttttatggattcatgcaaggaaactacccataatacaagaaaag
cccgtcacgggcttctcagggcgttttatggcgggtctgctatgtggtgctatctgactttttgctgttcagcagtt
cctgccctctgattttccagtctgaccacttcggattatcccgtgacaggtcattcagactggctaatgcaccc
agtaaggcagcggtatcatcaacaggcttacccgtcttactgtccctagtgcttggattctcaccaataaaa
aacgcccggcggcaaccgagcgttctgaacaaatccagatggagttctgaggtcattactggatctatca
acaggagtccaagcgagctcgtaaacttggtctgacagctctagctccggcaaaaaaacgggcaaggt
gtcaccaccctgccctttttctttaaaaccgaaaagattacttcgcgtttgccacctgacgtctaagaaaagg
aatattcagcaatttgcccgtgccgaagaaaggcccacccgtgaaggtgagccagtgagttgattgctac
gtaattagttagttagcccttagtgact

Table1

System coop 2	Sequence
CoopA2 (with T500)	TGGCGGCGCAGCGTCCGGCCCGCCTCACATTTGCTCAACCAAAGC CCGCCGAAAGGCGGGCTTTTCTGT
CoopB2 (with TrrnC	ACTGGCGCGAAATGTAGAGGTGGGCCGGACGAATCCTTAGCGAAAGC TAAGGATTTTTTTT
cooU2	ACATCGCAGGTTTCTGCCTGCCTGCGCCGCCACACAGTAGGAGAAATTCGATATG...

Coop 1A (with T500)	AATTTATTGAGGACGCTGCTTGTACGCTCTCGTATTGACGGCAC CCGCGTCGATGTG AGGGACTTGG
Coop 1B (with B100 2)	CAAGTCCGTGAAGTGTACGGGCAGCTTGATATTTCGACCAGTTG GAACTATTAATTTGGGACCA TTCATAGTGGTTCCGAAG
Coop 1U	AGTTCCGACGGGTCTCCTCTTTCGACTCCGCTTGAAAGAGG AGGTTTGTCATATG

Table Strains and plasmids used in this work.

Strains or plasmids	**Features**	**Ref.**
E. coli DH5□	Commercial	Invitrogen
E. coli DH5□-Z1	Commercial (DH5□□ *lacI*Q, PN25-*tetR*, SpR)	Clontech
E. coli MG1655-Z1	*lacI*Q, PN25-*tetR*, SpR	Gifted by M.B. Elowitz
ptrigR2	pSC101m ori, kanR, sfGFP-LAA	This work
ptrigR2St	pSC101m ori, kanR, sfGFP	This work
ptrigR1	pSC101m ori, kanR, sfGFP-LAA	This work
ptrigR11	pSC101m ori, kanR, sfGFP-LAA	This work
ptrigR11St	pSC101m ori, kanR, sfGFP	This work
ptrigR31	pSC101m ori, kanR, sfGFP-LAA	This work

ptrigR32	pSC101m ori, kanR, sfGFP-LAA	This work
pRAJ11	pUC ori, ampR-kanR, GFPmut3b	[1]
ptrigR31Hfq	pSC101m ori, kanR, sfGFP-LAA	This work
ptrigR31Fusion	pSC101m ori, kanR, sfGFP-LAA	This work
ptrigR11/2	sRNAs from system trigR11, 5' UTR from system trigR2 pSC101m ori, kanR, sfGFP-LAA	This work
ptrigR2/11	sRNAs from system trigR2, 5' UTR from system trigR11 pSC101m ori, kanR, sfGFP-LAA	This work
ptrigR31FFL	pUC ori, ampR, sfGFP-LAA (J23119:SRRb31)	This work

Table 5 Creating single copy RNA switches

pTKRED	pSC101 low copy, SpR, Temp sensitive	This work
pTK/CS	P15A, CamR landing pad	This work
pTKIP	pMB1 ampR , MCS site	This work
pTK/CS-landing pad	P15A, CamR landing pad in ygce	This work
pTKIP-RAJ11	pMB1 ampR, RAJ11	This work
E. coli MG1655-Z1	*lacI*Q, PN25-*tetR*, SpR	Gifted by M.B. Elowitz