Review

RNA aptamers as genetic control devices: The potential of riboswitches as synthetic elements for regulating gene expression

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RNA utilizes many different mechanisms to control gene expression. Among the regulatory elements that respond to external stimuli, riboswitches are a prominent and elegant example. They consist solely of RNA and couple binding of a small molecule ligand to the so-called “aptamer domain” with a conformational change in the downstream “expression platform” which then determines system output. The modular organization of riboswitches and the relative ease with which ligand-binding RNA aptamers can be selected in vitro against almost any molecule have led to the rapid and widespread adoption of engineered riboswitches as artificial genetic control devices in biotechnology and synthetic biology over the past decade. This review highlights proof-of-principle applications to demonstrate the versatility and robustness of engineered riboswitches in regulating gene expression in pro- and eukaryotes. It then focuses on strategies and parameters to identify aptamers that can be integrated into synthetic riboswitches that are functional in vivo, before finishing with a reflection on how to improve the regulatory properties of engineered riboswitches, so that we can not only further expand riboswitch applicability, but also finally fully exploit their potential as control elements in regulating gene expression.

Keywords: Engineered riboswitch · Regulatory circuits · RNA aptamer · RNA aptazyme · SELEX

1 Introduction

Working with RNA can be like opening a “goodie bag” – for one, you never know what you might find until you look, but once in a while you will discover something exciting and unknown you never had thought about before. Similarly, our knowledge of what RNA can do in a cell has changed dramatically since the “central dogma of molecular biology”, in which RNA served primarily as an information carrier, was proposed about 45 years ago [1]. And fortunately, our perception of what RNA might be capable of doing in a cell or in a test-tube is still expanding, since new regulatory functions of RNA are continuously being discovered [2]. Circular exonic RNA molecules, for example, are an abundant and differentially expressed RNA species that have recently been proposed to serve as molecular “sinks” for trans-acting short RNA regulators, as mRNA traps in regulating protein expression, or as interaction partners of RNA-binding proteins [3]. Another newly identified and apparently ubiquitous RNA species is long noncoding RNA. This quite heterogeneous population of RNA molecules participates in many different molecular and cellular processes involved in regulating nuclear organization, development, viability, immunity, and disease [4]. CRISPR/Cas systems are used by various bacteria and archaea as an adaptive immune

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; GFP, green fluorescent protein; miRNA, micro RNA; mRNA, messenger RNA; PPDA, pyrimido[4,5-d]pyrimidine-2,4-diamine; SD, Shine-Dalgarno; SELEX, systematic evolution of ligands by exponential enrichment; shRNA, short hairpin RNA; siRNA, short interfering RNA; sRNA, small RNA; UTR, untranslated leader region

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system to mediate defence against bacteriophages and other foreign nucleic acids. They use a “guide RNA” with complementarity to a specific DNA target site to direct a Cas9 nuclease which then initiates a double-strand break resulting in degradation of the target DNA molecule [5].

Many of these types of RNA-mediated regulation of gene expression have proven to be very attractive for biotechnological and even therapeutic applications. RNA interference [6], using miRNA, shRNA and siRNA, is employed routinely in the lab for loss-of-function studies in eukaryotes [7] and also shows potential as therapeutic molecule in disease treatment [8]. Circular RNAs have biotechnological potential due to their exceptional intracellular stability and their ability to titrate endogenous RNA species or to support internal ribosome entry site-mediated translation [3]. Finally, CRISPR/Cas9 systems are very successful at precisely manipulating the genomes of both pro- and eukaryotes [9, 10].

Sophisticated molecular systems that sense external stimuli and respond to these by modulating the output of a cellular process are another important application in biotechnology. Genetically, this problem can be tackled by several different approaches of which one is based on allosteric RNA elements termed riboswitches. Typically, riboswitches are genetic control elements that consist of two domains – an aptamer domain that binds a small molecule ligand and an expression platform that converts ligand binding into a change in gene expression by adopting an alternative RNA structure [11]. Interestingly, such allosteric aptamer-containing devices were designed and shown to be active in vitro [12, 13] and in vivo [14] about five years before the first naturally occurring riboswitches were discovered [15–17]. Since then, a plethora of different riboswitches have been identified in all three domains of life and have been reviewed comprehensively [18, 19]; see also the recent special edition on “Riboswitches” in Biochimica et Biophysica Acta – Gene Regulatory Mechanisms (2014) [133].

There are several reasons why riboswitches are an attractive tool in biotechnology. Most importantly, they consist solely of RNA. So far, protein components have not been found to be necessary for their activity. Therefore, they are easy to implement, because they involve the transfer of only a single genetic control element into an organism. They are modular, so that different aptamer domains can be combined with different expression platforms [20]. Finally, their use allows spatial, temporal and dosage control over target gene expression. It is therefore not astonishing that engineered riboswitches were introduced early to the fields of genetic engineering and synthetic biology [14, 21] and have seen widespread application since then [22–26].

This review is not intended to serve as a comprehensive overview of all aspects of riboswitch engineering. For example, we do not cover the wide fields of diagnostic and therapeutic aptamers and riboswitches, which have been addressed elsewhere [23, 27–30]; see also the September 2014 special issue on Aptamers published in Molecular Therapy – Nucleic Acids. Our aim is rather to present three topics we consider to be of prime importance for riboswitch biotechnology. We not only demonstrate how far the field has progressed since the early days of artificial in vitro “riboregulators” [12], but also present strategies we believe are important for isolating in vivo active riboswitches, as well as challenges that will be faced in designing more versatile and effective riboswitches. We hope this format will stimulate discussions that will lead to new and improved approaches and selection systems and, ultimately and hopefully, to the development of new and more efficient synthetic riboswitches.

2 Engineered riboswitches: An overview of the status quo

2.1 The toolbox of engineered riboswitches recapitulates the functionality of natural riboswitches

Natural riboswitches control translation, transcription, ribozyme activity, and splicing [15–17, 31, 32]. In the majority of these cases, they act in cis as part of the mRNA transcript. But they can also regulate in trans either as a processed, terminated riboswitch that functions like an sRNA [33] or as a full-length transcript containing a binding site for sequestering an activated RNA-binding protein [34, 35] or by acting as an antisense RNA [36]. In synthetic biology, creating complex and more realistic genetic circuits requires that we eventually identify and design “BioBricks” that not only reflect the entire spectrum of genetic regulatory elements, but that can also be tailored and fine-tuned to obtain the desired output level of the target gene. The first step towards achieving this goal is the construction of proof-of-principle applications to demonstrate that we are able to reproduce these genetic elements using artificial and/or heterologous components. The following section will provide an overview of such archetype constructs. More detailed descriptions can be found in several other reviews [25, 26].

In bacteria, engineered riboswitches most often target translation initiation, either by controlling access to the ribosomal binding site through helix slippage [37] or by sequestering this Shine-Dalgarno (SD) element (Fig. 1A) [38]. Regulation of gene expression by a theophylline-controlled translational switch was efficient enough to allow the control of chemotaxis in Escherichia coli [39]. In contrast, transcriptionally-controlled synthetic riboswitches have only recently been introduced to the toolbox by the Mör [40], Batey [41, 42], and Micklefield [43] groups. Here, a transcriptional terminator is formed depending on the binding state of the aptamer (Fig. 1B). It will be interest-
ing to see how these devices perform with respect to regulatory efficiency, basal activity, noise, and kinetic parameters, in comparison to analogous, but translationally controlled riboswitches in similar experimental settings.

The cleavage activity of small ribozymes, like the hammerhead ribozyme, was made ligand-dependent by inserting an aptamer domain into one of its three stem-loops [44]. Luckily, all three stem-loops of the hammerhead ribozyme are amenable to aptamer insertion [45–48], greatly facilitating the applicability of such “aptazymes”, because the “best fit” between ribozyme and target sequence can now be exploited. In bacteria, such aptazymes were used to liberate the ribosomal binding site after ligand-dependent ribozyme cleavage (Fig. 1C). With respect to the thermally-controlled riboswitches mentioned below in Section 2.3 [49], it will be interesting to see if the recently introduced “thermozymes” [50] can also be modified to include an additional aptamer control element. The examples listed here indicate that in bacteria engineered riboswitches function in regulatory settings that are also found for natural riboswitches.

In eukaryotes, translational regulation by engineered riboswitches functions differently. Here, insertion of the riboswitch into the 5’-untranslated leader region leads to inhibition of translation initiation, either by preventing binding of the small ribosomal subunit to the mRNA cap structure or by interfering with ribosomal subunit scanning for the AUG start codon (Fig. 2A). Here, aptamers which specifically recognize a Hoechst dye [14], malachite green [51], tetracycline [21, 52], neomycin [53], biotin or theophylline [54] (Fig. 2A) have been engineered to serve as regulatory elements. The riboswitches were active in yeast, Xenopus oocytes and mammalian cell culture [55]. Cap-independent translation initiation by an internal ribosome entry site has also been controlled by engineered riboswitches. However, up to now this has only been demonstrated in vitro [56].

The only naturally occurring eukaryotic riboswitches identified so far have been discovered in filamentous fungi, green algae and higher plants where they control gene expression via regulation of mRNA splicing [57–60]. Analogously, theophylline- or tetracycline-binding aptamers have been used to control constitutive and alternative splicing by placing them close to either the 5’-splice site, the 3’-splice site or the intron internal branch point (Fig. 2B) [61–63].
Aptazyme-mediated control of gene expression in yeast and in mammalian cell lines using hammerhead ribozymes (Fig. 2C) has been demonstrated by several groups [46, 48, 64–66]. Here, ligand-dependent ribozyme cleavage leads to mRNA degradation. Besides the hammerhead ribozyme, the hepatitis delta virus ribozyme has also been successfully applied to RNA engineering [67].

RNA interference is an important and widely used mechanism in eukaryotes to repress gene expression by base-pairing to mRNA molecules. RNA interference controls a broad range of developmental and physiological processes [68–70]. It is also a standard method in molecular biology with a possible therapeutic option which is being tested in clinical trials [71]. Consequently, attempts to control the biogenesis pathway of RNA interference were made by introducing theophylline-dependent aptamers [72, 73] or aptazymes [74] at sites of siRNA or shRNA molecules where their presence can interfere with...
processing by either Drosha or Dicer (Fig. 2D). So far, extremely high ligand concentrations are needed for efficient regulation. Optimization of the experimental strategies is clearly needed and will be challenging to see if endogenous miRNA molecules can also become subject to riboswitch control.

2.2 Riboswitch regulation is becoming more robust and versatile

While proof-of-principle applications are important for demonstrating the functionality of any kind of experimental approach, they do not, per se, demonstrate their general transferability to other systems, organisms or even across the kingdoms of life. Undoubtedly, the general applicability and the ease with which system functionality can be achieved is an important parameter for determining success and acceptance of an application as a tool by the respective community. So far, most of the regulatory strategies presented in the previous section were tested in the well-characterized model organisms E. coli for Gram-negative bacteria, Bacillus subtilis for Gram-positive bacteria, Saccharomyces cerevisiae for lower eukaryotes and mammalian cell lines for higher eukaryotes [19]. In developmental biology, biotechnology and in medicine, other organisms are frequently employed as physiological, developmental and disease models or as cell factories for the production of materials [75–80]. Since endogenous riboswitches have been identified in the genomes of many different organisms [81], it seem reasonable to assume that engineered riboswitches, based on similar regulatory principles, should also be active in these species.

In bacteria, the Gallivan lab, consequently, designed a set of theophylline-inducible translationally controlled riboswitches, initially termed A-E, which vary in the strength of their ribosomal binding sites and the stability of their secondary structures [38]. Later, riboswitches designated E* (supplemental material in [38]), F (clone 8.1 from [82]), and G (clone D2 from [83]) were added to the set. These riboswitches were then tested for activity in diverse bacterial species, including the developmental model organism and major producer of antibiotics, anticancer and antiviral drugs or enzymes, Streptomyces coelicolor [84], cyanobacteria, model organisms for photosynthesis and potential material-producing cell factories [85, 86] or tobacco chloroplasts, which are of interest in plant biotechnology [87]. The riboswitch set was also evaluated in important pathogens like Agrobacterium tumefaciens, Acinetobacter baumannii, Streptococcus pyogenes [38], Mycobacterium [88] and Francisella [89]. The tetracycline aptamer was shown to be functional in the anaerobic model organism Methanosarcina acetivorans, an archaeon [90]. In all organisms tested, at least one riboswitch was active enough to establish a conditional knockout of an endogenous gene. In some cases, the regulatory factors obtained were similar to those obtained with transcriptional regulation. Effector molecule penetration across several membranes was also effective enough to permit target gene expression control in chloroplasts and in intracellular infections models.

Theophylline was the preferred ligand of choice. It can be added at high concentrations (2–4 mM) to the medium without causing obvious toxicity. In contrast, tetracycline and neomycin are antibiotics in bacteria and the concentrations needed for riboswitch activity can be close to or above the respective minimal inhibitory concentrations. Unfortunately, anhydrotetracycline, a less toxic derivative of tetracycline, does not bind to the tetracycline aptamer [91]. Translational ON-switches are the most frequently used type of expression platform. They appear the easiest to integrate into the sequence of the target gene. And finally, there is no single “optimal” riboswitch. Depending on the species and the specific target gene, different riboswitches displayed the best regulatory properties. So far, several constructs have to be tested to find the right combination for a given application.

Regulation of gene expression by synthetic riboswitches is not limited to bacteria. Viral replication was inhibited in eukaryotic DNA (adenovirus, adeno-associated virus) and RNA viruses (measles) by flanking essential genes with theophylline-dependent aptazymes [92, 93].

Taken together, gene expression regulation by riboswitches has been demonstrated to be stringent, reversible, and dose-dependent in viruses, organelles, archaea, and bacteria from a broad taxonomic range of species, firmly establishing this technology as robust and universally applicable.

2.3 Riboswitches can be integrated into complex regulatory circuits

Genetic circuits are frequently more complex than simple one input/one output type networks. They can have complex architectures like toggle switches [94], oscillators [95], Boolean logic gates that integrate several external signals [96], and binary or graded responses [97]. They can also include positive [98] or negative [99] feedback loops or linearizers [100]. These examples of engineered circuits were all realized with protein-based transcription factors. Consequently, the interesting question is to determine if RNA-based devices can be used similarly to assemble multiple input or complex regulatory circuits.

Nature itself has come up with several combinatorial solutions to tackle diverse regulatory problems. In B. subtilis, the glmS riboswitch probably represents the simplest solution to regulation by multiple signals. The ligand-binding pocket of the aptamer domain not only binds intracellular glucosamine-6-phosphate as an activating agent. It also binds glucose-6-phosphate, which acts as an inhibitor of ribozyme activity. The riboswitch, thus, integrates metabolite information to sense the overall
metabolic state of the cell [101]. A second solitary element that senses and integrates two different input signals is the adenine riboswitch encoded by the add gene from Vibrio vulnificus. This translational regulatory element senses temperature and ligand concentration to confer efficient regulation over the range of physiologically relevant temperatures, allowing gene expression control to adapt to the differing temperatures experienced in the free-living and in the infectious environments [49]. In Bacillus clausii, two different riboswitches, which responded independently to S-adenosylmethionine and vitamin B12, respectively, were found in tandem in the 5’ untranslated leader region (UTR) of the metE mRNA. System output fits a Boolean NOR gate, because transcription is terminated in the presence of only one or of both ligands [102].

The Yokobayashi group was first to exploit this strategy in riboswitch engineering. They embedded aptamers that recognize theophylline and thiamine pyrophosphate, respectively, in tandem in the 5’-UTR of a bacterial mRNA and selected for translational riboswitches that responded to ligand presence like AND or NAND logic gates. Reporter gene activity was observed either only in the presence of both ligands (AND gate), or, in the case of the NAND gate, when neither or only one ligand was present [103]. In a subsequent, different experimental setup, they combined a transcriptional-OFF riboswitch with a translational-ON switch. Because both switches respond to the same ligand, thiamine pyrophosphate, albeit with different sensitivity, a band pass filter is the result, with maximum target gene expression obtained at intermediate ligand concentrations. At low ligand concentrations, gene expression is repressed translationally, while at high concentrations, premature transcription termination occurs [104]. Boolean logic gates can also be constructed with aptazymes. The Hartig group [105] combined ribozyme switches responsive to thiamine pyrophosphate and theophylline to yield AND, NOR, and ANDNOT gates. Win and Smolke [106] also generated an entire set of Boolean logic gates by combining theophylline- and tetracycline-responsive aptamers with ON- and OFF-responsive ribozymes. For example, such gates were constructed either by assembling two different aptazymes in series (AND gate and NOR gate) or by, very ingeniously, exploiting the possibility of joining aptamer-responsive control elements to all stems of the hammerhead ribozyme. Coupling two ON-switches responsive to different inputs to different ribozyme stems led to an output signal only if no or one of the ligands was present, thus generating a NAND gate [106].

3 What makes a riboswitch out of an aptamer?

So far, in vivo active riboswitches rely on a small set of ligands – theophylline, tetracycline, neomycin [53], 2,4-dinitrotoluene [107], ammeline, 5-aza-cytosine [108] or pyrimido(4,5-d)pyrimidine-2,4-diamine (PPDA) [43], although several dozen small molecule-binding aptamers have been selected by a process called SELEX (systematic evolution of ligands by exponential enrichment), an in vitro technology invented about a decade before the discovery of natural riboswitches [109, 110]. During SELEX, a huge combinatorial library of RNA (and sometimes DNA) molecules is subjected to affinity of a small RNA chromatography to the ligand of choice. Non-binding sequences are removed during washing steps, whereas sequences with a certain binding affinity to the target of interest are eluted. These molecules are amplified and subjected to further rounds of selection. During these iterative cycles, the selection pressure can be increased steadily, finally resulting in molecules with sometimes extraordinarily high binding affinities and specificities, the so-called aptamers (Fig. 3A). However, as stated above, only a handful of such small molecule-binding aptamers have made it into the toolbox of RNA engineers. This raises the questions (i) what makes a riboswitch out of an aptamer; and (ii) how can we identify further aptamers which can be used for riboswitch engineering.

A detailed analysis of neomycin-binding aptamers could shed some light on the properties such aptamers need in order to become regulatory molecules that are active in vivo. The neomycin-binding aptamer R23 was isolated by in vitro selection [111] and shows both high affinity and specificity for its ligand [111]. However, this aptamer – like many other small molecule-binding aptamers, too – showed no ligand-dependent regulation when tested for riboswitch activity in an assay for translational regulation (like in Fig. 2A) [53]. The subsequent analysis of the enriched aptamer pool (after seven rounds of selection) for sequences with regulatory activity using an in vivo screen, such as the one displayed in Fig. 3A, resulted in the isolation of a completely different aptamer, designated N1 and shown in Fig. 3B [53]. This new neomycin aptamer is active as riboswitch when inserted into the 5’-UTR of a reporter gene (Fig. 2A) [53], but also as sensing domain in the context of an allosteric ribozyme (Fig. 2C) [66]. Interestingly, N1 was not among the sequenced aptamers isolated after in vitro selection indicating that this aptamer was underrepresented in the pool enriched for binding aptamers [111].

A detailed genetic, biochemical and structural analysis of the aptamer N1 unravelled the molecular basis of this regulation. While the aptamer shows tight ligand binding with a $K_d$ in the low nanomolar range, this affinity was still very similar to that of the originally identified in vivo inactive aptamer R23 [112]. However, substantial
conformational changes concomitant to ligand binding were detected in N1, which were completely absent in the inactive aptamer R23 [113]. A short intervening sequence separating two short helical elements was identified as being important for regulation and therefore called “switching element”. It allows the ground state of the aptamer to retain an open, less structured conformation. Ligand binding then stabilizes a highly structured conformation, in which additional base pairs are formed. These elongate the two short helices and promote stacking of the upper onto the lower helix resulting in a long α-helical element (Fig. 3B). The ligand neomycin then acts like a clamp holding both the upper and lower helix together. This conformation then is able to efficiently interfere with ribosomal scanning in yeast. It seems that the interplay between an open ground state and a highly structured ligand-bound state is important for the regulatory activity of the aptamer.

Further aptamers with confirmed riboswitch activity, like the malachite green or the theophylline aptamers, show a similar open, less structured ground state and a highly structured ligand-bound conformation [114, 115].

The conclusion drawn from this analysis is that regulating aptamers have to possess two properties: first, high affinity ligand binding, and second, significant conformational changes and stabilization upon ligand binding. The first point can be addressed in any classical SELEX experiment. By increasing the stringency of the selection process, high affinity binding aptamers can be obtained with binding constants even in the picomolar range [116]. Tight binding, however, is necessary but not sufficient for regulation. The second requirement – the conformational changes within the aptamer upon ligand binding – cannot be addressed by in vitro selection. An additional cellular screening step, preferably directly in the organism of
choice, is necessary to identify aptamers with regulatory activity.

Several approaches have been undertaken which not only support the success, but also reinforce the importance of cellular screening for the identification of in vivo active regulatory devices. Gallivan and colleagues applied different screens to identify in vivo active riboswitches [83, 117], which proved to be extremely powerful in bacteria (see also Section 2.2). In vivo screening was also applied to identify aptamer-controlled ribozymes. Here, the communication module connecting the sensing domain (aptamer) with the catalytic domain (ribozyme) was randomized [118, 119]. Together with the neomycin screen described above, these examples underline the importance of this approach.

4 How can we further improve riboswitches?

Despite of the success engineered riboswitches are enjoying as regulatory devices, there are still two important problems that have to be overcome to allow us to build even better riboswitches. One is the lack of a large number of alternative aptamers as ligand-binding modules and the second is the large discrepancy between the in vitro affinities of aptamers for their ligands and the high concentrations of ligand needed in vivo to flip the corresponding switches.

Besides introducing an additional cellular screening step which allows the identification of regulating aptamers as specified above, reengineering the aptamer domain scaffolds of existing functional riboswitches to recognize novel ligands is also a promising approach to extend the ligand toolbox. The Micklefield group mutated a purine riboswitch to not recognize its natural ligand anymore and then screened this mutant pool for binding to synthetic, heterocyclic molecules. In the end, they obtained a ligand, PPDA, which controlled riboswitch activity efficiently [43, 108]. It will be interesting to see if this approach can be extended successfully to other natural riboswitches and, how different any potential novel ligands can be structurally from the respective native ligands. The many approaches to identify inhibitors of riboswitch activity for therapeutic applications might serve as starting points for finding such alternative ligands [120–122]. More extensive mutagenesis might contribute to this process, because natural sequence variants of riboswitches already display functional diversity, which is thought to reflect the adaptation to the idiosyncratic regulatory requirements of a specific gene [123]. Correspondingly, mutagenesis of residues that are not directly involved in ligand binding affect the regulatory properties of riboswitches [124, 125].

The ligand-binding site of the tetracycline aptamer is formed by a three-way-junction that is stabilized by long-range interactions and almost completely envelops the ligand [126]. This is reminiscent of natural riboswitches and distinguishes the tetracycline aptamer from the ligand-binding pockets of many aptamers which are much more open and solvent-exposed [127]. It might suggest that alternative aptamer selection protocols, like capillary electrophoresis, that do not require coupling of the ligand to a matrix and, thus, do not force surface exposure might be more advantageous for isolating in vivo functional aptamer domains [128–130].

A discrepancy between high affinity binding and efficient regulation has not only been observed for engineered riboswitches, but also for the tetrahydrofolate riboswitch. Although several ligand analogs and adenine derivatives were found to bind with high affinity to the riboswitch, they were not able to regulate its activity [131]. The mechanism(s) responsible for this effect are not clear at the moment, although for synthetic riboswitches, the fusion of an aptamer domain to an expression platform might result in a suboptimal interface between the two elements. Here, some optimization by mutagenesis and selection might be needed. A recent publication by the Gallivan lab might aid in this optimization process [132]. Mishler & Gallivan were able to recapitulate the approximately 1000-fold discrepancy between the concentrations needed for efficient binding of theophylline to its aptamer and the concentration needed for activating the activity.
riboswitch in E. coli in vitro in an S30 cell extract. This might finally allow in vitro screening and selection for more efficient switches requiring less ligand [132].

5 Conclusions

Riboswitch engineering has come a long way since the first applications were introduced approximately fifteen years ago. This technology has finally reached a stage in which it can not only complement other established mechanisms and strategies to regulate gene expression, but in which there are applications where we believe that it can even effectively replace these. The riboswitch engineer’s toolbox now provides a broad platform which should be able to offer flexible solutions to most synthetic biology applications requiring regulation of gene expression. Advances in selection technology and further improvement of our understanding of riboswitch structure and function will guide the design of better riboswitches to harness the full regulatory, diagnostic and therapeutic potential of aptamer-controlled regulatory devices.

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