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Aptamers for allosteric regulation

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Aptamers are useful for allosteric regulation because they are nucleic acid–based structures in which ligand binding induces conformational changes that may alter the function of a connected oligonucleotide at a distant site. Through this approach, a specific input is efficiently converted into an altered output. This property makes these biomolecules ideally suited to function as sensors or switches in biochemical assays or inside living cells. The ability to select oligonucleotide-based recognition elements *in vitro* in combination with the availability of nucleic acids with enzymatic activity has led to the development of a wide range of engineered allosteric aptasensors and aptazymes. Here, we discuss recent progress in the screening, design and diversity of these conformational switching oligonucleotides. We cover their application *in vitro* and for regulating gene expression in both prokaryotes and eukaryotes.

There is a great demand in chemical biology for tools that convert a specific input into a desired output, because such tools can find broad application in measuring the concentration of biomolecules, in modulating protein levels and activity or in controlling molecular machinery. Cells and organisms have evolved such molecular control mechanisms so as to be able to adjust rapidly and precisely to both extracellular and intracellular changes in the physicochemical environment. These changes are sensed by cellular networks of macromolecules, which activate molecular pathways at the transcriptional, translational or post-translational level. Inspired by these natural gates, which efficiently translate a change in input to an altered output, researchers have made strong efforts to design artificial sensors or switches using synthetic molecules or biomolecules. Strategies involving proteins and peptides have been pursued[1], but here we discuss recent examples involving nucleic acids, particularly aptamers[2], thereby demonstrating their suitability and versatility to generate regulatory elements.

An important reason that nucleic acids are used to engineer synthetic switches is that their secondary structure is mostly governed by Watson-Crick base-pairing interactions. As a result, the rational design of an oligonucleotide in which ligand binding induces a conformational change that alters function at a distant site can be fairly straightforward. In addition, a recognition element for a ligand of interest can be obtained with relative ease. Libraries of short nucleobases have been screened by a process called SELEX (systematic evolution of ligands by exponential enrichment)[3,4], which has resulted in aptamers that bind targets ranging from small molecules and proteins to protein complexes and even entire cells[5]. Aptamers can be as specific as antibodies and can bind their targets with similar affinity, but they are often smaller, easier to generate and more straightforward to modify chemically than their protein-based counterparts[2,6].

Importantly, the evolved aptamers can frequently be combined with other functional elements without altering their molecular recognition properties. If such an element is a natural or evolved nucleic acid enzyme, its activity can be regulated by the addition of its ligand (**Fig. 1**). A classical example is the ATP-induced activation of a hammerhead ribozyme (HHR) through replacement of one of the stem-loops of the enzyme with an ATP-binding aptamer[7]. As an alternative output, a second aptamer can be used to obtain a switch for which the structural changes upon binding of the first ligand alter the aptamer's ability to bind a second effector.

An early example of such a system was a label-free sensor that could bind the fluorophore Malachite Green after binding of the ligand flavin mononucleotide (FMN), leading to an increase in fluorescence[8]. The mechanisms involving altered enzymatic activity or binding of a second effector due to ligand binding also occur in natural regulatory RNA elements. Allosteric ribozymes control gene expression by cleaving RNA in response to specific metabolites, and riboswitches undergo a conformational change upon ligand binding that enhances or diminishes ribosome binding, thereby influencing the translation efficiency of the mRNA[9,10]. These examples illustrated the potential of allosteric aptamers for application *in vitro* and *in vivo* and have led to the engineering of a plethora of nucleic acid–based sensors and switches.

In our discussion of the latest developments in the field of allosteric aptamers, we include the expansion of the output domains for application *in vitro* (**Fig. 1**) and the establishment of DNA and RNA to create molecular computing circuits. Furthermore, we provide an overview of advances in both design and screening methods that have greatly increased the multitude of artificial RNA switches that regulate gene expression.

Outputs for *in vitro* applications

The first applications of aptamers *in vitro* involved individual aptamers that were chemically modified by fluorophores and found applications in systems where antibodies are traditionally used, such as ELISAs[11,12]. The time-consuming washing steps in such assays not only make them labor intensive, but also prevent analysis in real time. By contrast, allosteric aptamers generate an output only after they bind ligand, thereby eliminating washing steps. If a non-accumulative output is chosen, real-time measurements become possible.

A straightforward way in which to achieve an immediate output signal after ligand binding is by coupling aptamers to natural ribozymes or synthetic DNAzymes in a way that makes the catalytic activity of the enzyme ligand dependent—for example, by replacing one of the enzyme's stems with an adaptive aptamer of interest. Aptazymes that use this principle have been developed through rational and high-throughput design strategies[13], resulting in allosteric aptamers that respond to metal ions, small molecules, oligonucleotides and even proteins[14]. Aptamers have also been selected that recognize the conformation of an allosteric RNA[15,16]. One highly modular detection method relies on functionalization of the ribozyme's oligonucleotide substrate strand with pairs of fluorophore and quencher dyes, or a

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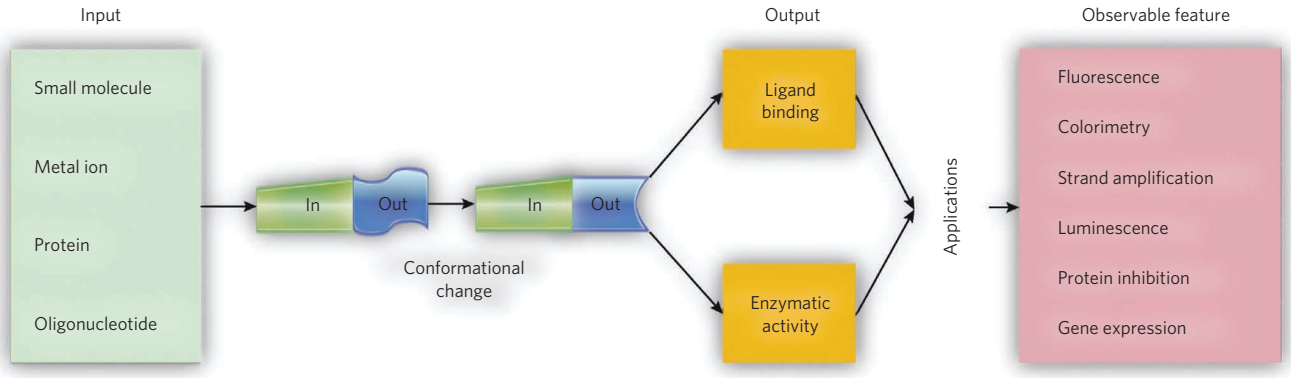


Figure 1 | Signal transduction by aptamer-mediated allostery. Different ligand types have been used as inputs to generate a conformational change that either alters the aptamer's ability to bind a second effector or changes its enzymatic activity. The resulting aptamers have been used as sensors, by coupling them to a detectable readout, or as switches that control protein expression or function.

fluorescence resonance energy transfer donor-acceptor pair. Ligand binding and subsequent strand cleavage resulting from the aptamer-modulated catalytic activity then leads to a change in fluorescence[17–20] (**Fig. 2a**). This strategy has allowed researchers not only to use reporter ribozymes for detecting ligands in solution, but also to use regulatory aptamer–protein complexes to screen for small molecules that inhibit this interaction[21]. An allosteric aptazyme based on hairpin ribozyme regulation by the anti–α-thrombin aptamer was used to show that even protein-protein interactions can be accurately monitored[22]. This approach was further extended to sense the activity status of a protein, as was shown for the L-tryptophan–activated Trp-RNA–binding attenuation protein[23].

Another example used small, metastable RNA hairpins containing a short overhang or 'toehold' at the 5′ end[24]. The addition of an initiator sequence that was complementary to the toehold sequence and to a part of the nucleotides in the hairpin led to unfolding of the hairpin. This resulted in a 3′ overhang that served as an initiator for unfolding of a second hairpin, yielding a new 3′ overhang. Addition of the initiator to a solution containing four different hairpins, one labeled with a fluorophore-quencher pair, started a cross-catalytic circuit in which fluorescence quenching decreased exponentially. The approach was extended to use mRNAs as initiator sequences to amplify fluorescence, enabling simultaneous imaging of multiple mRNAs in fixed zebrafish embryos[25]. This demonstrated the ability of these metastable hairpins to amplify signals in biological probes.

Synthetic DNAzymes have been coupled to gold nanoparticles (AuNPs) to create sensors with a colorimetric output. Analyte binding increases the enzymatic activity and thereby reduces the aggregation state of the AuNPs, which induces a color change from blue to red (**Fig. 2b**). This mechanism was first shown to function for adenosine and cocaine as a proof of principle[26,27]. Sensors for metal ions such as Pb^{2+}, Hg^{2+} and uranyl that function by the same principle have also been developed[11], although in these cases the metal functions as a cofactor and not as an allosteric effector. The power of these colorimetric systems lies in their ease of application, as has been shown by dipstick tests to allow on-site detection of cocaine levels in blood serum[28] and Pb^{2+} levels in paint[29].

In addition to the catalysis of phosphodiester transfer reactions that lead to strand cleavage, ribozymes that catalyze other reactions have also been incorporated into allosteric designs. For example, a Diels-Alderase ribozyme was made theophylline responsive to generate colored or fluorescent reaction products of the Diels-Alder reaction upon target binding[30]. In addition, synthetic RNA ligases have been used to create sensors for which ligand binding induces strand ligation, thereby making the amount of ligation product a measure of the ligand concentration[31,32]. A drawback of these systems is their relatively low sensitivity due to the linear increase in signal after ligand binding, which has led to the addition of a polymerase to exponentially amplify the reaction product.

Recently, RNA enzymes that replicate each other were developed, thereby allowing an RNA ligase–based sensor design that does not require the addition of a polymerase for exponential amplification. Two RNA enzymes catalyze each other's synthesis from a total of four RNA substrates[33,34] (**Fig. 2c**). When a hairpin in the RNAzyme structure was replaced with the theophylline- or FMN-binding aptamers, RNA replication was observed only in the presence of ligand[35]. In the original design, the amplification rate was reduced—and no longer dependent on the ligand concentration—at low analyte concentrations, thereby complicating the RNAzyme's application as a quantitative analytical tool in most biochemical assays. More recently, however, quantitative ligand detection was achieved by dividing amplification into two steps. In step 1, ligand binding leads to enzyme activation, after which the resulting ligation product serves as the enzyme for further amplification in step 2 (ref. 36). Although the assay so far has been established for theophylline detection, the straightforward design of these self-replicating RNAs holds promise for extension to more physiologically relevant analytes in biological samples.

Horseradish peroxidase is essential for diagnostic assays involving antibodies such as western blots and ELISAs. In 1998, G-quadruplex structures were shown to show peroxidase activity after binding of hemin[37]. The resulting horseradish peroxidase (HRP)–mimicking DNAzyme has since been used as an output module in multiple allosteric aptamers, all based on the principle that ligand binding enables the formation of the G-quadruplex, which subsequently enhances enzymatic activity[38,39] (**Fig. 2d**). Most commonly, DNAzyme activity is monitored through the conversion of 2,2′-azido-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$) to the colored $ABTS^{\bullet-}$ radical anion, although the oxidation of luminol can also be observed using chemiluminescence[40]. The G-quadruplex structure of the anti–α-thrombin aptamer also shows enzymatic activity[41]. Importantly, the activity depends on binding of thrombin, which suggests that aptamers that contain G-quadruplexes potentially have a built-in readout for biochemical assays.

The HRP-mimicking DNAzyme has also been used to create metal sensors. Addition of Pb^{2+} or Hg^{2+} to a K^+-stabilized G-quadruplex results in a structural change that leads to hemin release and a concomitant decrease in enzymatic activity, allowing the detection of nanomolar levels of these toxic metal ions[40,42]. In a different approach, the HRP-mimicking DNAzyme was split into two halves, and then recognition sequences for Hg^{2+} or adenosine

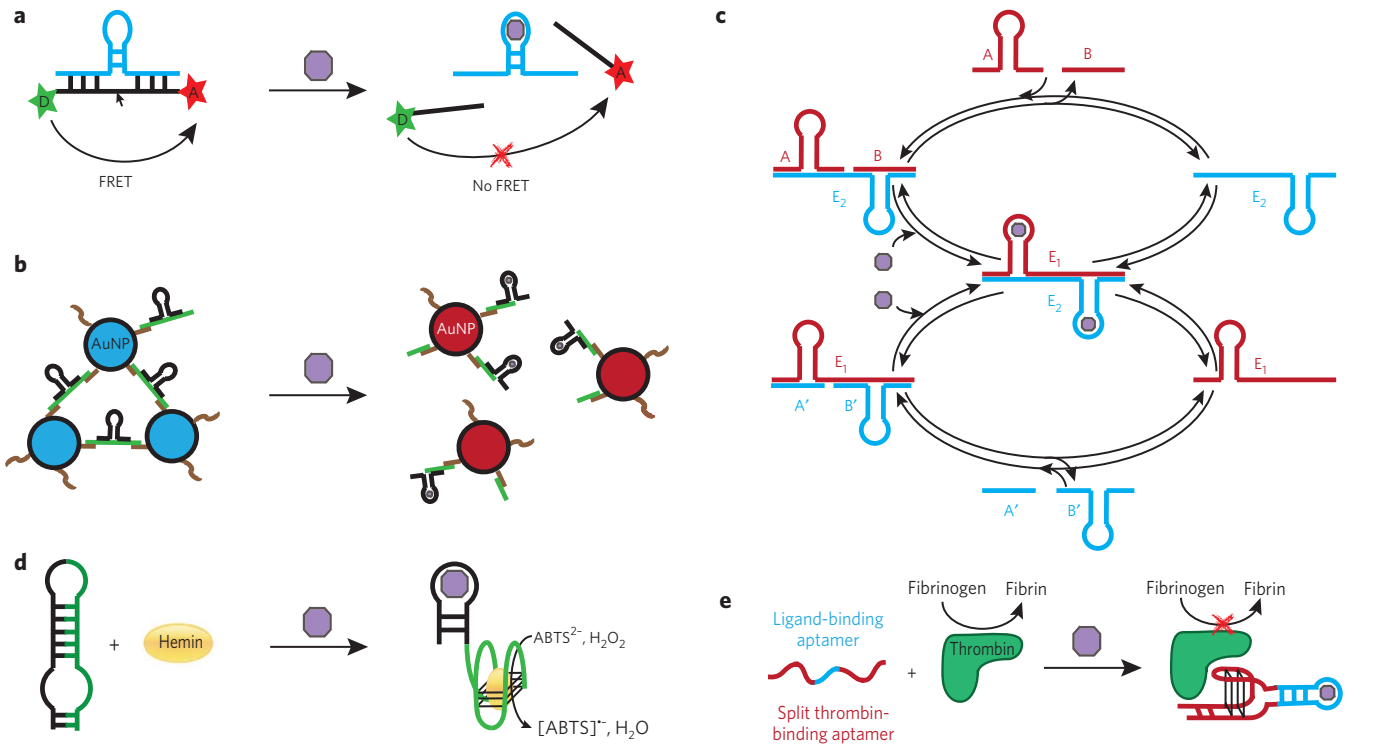


Figure 2 | Designs of allosteric aptasensors for application *in vitro*. (**a**,**b**) Schematic representation of allosteric ribozymes or DNAzymes for which ligand binding induces strand cleavage. Cleavage is detected through a change in fluorescence (**a**) or a shift in color from blue to red (**b**). (**c**) Self-replicating RNAs that catalyze each other's synthesis. At the start of the reaction, substrates A, B, A′ and B′ and either RNAzyme E_1 or E_2 are present. If E_1 is present, ligand binding to this oligonucleotide enables ligation of A′ and B′ to form E_2. The latter catalyzes ligation of A and B in the presence of ligand to form E_1. The presence of ligand therefore leads to exponential amplification of E_1 and E_2. (**d**) Schematic illustration of a DNA hairpin that consists of a ligand-binding domain fused to an HRP-mimicking DNAzyme. Addition of ligand allows binding of hemin to the HRP-mimicking DNAzyme, leading to catalysis of $ABTS^{2-}$ into the colored $[ABTS]^{\bullet-}$. (**e**) Allosteric aptasensor design created by fusion of a ligand-binding aptamer to a split variant of the α-thrombin aptamer. Ligand binding stabilizes a conformation that enables the aptasensor to bind to thrombin, thereby inhibiting the protein's activity.

were inserted between these halves[43,44]. Both the full-length and split G-quadruplex sensors showed a strong signal change after addition of ligand, but their OFF-switching character might turn out to be a disadvantage for application in some assays. A complex design in which the HRP-mimicking DNAzyme was used involved four separate DNA strands[45]. Addition of ligand triggered a 'DNAzyme cascade' in which a DNAzyme was formed from two strands, which then recognized and cleaved a third strand, leading to the formation of two HRP-mimicking DNAzymes. A fourth strand prevented peroxidase activity in the absence of ligand by hybridizing with the third strand. Although technically not allosteric, the assembly from four parts yields a highly modular design that allows the facile exchange of input domains for a ligand-induced change in enzymatic activity.

Compared to the variety of engineered aptazymes, there are relatively few examples in which ligand binding alters the oligonucleotide's ability to bind a second effector molecule. The design of an allosteric aptamer that binds Malachite Green in the presence of ligand showed great promise to detect small metabolites in cells. Being label free, the aptamer can be endogenously expressed, and this can be followed by cellular dye uptake to enable sensing. Although there has been some recent progress in the palette of fluorophores that light up upon RNA binding[46,47], no examples of intracellular application of these sensor designs are available yet, which illustrates the challenges to be overcome in achieving this rewarding goal.

Besides coupling of two aptamers that bind small molecules, protein-binding aptamers have been made ligand dependent by fusion to a recognition element for a small effector. For this purpose, the G-quadruplex structure that binds to thrombin was also split in two parts and combined with the adenosine-binding aptamer to generate allosteric regulators that either inhibit or enhance thrombin activity upon ligand binding[48] (**Fig. 2e**). In a similar fashion, fusion of the aptamers for vascular endothelial growth factor or a protein tyrosine phosphatase to Russell's viper venom factor X activator (RVV-X) led to allosteric aptamers that inhibited RVV-X upon binding of their respective ligands[49]. Despite the allosteric switching of the aptamers in these examples, the read-outs of the assays are blood-clotting time and precipitation of microspheres that bind to clotted fibrin, which will probably complicate widespread application. However, these switches clearly show the potential of allosteric aptamers to regulate protein activity in a ligand-dependent fashion.

Nucleic acid–based logic gates

In addition to the application of allosteric aptamers *in vitro* to detect biomolecules, the input and output domains provided by aptamers and nucleic acid–based enzymes also serve as ideal building blocks for nanoscale molecular computation. Although such circuits are unlikely to replace the current von Neumann cycle–based microprocessors in the near future, DNA computation might find applications in both the biological and materials sciences and has the potential to provide control components for autonomous agents in therapeutics.

Early work included the use of nucleic acid catalysts to generate Boolean operators based on RNA, either by rational design[50] or assisted by computer modeling[51]. The potential of these logic gates was demonstrated by their incorporation in a molecular automaton that could play flawless tic-tac-toe against a human opponent[52]. These gates had oligonucleotides as their inputs and a change in

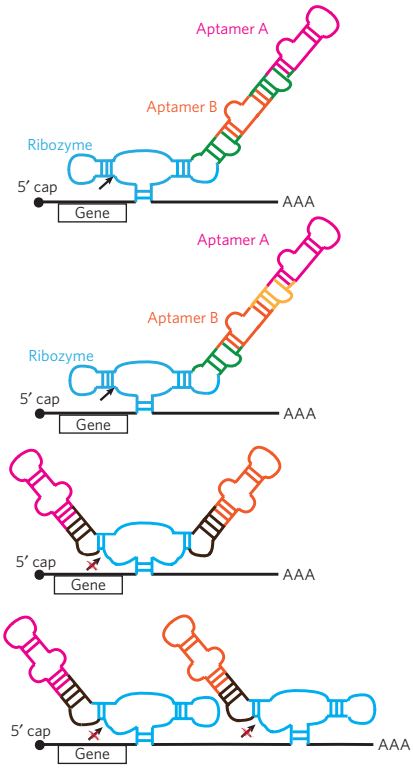
fluorescence due to strand cleavage as their output, but the approach was extended to yield gates in which strand cleavage altered the functional state of an aptamer, thereby releasing a small molecule or changing the enzymatic activity[53]. Although this strategy has not been applied inside cells yet, one can envision its application to release drugs or inhibit proteins only in the presence of a specific set of inputs. More recently, the split DNAzyme that forms part of a cascade (see above)[45] has been used as a logic gate building block[54]. The core of this design involves two DNA strands that, upon binding of a specific input sequence, self-assemble into an active DNAzyme, leading to cleavage of a fluorophore and quencher-labeled output strand. A library of split DNAzymes was created that could combine into various enzymes, depending on the provided input strand. Each output strand was labeled with a different fluorophore, allowing complex gating in which each fluorescence emission spectrum was the exclusive result of a specific input sequence. Gating was demonstrated both in parallel and in serial, thereby in principle allowing the construction of negative or positive feedback loops to gain control over the amount of released output.

The input of allosteric aptamer-based logic gates is not limited to oligonucleotides that hybridize with a DNAzyme to alter its conformation and enzymatic activity. The aptamers for adenosine and thrombin have been fused together with an 11-nucleotide linker to create an OR gate and an AND gate[55]. In the absence of ligand, two complementary DNA sequences bound both of the aptamer strands, whereas ligand binding of thrombin or adenosine resulted in displacement of the respective complementary strand, yielding a change in fluorescence. The earlier discussed self-replicating RNAs were also shown to function as an AND gate, as a combination of the FMN and theophylline-dependent enzyme in a single reaction yielded exponentially amplified product only in the presence of both ligands[35].

One of the first intracellular applications of logic gates based on allosteric aptamers involved ligand-dependent control over gene expression in yeast. Central to this design was the full-length HHR, for which fusion to an aptamer sequence and placement in the 3′ untranslated region (UTR) of a target gene allows ligand-induced control over the expression of the target gene[56]. Depending on the connecting module between the aptamer and the ribozyme, activity was either enhanced or decreased in the presence of ligand. Importantly, the use of a connecting module allowed facile exchange of a theophylline aptamer with a tetracycline aptamer, showing that, at least for small molecules, the design is fairly modular. By fusing more aptamers to the ribozyme, and by connecting them both in different positions and in series or in parallel, the authors obtained AND, OR, NAND and NOR gates[57] (**Fig. 3**). Although for some gates the difference in gene expression between a single and a dual input was rather modest (about four-fold), the approach illustrates the potential for using aptazyme-based logic gates to generate a desired response to a given combination of inputs.

Intracellular application of allosteric aptamers

Adaptive aptamers that inhibit intracellular proteins or protein domains have been applied in various contexts *in vivo* for more than a decade—a concept that is often referred to as the 'intramer approach'[58–61]. The first example of an intracellularly applied allosteric aptamer was also created more than ten years ago to regulate gene expression[62] and inspired researchers to design RNA-based switches. These switches function similarly to natural riboswitches—they undergo a conformational change upon ligand binding that alters the translation efficiency—or contain allosteric ribozymes for which effector binding yields cleavage of an RNA of interest. The intracellular application of engineered allosteric regulation based on natural riboswitches has been thoroughly reviewed[63–67]; here we focus on the most recent strategies to alter gene expression with aptazymes in relation to the organism in which the gene is regulated.



AND gate

A	B	Enzyme	Output
0	0	ON	0
1	0	ON	0
0	1	ON	0
1	1	OFF	1

OR gate

A	B	Enzyme	Output
0	0	ON	0
1	0	OFF	1
0	1	OFF	1
1	1	OFF	1

NAND gate

A	B	Enzyme	Output
0	0	OFF	1
1	0	OFF	1
0	1	OFF	1
1	1	ON	0

NOR gate

A	B	Enzyme	Output
0	0	OFF	1
1	0	ON	0
0	1	ON	0
1	1	ON	0

Figure 3 | Modular logic gate designs based on the HHR. Placement of an allosteric ribozyme in the 3′ end of a gene of interest allowed ligand-induced removal of the poly(A) tail, providing control over gene expression. Depending on the connecting modules that are used to fuse aptamers to different stems of the ribozyme, ligand binding induces switching of the HHR from the active to the inactive state or vice versa. For the AND gate, binding of ligands A and B is required for ribozyme inactivation, leading to gene expression. The ribozyme of the OR gate has a similar design, but the connecting module between the two aptamers was devised in such a way that binding of ligand A, B or both all led to deactivation of the enzyme. The NAND gate is deactivated before ligand binding and activates only when both ligands have bound to their respective aptamers. Placing two ribozymes with single inputs in serial creates a NOR gate for which either ligand A or B suffices for strand cleavage and thus gene downregulation. Adapted from ref. 56. Reprinted with permission from the American Association for the Advancement of Science.

Gene regulation in prokaryotes. Most allosteric ribozymes that are used in *in vitro* assays contain the minimal motif of the HHR. This truncated version requires Mg^{2+} concentrations that exceed the intracellular free Mg^{2+} levels, which complicates its application inside cells. Elucidation of the crystal structure of the full-length HHR revealed that tertiary interactions of stem I and stem II are important for ribozyme activity and enable cleavage at physiological concentrations of Mg^{2+} (ref. 68). This has strongly facilitated the success of using aptazymes that rely on ribozyme cleavage *in vivo* and has led to different mechanisms for regulating prokaryotic gene expression (**Fig. 4a**,**b**). By coupling a theophylline-responsive, full-length HHR to a strand that is complementary to the ribosome binding site (RBS), gene expression is prohibited in absence of ligand. Both theophylline and thiamine have been used to activate the ribozyme and liberate the RBS, thereby enabling gene expression[69–71] (**Fig. 4a**).

An alternative approach that has the potential to regulate bacterial gene expression was achieved by inserting an aptazyme into 16S RNA of an orthogonal 16S rRNA–mRNA pair in *Escherichia coli*[72] (**Fig. 4b**). The addition of thiamine resulted in ribosome activation, leading to cleavage of 16S rRNA and subsequently to an up to 80%

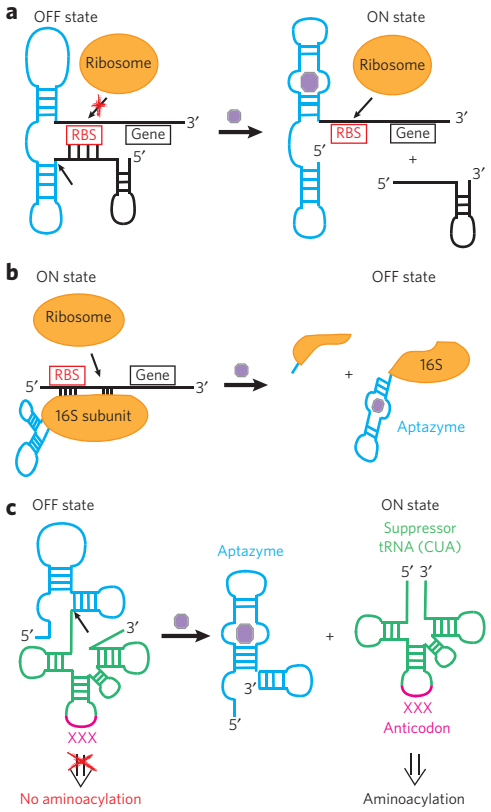


Figure 4 | Allosteric ribozymes for regulation of gene expression in prokaryotes. (**a**) Allosteric control over ribosome docking to bacterial mRNA. Introduction of the full-length HHR in combination with a strand that hybridizes to the RBS at the 5′ UTR of prokaryotic mRNA prevented gene expression when no effector was present. Addition of ligand induces strand cleavage, thereby liberating the RBS and enabling ribosome docking. Adapted from refs. 69,70. (**b**) Regulation of the stability of rRNA of 16S ribosomal subunit by an aptazyme. Addition of thiamine to bacteria expressing an orthogonal mRNA-rRNA pair yielded cleavage of the 16S rRNA, resulting in sufficient destabilization to prevent gene expression. Adapted from ref. 72. Reprinted with permission from Elsevier. (**c**) Fusion of an HHR to the 5′ site of a suppressor tRNA that recognizes the amber stop codon. Ligand binding induces cleavage, allowing aminoacylation of the tRNA and subsequent incorporation of a serine, leading to synthesis of the amber-mutated gene. Adapted from ref. 72.

decrease in gene expression. As application of this system requires the availability of an orthogonal sRNA-16s rRNA pair, application in its present form is limited to prokaryotes. Gene expression in bacteria has also been regulated by coupling an aptazyme to a suppressor tRNA, thereby allowing aminoacylation only after ligand binding and subsequent release of the ribozyme from the tRNA[73,74] (**Fig. 4c**). This means that the ribosome stalls at the amber codon of an amber-mutated reporter gene in the absence of ligand, whereas ribozyme activation after addition of ligand enables synthesis of the full-length protein, leading to a large increase in protein synthesis. This system was first shown in a cell-free translation system using a minimal motif HHR[73], but more recently has been demonstrated in *E. coli* with a theophylline-dependent full-length HHR[74].

One of the problems with the intracellular application of aptazymes is the relatively small number of ligands that have been successfully applied to induce gene regulation. This lack of ligands is especially true for bacteria, where antibiotics such as tetracycline and neomycin, which are often used in eukaryotic systems, cannot be used. The palette of available ligands has been enriched by looking at the crystal structure of an existing riboswitch and selectively randomizing two positions of the *add* A riboswitch to yield a system that responded to ammeline (4,6-diamino-2-hydroxy-1,3,5-triazine) and azacytosine and no longer to adenine[75].

A recent application-driven approach involved bacteria containing a riboswitch that was responsive to the toxin atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-*S*-triazine)[76,77]. Aptamers were introduced in the 5′ UTR of the *cheZ* gene, which controls *E. coli* motility, in a strain that also contained an atrazine-metabolizing gene. This generated bacteria that followed and degraded atrazine by chemotaxis, allowing their application in contaminated sites. In addition, the controllable movement of *E. coli* showed the potential of aptamer-based allostery to program organisms to autonomously carry out complex, user-defined tasks.

Gene regulation in eukaryotes. Compared to prokaryotes, eukaryotic cells use many more processing steps between DNA transcription and protein translation. These different levels of regulation not only allow the cell to precisely control gene expression, but also provide points at which aptamers can be applied to allow effector-induced regulation of protein levels (**Fig. 5**).

Regulation of protein expression at the transcriptional level in yeast was achieved by fusing a tetramethylrosamine (TMR)-binding aptamer with an RNA-based transcriptional activator[78]. The connecting module between the two aptamers was optimized by screening a small combinatorial library (~10^4 mutants), yielding a clone that was ten-fold more active in the presence of TMR.

The allosteric variant of the full-length HHR[69] was applied to control gene expression in mammalian cells[79]. However, instead of liberating the RBS upon cleavage, placement in the 5′ UTR of a gene allowed cleavage-induced loss of the 5′ cap, which decreases ribosome recruitment by reducing mRNA stability. Removal of additional start codons and optimization of the linker between aptamer and ribozyme through intracellular screening yielded mutants that showed a reduction in gene expression by a factor of 6.

Placement of a ribozyme in the 3′ UTR region of eukaryotic RNA allows removal of the poly(A) tail after cleavage, which also reduces mRNA stability. In a first application, a full-length HHR was used as a logic gate to regulate gene expression in yeast[56], whereas a second study applied the same switches to regulate T-cell growth in response to drug input[80]. The allosteric switching behavior was demonstrated in a mouse T-cell line and in primary human T-cells. Injection of a hydrogel matrix harboring clonal cell lines containing the transgene regulatory system into mice even allowed the control of T-cell proliferation *in vivo*.

Recently, an internal ribosome entry site (IRES) of mRNA was modulated in a ligand-dependent fashion to control protein synthesis *in vitro*[81]. After screening for sequences that were important for successful translation within the IRES of the *Plautia stali* intestinal virus, two additional complementary RNA strands were inserted together with the theophylline aptamer. In the absence of theophylline, one of the inserted strands hybridizes with the IRES sequence, thereby prohibiting efficient translation of the mRNA. Theophylline binding forms a loop that induces hybridization of the two inserted strands, leading to a ten-fold increase in protein expression. The modularity of the design was shown by exchanging the theophylline aptamer for the FMN, tetracycline or sulforhodamine B aptamers without altering the ON-switching behavior upon ligand binding.

Regulation of intron splicing by the insertion of aptamer sequences can also be an attractive strategy for controlling gene expression, as the conformation of the pre-mRNA can influence splicing efficiency[82]. The spliceosome requires different recognition elements for assembly, and their availability can be made ligand dependent using an aptamer. Insertion of the theophylline-binding aptamer at the 3′ splice site of a model pre-mRNA resulted in theophylline-induced repression of splicing[83]. This study was performed in nuclear extracts, but control over splicing *in vivo* can also be achieved by

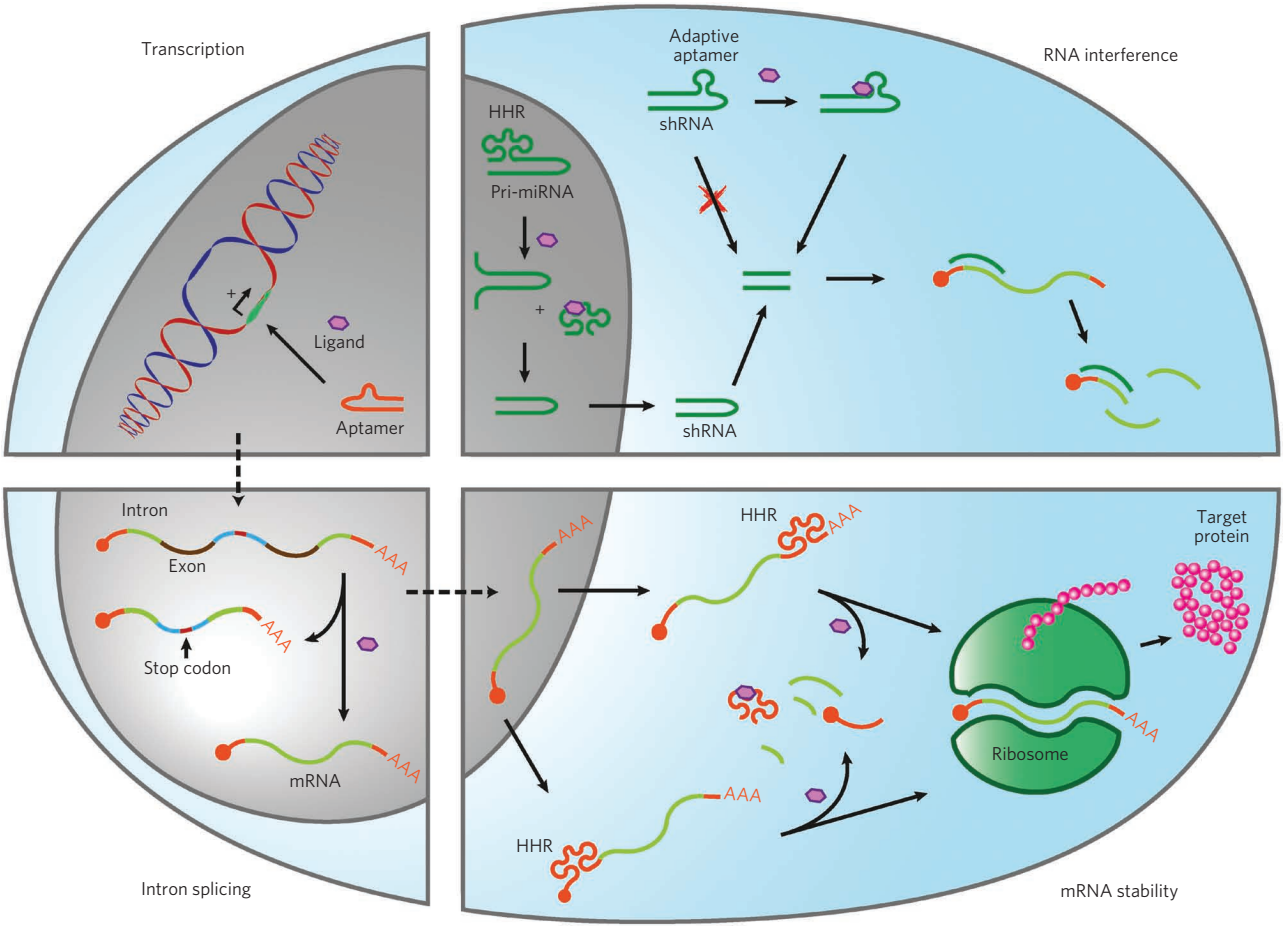


Figure 5 | Control over eukaryotic gene expression by artificially designed allosteric RNAs. Aptamers have been integrated at different points in the natural eukaryotic systems that control processing of nucleic acids to allow effector-mediated control over protein concentration by allosteric mechanisms. At the transcriptional level, protein expression can be artificially regulated by an aptaswitch consisting of a transcriptional activating RNA and a tetramethylrosamine (TMR)-binding aptamer. Binding of TMR induces a conformational change, leading to an increase in transcription of the target gene. The RNA interference pathway can also be modulated. In this approach, allosterically regulated HHRs are fused to Pri-miRNA to allow ligand-induced control of the generation of miRNA. In addition, insertion of adaptive aptamers into shRNA results in control over processing of the respective shRNA by dicer to form siRNAs. Aptamers can also be used to control intron splicing. The pre-mRNA contains two introns that surround an exon with a premature stop codon. Depending on the position of aptamer insertion in the intron(s), ligand binding either enhances or diminishes removal of the exon containing the premature stop codon. Here, ligand binding enhances generation of mRNA with the correct open reading frame. The stability of mRNA can be regulated by placing allosteric ribozymes in either the 5′ or the 3′ UTR of the mRNA. Effector-induced cleavage of the cap or poly(A) tail strongly reduces the stability of the mRNA, leading to its degradation and a subsequent decrease in gene expression.

sequestering the branch-point sequence of the intron upon binding of theophylline to its target aptamer[84]. In both cases however, splicing was not completely inhibited, leading to modest signal changes of 2–5 times the normal value. Larger differences in gene expression were obtained by inserting a tetracycline-binding aptamer at the 5′ splice site of pre-mRNA in yeast[85]. The consensus sequence of the splice site was within the aptamer stem, resulting in tetracycline-dependent splicing of the respective pre-mRNA. By placing multiple tetracycline-dependent splice sites in one gene, GFP expression was reduced by a factor of 32.

In addition to small molecules, proteins have also been used as effectors for regulating splicing[86]. Multiple RNA aptamers were inserted at 12 different positions before and after an exon that contained a stop codon. Depending on the position, ligand binding either improved or diminished removal of the stop codon–containing exon, thereby regulating the expression of the correct open reading frame. Various protein-binding aptamers were used as input domains to demonstrate modularity, but there were opposing effects on gene expression when the aptamers that bind the bacteriophage coat protein MS2 and β-catenin were inserted in the same intronic location. Although relatively modest 2–5-fold changes in expression were observed, an AND gate was created by using two therapeutic inputs to trigger programmed cell death. This, together with regulation of T-cell proliferation[80] and gene knockdown in yeast cells[87], represents one of a few examples in which researchers have regulated genes with physiologically relevant functions instead of demonstrating proof of principle with GFP or luciferase.

The RNA interference machinery in mammalian cells can also be made ligand dependent. GFP expression was upregulated by coupling a theophylline-responsive aptazyme to a structural analog of primary microRNA (pri-miRNA)[88]. Strand cleavage resulted in processing of the pri-miRNA by the RNase III enzyme drosha, which enabled downstream processing. This induced RNA interference and subsequent knockdown of gene expression.

Further downstream in the interference pathway, cleavage of short hairpin RNA (shRNA) by the endonuclease dicer was made ligand dependent in two ways[89,90]. In both approaches, a theophylline aptamer was inserted at the loop of an shRNA, thereby preventing cleavage of the hairpin and formation of small interfering RNA (siRNA) in the presence of ligand. In the first approach,

the anti-theophylline aptamer was fused directly to the shRNA. This yielded a switch for which GFP silencing was similar to that for wild-type shRNA in the absence of theophylline, whereas there was a three-fold increase in GFP expression after addition of the effector[89]. The second approach involved a more modular design. Here, a connector was fused between the shRNA stem and the theophylline aptamer[90]. This connector was complementary to part of the shRNA stem so that ligand binding switched the modified shRNA from an active to an inactive conformation. Two levels of modularity were achieved by using the connector. First, the input domain was easily exchanged, yielding switches that responded to three different inputs. Second, altering the amount of complementary nucleotides between the shRNA stem and the connector regulated the level of silencing in the absence of ligand. This allowed the switching behavior to be tuned and in principle means that the switch could be tailored for specific applications.

Intracellular screening. Progress in the intracellular application of aptamer-mediated allostery has been made not only in regulating gene expression but also in the selection of switches that show intracellular activity. Because cleavage rates and switching behavior of aptamers *in vitro* are often lower inside living cells[91], researchers moved from *in vitro* selection[92,93] to intracellular screening.

Dual intracellular screening in *E. coli* was used to select for riboswitches that reduced expression upon ligand binding[94]. The RBS was incorporated either within a thiamine pyrophosphate (TPP) aptamer or in the stem that connects with the 5′ UTR. Libraries of 5×10^3–10^4 mutant variants with randomized regions in the stem or between the stem and the start codon were screened, yielding riboswitches for which ligand binding blocked the Shine-Dalgarno sequence and switched off gene expression. As stabilization of the base stem is observed in both natural and synthetic riboswitches, this screening strategy should also be applicable to generate OFF switches that respond to other ligands. Another interesting intracellular screening technique used chemotactic bacterial motility as a read-out in searching for riboswitches[95]. A library that contained a randomized region between the theophylline aptamer and the ribosome binding site was transformed into *E. coli* and spotted onto semi-solid selection medium with or without theophylline. *E. coli* mutants that moved only in the presence of theophylline were amplified, yielding switches with up to 25-fold activation. Although this is a fast method for screening relatively large libraries (>10^6 members), it can be difficult to detect the strongest activators of gene expression by this approach. Alternatively, fluorescence-assisted cell sorting (FACS) has been used to select riboswitches that induce gene expression in the presence of theophylline[96,97], which in one case resulted in a mutant that showed an impressive 96-fold activation of gene expression[96].

Yeast is also eligible for intracellular evolution of aptamer-mediated allostery[98]. In a recent study a 74-nucleotide-long RNA pool containing a randomized region flanked by constant parts was screened for neomycin binding in six rounds of *in vitro* selection. Next, the resulting variants were inserted at the 5′ UTR of a gene encoding GFP and screened for variants in which addition of neomycin reduced GFP expression. The selected mutant variants showed high sequence overlap, and the strongest inhibitor showed downregulation of GFP expression by a factor of 7.5. Interestingly, the sequences of the obtained mutants showed no obvious similarities to neomycin aptamers that were selected *in vitro*[99], but instead showed sequence overlap with natural neomycin binding sites.

A disadvantage of using *E. coli* or yeast in intracellular screenings is that the transformation efficiency generally limits the pool size to 10^6–10^7 variants, which is small compared to the traditional 10^{14}–10^{16} pool size that is used during *in vitro* SELEX. Mammalian cells are easier to transfect, but in these cells it is difficult to deliver a single mutant into an individual cell. To circumvent this problem, mammalian cells were used to screen for allosteric HHRs; this was followed by extraction of the RNA and *in vitro* amplification of only the cleaved mutants[100]. Two libraries of HHRs with randomized regions in either stem II or loop II were subjected to five rounds of selection, leading to mutants that showed strong inhibition of gene expression. Although their *in vivo* performance was comparable to that of the wild-type HHR, the selected ribozymes were around 100 times slower *in vitro*, which probably reflected the challenges involved in increasing selection stringency inside cells.

Discussion and outlook

The multitude of aptasensors and switches that have been developed for *in vitro* and intracellular application demonstrate the attractiveness of nucleic acids as building blocks for generating allosteric effectors. *In vitro*, one can now connect a variety of readouts with ribozyme or DNAzyme activity and combine this output with a selected aptamer to in principle detect any ligand of interest with high sensitivity. Intracellularly, almost all levels at which nucleic acids are involved in gene expression have been combined with allosteric aptazymes to make protein expression ligand dependent.

However, there appears to be only a rather limited variation of aptamer-target pairs that are used again and again by researchers who seek to obtain proof of principle for their respective new aptasensor designs; the most frequently applied aptamer–small molecule target pairs sense ATP, theophylline, tetracycline and FMN, and the aptazyme world appears to know even fewer proteins: thrombin and perhaps platelet-derived growth factor. This is related to the fact that early designs of aptameric switches can be considered as stand-alone where, for example, the connection of subdomains was optimized specifically for these domains, and adaptation of the functional mechanism to an alternative, more applicable input would have required renewed optimization.

In recent years there has been an increasing trend toward modular designs in which the input domains could be more easily exchanged, resulting in a larger number of functional inputs. In addition, examples have become available in which not the design but the application of the allosteric switch stood central, leading to aptasensors that go beyond proof of principle—such as the atrazine-metabolizing bacteria[77]—or are more tailored to answer biological questions. Indeed, to develop the atrazine-metabolizing bacteria, the investigators went through a great deal of effort by doing *in vitro* selection, bacterial screening and functional analysis of a brand-new riboswitch for a real problem—chemical toxin detection, which represents a good example for other researchers to take up more demanding challenges in the future. If such application-driven research can be further combined with the available modular, well-behaved aptamers and the variety of available logic gates, one can envision the creation of more complex and interwoven aptazyme-directed operations that will greatly facilitate diagnostic and therapeutic research, both *in vitro* and *in vivo*.

The successful applications of allosteric aptamers that modulate protein expression in *E. coli*, yeast and mammalian cells underlines their potential as signal transducers in synthetic biology. However, examples of post-translational regulation by oligonucleotides are scarce. Inhibition of thrombin activity after adaptive binding of a ligand to an aptamer was used as a readout in an *in vitro* assay[48], and a similar example exists for a sniper venom protein[49]. Neomycin-induced control over aptamer binding to a DNA-repair protein has also been demonstrated, but only *in vitro*[101]. It would be interesting to use these aptamers for ligand-induced control over protein activity in an intracellular setting. If such designs could be made in a modular fashion, exchange of both input and output domains would be straightforward and would allow one to mix and match a specific effector to inhibit a protein of interest. In this way, aptamer-based allostery could be used to modulate protein activity on multiple levels and to induce effects at different time scales. Combined

with logic gate functions, this would enable fully nucleic acid–based constructs to generate complex intracellular switching pathways, allowing one to study protein networks inside living cells with high spatiotemporal resolution.

References

1. Golynskiy, M.V., Koay, M.S., Vinkenborg, J.L. & Merkx, M. Engineering protein switches: sensors, regulators, and spare parts for biology and biotechnology. *ChemBioChem* **12**, 353–361 (2011).
2. Mayer, G. The chemical biology of aptamers. *Angew. Chem. Int. Edn Engl.* **48**, 2672–2689 (2009).
3. Tuerk, C. & Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505–510 (1990).
4. Ellington, A.D. & Szostak, J.W. *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**, 818–822 (1990).
5. Famulok, M., Hartig, J.S. & Mayer, G. Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy. *Chem. Rev.* **107**, 3715–3743 (2007).
6. Famulok, M. & Mayer, G. Aptamers as tools in molecular biology and immunology. *Curr. Top. Microbiol. Immunol.* **243**, 123–136 (1999).
7. Tang, J. & Breaker, R.R. Rational design of allosteric ribozymes. *Chem. Biol.* **4**, 453–459 (1997).
8. Stojanovic, M.N. & Kolpashchikov, D.M. Modular aptameric sensors. *J. Am. Chem. Soc.* **126**, 9266–9270 (2004).
9. Nudler, E. Flipping riboswitches. *Cell* **126**, 19–22 (2006).
10. Winkler, W.C. & Breaker, R.R. Genetic control by metabolite-binding riboswitches. *ChemBioChem* **4**, 1024–1032 (2003).
11. Liu, J., Cao, Z. & Lu, Y. Functional nucleic acid sensors. *Chem. Rev.* **109**, 1948–1998 (2009).
12. Tombelli, S., Minunni, M. & Mascini, M. Analytical applications of aptamers. *Biosens. Bioelectron.* **20**, 2424–2434 (2005).
13. Soukup, G.A. & Breaker, R.R. Nucleic acid molecular switches. *Trends Biotechnol.* **17**, 469–476 (1999).
14. Famulok, M. Allosteric aptamers and aptazymes as probes for screening approaches. *Curr. Opin. Mol. Ther.* **7**, 137–143 (2005).
15. Mayer, G., Raddatz, M.S., Grunwald, J.D. & Famulok, M. RNA ligands that distinguish metabolite-induced conformations in the TPP riboswitch. *Angew. Chem. Int. Edn Engl.* **46**, 557–560 (2007).
16. Rentmeister, A., Mayer, G., Kuhn, N. & Famulok, M. Conformational changes in the expression domain of the *Escherichia coli* thiM riboswitch. *Nucleic Acids Res.* **35**, 3713–3722 (2007).
17. Vaish, N.K. *et al.* Monitoring post-translational modification of proteins with allosteric ribozymes. *Nat. Biotechnol.* **20**, 810–815 (2002).
18. Srinivasan, J. *et al.* ADP-specific sensors enable universal assay of protein kinase activity. *Chem. Biol.* **11**, 499–508 (2004).
19. Chiuman, W. & Li, Y. Simple fluorescent sensors engineered with catalytic DNA 'MgZ' based on a non-classic allosteric design. *PLoS ONE* **2**, e1224 (2007).
20. Sekella, P.T., Rueda, D. & Walter, N.G. A biosensor for theophylline based on fluorescence detection of ligand-induced hammerhead ribozyme cleavage. *RNA* **8**, 1242–1252 (2002).
21. Hartig, J.S. *et al.* Protein-dependent ribozymes report molecular interactions in real time. *Nat. Biotechnol.* **20**, 717–722 (2002).
22. Najafi-Shoushtari, S.H. & Famulok, M. DNA aptamer-mediated regulation of the hairpin ribozyme by human alpha-thrombin. *Blood Cells Mol. Dis.* **38**, 19–24 (2007).
23. Najafi-Shoushtari, S.H., Mayer, G. & Famulok, M. Sensing complex regulatory networks by conformationally controlled hairpin ribozymes. *Nucleic Acids Res.* **32**, 3212–3219 (2004).
24. Yin, P., Choi, H.M., Calvert, C.R. & Pierce, N.A. Programming biomolecular self-assembly pathways. *Nature* **451**, 318–322 (2008).
25. Choi, H.M. *et al.* Programmable *in situ* amplification for multiplexed imaging of mRNA expression. *Nat. Biotechnol.* **28**, 1208–1212 (2010).
26. Liu, J. & Lu, Y. Adenosine-dependent assembly of aptazyme-functionalized gold nanoparticles and its application as a colorimetric biosensor. *Anal. Chem.* **76**, 1627–1632 (2004).
27. Liu, J. & Lu, Y. Smart nanomaterials responsive to multiple chemical stimuli with controllable cooperativity. *Adv. Mater.* **18**, 1667–1671 (2006).
28. Liu, J., Mazumdar, D. & Lu, Y. A simple and sensitive 'dipstick' test in serum based on lateral flow separation of aptamer-linked nanostructures. *Angew. Chem. Int. Edn Engl.* **45**, 7955–7959 (2006).
This application involves gold nanoparticles functionalized with allosteric aptamers in a 'dipstick' test to detect cocaine levels in serum with the naked eye.
29. Mazumdar, D., Liu, J., Lu, G., Zhou, J. & Lu, Y. Easy-to-use dipstick tests for detection of lead in paints using non-cross-linked gold nanoparticle-DNAzyme conjugates. *Chem. Commun. (Camb.)* **46**, 1416–1418 (2010).
30. Helm, M., Petermeier, M., Ge, B., Fiammengo, R. & Jaschke, A. Allosterically activated Diels-Alder catalysis by a ribozyme. *J. Am. Chem. Soc.* **127**, 10492–10493 (2005).
31. Robertson, M.P. & Ellington, A.D. *In vitro* selection of an allosteric ribozyme that transduces analytes to amplicons. *Nat. Biotechnol.* **17**, 62–66 (1999).
32. Cho, E.J., Yang, L., Levy, M. & Ellington, A.D. Using a deoxyribozyme ligase and rolling circle amplification to detect a non-nucleic acid analyte, ATP. *J. Am. Chem. Soc.* **127**, 2022–2023 (2005).
33. Kim, D.E. & Joyce, G.F. Cross-catalytic replication of an RNA ligase ribozyme. *Chem. Biol.* **11**, 1505–1512 (2004).
34. Lincoln, T.A. & Joyce, G.F. Self-sustained replication of an RNA enzyme. *Science* **323**, 1229–1232 (2009).
35. Lam, B.J. & Joyce, G.F. Autocatalytic aptazymes enable ligand-dependent exponential amplification of RNA. *Nat. Biotechnol.* **27**, 288–292 (2009).
Elegant example in which loop replacement yields allosteric regulation of a self-replicating RNA, providing an accumulative output in response to ligand without requiring a polymerase.
36. Lam, B.J. & Joyce, G.F. An isothermal system that couples ligand-dependent catalysis to ligand-independent exponential amplification. *J. Am. Chem. Soc.* **133**, 3191–3197 (2011).
37. Travascio, P., Li, Y. & Sen, D. DNA-enhanced peroxidase activity of a DNA-aptamer-hemin complex. *Chem. Biol.* **5**, 505–517 (1998).
38. Pelossof, G., Tel-Vered, R., Elbaz, J. & Willner, I. Amplified biosensing using the horseradish peroxidase-mimicking DNAzyme as an electrocatalyst. *Anal. Chem.* **82**, 4396–4402 (2010).
39. Teller, C., Shimron, S. & Willner, I. Aptamer-DNAzyme hairpins for amplified biosensing. *Anal. Chem.* **81**, 9114–9119 (2009).
40. Li, T., Wang, E. & Dong, S. Lead(II)-induced allosteric G-quadruplex DNAzyme as a colorimetric and chemiluminescence sensor for highly sensitive and selective Pb^{2+} detection. *Anal. Chem.* **82**, 1515–1520 (2010).
41. Li, T., Wang, E. & Dong, S. G-quadruplex-based DNAzyme for facile colorimetric detection of thrombin. *Chem. Commun. (Camb.)* 3654–3656 (2008).
42. Li, T., Dong, S. & Wang, E. Label-free colorimetric detection of aqueous mercury ion (Hg^{2+}) using Hg^{2+}-modulated G-quadruplex-based DNAzymes. *Anal. Chem.* **81**, 2144–2149 (2009).
43. Lu, N., Shao, C. & Deng, Z. Rational design of an optical adenosine sensor by conjugating a DNA aptamer with split DNAzyme halves. *Chem. Commun. (Camb.)* 6161–6163 (2008).
44. Lu, N., Shao, C. & Deng, Z. Colorimetric Hg^{2+} detection with a label-free and fully DNA-structured sensor assembly incorporating G-quadruplex halves. *Analyst (Lond.)* **134**, 1822–1825 (2009).
45. Elbaz, J., Moshe, M., Shlyahovsky, B. & Willner, I. Cooperative multicomponent self-assembly of nucleic acid structures for the activation of DNAzyme cascades: a paradigm for DNA sensors and aptasensors. *Chemistry* **15**, 3411–3418 (2009).
46. Constantin, T.P. *et al.* Synthesis of new fluorogenic cyanine dyes and incorporation into RNA fluoromodules. *Org. Lett.* **10**, 1561–1564 (2008).
47. Furutani, C., Shinomiya, K., Aoyama, Y., Yamada, K. & Sando, S. Modular blue fluorescent RNA sensors for label-free detection of target molecules. *Mol. Biosyst.* **6**, 1569–1571 (2010).
48. Yoshida, W., Sode, K. & Ikebukuro, K. Aptameric enzyme subunit for biosensing based on enzymatic activity measurement. *Anal. Chem.* **78**, 3296–3303 (2006).
49. Chelyapov, N. Allosteric aptamers controlling a signal amplification cascade allow visual detection of molecules at picomolar concentrations. *Biochemistry* **45**, 2461–2466 (2006).
50. Stojanovic, M.N., Mitchell, T.E. & Stefanovic, D. Deoxyribozyme-based logic gates. *J. Am. Chem. Soc.* **124**, 3555–3561 (2002).
51. Penchovsky, R. & Breaker, R.R. Computational design and experimental validation of oligonucleotide-sensing allosteric ribozymes. *Nat. Biotechnol.* **23**, 1424–1433 (2005).
52. Stojanovic, M.N. & Stefanovic, D. A deoxyribozyme-based molecular automaton. *Nat. Biotechnol.* **21**, 1069–1074 (2003).
53. Kolpashchikov, D.M. & Stojanovic, M.N. Boolean control of aptamer binding states. *J. Am. Chem. Soc.* **127**, 11348–11351 (2005).
54. Elbaz, J. *et al.* DNA computing circuits using libraries of DNAzyme subunits. *Nat. Nanotechnol.* **5**, 417–422 (2010).
A biocomputing platform with modular architecture based on a library of DNAzyme subunits, pre-designed substrates and different inputs is described.
55. Yoshida, W. & Yokobayashi, Y. Photonic Boolean logic gates based on DNA aptamers. *Chem. Commun.* **2007**, 195–197 (2007).
56. Win, M.N. & Smolke, C.D. A modular and extensible RNA-based gene-regulatory platform for engineering cellular function. *Proc. Natl. Acad. Sci. USA* **104**, 14283–14288 (2007); erratum **106**, 15514 (2009).
57. Win, M.N. & Smolke, C.D. Higher-order cellular information processing with synthetic RNA devices. *Science* **322**, 456–460 (2008).

58. Klug, S.J., Huttenhofer, A., Kromayer, M. & Famulok, M. In vitro and in vivo characterization of novel mRNA motifs that bind special elongation factor SelB. *Proc. Natl. Acad. Sci. USA* **94**, 6676–6681 (1997).
59. Choi, K.H. *et al.* Intracellular expression of the T-cell factor-1 RNA aptamer as an intramer. *Mol. Cancer Ther.* **5**, 2428–2434 (2006).
60. Famulok, M., Blind, M. & Mayer, G. Intramers as promising new tools in functional proteomics. *Chem. Biol.* **8**, 931–939 (2001).
61. Lee, H.K. *et al.* B-catenin regulates multiple steps of RNA metabolism as revealed by the RNA aptamer in colon cancer cells. *Cancer Res.* **67**, 9315–9321 (2007).
62. Werstuck, G. & Green, M.R. Controlling gene expression in living cells through small molecule-RNA interactions. *Science* **282**, 296–298 (1998).
63. Wieland, M. & Hartig, J.S. Artificial riboswitches: synthetic mRNA-based regulators of gene expression. *ChemBioChem* **9**, 1873–1878 (2008).
64. Suess, B. & Weigand, J.E. Engineered riboswitches: overview, problems and trends. *RNA Biol.* **5**, 24–29 (2008).
65. Win, M.N., Liang, J.C. & Smolke, C.D. Frameworks for programming biological function through RNA parts and devices. *Chem. Biol.* **16**, 298–310 (2009).
66. Saito, H. & Inoue, T. Synthetic biology with RNA motifs. *Int. J. Biochem. Cell Biol.* **41**, 398–404 (2009).
67. Topp, S. & Gallivan, J.P. Emerging applications of riboswitches in chemical biology. *ACS Chem. Biol.* **5**, 139–148 (2010).
68. Khvorova, A., Lescoute, A., Westhof, E. & Jayasena, S.D. Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. *Nat. Struct. Biol.* **10**, 708–712 (2003).
69. Wieland, M. & Hartig, J.S. Improved aptazyme design and in vivo screening enable riboswitching in bacteria. *Angew. Chem. Int. Edn Engl.* **47**, 2604–2607 (2008).
70. Ogawa, A. & Maeda, M. An artificial aptazyme-based riboswitch and its cascading system in *E. coli*. *ChemBioChem* **9**, 206–209 (2008).
71. Wieland, M., Benz, A., Klauser, B. & Hartig, J.S. Artificial ribozyme switches containing natural riboswitch aptamer domains. *Angew. Chem. Int. Edn Engl.* **48**, 2715–2718 (2009).
72. Wieland, M., Berschneider, B., Erlacher, M.D. & Hartig, J.S. Aptazyme-mediated regulation of 16S ribosomal RNA. *Chem. Biol.* **17**, 236–242 (2010).
73. Ogawa, A. & Maeda, M. A novel label-free biosensor using an aptazyme-suppressor-tRNA conjugate and an amber mutated reporter gene. *ChemBioChem* **9**, 2204–2208 (2008).
74. Berschneider, B., Wieland, M., Rubini, M. & Hartig, J.S. Small-molecule-dependent regulation of transfer RNA in bacteria. *Angew. Chem. Int. Edn Engl.* **48**, 7564–7567 (2009).
Demonstration that aminoacylation of a suppressor tRNA can be used to regulate the expression of an amber-mutated gene.
75. Dixon, N. *et al.* Reengineering orthogonally selective riboswitches. *Proc. Natl. Acad. Sci. USA* **107**, 2830–2835 (2010).
76. Topp, S. & Gallivan, J.P. Guiding bacteria with small molecules and RNA. *J. Am. Chem. Soc.* **129**, 6807–6811 (2007).
77. Sinha, J., Reyes, S.J. & Gallivan, J.P. Reprogramming bacteria to seek and destroy an herbicide. *Nat. Chem. Biol.* **6**, 464–470 (2010).
An application-driven approach in which bacteria are reprogrammed to move into the direction of a toxin and then to take up and metabolize this toxin.
78. Buskirk, A.R., Landrigan, A. & Liu, D.R. Engineering a ligand-dependent RNA transcriptional activator. *Chem. Biol.* **11**, 1157–1163 (2004).
79. Ausländer, S., Ketzer, P. & Hartig, J.S. A ligand-dependent hammerhead ribozyme switch for controlling mammalian gene expression. *Mol. Biosyst.* **6**, 807–814 (2010).
The first example of use of the full-length hammerhead ribozyme to control gene expression in mammalian cells.
80. Chen, Y.Y., Jensen, M.C. & Smolke, C.D. Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems. *Proc. Natl. Acad. Sci. USA* **107**, 8531–8536 (2010).
81. Ogawa, A. Rational design of artificial riboswitches based on ligand-dependent modulation of internal ribosome entry in wheat germ extract and their applications as label-free biosensors. *RNA* **17**, 478–488 (2011).
82. Buratti, E. & Baralle, F.E. Influence of RNA secondary structure on the pre-mRNA splicing process. *Mol. Cell. Biol.* **24**, 10505–10514 (2004).
83. Kim, D.S., Gusti, V., Pillai, S.G. & Gaur, R.K. An artificial riboswitch for controlling pre-mRNA splicing. *RNA* **11**, 1667–1677 (2005).
84. Kim, D.S., Gusti, V., Dery, K.J. & Gaur, R.K. Ligand-induced sequestering of branchpoint sequence allows conditional control of splicing. *BMC Mol. Biol.* **9**, 23 (2008).
85. Weigand, J.E. & Suess, B. Tetracycline aptamer-controlled regulation of pre-mRNA splicing in yeast. *Nucleic Acids Res.* **35**, 4179–4185 (2007).
86. Culler, S.J., Hoff, K.G. & Smolke, C.D. Reprogramming cellular behavior with RNA controllers responsive to endogenous proteins. *Science* **330**, 1251–1255 (2010).
This paper describes ligand-induced control over gene expression through regulation of splicing activity using proteins as inputs, resulting in the ability to rewire cellular networks.
87. Kötter, P., Weigand, J.E., Meyer, B., Entian, K.D. & Suess, B. A fast and efficient translational control system for conditional expression of yeast genes. *Nucleic Acids Res.* **37**, e120 (2009).
88. Kumar, D., An, C.I. & Yokobayashi, Y. Conditional RNA interference mediated by allosteric ribozyme. *J. Am. Chem. Soc.* **131**, 13906–13907 (2009).
89. An, C.I., Trinh, V.B. & Yokobayashi, Y. Artificial control of gene expression in mammalian cells by modulating RNA interference through aptamer-small molecule interaction. *RNA* **12**, 710–716 (2006).
90. Beisel, C.L., Bayer, T.S., Hoff, K.G. & Smolke, C.D. Model-guided design of ligand-regulated RNAi for programmable control of gene expression. *Mol. Syst. Biol.* **4**, 224 (2008).
91. Suess, B. *et al.* Conditional gene expression by controlling translation with tetracycline-binding aptamers. *Nucleic Acids Res.* **31**, 1853–1858 (2003).
92. Koizumi, M., Soukup, G.A., Kerr, J.N. & Breaker, R.R. Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP. *Nat. Struct. Biol.* **6**, 1062–1071 (1999).
93. Piganeau, N., Thuillier, V. & Famulok, M. In vitro selection of allosteric ribozymes: theory and experimental validation. *J. Mol. Biol.* **312**, 1177–1190 (2001).
94. Muranaka, N., Abe, K. & Yokobayashi, Y. Mechanism-guided library design and dual genetic selection of synthetic OFF riboswitches. *ChemBioChem* **10**, 2375–2381 (2009).
95. Topp, S. & Gallivan, J.P. Random walks to synthetic riboswitches—a high-throughput selection based on cell motility. *ChemBioChem* **9**, 210–213 (2008).
96. Lynch, S.A. & Gallivan, J.P. A flow cytometry-based screen for synthetic riboswitches. *Nucleic Acids Res.* **37**, 184–192 (2009).
A fast intracellular screening approach involving fluorescence-assisted cell sorting, yielding one of the strongest selected switches for gene regulation so far.
97. Fowler, C.C., Brown, E.D. & Li, Y.A. FACS-based approach to engineering artificial riboswitches. *ChemBioChem* **9**, 1906–1911 (2008).
98. Weigand, J.E. *et al.* Screening for engineered neomycin riboswitches that control translation initiation. *RNA* **14**, 89–97 (2008).
99. Wallis, M.G., von Ahsen, U., Schroeder, R. & Famulok, M. A novel RNA motif for neomycin recognition. *Chem. Biol.* **2**, 543–552 (1995).
100. Chen, X., Denison, L., Levy, M. & Ellington, A.D. Direct selection for ribozyme cleavage activity in cells. *RNA* **15**, 2035–2045 (2009).
101. Vuyisich, M. & Beal, P.A. Controlling protein activity with ligand-regulated RNA aptamers. *Chem. Biol.* **9**, 907–913 (2002).

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