Expanding the toolbox of synthetic riboswitches with guanine-dependent aptazymes

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Abstract

Artificial riboswitches based on ribozymes serve as versatile tools for ligand-dependent gene expression regulation. Advantages of these so-called aptazymes are their modular architecture and the comparably little coding space they require. A variety of aptamer-ribozyme combinations were constructed in the past 20 years and the resulting aptazymes were applied in diverse contexts in prokaryotic and eukaryotic systems. Most in vivo functional aptazymes are OFF-switches, while ON-switches are more advantageous regarding potential applications in e.g. gene therapy vectors. We developed new ON-switching aptazymes in the model organism Escherichia coli and in mammalian cell culture using the intensely studied guanine-sensing xpt aptamer. Utilizing a high-throughput screening based on fluorescence-activated cell sorting in bacteria we identified up to 9.2-fold ON-switches and OFF-switches with a dynamic range up to 32.7-fold. For constructing ON-switches in HeLa cells, we used a rational design approach based on existing tetracycline-sensitive ON-switches. We discovered that communication modules responding to tetracycline are also functional in the context of guanine aptazymes, demonstrating a high degree of modularity. Here, guanine-responsive ON-switches with a four-fold dynamic range were designed. Summarizing, we introduce a series of novel guanine-dependent ribozyme switches operative in bacteria and human cell culture that significantly broaden the existing toolbox.

Key words: aptazyme; engineered riboswitch; hammerhead ribozyme; regulation of gene expression; synthetic biology.

1. Introduction

Ligand-dependent small self-cleaving ribozymes have emerged in the recent years as valuable tools to control gene expression. These so-called aptazymes are small, modular and do not require regulatory protein factors. Their favorable properties make aptazymes promising instruments in synthetic biology and in clinical applications (1–3). Aptazymes are commonly composed of three parts. A ligand-binding aptamer domain is connected to a ribozyme platform via a communication module. When a ligand binds to the aptamer, the induced conformational change is transmitted through the communication module to the ribozyme, triggering a change in cleavage activity. Such allosteric ribozymes have been utilized to control the accessibility of the ribosome binding site in a transcript of a prokaryotic gene (4–6) or the stability of a eukaryotic mRNA (7–10). Since the first description of an aptazyme in 1997 (11), a variety of different aptamer-ribozyme combinations has been constructed. Commonly used ribozyme platforms are the hammerhead ribozyme (HHR) (4, 5, 7, 9, 10), the hepatitis delta virus ribozyme (8) and the twister ribozyme (6). Available ligands include, among others, the antibiotics neomycin (6, 12, 13) and tetracycline.
(10), the vitamin derivative thiamine pyrophosphate (5, 6) or purines like guanine (7, 8) or theophylline (4, 8).

Most developed aptazymes to date are OFF-switches, i.e. the gene expression is down-regulated when the respective ligand is added to the system. However, in many applications ON-switches are more advantageous since they allow to turn on the gene expression at a specific time point upon addition of the ligand. In a gene therapeutic application this would allow to express the transgene only when needed and thus reduce adverse effects of the transgene. For instance, it was shown in an AAV-system that higher yields can be achieved during vector production when transgene cytotoxicity is reduced by aptazyme-mediated down-regulation of the transgene expression (3). In more simple systems, such as the prokaryotic model organism Escherichia coli, ON-switches are useful to generate conditional hypomorphic mutants in cases when null mutants are lethal (14) or to control bacterial chemotaxis (15).

In our study, we extend the repertoire of functional ON-switches by making use of the guanine-sensing xpt aptamer derived from Bacillus subtilis (16). The xpt aptamer shows ideal characteristics for the construction of aptazymes as it has nanomolar affinity towards its ligand (16), displays high interchangeability in natural (17) as well as artificial riboswitches (7), the ligand guanine is non-toxic, and powerful OFF-switches have already been constructed for the use in mammalian cell cultures (7–9). The GuaMHDV aptazyme constructed by the Yokobayashi group (3, 8) and the Gua3 aptazyme from the Farzan group (9) were already successfully tested in an AAV-based transgene system which underlines the applicability of the guanine switches (3). First, we started to develop new aptazymes in E. coli since it is a simple model system, no guanine-dependent aptazymes are known for this system so far, and high-throughput screening methods are available that assist in the identification of functional aptazymes from large libraries (18). In mammalian cell cultures, however, there is a lack of powerful screening methods. Therefore, we decided to start from already known ON-switches that are triggered by tetracycline (10) and rationally reengineer the switches towards the recognition of guanine.

2. Materials and methods

2.1 Expression systems and plasmid construction in E. coli

For our study in E. coli we used a pET16b (Novagen) plasmid system that features an ampicillin resistance and egfp as a reporter gene under the control of a T7 promoter. A Schistosoma mansoni Type 1 HHR was placed in the 5’ untranslated region (5’ UTR) of the egfp gene. The xpt aptamer was inserted by whole-plasmid polymerase chain reaction (PCR) with Phusion Hot Start 2 Polymerase (NEB) using primers with the aptamer and the randomized communication module in the 5’ overhangs (Sigma-Aldrich; Supplementary Table S3). The template was digested using DpnI (NEB). The PCR products were band purified after gel electrophoresis (ZymocleanTM Gel DNA Recovery Kit) and ligated with Quick Ligation (NEB). The ligation product was concentrated using the Clean and Concentrate Kit (Zymo Research) and transformed into E. coli BL21(DE3) gold (Stratagene) electrocompetent bacteria by electroporation. Bacteria were grown in liquid LB medium containing 100 μg/ml carbenicillin (Roht) at 37°C overnight. All constructs were verified by DNA sequencing (Eurofins Genomics/GATC Biotech).

2.2 Sample preparation for cell sorting

Precultures of the libraries and controls were grown in 5 ml LB medium supplemented with 100 μg/ml carbenicillin at 37°C overnight. From the precultures, 10 μl were used to re-inoculate in 2.5 ml M9 minimal medium supplemented with 0.4% (w/v) glucose and 100 μg/ml carbenicillin in the absence (0 mM) or presence (1 mM) of guanosine. Cultures were cultivated shaking overnight at 37°C. The next day, cells were washed twice in PBS, resolved in PBS and adjusted to an OD_{600} of approximately 0.15.

2.3 Cell sorting

Samples were sorted with a FACSaria IIu instrument (BD Biosciences, FlowKon facility) using a nozzle size of 70μm. The eGFP signal was measured using a 488 nm argon laser for excitation and fluorescence was detected through a 530/30 bandpass filter. The bacteria were sorted for low eGFP expression in the presence (OFF-switches) or absence (ON-switches) of guanosine on the first day (150 000 events). The sorted cells were incubated at 37°C overnight in the respective other condition and sorted for high eGFP expression the next day (5000 events). The bacteria were plated directly after the second sort on LB agar plates supplemented with 100 μg/ml carbenicillin.

2.4 Analysis of single clones

Single colonies were picked into 96-well deepwell plates containing 400 μl LB medium supplemented with 100 μg/ml carbenicillin and incubated overnight shaking at 37°C. For each sample, two new wells containing 400 μl M9 minimal medium supplemented with 0.4% (w/v) glucose and 100 μg/ml carbenicillin either in the absence (0 mM) or presence (1 mM) of guanosine were inoculated with 10 μl of each pre-culture. The bacterial cultures were grown for 20 h at 37°C. For bulk measurement 100 μl of each culture was transferred into 96-well microtiter plates and analyzed with an Infinite M200 Microplate Reader (Tecan). Fluorescence of the expressed eGFP (excitation = 488 nm; emission = 535 nm) as well as OD_{600} was measured. Fluorescence data was normalized with the respective optical density for each well. E. coli transformed with a plasmid bearing a truncated form of the eGFP gene were used to determine the background fluorescence of the bacteria. The background fluorescence was subtracted from the normalized fluorescence of the individual clones. Plasmid DNA of candidate clones was isolated using a Zippy Miniprep Kit (Zymo Research) and sequenced (Eurofins Genomics/GATC Biotech).

2.5 Flow cytometry analysis

Bacterial cultures were grown in 96-well deepwell plates as described for the bulk measurements. The cultures were pelleted, washed twice with PBS and resuspended in 200 μl PBS. The bacteria were diluted 1:100 in PBS and analyzed with a FACSVerse instrument (BD Biosciences, FlowKon facility). The GFP signal was measured using 488 nm argon laser for excitation and fluorescence was detected through a 530/30 bandpass filter. A total of 20 000 events were recorded per sample.

2.6 In vitro transcription

Aptazyme constructs were amplified by Taq-PCR with primers inserting a T7 promoter to start transcription in vitro. The total volume of PCR reaction was 200 μl. PCR products were precipitated adding 1/10 volume of 3 M sodium acetate pH 5.7 and 3 volumes of 100% ethanol. Pelleted DNA was resuspended in 40 μl MilliQ and directly used for in vitro transcription [Thermo Scientific transcription buffer, 90 nM ATP, 2 mM CTP, 2 mM GTP and 2 mM UTP, 80 U RiboLock RNase Inhibitor
HHR was placed in the 3′ magnesium concentrations needed for transcription. RNAszyme, thereby prevents self-cleavage in the presence of high magnesium concentrations needed for transcription. RNAs were purified by an 8% preparative PAGE gel.

2.7 Measurement of in vitro ribozyme activity
A cleavage reaction of the aptazyme constructs was performed in 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, either in the presence or absence of guanine. The amount of guanine corresponding to a 1 mM solution was dissolved directly in the reaction buffer. Due to solubility problems the solution was used as a suspension, resulting in slightly reduced concentrations. The final concentration of RNA was 50 nM. The sample was heated to 95°C, then slowly cooled down to 25°C. To start the cleavage reaction magnesium chloride solution was added to a final concentration of 1 mM. The time course of the cleavage reaction was followed by sampling just before starting the reaction and after defined time points. Samples were quenched with PAGE loading dye and loaded on an analytical 8% PAGE gel. Bands were visualized by phosphomaging and quantified using ImageQuantTL software (GE Healthcare). Following equation was used to fit the data:

\[ F_t = F - AF \times e^{-kt} \]

with \( F_t \) as the fraction cleaved at time point \( t \), \( F \) the maximal fraction cleaved and \( AF \) the difference between the maximal fraction cleaved and the fraction cleaved at time point 0.

2.8 Expression systems and plasmid construction for mammalian cell culture
Luciferase measurements were conducted using the psicCHECK™-2 plasmid (Promega) harboring a firefly luciferase (luc+) and a Renilla luciferase (hRluc) gene. A S. mansoni Type 3 HHR was placed in the 3′ UTR of the hRluc gene by standard molecular cloning procedures as described above. The xpt aptamer was inserted using the primers listed in Supplementary Table S3. All constructs were verified by sequencing (Eurofins Genomics/GATC Biotech).

2.9 Cell culture, transient transfection and luciferase assay
HeLa cells were grown in Dulbecco’s modified Eagle’s serum (DMEM, Thermofisher) supplemented with 1% (v/v) Penicillin-Streptomycin (Thermofisher) and 10% (v/v) fetal calf serum (FCS, Thermofisher) (following called medium) in a humidified incubator at 37°C and 5% CO2. For the luciferase assay, 25,000 cells per well were seeded in 96-well microtiter plates in 100 μl medium. The next day, transfection was conducted with Lipofectamine® 3000 (Thermofisher) according to manufacturer’s protocol (100 ng DNA and 0.15 μl Lipofectamine® 3000 per well). After 4 h transfection, the medium was exchanged for medium with or without 500 μM guanosine (Sigma-Aldrich). For dose dependency measurements, the cells were incubated in the presence of 0 μM, 30 μM, 50 μM, 70 μM, 90 μM, 100 μM, 110 μM or 150 μM guanosine. After 24 h, the medium was exchanged and the Dual-Clo Luciferase Assay (Promega) was performed according to manufacturer’s instructions. The relative hRluc activity was determined by referencing it to the luc− activity. Mean values of triplicates were normalized to the values of the psiCHECK™-2 plasmid without ribozyme insertion.

3. Results
3.1 HHR Type 1-based artificial riboswitches in E. coli—high-throughput screening
For the development of guanine-sensing switches in E. coli a Type 1 HHR was chosen since powerful OFF- as well as ON-switches have already been created in E. coli using this scaffold (4, 5). A similar library design that previously led to the identification of thiamine pyrophosphate- and theophylline-dependent aptazymes was chosen (4, 5). Stem III of the Type 1 HHR was partially replaced by stem P1 of the xpt aptamer. The stem connecting aptamer and ribozyme constitutes of four or six randomized nucleotides as communication module (Figure 1a). The aptazyme library was placed in the 5′ UTR of an eGFP reporter gene. The accessibility of the ribosome binding site is controlled by the cleavage activity of the ribozyme resulting in a translational control of gene expression (Supplementary Figure S1a).

Previously, a Fluorescence-Activated Cell Sorting (FACS)-based high-throughput screening has been established for the screening of large aptazyme libraries in E. coli (18). We employed this method to screen the libraries for functional ON-switches. In this setup, E. coli transformed with plasmids bearing the respective aptazyme library were cultivated in the absence of the ligand guanine. Since guanine has a very low solubility in aqueous solutions, we employed guanosine for these experiments instead. Guanosine is taken up by nucleoside transporters into the bacterial cell and degraded by a purine nucleoside phosphorylase which releases guanine (19, 20). Previous studies revealed that only guanine but not guanosine is recognized by the xpt aptamer (16). In addition, crystal structures of the aptamer bound to guanine showed that guanine is completely contacted by RNA, preventing the recognition of an additional ribose moiety in the binding pocket (21). Using a FACS instrument, the cultivated bacterial cells were sorted for low eGFP expression in the absence of the ligand. In a subsequent step, the sorted bacteria were cultivated in the presence of guanosine and sorted for high eGFP fluorescence. With this method, several ON-switching aptazyme sequences were isolated (Figure 1b and Supplementary Figure S2a). The switches displayed fold changes between 2.1-fold and 9.2-fold. Sequencing revealed that most of the identified communication modules were unpaired or contained weak wobble or AU base pairs (Figure 1b and Supplementary Figure S2a).

We also used the high-throughput screening to search for guanine-dependent OFF-switching aptazyme sequences since none are known in E. coli. For this purpose, the culturing conditions for the E. coli libraries were reversed. Bacteria were first sorted for low eGFP expression in the presence of ligand and subsequently sorted for high expression in the absence of guanosine. We could identify several OFF-switches with dynamic ranges of up to 32.7-fold (Figure 1b and Supplementary Figure S2b). The communication modules of all OFF-switches were fully paired with canonical base pairs, while most of the base pairs constituted of the more stable GC pairs.
3.2 Dose-dependent regulation of gene expression and single-cell analysis

For further characterization of the newly identified switches two ON-switches Gua_eco1 and Gua_eco2 as well as two OFF-switches Gua_eco3 and Gua_eco4 were chosen (Figure 1b). We investigated the concentration dependency of the switches in comparison to a constitutively active HHR. All switches showed a clear dose dependency with a maximum effect obtained at approximately 500 μM guanosine (Figure 1c) with EC50 values for half-maximum effects at 267 and 274 μM for the two ON-switches and 169 and 229 μM for the two OFF-switches, respectively. One of the ON-switches (Gua_eco1) reached a maximum eGFP expression in the induced state comparable to a control consisting of a constitutively active ribozyme. This finding demonstrates that the presence of the aptamer does not impair the catalytic activity of the ribozyme in general. The switches were further investigated on the single-cell level, using flow cytometry, to exclude the possibility that the observed switching behavior is due to a bulk effect. The resulting histograms demonstrate that the identified switches also perform well on the single-cell level (Figure 1d). Dynamic ranges were determined using the mean fluorescence of the flow cytometry data. The switches reached a high degree of gene expression control in these measurements (up to 41.4-fold regulation in case of the sequence Gua_eco4), emphasizing again the performance of the newly identified switches. Quantifications of the flow cytometry data are summarized in Supplementary Table S1.

3.3 Characterization of cleavage activity in vitro

In order to better understand the mode of action of these aptazymes, cleavage kinetics were carried out with isolated RNAs. The four sequences showing best switching performances in bacteria were in vitro transcribed utilizing a blocking strand in order to prevent co-transcriptional cleavage, PAGE-purified and kinetically characterized. The cleavage rate was followed over time in absence or presence of guanine. Results are shown in Figure 2, fitting parameters and the calculated k_{obs} values in Supplementary Table S2. For both investigated ON-switches the cleavage activity clearly enhances in the presence of the ligand. This is in accord to the induction of gene expression upon...
activating the ribozyme in vivo, resulting in an accessible ribosome binding site. However, the two characterized OFF-switches show only modest or no ligand-dependent change in cleavage activity. Hence the in vitro kinetics are not able to explain the drastic control of gene expression in case of the OFF-switches. The phenomenon is well known in the field: It was often observed that sequences engineered in vitro turned out to be non-functional in vivo and vice versa (see e.g. (22, 23)). This is the main reason why we screen for functional switches in vivo. The lack of in vitro functionality again demonstrates that both situations are not comparable. A likely reason is different folding of the RNAs and hence different kinetic properties or the involvement of currently unknown RNA-binding proteins in vivo. This highlights the importance of efficient in vivo screening methods to generate functional ribozyme-based artificial riboswitches.

3.4 HHR Type 3-based artificial riboswitches in HeLa cells—rational re-engineering

So far, only few screening methods have been established for sampling aptazyme libraries in mammalian cell cultures (24, 25). These techniques are hampered by a medium-throughput and are rather costly and laborious. However, in order to obtain switches functional in human cell culture such screening approaches seem valuable since attempts to transfer sequences identified in E. coli or Saccharomyces cerevisiae into mammalian cells have frequently failed (10, 26). On the other hand, one successful example of rationally designing communication modules in order to obtain ON-switches in mammalian cell culture has been reported by the Süß group (10). We wondered whether it might be possible to transfer the described modules to guanine aptamer-controlled ribozymes. As a starting point the ON-switches TetK4 and TetK19 were used. These ON-switches are Type 3 HHR-based sequences that are activated upon tetracycline addition. Tertiary loop-loop interactions between stem I and stem II crucial for the full activity of the HHR are controlled by the aptamer via the rationally designed communication modules (27, 28). For the tetracycline-dependent ON-switches, the full length S. mansoni HHR N79 (29) is connected via stem III to the 3’ UTR of the gene of interest. Thus, an active ribozyme cleaves off the polyA tail of the transcript resulting in degradation of the mRNA. The tetracycline aptamer was inserted into stem I of the ribozyme by Süß et al. A communication module was developed based on the rationale that the loop-loop

Figure 2. Cleavage activities of aptazyme sequences in vitro. Percentage of cleaved fraction for ON-switches (a) Gua_eco1, (b) Gua_eco2 and OFF-switches (c) Gua_eco3, (d) Gua_eco4 in absence (black line) and presence (grey dashed lines) of guanine.
interactions between stem I and stem II are impaired. Binding of tetracycline stabilizes the communication module further and renders the ribozyme inactive. The mRNA stays intact and gene expression can occur (Supplementary Figure S1b).

In our approach the two most potent switches, TetK4 and TetK19 were chosen and the tetracycline aptamer was replaced by the \textit{xpt} aptamer. Two basepairs in the P1 stem of the \textit{xpt} aptamer were kept similar to the aptazymes constructed by Yokobayashi et al. (7, 8) (Figure 3a). The sequences were inserted into the 3' UTR of a \textit{Renilla} luciferase reporter and the expression of \textit{Renilla} luciferase was normalized to the luminescence of a firefly luciferase constitutively expressed from the same vector. The resulting Gua_K4 switch showed a slight switching behavior with 1.4-fold higher Renilla luciferase expression in the presence of guanosine (Figure 3b). In contrast, no such effect was observed with Gua_K19. Since the K19 communication module is one base pair longer than the K4 communication module a shorter or a less stable stem might be more optimal in case of the \textit{xpt} aptamer. On the basis of this observation, we designed new constructs by selecting communication modules that are either destabilized or shortened versions of Gua_K4 and Gua_K19 (Figure 3a and Supplementary Figure S3a). Here, several constructs displayed improved switching efficiencies between 1.6-fold and 4.0-fold (Figure 3b and Supplementary Figure S3b). The best performing switches, Gua_K3 and Gua_K9, differed from Gua_K4 only by the introduction of a destabilizing wobble base pair. Both switches induce gene expression 4.0-fold at 500 \( \mu \)M guanosine but Gua_K3 has a lower basal expression level. Further characterization of Gua_K3, Gua_K4.1, Gua_K9 and Gua_K18 revealed that all switches exhibit a dose dependency with EC50 concentrations between 35 \( \mu \)M and 78 \( \mu \)M guanosine (Figure 3c). Inactive variants of the switches were generated by introducing a A\textsuperscript{\rightarrow}G mutation in the active site of the ribozyme (Figure 3a). None of the inactive variants could induce gene expression (Figure 3c) proving that the observed regulatory effect is indeed caused by the ribozyme activity.

4. Discussion

4.1 Development of new guanine-dependent ON- and OFF-switches in \textit{E. coli}

In the present study, we utilized a previously established FACS-based high-throughput screening approach (18) to identify guanine-sensing ON- and OFF-switches from an aptazyme library. We successfully identified several ON- as well as OFF-switching aptazymes, representing the first guanine-dependent ribozymes functional in bacteria. The ON-switches reached dynamic ranges of up to 9.2-fold, while the OFF-switches reduced gene expression up to 32.7-fold. Thus, a single aptazyme library design resulted in both, ON- as well as OFF-switches, solely dependent on the nature of the communication module. This result is similar to screening efforts with thiamine pyrophosphate-dependent aptazymes (4). In both, the previous and the present study, a rather unpaired communication module gave rise to ON-switches, while a fully canonical base-paired communication module led to OFF-switches. Very similar communication sequences obtained for thiamine-dependent aptazymes were identified as hits in the FACS-based screening (see Supplementary Figure S2b). This also hints towards the

![Figure 3. Rational design of guanine-sensing aptazymes in HeLa cells. (a) The tetracycline aptamer in the TetK4/K19 constructs (10) is replaced by the guanine-sensing xpt aptamer (16), while the respective communication module (green) remains identical. Additional tested communication modules (K3, K4.1, K9, K18) are presented as well. Nucleotides involved in the stem I-stem II tertiary interactions of the ribozyme are encircled in red. A ribozyme inactivating A\textsuperscript{\rightarrow}G mutation is depicted. (b) Dual luciferase assay of the constructs shown in (a). Transfected cells were incubated in the absence or presence of 500 \( \mu \)M guanosine. Experiments were performed in triplicates and error bars represent standard deviation. A psiCheck2 plasmid without aptazyme serves as control. (c) Dose dependency of the Gua_K3, Gua_K4.1, Gua_K9 and Gua_K18 switches. The relative luciferase activity under control of the respective switch and an inactivated variant is shown under varying guanosine concentrations. A sigmoidal fit was used to calculate the EC50-value. Experiments were performed in triplicates and error bars represent standard deviation.](image-url)
possibility that the thM and the xpt aptamer may be interchangeable in this context. In general, the successful identification of several switching sequences in this study demonstrates that the FACS-assisted screening method is a powerful technique to identify new aptazymes in E. coli.

Due to the lack of sufficient high-throughput screening methods in mammalian cell culture, we used a rational design approach to develop a guanine-dependent ON-switch. We replaced the tetracycline aptamer of the TetK4 ON-switch described by Süss et al. (10) with the natural xpt aptamer and obtained an ON-switch that is triggered by guanine. However, the new guanine ON-switch was less efficient than its parental switch. We improved the performance of the switch by using the less stable communication modules K3 and K9 and could thus create ON-switches that induce gene expression 4.0-fold with EC50-values of 35–78 μM. These concentrations of half-maximum effect are in a similar range as previously described guanine-dependent aptazymes (3, 7). Thus, the application of guanosine instead of guanine is not limiting the switching performance. It is notably that the corresponding tetracycline-dependent constructs TetK3 and TetK9 could not induce gene expression significantly (10). Hence, the utilized strategy of switching stem I/II interactions in Type 3 HHRs seems to be transferable but individual adjustments are necessary for different aptamers. This is in line with previous studies on the modularity of natural riboswitches (17). The Batey group created riboswitch chimeras in which they combined the expression platform of selected natural riboswitches with a number of natural and synthetic aptamers. These riboswitch chimeras were functional although some showed lower dynamic ranges compared with the original riboswitch. Nevertheless, the switching efficiency of the chimeras could be optimized by altering the stability of the P1 helix. In the context of artificial riboswitches in mammalian cell culture, a few additional examples for modularity exist. The guanine-sensing OFF-switch RzGuaM5 of the Yokobayashi group was created by replacing a theophylline aptamer of the RzTheoM5 switch with the xpt aptamer (7, 30). In another study, the theophylline aptamer of an ON-switching aptazyme was replaced by a tetracycline aptamer resulting in a new switch with even increased dynamic ranges (2). In conclusion, existing natural and synthetic riboswitches can serve as starting points for the development of new switches that are triggered by a different ligand. Adjustments of the communication module could be necessary in order to increase the performance of the new switches. However, there is still a need for novel, sophisticated aptazyme screening techniques in mammalian cell cultures in order to be able to develop completely new aptazymes with aptamers that do not behave as modular as the presented and described examples.

5. Availability
All plasmids are available upon request. Sequences of all plasmids are available at SYNBIO online.

Supplementary data
Supplementary Data are available at SYNBIO online.

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