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Ligand-dependent ribozymes

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The discovery of catalytic RNA (ribozymes) more than 30 years ago significantly widened the horizon of RNA-based functions in natural systems. Similarly to the activity of protein enzymes that are often modulated by the presence of an interaction partner, some examples of naturally occurring ribozymes are influenced by ligands that can either act as cofactors or allosteric modulators. Recent discoveries of new and widespread ribozyme motifs in many different genetic contexts point toward the existence of further ligand-dependent RNA catalysts. In addition to the presence of ligand-dependent ribozymes in nature, researchers have engineered ligand dependency into natural and artificial ribozymes. Because RNA functions can often be assembled in a truly modular way, many different systems have been obtained utilizing different ligand-sensing domains and ribozyme activities in diverse applications. We summarize the occurrence of ligand-dependent ribozymes in nature and the many examples realized by researchers that engineered ligand-dependent catalytic RNA motifs. We will also highlight methods for obtaining ligand dependency as well as discuss the many interesting applications of ligand-controlled catalytic RNAs.

INTRODUCTION

Ribozymes are RNA molecules that are catalytically active. The first two examples of naturally occurring ribozymes were reported in the early 1980s by Cech, who was studying the splicing in the unicellular eukaryote *Tetrahymena thermophila*,[1] and by Altman, who was studying the endoribonuclease RNase P.[2] Group I introns are ribozymes that can self-splice in the presence of a bound guanosine cofactor,[3] whereas RNase P is a ribonucleoprotein that processes transfer RNA (tRNA) molecules from a longer precursor.[4] The catalytic activity of RNase P was shown to reside in the RNA moiety of the complex.[2] Today we know that ribozymes are a diverse and widespread group of catalytically active biomolecules that are involved in different biological processes. The group II introns are a second family of self-splicing ribozymes which do not require a guanosine cofactor but operate with a mechanism similar to the spliceosome-catalyzed removal of introns.[5] The ribosome is also a ribozyme: when the crystal structure of the 50S ribosome subunit of *Haloarcula marismortui* was published, it was conclusively revealed that the peptidyl transferase activity of the ribosome is not mediated by any ribosomal proteins but by ribosomal RNA (the 23S component in bacteria and the 28S component in eukarya and archaea).[6] Another family of naturally occurring catalytic RNAs with diverse and widespread appearance is the one of small endonucleolytic ribozymes.[7] This family is composed of RNA motifs that have a size in the range of 50–150 nucleotides (nt) and that show intrinsic RNA cleavage activity. The cleavage takes place via an internal phosphoester transfer reaction. Starting from 1986, when the first autolytic processing of a plant subviral particle RNA genome (tobacco ringspot virus satellite RNA) was observed[8] an increasing number of self-cleaving RNA motifs was described. Until today, nine distinct classes of small self-cleaving ribozymes have been characterized: hammerhead (HHR),[8,9] hairpin,[10] hepatitis delta virus (HDV),[11] Varkud satellite (VS),[12] *glmS*,[13] twister,[14] twister sister, pistol, and hatchet.[15] The physiological functions of most of the identified motifs remain unknown. The three known hairpin motifs, the VS, and a some of the HHRs and HDV-like motifs are

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involved in the processing of concatemeric copies of RNA genomes of subviral particles (viroids, satellites, and HDV).[7] Moreover, many HHR and HDV-like ribozymes were found, respectively, in short interspersed nuclear elements (SINE) and long interspersed nuclear elements (LINE), implicating a role in the transposition process.[7,16,17] Some HHRs were found in intronic sequences indicating a potential role in messenger RNA (mRNA) processing.[18] Remarkably, the bacterial *glmS* ribozyme facilitates a riboswitch-based negative feedback regulation of the biosynthesis of an important precursor for the bacterial cell wall.[19] All natural ribozymes described until now catalyze phosphodiester cleavage or ligation reactions, with the important exception of the ribosome, which catalyzes peptide bond formation.[20] Nevertheless, RNA and DNA are potentially capable of catalyzing a broad spectrum of reactions: in addition to the naturally occurring catalytically active motifs, an outstanding number of artificial ribozymes and deoxyribozymes (or DNAzymes) were developed within the past 25 years. The first artificial ribozyme was reported in 1990.[21] Since that time, *in vitro* selection has been used to generate many artificial ribozymes with a wide range of catalytic activities.[22]

Similar to protein enzymes, the catalytic activity of ribozymes can be regulated by external stimuli. While only a few examples of naturally occurring ribozymes being regulated by external stimuli are known, a huge diversity of engineered ribozymes has been developed to respond to molecular interactions in the last decades. In principle, the catalytic activity of such ribozymes can be modulated using different types of stimuli. Most of the time, the regulation is realized using ligands as modulators. The identity of such activity-modulating ligands can vary from monoatomic ions, organic compounds of low and intermediate molecular weight, peptides, proteins, to oligonucleotides. However, examples of light- and temperature-dependent ribozymes have been reported as well.[23–26] Allosteric control of ribozymes can be artificially achieved by connecting an aptamer to the catalytically active motif, thereby generating a so-called aptazyme. Aptamers are nucleic acid motifs that are able to bind a ligand with high affinity and specificity. In case of an aptazyme, binding of the ligand to the aptamer induces a conformational change that modulates the activity of the catalytic domain. This review focuses on both natural as well as artificial ligand-dependent ribozymes, discussing their potential biological functions, the methods for their generation, and the possible applications. The study on ligand-dependent ribozymes contributes significantly to our understanding of the functional roles of noninformational RNAs.[27] In addition, the engineering endeavors provide innovative tools for biotechnological applications such as the development of RNA-based biosensors and artificial genetic switches. These tools open up new possibilities for future diagnostic and therapeutic strategies.

NATURALLY OCCURRING LIGAND-DEPENDENT RIBOZYMES

Many naturally occurring ribozymes carry out their biological functions taking advantage of interactions with different classes of molecular partners. In this regard, we can distinguish three general situations. In the first case, the molecular partner is a real cofactor that is needed to carry out the chemical reaction catalyzed by the ribozyme. Some examples are the group I introns, the *glmS* ribozyme (see discussion below), and the HDV ribozyme, which uses Mg^{2+} ions for both the deprotonation of the nucleophile and the stabilization of the phosphorane transition state during the *trans*-esterification reaction.[28] In the second case, the molecular partners are proteins (sometimes dispensable) that are associated with the ribozyme and that can have either structural or regulatory roles. Some examples are the RNase P ribozyme, some group I introns (see discussion below), and the ribosome.[29] Finally, we have one case of a group I intron in which the ligand regulates ribozyme activity in a truly allosteric manner (see below).

The RNase P ribozyme is found in bacteria, archaea, and eukaryotes and it is composed of a conserved RNA subunit of 350–400 nucleotides and one or more protein partners.[4] Similarly to other ribozymes, the RNase P ribozyme also requires divalent ions, preferably Mg^{2+}.[30] Under high ionic strength conditions, the RNase P RNA can catalyze *in vitro* the tRNA processing independently of proteins.[2] However, the protein greatly increases the rate of the reaction.[31] In bacteria the protein partner is a small protein of 14 kDa (RnpA) that stabilizes the active RNA conformation, helps substrate recognition, and metal ion binding.[4] The minimal holoenzyme of archaea and eukaryotes includes at least four conserved proteins.[32] Human and yeast RNase P can present up to 10 protein partners.[4,32] The roles of the protein component in eukaryotes remain unclear: they are probably involved in the stabilization of the tertiary interaction of the RNA component, in the regulation and intracellular localization of the enzyme, and in the processing of different types of RNA substrates.[4] Moreover, the protein

component of the human RNase P is essential in the activation of transcription by RNA polymerases I and III.[33,34] RNase P was shown to be involved in the maturation of different RNAs apart from tRNAs [e.g., ribosomal RNAs (rRNAs), protein-coding mRNAs, transfer-messenger RNA (tmRNA), riboswitches, viral RNA, and small nucleolar RNAs (snoRNAs)].[4] Protein-only RNase P was also reported in eukaryotic organelles (chloroplast and mitochondria) and in the nuclei of *Arabidopsis thaliana* and *Trypanosoma brucei*.[35]

The family of group I intron ribozymes that promote self-splicing activity utilizes guanosine derivatives such as GTP as cofactors[36] (Figure 1(a)). Group I introns are found in bacteria, mitochondrial and chloroplast genomes of some eukaryotic organisms (fungi, plants, and unicellular eukaryotes), and in the rRNA genes of the nuclear genome of some unicellular eukaryotes.[37] The first motif was discovered within the ribosomal RNA of *T. thermophila*. Here, the 414 nucleotides long intron is removed autonomously from a 6.4-kbp precursor to obtain the 26S rRNA.[1] The product of the first self-splicing is catalytically active and can act as a ribozyme as well, undergoing multiple rounds of self-splicing.[3] Although *in vitro* the splicing reaction of the group I introns does not require additional factors, it is thought that *in vivo* further protein factors could be required.[3] For instance, it was shown that the activity of the mitochondrial group I intron in *Aspergillus nidulans* is facilitated by the maturase I-AniI.[38]

Apart from the widespread occurrence of self-splicing group I introns, a rare example of a truly allosteric group I self-cleaving intron was discovered in *Clostridium difficile* by Breaker and coworkers.[39] In addition to GTP, the activity of this self-splicing intron was shown to be regulated by cyclic-di-GMP (c-di-GMP). The cyclic dinucleotide is a ubiquitous bacterial second messenger that plays important roles in facilitating adaptation and life style changes.[40] It was noted that an aptamer recognizing c-di-GMP in certain riboswitches was found to be associated with the group I intron. In the absence of c-di-GMP the ribozyme-processed mRNA lacks a ribosome-binding site (RBS; Figure 1(b)), whereas in the presence of c-di-GMP the ribozyme selects an alternative splice site. This modulation results in the production of the fully spliced RNA containing a strong RBS and start codon (Figure 1(b)). The combination of a c-di-GMP aptamer and the group I intron as expression platform represents an interesting example of a riboswitch that switches on self-splicing to yield a functional mRNA in response to c-di-GMP.[39] Moreover, it exemplifies that nature as well makes use of the highly modular architecture of functional RNAs for the conditional control of fundamental processes such as gene expression.

Another important example of a natural ligand-dependent ribozyme that acts as riboswitch is the bacterial *glmS* motif. The *glmS* ribozyme was discovered as a conserved element residing in the 5′-UTR of the *glmS* mRNA in many Gram-positive bacteria including *Bacillus subtilis*. Later, comparative sequence analyses revealed that the *glmS* ribozymes have a broader phylogenetic distribution (including representatives found in the Deinococcus-Thermus phylum).[41] The *glmS* gene encodes the glutamine: fructose-6-phosphate amidotransferase, an enzyme that generates glucosamine-6-phosphate (GlcN6P) from fructose-6-phosphate and glutamine. GlcN6P is an important precursor in the biosynthesis of the bacterial cell wall. The self-cleavage of the *glmS* RNA motif was shown to be promoted by GlcN6P.[13] The crystal structures of the *glmS* ribozyme in the absence and in the presence of GlcN6P revealed that the nucleophile (2′O) and leaving group are already aligned prior to ligand binding, indicating an outstandingly rigid structure of the active site and the metabolite-binding pocket.[42] However, the cleavage reaction is very slow in the absence of the GlcN6P. In fact, the ligand was shown to be directly involved in the catalytic mechanism with its protonated amino group acting as the general acid and its C1-OH stabilizing the negative charge developing on the scissile phosphate.[42] Hence, the GlcN6P acts as a cofactor rather than an allosteric effector of ribozyme catalysis.[43] This is in contrast to most other riboswitch mechanisms where ligand binding to the aptamer domain alters gene expression via a rearrangement in the expression platform. The mechanistic basis of the ribozyme's impact on gene expression was described subsequently by Winkler and coworkers.[19] They found that the GlcN6P-induced cleavage within the 5′-UTR of the *glmS* mRNA results in a destabilization and degradation of the message by the RNA 5′-exonuclease RNase J (Figure 1(c)).

In principle, with the exception of the rare c-di-GMP-dependent group I intron, all naturally occurring ligand-dependent ribozymes known to date rely on molecular interaction partners (metal ions, guanosine, GlcN6P, RNase P, and ribosomal proteins) that act as cofactors rather than allosteric modulators. In addition, it was shown that RNA-targeting antibiotics can modulate (i.e., most often inhibit) ribozyme activities.[44,45] Most prominently, the ribosome is a major target of antimicrobial compounds.[46] Small endonucleolytic ribozymes such as the HHR have been described as targets of antibiotics as well.[47,48]

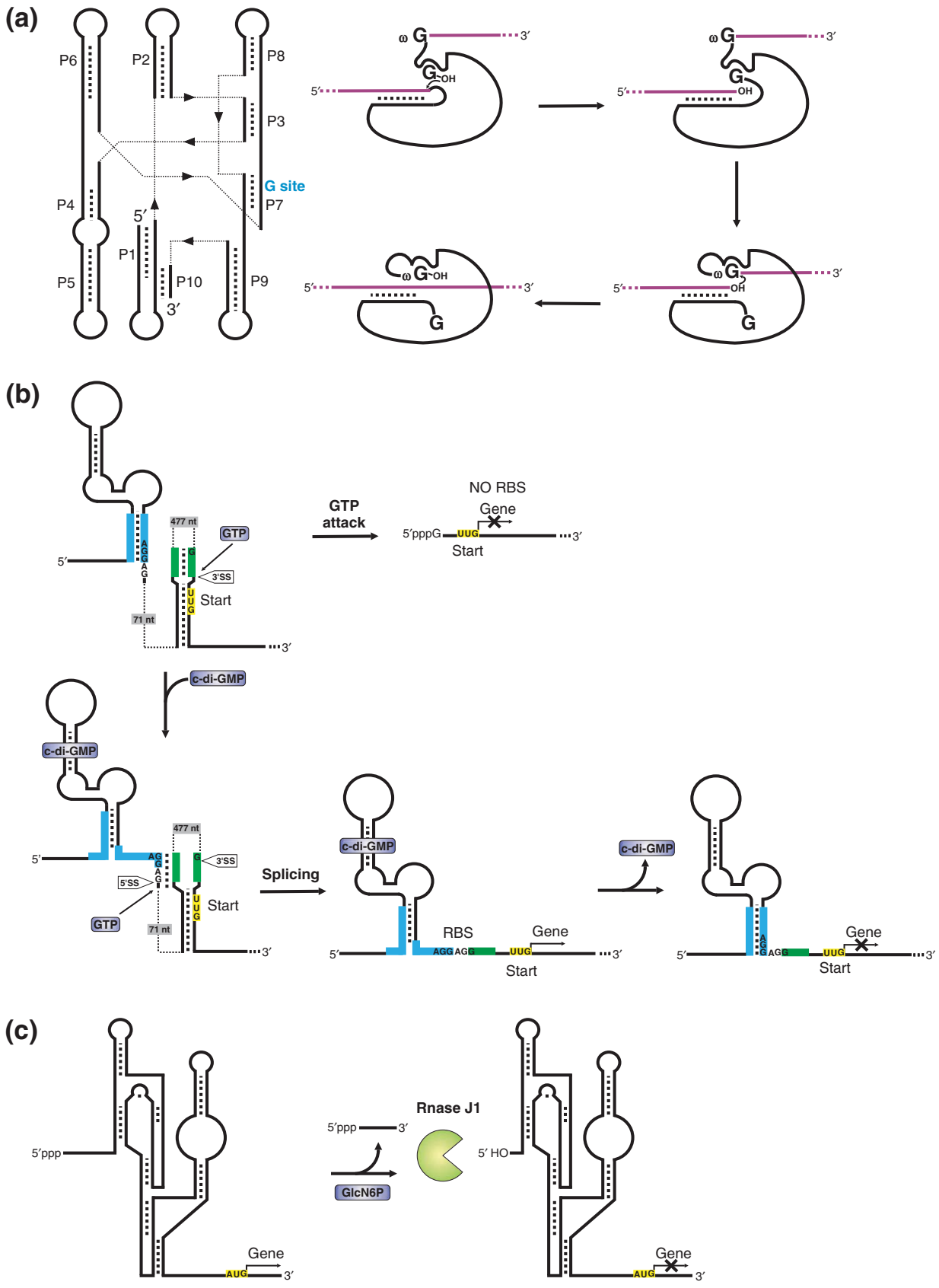

FIGURE 1 | Legend on next page.

In this regard, HHR inhibitors were used for constructing early examples of ribozyme-based switches of gene expression (for more details see below).[49] In conclusion, currently only a few examples of naturally occurring ligand-dependent ribozymes have been described. However, it has been speculated recently that the large variety and widespread distribution of ribozymes and candidate riboswitches will very likely reveal more examples of ligand-dependent ribozymes occurring in nature.[14,50,51]

ARTIFICIAL LIGAND-DEPENDENT RIBOZYMES

In parallel to the discovery of naturally occurring ligand-dependent ribozymes, researchers have generated a huge diversity of catalytic motifs whose activity is artificially controlled by different types of stimuli.[23] The first example of a designed modulation of the ribozyme activity was reported in 1995.[52] Here the control of the catalytic activity was performed through an effector and a facilitator DNA oligonucleotide that permitted the formation of a correctly folded and catalytically active form of the HHR. In the same year the first modulation of a hairpin ribozyme was realized by connecting an RNA domain binding to the bacteriophage R17 coat protein to the catalytic motif.[53] Protein binding enhanced the cleavage rate of the hairpin ribozyme twofold and the ligation reaction 16-fold. The protein-binding domain was introduced in the form of an extension of helix 4 of the hairpin ribozyme. Since then, huge efforts were put into the development of catalytic RNA motifs that could be employed to detect and respond to different types of ligands in the test tube (*in vitro*) and in cells or even whole organisms (*in vivo*). Although many early artificial ligand-dependent ribozymes were certainly constructed with both *in vitro* and *in vivo* applications in mind, the first achievements in this field were demonstrated *in vitro*. It was soon realized that ligand-dependent ribozymes are innovative tools with potential uses as basic components in biosensors for environmental monitoring and diagnostic as well as potential therapeutic purposes. However, it turned out that many ligand-dependent ribozymes were not easily transferred from the test tube to function within cellular environments. Recent efforts are often focused on the generation of ligand-dependent ribozymes specifically for their application *in vivo*. This is why we structured the following content into ribozymes developed for *in vitro* and *in vivo* applications. We will discuss artificial ligand-dependent ribozymes responding to different ligands with a particular attention to the methods for obtaining ligand dependency and we will describe possible applications.

Artificial Ligand-Dependent Ribozymes *In Vitro*

Apart from the early examples mentioned above, a first group of ligand-dependent ribozymes, called aptazymes (i.e., ribozymes coupled with aptamers that recognize small molecular ligands or proteins), were systematically developed starting in 1997.[54] Aptazymes present a tripartite functional organization (Figure 2): a sensor domain (which is responsible of the binding of the effector molecule), an actuator or catalytic domain (the ribozyme), and the so-called communication module, a bridge element that connects the sensor and the actuator domains.[54] The communication module is responsible for transmitting the information of ligand binding from the sensor domain to the catalytic domain. It assumes a critical role because it determines whether the molecular stimulus will behave as an inducer or a repressor via either stabilization or destabilization of the catalytic domain.

Ribozymes can easily be engineered in a way to be modulated by oligonucleotide effectors. Similar to small molecule- and protein-sensing aptazymes, such systems can be used for analytical purposes. However, there are important differences concerning the design of such functional motifs because the strict separation of sensor and catalytic domains and their connection

FIGURE 1 | Naturally occurring ligand-dependent ribozymes. (a) Group I intron ribozymes promote self-splicing activity using guanosine nucleotides as cofactors. The consensus secondary structure of the group I introns consists of 10 paired regions (P1–P10). The guanosine cofactor (G) binds to the G site located in helix P7 (in blue). The processing of the group I introns starts with the nucleophilic attack of the 3′-OH of the exogenous G cofactor to the phosphodiester bond at the 5′ splice site (5′SS) in P1. The terminal G (ωG) of the intron replaces the G cofactor in the G site. Afterward a second *trans*-esterification takes place with the 3′-OH of the upstream exon attacking the phosphodiester bond in the 3′ splice site (3′SS), leading to the ligation of the two exons. (b) Mechanism of gene regulation by the c-di-GMP-dependent group I intron of *Clostridium difficile*. In the absence of c-di-GMP (top) the GTP cofactor attacks the 3′SS generating an mRNA that lacks a ribosome-binding site (RBS). In the presence of c-di-GMP (bottom) the ribozyme selects alternative splice sites (5′SS and 3′SS). The splicing results in the production of an mRNA containing a strong RBS and start codon (UUG). (c) The *glmS* ribozyme exhibits self-cleavage in the presence of glucosamine-6-phosphate (GlcN6P), which is used as a cofactor. The cleavage of the mRNA generates a free 5′ hydroxyl group that allows mRNA degradation by RNase J.

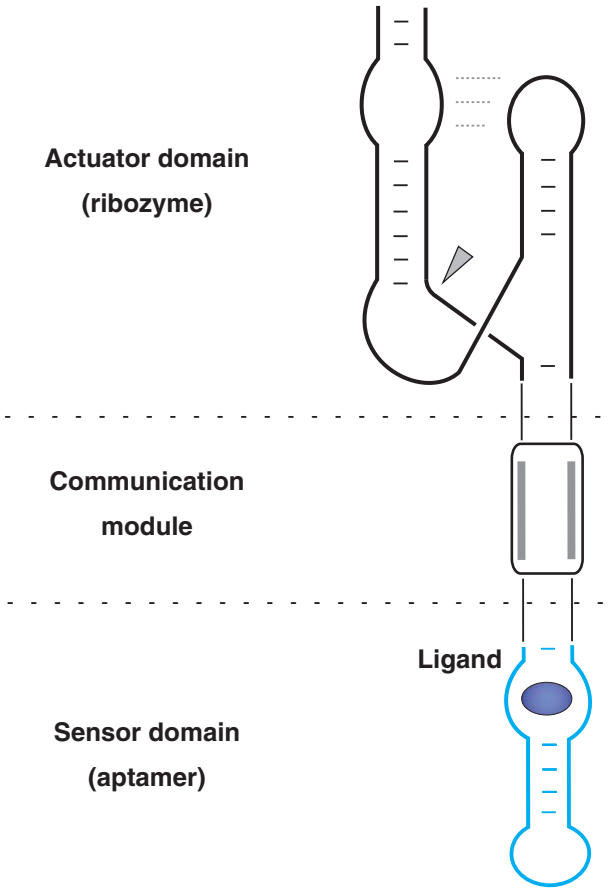

FIGURE 2 | Tripartite organization of aptazymes. The actuator domain, here a hammerhead ribozyme, is represented in black. The cleavage site is indicated with a gray arrowhead. The sensor domain and the effector ligand are represented in light and dark blue, respectively. The communication module, which functionally connects the actuator and the sensor domain, is displayed in gray.

utilizing a communication module is not necessary. This is due to the fact that molecular interactions can be designed following simple Watson–Crick base pairing rules, allowing to easily program interactions of ribozymes with effector oligonucleotides.

In Vitro Generation of Aptazymes

As the generation of the communication module is a critical step in aptazyme development, different experimental approaches have been developed. A general method for obtaining aptazymes was described for the first time in the pioneering work of Tang and Breaker.[54] ATP-dependent aptazymes based on a minimal *in trans*-cleaving HHR were generated using a so-called modular rational design approach. It allows integrating the different functional domains by taking advantage of the modular nature of functional RNAs by using in a judicious way Watson–Crick base-pairing rules and considerations about thermodynamic stability of RNA duplexes and their conformational changes. In this way, many rationally designed aptazymes detecting several different types of ligands were generated *in vitro* based on the HHR,[54,55] HDV,[56] as well as artificial ribozymes.[57–59] Moreover, the modular rational design approach can be assisted by computational methods.[60,61]

After these initial studies the field moved rapidly toward more combinatorial designs that require less *a priori* knowledge regarding the structure and conformational changes of the RNA motifs utilized. The first *in vitro* selection method was presented by Soukup and Breaker to isolate flavin mononucleotide (FMN)-dependent aptazymes.[62] In this iterative approach, the different populations (catalytically active or inactive) of ribozymes were separated using polyacrylamide gel electrophoresis (PAGE). According to this protocol, the first step is the generation of a pool of RNA molecules in which the ribozyme and the aptamer domains are connected by a number of randomized nucleotides. To isolate communication modules that direct allosteric induction, first a negative selection is performed in which the RNAs that cleave in the absence of the ligand are removed. The pool fraction that remained uncleaved during the first selection is subjected to a positive selection in the presence of the ligand in which only the motifs that perform cleavage are retained. To isolate communication modules that direct allosteric inhibition, the pool was transcribed in the presence of the ligand and only the motifs that are not cleaved are selected (negative selection). The remaining RNA precursors are then subjected to positive selection in the absence of ligand. After multiple rounds of selection, highly sensitive aptazymes that are positively or negatively controlled by the ligand were generated. The selected communication modules could be used in a modular fashion in order to rapidly generate new aptazymes simply by swapping the respective sensor domains (demonstrated for theophylline and ATP aptamers) and conserving the catalytic and communication module domains. *In vitro* selection approaches can be used also to generate new ligand-binding domains. For this purpose, mutations[63] or randomized sequences[64,65] can be introduced into the sensor domain of a functional aptazyme. Then the selection of new ligand-binding domain can be performed based on the modulation of a catalytic activity of the ribozyme. Rational design and *in vitro* selection approaches can be combined assembling in a modular way actuators and sensor domains via previously selected communication modules.[66–68]

In vitro selection and mixed approaches proved to be very successful because they allowed the generation of a broad variety of aptazymes active *in vitro* using different types of actuator platforms (e.g., HHR,[62–64,69] hairpin,[70] HDV, X motif, group I intron,[68] and artificial ribozymes).[71] An interesting application of this approach was described by Sen and coworkers who used an *in vitro* selected aptamer specific for binding one but not the other of two isomers of a photoconvertible compound for the construction of an allosteric HHR whose catalysis is controlled by irradiation with visible versus ultraviolet light.[72]

In Vitro *Applications of Aptazymes and Readouts*

The second critical aspect of using aptazymes for most *in vitro* applications is the functional connection of the modulated ribozyme catalytic activity to a readout that quantitatively reports the binding of the ligand to the nucleic acid. In first proof-of-concept studies, the aptazymes were catalyzing cleavage or ligation reactions and the binding of the ligand was evaluated by direct quantification of radioactively labeled products of the reactions. The immobilization of such aptazymes in arrays allowed the parallel analysis of several different analytes in a complex mixture[73,74] (Figure 3(a)). Detection of ligand-dependent cleavage or ligation reactions was also performed by quartz crystal microbalance (QCM)[75] (Figure 3(b)) and cyclic voltammetry.[76] In addition, an example of an aptazyme that coverts a small-molecule input into an RNA strand output through a cascade of enzymatic reactions was reported.[77] Amplification of the signal can be achieved using *in trans* constructs that allow multiple turnover reactions. Joyce and coworkers developed an advanced design in which two synthetic ligand-dependent ribozymes catalyze each other's synthesis using four RNA strands[58] (Figure 3(c)). The ligation reaction took place only in the presence of the ligand (theophylline or FMN) resulting in an amplification of the signal. This system can be regarded as performing a Boolean logical AND operation upon mixing theophylline- and FMN-dependent replicases.[58]

The catalysis of RNA cleavage and ligation reactions can be used to develop readout systems that monitor changes in activity by fluorescence using fluorescence resonance energy transfer (FRET) donor–acceptor dye pairs[78] (Figure 3(d)). This approach was used to develop functional assays based on aptazymes to detect posttranslational modifications of proteins,[79] protein kinase activity,[80] and the presence of small molecules in solution.[81] Such an approach allowed using HHR-based aptazymes not only to detect protein–nucleic acid and protein–protein interactions but also as sensors for high-throughput screening of small molecules that inhibit such interactions.[55,82] Ogawa and Maeda used aptazymes to regulate the state of aggregation of gold nanoparticles, thus inducing a visible color shift of the solution upon ligand binding[83] (Figure 3(e)). Using this approach, different logic gates were generated. Synthetic ribozymes that catalyze reactions other than phosphoester transfers can be used to develop readout systems that allow the direct detection of changes in substrate and product concentrations. Such an example is represented by a theophylline-responsive Diels–Alderase aptazyme that converts the fluorescent substrate anthracene in a nonfluorescent product upon ligand activation[71] (Figure 3(f)).

In Vitro *Generation of Oligonucleotide-Dependent Ribozymes*

The design of oligonucleotide-dependent ribozymes is normally performed following simple Watson–Crick base-pairing rules. In the most common approach, a sensor domain is appended to the catalytic domain in a way that it forces the ribozyme to assume a catalytically inactive conformation. The effector oligonucleotide binds to the sensor domain, thereby disrupting the inhibitory structure and increasing the activity of the ribozyme (Figure 4(a)). A high diversity of positively regulated motifs able to sense oligonucleotides *in vitro* was generated using such general designs.[52,84,85] An interesting complementation to this principle was introduced by Müller and coworkers with the inactivation of the hairpin ribozyme by mutating an essential nucleotide. The activity of the ribozyme was reestablished by addition of an effector oligonucleotide containing the correct sequence. The added RNA strand displaced the nonfunctional intramolecular strand from the hairpin, thereby activating the ribozyme[86] (Figure 4(b)). Although the concept is very appealing, such approaches are restricted to oligonucleotides that are to a large degree complementary to parts of the sequence of the catalytic domain. Because an interesting application of oligonucleotide-dependent ribozymes is their utilization in nucleic acids detection, designs that allow flexibility in the choice of ligand sequences are desirable. More recent designs are proposed to be particularly flexible and modular with respect to the range of sequences to be detected[87–89] (Figure 4(c)). Here, the sensor strand is not directly interacting with the catalytic domain, allowing the system to be adaptable to different target–effector sequences. Moreover, oligonucleotide-mediated repression of the ribozyme activity was

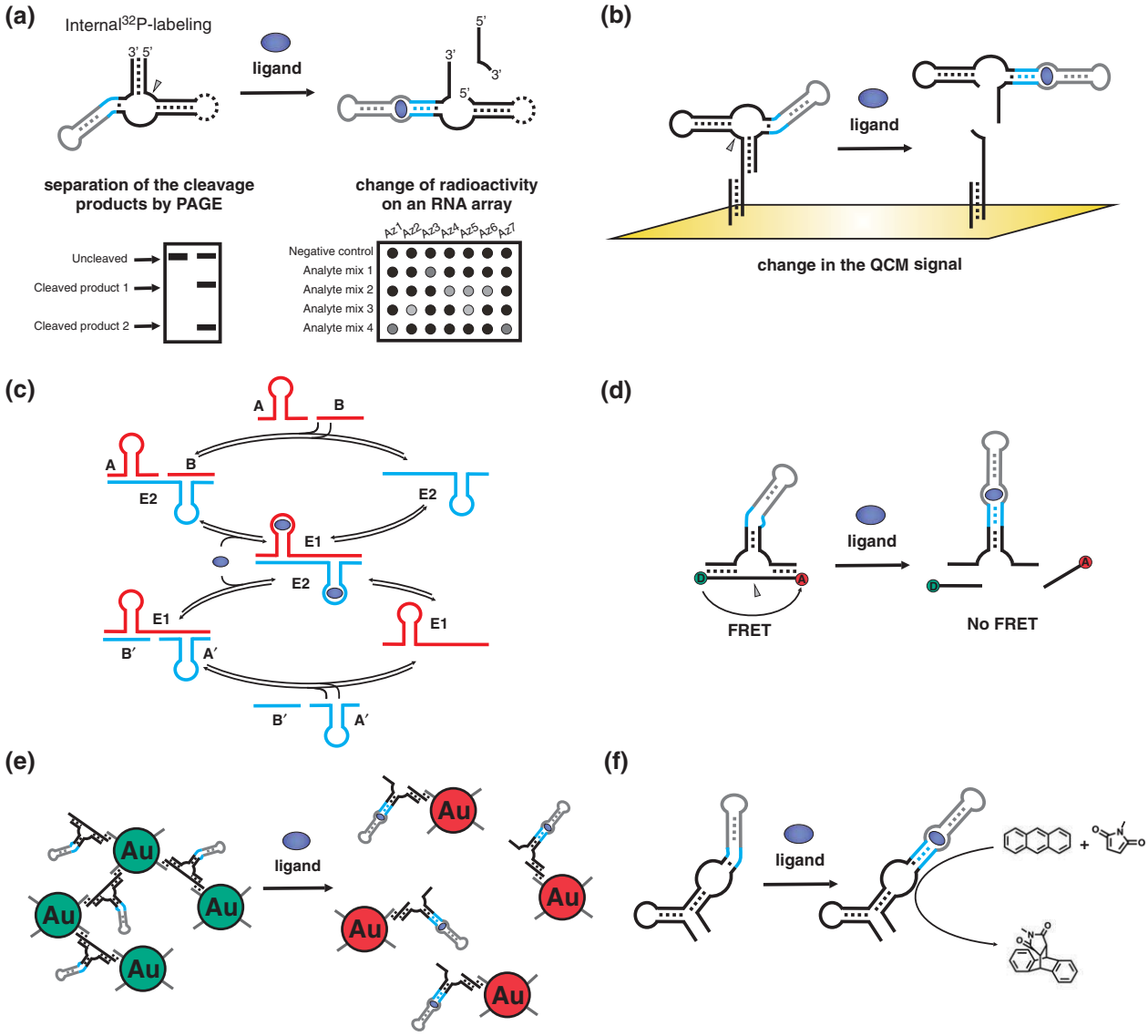

FIGURE 3 | *In vitro* applications of aptazymes and readout systems. (a) Ligand binding induces the cleavage of a radioactively labeled RNA. The detection of the cleavage products is performed by polyacrylamide gel electrophoresis (PAGE) (bottom left) or on array (bottom right). The use of arrays allows the parallel analysis of different analytes in a mixture. (b) The detection of cleavage or ligation products can be performed by quartz crystal microbalance (QCM). (c) Amplification of the signal can be obtained using *in trans* constructs. An amplification system in which two synthetic ligand-dependent ligases catalyze each other's synthesis was developed using a total of four RNA strands. (d) Fluorescence resonance energy transfer (FRET) can be used to monitor changes in the cleavage activity of an aptazyme by fluorescence using donor–acceptor dye pairs. (e) Aptazymes can regulate the state of aggregation of gold nanoparticles, thus inducing a visible color shift of the solution upon ligand binding. (f) Using a ligand-dependent Diels–Alderase aptazyme, the presence of the ligand can be detected monitoring the conversion of the fluorescent substrate anthracene into the nonfluorescent cycloaddition product.

realized.[88] An alternative approach, the so-called expansive regulation, was developed by the group of Sen (Figure 4(d)).[90] In this design, an effector oligonucleotide binds to both elements of a bipartite ribozyme/DNAzyme–substrate complex, forming a stable three-way junction. The activation of the catalytic motif is due to an enhancement of the stability of the enzyme–substrate complex.[90] This approach was demonstrated to be broadly applicable to various catalytic RNA motifs that recognize their substrates via Watson–Crick base pairs. The expansive regulation approach was used also to realize small-molecule-responsive ribozymes (ATP and FMN).[91] Similar to the oligo-dependent design, the effector molecules exercise their activation effect by stabilizing the enzyme–substrate complex rather than modulating the chemical step of catalysis.

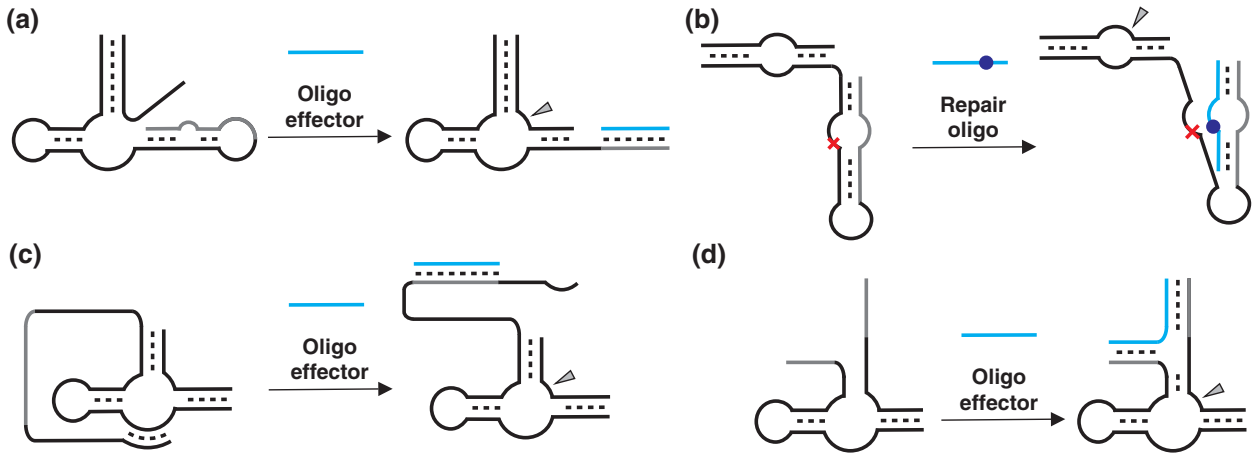

FIGURE 4 | Common designs for oligonucleotide-dependent ribozymes. (a) In the inhibitor control strategy the effector oligonucleotide binds to the sensor domain disrupting an inhibitory structure and increasing the activity of the ribozyme. (b) In the repair strategy a repair RNA strand displaces a nonfunctional intramolecular strand, thereby activating the ribozyme. (c) An alternative strategy consists in using a sensor strand that is not directly interacting with the catalytic domain, allowing the system to be adaptable to different target–effector sequences. (d) In the expansive regulation model, the activation of the catalytic activity is due to an enhancement of the stability of the enzyme–substrate complex exerted by the effector oligonucleotide.

Regarding the methods for obtaining oligonucleotide-dependent ribozymes, most of the devices were generated using a modular rational design, because in this type of ribozymes both the recognition of the effector and structural conformational exchanges are governed solely by well-understood Watson–Crick pairing rules. Penchovsky and Breaker reported a flexible computational strategy for rationally designing allosteric ribozyme constructs that exhibit robust allosteric activation or repression upon the addition of specific oligonucleotides.[92] However, a few examples of oligonucleotide-dependent ribozymes were developed by *in vitro* selection protocols. Robertson and Ellington selected an allosteric ribozyme ligase from a random sequence population that is activated by an oligonucleotide effector using a protocol similar to the one developed by Bartel and Szostak for the isolation of new ribozymes from a large pool of random sequences.[85,93] Komatsu et al. inserted a random sequence in a loop region of the hairpin ribozyme and performed multiple cycles of positive and negative selections until they obtained engineered hairpin ribozyme motifs that are catalytically active in the presence of an effector oligonucleotide.[94]

Applications of Oligonucleotide-Dependent Ribozymes In Vitro

An immediate application of oligonucleotide-dependent ribozymes is nucleic acid detection and quantification. For example, the oligonucleotide-dependent ribozyme of Taira and coworkers (termed maxizyme) was used *in vitro* to specifically detect the presence of the BCR-ABL mRNA whose translated product causes chronic myeloid leukemia.[84] The detection was performed through the direct visualization of radioactively labeled cleavage products of the maxizyme. In cultured cells transfected with the maxizyme only in leukemic cells presenting the BCR-ABL fusion gene showed apoptosis. In addition, a HHR that detects a specific sequence of the human telomerase mRNA was developed using an *in silico* selection approach.[95] In another example, an oligonucleotide-dependent hairpin ribozyme developed by Famulok and coworkers contains a sensor domain that is complementary to the trp leader mRNA, which binds the L-tryptophan-activated *trp* RNA-binding attenuation protein (TRAP). The ribozyme was used not only to detect the specific mRNA but also to measure the minimal tryptophan concentration required for TRAP activation *in vitro*. This was possible because it was shown that the activity of the ribozyme could be reverted by the TRAP/tryptophan-mediated sequestration of the *trp* leader mRNA.[88] The group of Famulok also engineered a hairpin ribozyme to detect microRNAs (miRNAs).[96] The advantage of using such a tool compared to *in situ* hybridization or molecular beacons is the possibility of signal amplification. The hairpin ribozyme cleaves a short RNA substrate that is labeled with a fluorophore/quencher pair, allowing detection in real time by FRET. The authors used a design that can be easily adapted to different effector oligonucleotides.[88] In addition to the application of detecting

oligonucleotides, DNA- and RNA-dependent ribozymes were shown to be suited for the purpose of molecular computing. Penchovsky and Breaker used their automated design method to generate oligonucleotide-responding AND, OR, NOT, and YES Boolean logic gates based on the HHR.[92] More recently, Penchovsky used oligonucleotide-dependent HHRs to implement complex circuits as a three-input AND logic gate, a two-input multiplexer, and a 1-to-2 decoder.[97]

Artificial Ligand-Dependent Ribozymes *In Vivo*

The driving force for the development of ligand-dependent ribozymes *in vivo* is the possibility of using such systems as RNA-based switches of gene expression. Such tools could be very helpful in biotechnology, diagnostics, and future therapy applications.

Aptazymes for Regulating Gene Expression In Vivo

Owing to their small size, robust folding, and high catalytic activity small self-cleaving ribozymes have proven as useful expression platforms for the development of artificial riboswitches. The utilization of aptazyme-based synthetic switches in biotechnology has many advantages including an increased robustness due to *in cis* regulation. In the case of *in trans*-based regulation systems, such as transcription factor control, the presence and the appropriate concentration of the *in trans*-regulating factor is important in order to assure the functionality of the genetic control mechanism. In addition, an RNA-only mechanism of controlling gene expression is often advantageous because regulatory proteins often used are potentially immunogenic and require large coding spaces. In addition, RNA-based systems are characterized by a high degree of modularity and are in many aspects programmable, making the adaptation of a given system to new applications potentially much easier than comparable protein-based mechanisms.[98] The first example of an artificial riboswitch was constructed by inserting a small-molecule aptamer into the 5′-UTR of an mRNA.[99] The artificial riboswitch was shown to repress translation upon ligand addition in cell extract as well as in mammalian cells, presumably by blocking either scanning or the ribosome–mRNA interaction. Interestingly, this artificial system preceded the discovery of riboswitches as a widespread mechanism for gene expression control in nature.

The first example of an artificial riboswitch based on a ribozyme was introduced by Ellington and coworkers in 2002 (see below).[100] Subsequently, research in this field has mostly focused on two aspects: (1) the development of methods for the generation of aptazymes functional *in vivo* and (2) the development of strategies and designs that allow utilizing the cleavage reaction catalyzed by aptazymes to control gene expression in different organisms. First, we will discuss methods introduced in order to obtain aptazymes that are functional with regard to controlling gene expression *in vivo*. Afterward we will describe main strategies in bacteria and eukaryotes for aptazyme-based control of gene expression.

Methods for Generating Aptazymes for In Vivo *Control of Gene Expression*

Early examples of aptazyme-based riboswitches were realized using a rational design approach.[100,101] The first example was presented by Ellington and coworkers in 2002 utilizing a group I intron. They obtained a ligand-dependent version by inserting a theophylline aptamer into stems P5 and P6, thereby making the splicing reaction dependent to the addition of the caffeine derivative.[100] Instead of a rational design, an *in vitro* selection approach was used by the group of Suess to generate tetracycline-dependent aptazymes that were shown to be active in yeast.[102] As discussed previously, modular rational design, *in vitro* selection, and mixed approaches revealed to be a rapid and efficient method to generate new aptazymes, but only few examples were shown to be active when expressed and characterized *in vivo*. It was observed that even very pronounced ligand-induced changes of cleavage rates did not necessarily exhibit activity in reporter gene assays *in vivo*.[100,103] This can be due to the fact that the *in vitro* conditions do not accurately reproduce the cellular environment and that *in vitro* and *in vivo* RNA folding pathways might be different.[104] The group of Keasling used mechanistic modeling and kinetic RNA folding simulations to engineer aptazyme-based RNA devices to control gene expression in *Escherichia coli*.[105] The results showed that the device behavior depends on the interaction of multiple factors in the cell. In particular, ribozyme folding, catalysis, and the RNA half-life had the largest effects on device performance. Predicted expression levels were in perfect agreement with experimental results.

Alternative *in vivo* approaches (screening and selection) are more suited for applications in different model organisms and in particular for gene expression control purposes as they directly link the activity of the aptazyme to a measurable gene expression

output. In one *in vivo* screening the aptazyme is used as a regulator of the expression of a reporter gene whose levels can be easily and rapidly measured. A library of aptazyme motifs in which the sensor and the catalytic domains are connected via a randomized communication module is generated, and the different connection sequences are screened directly *in vivo* for differential expression of the reporter gene in the presence and in the absence of the ligand. Such an approach was first developed by our group in bacteria.[106] Here the aptazyme works as an artificial riboswitch using a design in which the ribozyme sequesters the RBS, enabling control of translation initiation. A library of aptazyme motifs was generated attaching the theophylline aptamer to a full-length HHR via a randomized communication module containing three randomized nucleotides. The library was transformed in *E. coli* and isolated clones were screened for differential expression of the reporter gene in the presence and in the absence of the ligand. The use of the eGFP as a reporter gene allows rapid screening of the clones using simple bulk fluorescence measurements of the cultures. *In vivo* screening approaches were used also for the development of aptazyme-based switches in mammalian cells.[107,108]

Many efforts have been made to increase the throughput of the *in vivo* methods to generate aptazymes suitable to be used as molecular switches. The first bottleneck is the efficiency of transformation (or transfection) of the combinatorial library into the host organism. Second, an increased size of the library requires efficient methods (fast, cheap, and easily handled) to screen all members of the library. *In vivo* selection methods that link gene expression levels to selectable phenotypes such as cell motility, viability, or fluorescence (via FACS) can be used to enrich the library with members that show the expected behavior. Such approaches were already successfully used for the development of artificial switches based exclusively on aptamers (i.e., without the employment of a catalytic RNA domain).[109–111]

Recently, we generated a neomycin-dependent aptazyme that is able to control gene expression in yeast using an *in vivo* selection approach.[112] Here the aptazyme controls the expression of the GAL4 transcription factor that, in the employed yeast strain, is a transcriptional activator of the chromosomally encoded genes *HIS3*, *URA3*, and *lacZ*. The positive selection results in the enrichment of cells producing high levels of GAL4 and is realized by adding to the culture 3-amino-1,2,4-triazol (3-AT) as an inhibitor of the *HIS3* gene product. The negative selection instead results in the enrichment of cells producing low levels of GAL4 and is performed by adding to the culture 5-fluoroooratic acid (5-FOA) that is converted into the toxic compound 5-fluorouracil by the product of the *URA3* gene. *lacZ* is finally used as a reporter gene for the quantification of GAL4 expression. The group of Smolke established an *in vivo* selection approach in yeast that integrates FACS and next-generation sequencing (NGS).[113] The library was first sorted to enrich for cells exhibiting a reduced fluorescence reporter expression. The cells were then grown in the absence and in the presence of the ligand and sorted by FACS into eight different bins according to the level of the reporter gene expression. The library members in each of the eight bins were recovered, barcoded, and sequenced by NGS. The activity of the aptazymes was finally computed from the NGS counts in each of the eight bins.

The preceding methods allow the construction of ligand-dependent ribozymes for controlling gene expression. In order to control gene expression *in vivo*, several design strategies have been proposed that take advantage of the catalytic activity of an aptazyme and its modulation by a ligand. In the following, we will discuss the variety of different approaches that make use of ribozymes as expression platforms in bacteria and eukaryotic organisms.

Aptazymes for Gene Regulation in Bacteria

In bacteria, naturally occurring ligand-dependent ribozymes are present and were shown to have important regulatory functions in response to metabolite levels.[19,39] An advantage of artificial riboswitches based on catalytic activity is the possibility to apply them in a modular fashion to diverse genetic contexts. A variety of designs were proposed that enable regulating gene expression in bacteria based on ligand-dependent cleavage or splicing activity. Examples for these will be discussed in the following. As already mentioned, the first artificial control of gene expression in bacteria by means of a ribozyme was performed by the group of Ellington.[100] Here a theophylline-dependent group I intron was inserted in an interrupted thymidylate synthase (*ts*) gene in place of the parental T4 self-splicing intron. When this gene was introduced into an *E. coli* strain that lacked a functional *ts* gene, the bacteria were able to grow on a medium lacking thymidine only when theophylline was present (Figure 5(a)). Theophylline was demonstrated to activate the group I intron self-splicing, generating a functional *ts* gene.

Ogawa and Maeda introduced for the first time an artificial theophylline riboswitch into the 5′-UTR based on a minimal HHR using a novel design in

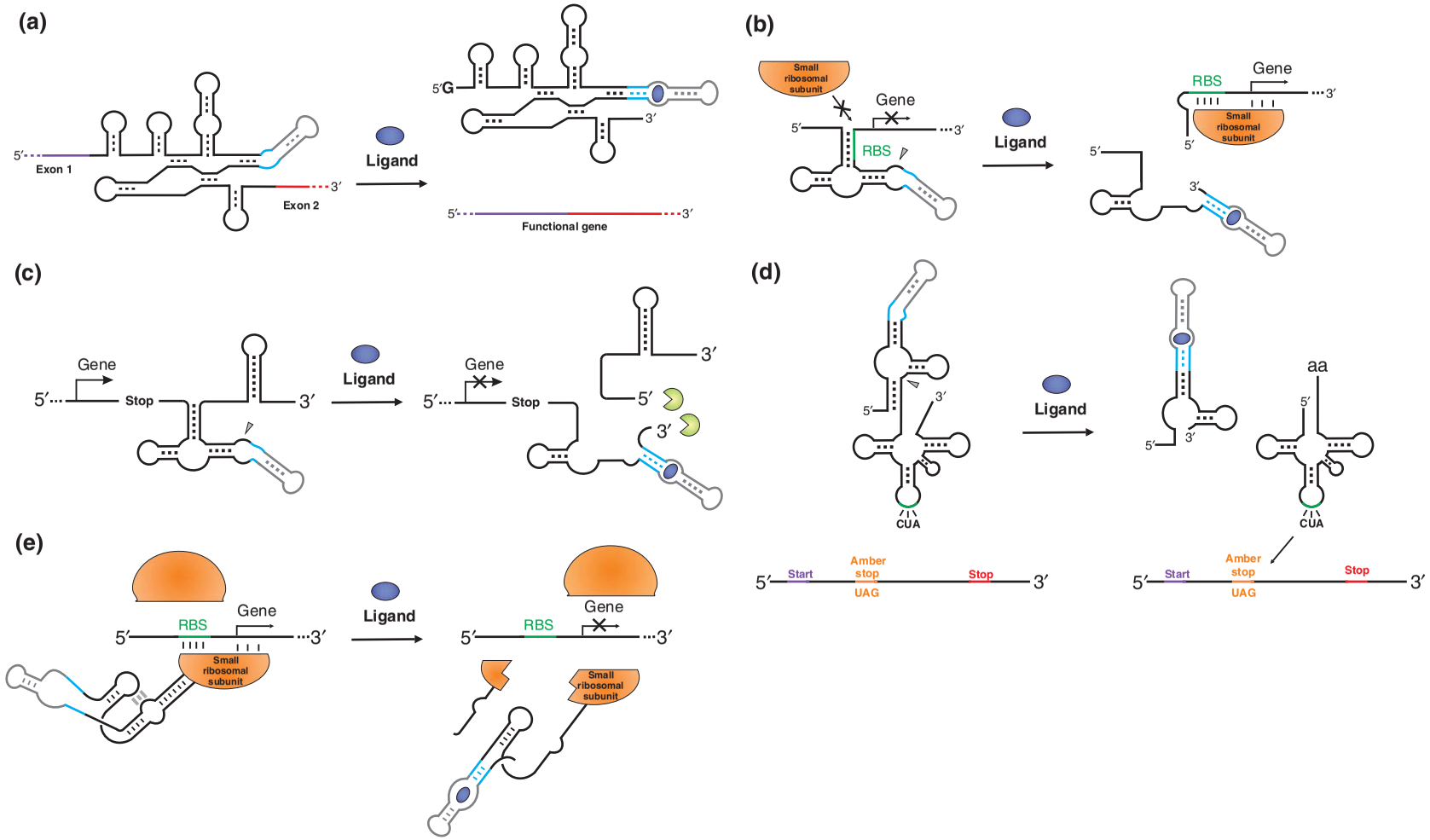

FIGURE 5 | Strategies for aptazyme-mediated gene regulation in bacteria. (a) In the first approach a ligand-dependent group I intron is inserted into an interrupted gene. The addition of the ligand results in correct splicing and in the formation of a functional gene. (b) In an alternative strategy, the ribozyme motif sequesters the ribosome-binding site (RBS), enabling control of translation initiation. (c) The ligand-dependent cleavage within the 3′-UTR using an aptazyme can be used to control gene expression in bacteria. This is probably due to the fact that secondary structures in the 3′-UTR of bacterial mRNAs were shown to have a protective function against RNase degradation. (d) Aptazyme-mediated control can be performed with tRNAs. When an amber stop codon is placed into the gene of interest, gene expression can be controlled via ligand-dependent activation of the amber suppressor tRNA. (e) Aptazymes can control in a ligand-dependent way the integrity of an orthogonalized 16S rRNA.

which the ribozyme motif sequesters the RBS, enabling control of translation initiation in bacteria[114,115] (Figure 5(b)). This artificial riboswitch, which mimics the mode of action of some naturally occurring riboswitches, was first shown to be active in bacterial cell extracts[114] and later *in vivo* using an efficient cascading system.[115] A similar strategy was used by our group based on a fast-cleaving HHR in order to develop theophylline[106,116] and thiamine pyrophosphate (TPP)-dependent[117] artificial riboswitches through *in vivo* screening of suited communication modules. In addition to insertion of such systems into the 5′-UTR of bacteria, aptazymes can be used to cleave within the 3′-UTR in a ligand-dependent manner.[118] Although pronounced changes of gene expression were observable, a detailed explanation of the mechanism of regulation necessitates further investigation. Because it is known that extended base-paired regions in the 3′-UTR (e.g., terminator stems) protect mRNAs from degradation, a ribozyme-mediated removal of such mRNA-stabilizing features would explain the observed gene expression changes[119] (Figure 5(c)).

An advantage of ligand-dependent ribozymes is their highly modular application to diverse genetic processes. For example, it was demonstrated that genetic control is not only limited to controlling gene expression via insertion into mRNAs but tRNA function can be regulated as well. This strategy consists of coupling the aptazyme with a suppressor tRNA. In this approach, the binding of the ligand induces the release and subsequent processing and aminoacylation of the tRNA. When an amber codon is placed into the gene of interest, expression can be controlled via ligand-dependent activation of the amber suppressor tRNA (Figure 5(d)). This approach was first demonstrated in a cell-free extract system[120] and subsequently realized *in vivo*.[121] Utilizing two different amber suppressor tRNAs under control of two distinct ligand-dependent HHR ribozymes, we were able to apply the principle of tRNA control to regulating posttranscriptionally the identity of the amino acid incorporated in protein synthesis.[122] In an extension, we combined the ribozyme-based control of mRNA and tRNA functionality in order to perform Boolean logics computation in *E. coli*: two different HHR switches were used to control independently an amber suppressor tRNA and translation initiation via RBS masking.[123] This allowed us to obtain AND, NOR, and ANDNOT logic gates. In addition to controlling mRNA and tRNA utilization, we achieved control over the integrity of the 16S rRNA in *E. coli* by incorporating a TPP-dependent HHR in different positions in an orthogonalized 16S rRNA[124] (Figure 5(e)). TPP-dependent activation of HHR cleavage activity resulted in the degradation of the small ribosomal subunit.

Ligand-Dependent Ribozymes for Gene Regulation in Eukaryotes

Although ribozymes appear to be widespread in genomes of eukaryotic organisms,[14,51] so far no example of a naturally occurring allosteric ligand-dependent ribozyme is known in this domain. However, a few examples of riboswitches that operate without ribozyme activity have been identified in plants and fungi.[125] In 2004, Mulligan and coworkers have introduced HHRs at different locations into a mammalian mRNA, demonstrating that the ribozyme is able to efficiently cleave and hence shut down the expression of the respective gene.[49] Efficient suppression of the gene expression was achieved by inserting the ribozyme at different positions in the 5′-UTR and 3′-UTR as well as in intronic regions. Although the introduced catalytic motifs were extended forms of the HHR and not ligand-binding aptazymes, gene expression was restored by addition of effectors that inhibit the HHR activity. Impressively, this approach was demonstrated to allow switching of gene expression in mice by adeno-associated virus (AAV)-mediated delivery of reporter genes under control of the HHR. In the Apicomplexa organisms *Toxoplasma gondii* and *Plasmodium falciparum* similar results were also obtained.[126] Moreover, the principle of inhibition of the HHR catalytic activity by toyocamycin was utilized for achieving photochemical gene regulation in mammalian cell culture with photo-caged derivatives of this nucleoside inhibitor.[127] However, the mechanism of HHR inhibition is based on the incorporation of toyocamycin into RNA and not on the specific recognition of the inhibitor by a sensor domain. Because the incorporation of nucleoside analogs into all cellular RNA is likely to provoke strong side effects, more specific approaches are needed in order to develop this approach into therapeutically useful systems. However, the works provide early examples for ribozyme-based gene expression control in whole organisms.

In 2007 the group of Smolke generated a series of ligand-dependent HHR-based switches in yeast via rational design.[101] Theophylline- and tetracycline-dependent HHRs were inserted into the 3′-UTR of a reporter gene. Here the regulated cleavage reaction allows the removal of the poly(A)-tail, which in turn results in a destabilization of the mRNA (Figure 6(a)). Although the performances of the switches were rather modest, this work represents an extensive example of the use of the modular rational design

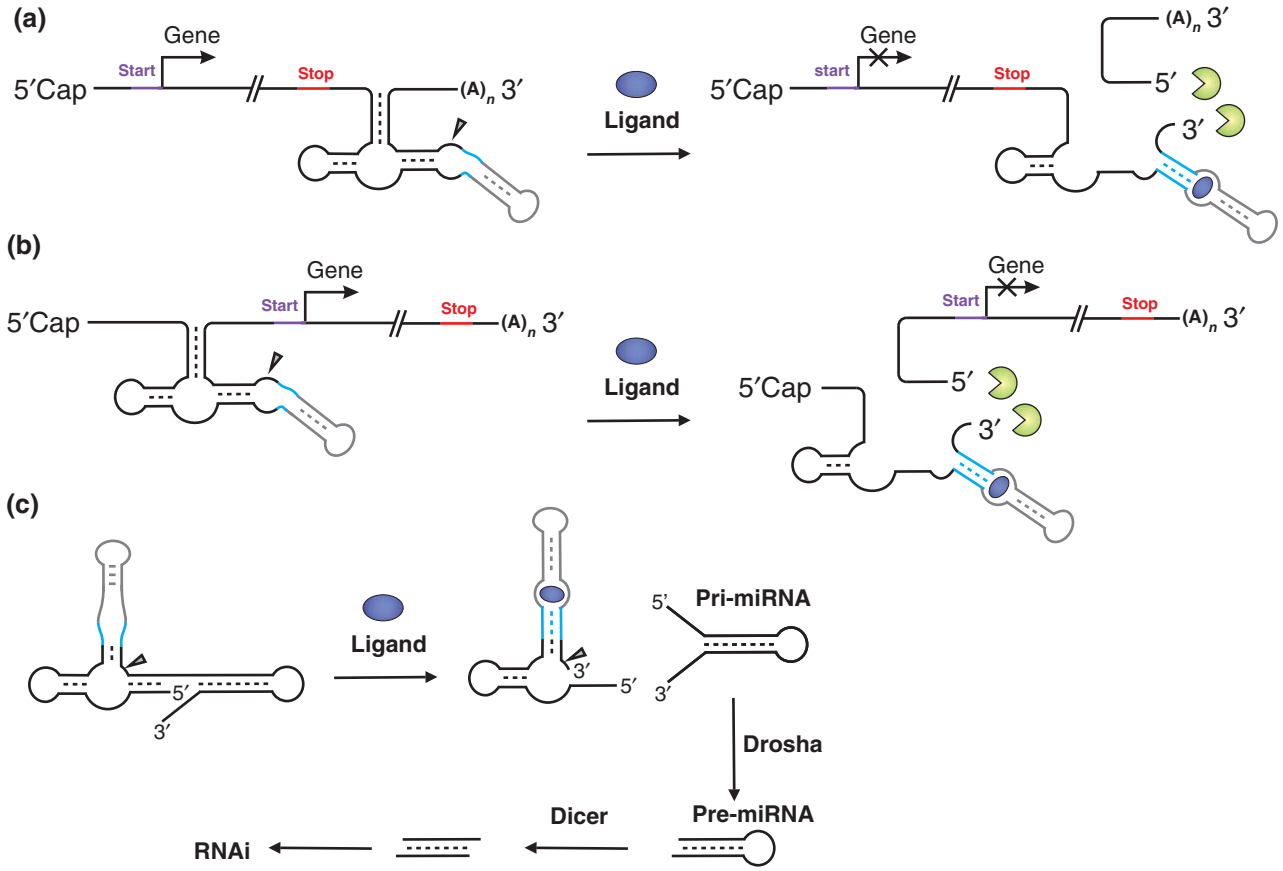

FIGURE 6 | Strategies for aptazyme-mediated gene regulation in eukaryotes. Allosteric control of gene expression can be achieved by inserting an aptazyme either (a) into the 3′-UTR or (b) into the 5′-UTR of the gene of interest. In the first strategy the regulated cleavage reaction results in removal of the poly(A)-tail, whereas in the 5′-UTR the 5′-cap is removed. In both cases mRNA is rapidly degraded upon ribozyme cleavage. (c) In an alternative strategy the ligand-induced cleavage releases a pri-miRNA species that is sequentially processed by Drosha and Dicer leading to RNA interference and conditional knockdown of gene expression.

approach for *in vivo* applications in eukaryotes. Artificial riboswitches in mammalian cells were obtained inserting a theophylline-dependent HHR in the 5′-UTR of a reporter gene (eGFP).[108] Here the ribozyme controls the stability of the mRNA via removal of the 5′-cap (Figure 6(b)). The communication modules were generated using both rational design and *in vivo* screening approaches. Although the insertion of the aptazyme in the 5′-UTR results to be effective to control gene expression, it requires the absence of start codons in the sequence of the RNA motif. For this reason, most of the artificial riboswitches in eukaryotes were developed inserting an engineered ribozyme (HHR or HDV) in the 3′-UTR of the gene of interest.[102,107,112,113,128–130] This strategy was applied to regulate T-cell proliferation in response to theophylline or tetracycline.[131] For this purpose, multiple copies of a ligand-dependent HHR were inserted into the 3′-UTR of the gene that encodes for the proliferative cytokine IL-2. The switches were shown to be active not only in mouse and human T-cell lines but also in mice. More recently, ligand-dependent ribozymes were used to arrest the cell cycle in U2-OS cells by controlling key regulators of the G0/1 or G2/M phases.[132] In another interesting application, ligand-dependent HHRs that can control mRNA stability were used to regulate the expression of an adenovirus and a measles virus structural gene, inhibiting viral genome replication and infectivity, respectively.[133,134] Such approaches could be used in the future for studying gene functions *in vivo* and for controlling oncolytic viruses for cancer therapies.

The first *in vivo* implementation of Boolean logic gates based on aptazymes that sense two small molecular inputs was reported by the group of Smolke in yeast.[128] Two-input logic gates were generated either by connecting two aptamer sensor domains (in series or in parallel) to a single HHR scaffold or by inserting two different one-input dependent HHRs into the 3′-UTR of the reporter

gene. However, the use of HHRs as a single scaffold for two-input riboswitches poses some restrictions due to a requirement of stem I/II tertiary interactions.[135] Utilizing the HDV ribozyme as expression platform the group of Yokobayashi generated a NOR logic gate in mammalian cells by inserting theophylline- and guanine-dependent HDV aptazymes into the 3′-UTR of the reporter gene.[107] The guanine switch was subsequently applied for optimizing transgene expression in an AAV construct.[136] An alternative strategy to control gene expression in mammalian cells consists in coupling a ligand-dependent HHR to a pri-miRNA sequence.[137,138] The ligand-induced cleavage of the aptazyme releases the pri-miRNA species that is sequentially processed by Drosha and Dicer resulting in RNA interference and conditional gene expression knockdown (Figure 6(c)).

Ligands Utilized in Ribozyme-Based Gene Expression Switches

In spite of the fact that different strategies for artificial gene expression regulation are available in bacteria, only three small-molecule ligands have been used until now in aptazyme setups (theophylline, 3-methylxanthine, and TPP).[100,106,115,117] Small-molecule ligands used so far for aptazyme-mediated gene regulation in eukaryotes are theophylline, guanine, tetracycline, and neomycin.[101,102,107,108,112,129,130] In addition to ligands of low molecular weight, protein-responsive ribozyme-based switches have been developed in eukaryotic organisms. A general design strategy for the development of such protein-responsive RNA devices that regulate mRNA stability was introduced recently by the Fussenegger group. It relies on a design that reengineers the critical tertiary stem I/II interactions in the HHR.[139] The switches were first selected in *E. coli* utilizing a FACS-based approach and subsequently adopted to a mammalian expression system.

Oligonucleotide-Dependent Ribozymes for Regulating Gene Expression In Vivo

To our knowledge, the use of oligonucleotide-dependent ribozymes *in vivo* is restricted to two examples. The group of Perreault inserted an oligonucleotide-dependent HDV ribozyme into the 5′-UTR of a reporter gene.[140] The oligonucleotide was able to activate the cleavage activity of the ribozyme resulting in a destabilization of the mRNA and lower gene expression in mammalian cells. Another example is an engineered HHR controlling translation initiation via RBS masking that is dependent on a small *trans*-acting RNA (taRNA) in *E. coli*.[141] In principle, these approaches are very interesting because they would allow monitoring and sensing mRNA levels in real time in the intact cell. In addition, with regard to synthetic biology, easy rewiring of expression networks should be possible. However, practical aspects such as a necessary excess of the effector oligonucleotide for efficient regulation as well as (at least in our case) restrictions regarding the taRNA sequence so far limit such approaches to the proof-of-concept stage.

CONCLUSION

To date, we are aware only of a few examples of naturally occurring ribozymes that are regulated via truly allosteric ligand interactions. However, a huge diversity of ligand-dependent natural and artificial ribozymes has been constructed by researchers throughout the last two decades. The many examples proved that RNA is a highly modular natural polymer. It is characterized by a hierarchical order that allows reassembling functional RNA domains from different origins into sequences with novel or extended properties. In many examples, relatively few optimization efforts are necessary in order to functionally link two RNA modules, often limited to regions that connect individual domains. With reference to aptazyme, the connection of the ligand-sensing aptamer domain with the catalytically active ribozyme domain is realized by optimizing communication modules linking both domains. Given the relative ease of engineering ligand dependency into catalytic RNA motifs, it seems very likely that in nature such systems have developed to a much larger extent than previously recognized. Hence, we should expect the discovery of further examples of ligand-controlled ribozymes in biological systems. The discovery of new naturally occurring ligand-dependent ribozymes will extend our knowledge about how functional RNAs take part in regulatory processes and, very likely, will broaden the already versatile toolbox for engineering RNA devices with novel and improved properties. In addition, new natural ligand-dependent ribozymes could represent potential therapeutic targets.[142]

The possibility of constructing ligand-dependent ribozymes is particularly interesting for future applications in biotechnology and medical technology. The use of a ligand-sensing domain enables external control of the system or (in combination with suited reporters) the detection and quantification of an analyte. The use of a self-cleaving ribozyme enables interference with genetic

processes when such devices are inserted into RNAs that participate in gene expression. The use in the control of gene expression in future therapeutic approaches is one of the most fascinating applications of RNA switches. For instance, they could be used for controlling transgene expression in diverse genetic contexts. However, in order to be broadly applicable for such purposes, additional important developments are necessary. Although artificial ligand-dependent ribozymes were proposed to be promising tools for advanced applications, until now mostly proof-of-concept studies were performed. Besides few examples, ligand-dependent ribozymes still need to demonstrate their applicability both *in vitro* and *in vivo*.

With respect to the use of ligand-dependent ribozymes for the construction of biosensors for environmental and diagnostic monitoring, the persistent presence of the functional RNA sensor at the site of interest is necessary and convenient but robust read-out systems need to be implemented. In addition, efficient ways of developing ribozymes that are regulated by the analytes that should be detected are necessary. Regarding the use of aptazymes as tools for the generation of artificial genetic switches *in vivo*, we have discussed that the spectrum of available ligands nowadays is rather limited. Many possible future uses will require additional ligand specificities with more favorable properties. In particular, for *in vivo* studies and therapeutic applications, ribozymes that respond to biocompatible ligands need to be engineered. In addition, it would be beneficial to develop ribozyme-based switches that respond to the presence of the effector ligand with either a more digital or a more progressive behavior. In conclusion, we have discussed that research on natural and artificial ligand-dependent catalytic RNA motifs is a very vibrant field with numerous exciting examples. We are confident that this field will continue to produce innovative tools for a diverse range of applications. Given that the major challenges mentioned above can be tackled, the use of ligand-dependent ribozymes in innovative biotechnological, diagnostic, and even therapeutic applications is envisioned.

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