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# Conditional Control of Mammalian Gene Expression by Tetracycline-Dependent Hammerhead Ribozymes

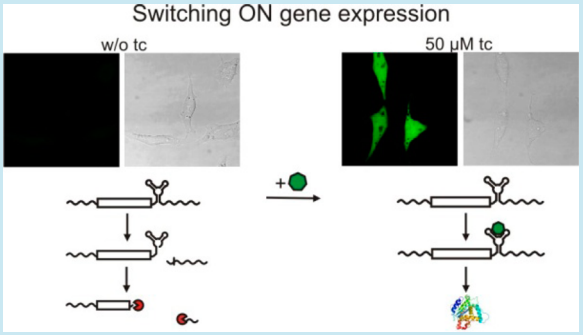
Kim Beilstein,[†] Alexander Wittmann,[†] Manuel Grez,[‡] and Beatrix Suess*,[†]

[†]Department of Biology, Technical University Darmstadt, Schnittspahnstr. 10, 64287 Darmstadt, Germany

[‡]Institute for Tumor Biology and Experimental Therapy, Georg−Speyer−Haus, Paul-Ehrlich-Str. 42-44, D-60596 Frankfurt, Germany

Supporting Information

**ABSTRACT:** Robust synthetic devices are requisite for the construction of synthetic genetic circuits and important scientific and technological tools to control cellular processes. We developed tetracycline-dependent ribozymes, which can switch on gene expression up to 8.7-fold upon addition of tetracycline. A tetracycline aptamer was grafted onto the hammerhead ribozyme in such a way that ligand binding to the aptamers destroys a loop−loop interaction within the ribozyme thereby inhibiting ribozyme cleavage and allowing gene expression. The advantage of the presented regulatory system is its independence of any regulatory proteins. The stable integration of the ribozyme into the genome of HeLa cells indicates a low background activity in the absence of ligand. Furthermore, the ligand concentration required to robustly flip the switch does not affect cell viability and therefore allows a long-term application of the system. These properties turn the tetracycline-dependent ribozymes into a very promising tool for conditional gene expression in mammalian cells.




**KEYWORDS:** *synthetic biology, engineered riboswitch, aptamer, tetracycline, ribozyme, HeLa cells*

Natural riboswitches have proven that direct interaction of intracellular metabolites with RNA sequences can influence gene expression through RNA self-cleavage.[1] The *glmS* riboswitch is located in the 5′ untranslated region (UTR) of the *glmS* gene whose gene product catalyzes the production of glucosamine-6-phosphate. At sufficient concentration of glucosamine-6-phosphate, the riboswitch cleaves itself, which leads to rapid mRNA degradation and ultimately lowers production of the enzyme.[2] Based on this mechanism, several synthetic approaches were undertaken to create conditional gene expression systems and provided proof-of-concept studies for ligand-mediated ribozyme cleavage via a coupled aptamer domain.[3−12] Many of these approaches resulted in ligand-responsive ribozymes that demonstrated activities *in vitro*[8] and in several model organisms.[3−7,10,11,13−15]

In bacteria, regulation was achieved by sequestration of the ribosomal binding site. The Shine−Dalgarno sequence was integrated into the fold of the self-cleaving hammerhead ribozyme in such a way that ribosomal access was not possible any more. Ribozyme cleavage releases the Shine−Dalgarno sequence; consequently, translation initiation can occur.[7] Grafting a theophylline aptamer to stem II of the hammerhead ribozyme via a randomized linker, followed by *in vivo* screening, resulted in ribozymes that triggered cleavage allosterically.[7] A functional switch was also obtained by exchanging the theophylline aptamer against the binding domain of the natural thiamine pyrophosphate (TPP) riboswitch, concomitantly demonstrating the modularity of these switches.[11]

The mechanism differs for application of allosteric ribozymes in eukaryotic systems. Here, the ribozymes are embedded into 5′ or 3′ untranslated regions of mRNAs, and self-cleavage leads to degradation of the mRNA and the inhibition of gene expression.[16] The addition of the theophylline or the tetracycline aptamer to the hammerhead ribozyme but also to the HCV ribozyme resulted in ligand-dependent modulation of reporter gene expression both in yeast[4,9] and mammalian systems.[3,13,14] One has to take care that the insertion of the ribozyme into the 5′ UTR often influence gene expression already in the absence of a ligand due to sterical hindrance of the RNA structure.[4] Therefore, the most applications target the 3′ UTR where no influence on gene expression level have been observed so far.

The advantages that such a ribozyme approach offers in the development of novel genetic control devices for diverse applications in synthetic biology are obvious: ligand-responsive ribozymes allow rapid control of gene expression by simple integration of the regulatory element within the UTR. Moreover, in contrast to well-established promoter based systems, like the Tet on/off system,[17] there is no need to coexpress additional factors such as regulatory proteins, which often represent a burden for the cell and can lead to unwanted

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immune responses.[18,19] Given that the 5′ and 3′ UTR sequences normally contain no or only a few sequence constrains, it should be straightforward to integrate regulatory elements into any UTR. While this seems to be the case if one wants to observe regulation at all, the regulatory parameters obtained are not always optimal, frequently due to high basal activity. In addition, the ligand concentrations needed to achieve regulation can be quite high, sometimes even reaching the limits of solubility and toxicity (reviewed in ref 20). Consequently, current research has to focus on improving these regulatory devices toward efficiency, improved regulatory parameters and more flexible applicability.

Here, we present the novel design of tetracycline-responsive ribozymes which allow ligand-dependent induction of gene expression in human cell lines. This ON-design is based on the disruption of a loop−loop interaction within the hammerhead ribozyme, which is essential for correct folding under cellular conditions.[21] The engineered ribozymes induce gene expression in a dose-dependent manner of the ligand, displaying at the same time remarkably little background activity, when stably integrated into the genome of HeLa cells. Furthermore, the ligand concentration required to switch ON gene expression does not affect cell viability and therefore allows a long-term application of the system. These properties turn the tetracycline-dependent ribozymes into a very promising tool for conditional gene expression in mammalian cells.

## RESULTS AND DISCUSSION

**Rational Design of Tetracycline-Dependent Hammerhead Ribozymes.** In this study, we engineered tetracycline-dependent hammerhead ribozymes to control gene expression in mammalian cells by combining the full length hammerhead ribozyme N79 from *Schistosoma mansoni*[16] with a tetracycline-binding aptamer.[22] We used this ribozyme as catalytic domain since it was successfully applied to repress gene expression in mammalian cells and mice.[16] We developed an ON-type strategy in which the hammerhead ribozyme leads to self-cleavage and subsequent mRNA degradation in the absence of tetracycline. Binding of the ligand to the aptamer domain then inhibits cleavage activity and mRNA translation can occur (Figure 1A).

The basic idea for the rational design was the fact that tertiary loop−loop interaction of the hammerhead ribozyme has been shown to be important for proper folding of the catalytic core of the ribozyme[21] and essential for cleavage activity under cellular magnesium concentration.[23,24] With this, we disrupted the internal loop of the hammerhead ribozyme domain by integrating three nucleotides (71U, 72A, 73C, encircled in Figure 1B) that are essential for loop−loop interaction in a new designed closing stem P1 of the aptamer domain. Thereby, P1 should be weakend enough to allow loop−loop interaction in the absence of the ligand resulting in self-cleavage activity. Ligand binding then stabilizes the closing stem;[25] hence, the three loop nucleotides are sequestered within P1 and are no longer available for loop−loop interaction. In this way, we designed several ribozyme constructs that differ in sequence, length, and stability of P1 (K3−K20, Figure 2A). Note, the strategy we used slightly differed from a design of a previous study in which we selected for tetracycline-dependent OFF switches (Supporting Information Figure S1,[4]). However, we were not able to isolate any candidates with ON switch function using the old design (data not shown).

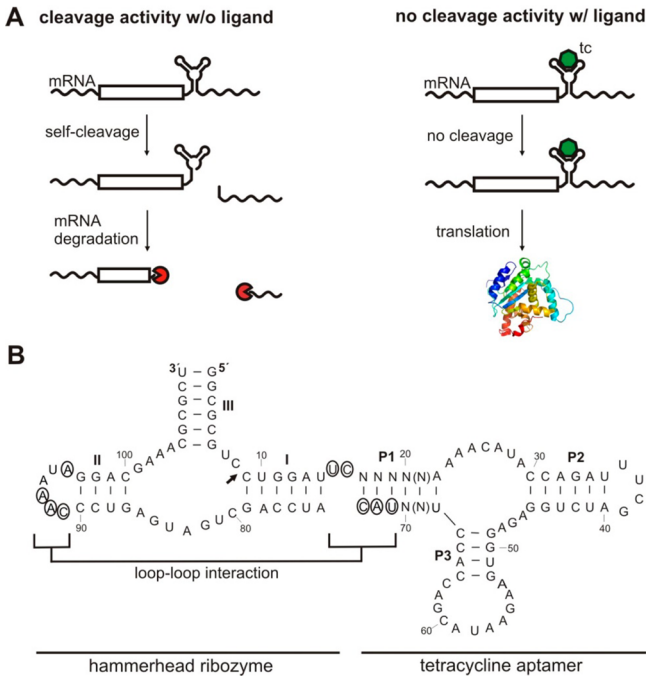


**Figure 1.** Mechanism and structure of tetracycline-dependent ribozyme regulation. (A) Integration of a ribozyme into the 3′ UTR of a mammalian mRNA leads to tetracycline-dependent regulation of gene expression. In the absence of the ligand, self-cleavage of the ribozyme occurs, leading to rapid mRNA degradation. Binding of tetracycline to the aptamer domain triggers a conformational change that results in the loss of the cleavage activity. As a consequence, translation takes place. (B) Nucleotide sequence of the ribozyme design used in this study. The three stems of the hammerhead ribozyme are labeled with I, II, and III. The cleavage site is indicated as an arrow. The three stems of the tetracycline aptamer are labeled P1, P2, and P3. A part of the internal loop of the hammerhead ribozymes is integrated into a new closing stem P1 of the aptamer. Nucleotides important for the loop−loop interaction of the ribozyme are encircled.

We performed reporter gene assays in HeLa cells to evaluate the regulatory potential of the different constructs and, therefore, inserted the ribozyme constructs into the 3′ UTR of a *Renilla* luciferase gene (hRluc) encoded by the expression vector psiCHECK-2 (Figure 2B). The insertion site was 19 nt behind the stop codon. The ribozymes were flanked by insulator sequences ($CAAA_3$ spacer) on both sides to prevent local missfolding. As controls, we used both a catalytically active (hh) and an inactive (hhi) variant of the hammerhead ribozyme (see Supporting Information Figure S2 and ref 4) and the expression vector without any ribozyme insertion (psi). Transfected HeLa cells were incubated for 2 days in the absence and presence of 50 $\mu$M tetracycline before luminescence was measured. The values were normalized against the activity of a firefly luciferase that was encoded on the same vector. The data of the luciferase assay are summarized in Table 1.

We identified four out of 17 ribozymes tested that mediate tetracycline-inducible hRluc expression with a dynamic range larger than 3-fold (indicated in bold, in Table 1, Figure 2C). Self-cleavage activity of the hh strongly reduced hRluc expression, an insertion of the inactive variant hhi resulted in reporter gene activity similar to the control vector without ribozyme (psi, Figure 2C). Interestingly, the four inducible ribozymes contain solely Watson−Crick or wobble base pairs.


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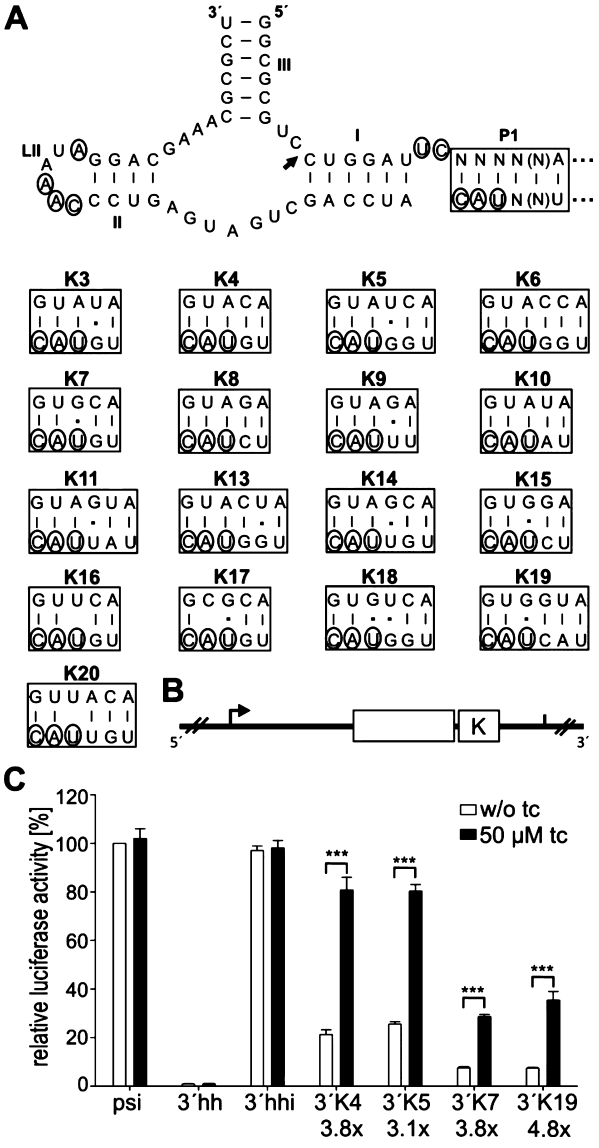


**Figure 2.** Rational design of tetracycline-dependent ribozymes. (A) The sequences of the closing stem P1 of different constructs are given. Encircled nucleotides are important for loop−loop interaction of the ribozyme. (B) Schematic representation of the vector used for the reporter gene assay. *Renilla* luciferase (hRluc, white box) is expressed under the control of a SV40 promoter (angulate arrow). The different ribozyme constructs are inserted in the 3′ UTR of the hRluc gene (white box, labeled with K). (C) Analysis of luciferase activities of transfected HeLa cells after 48 h incubation in the absence (w/o tc, white) and presence of 50 $\mu$M tetracycline (50 $\mu$M tc, black). Catalytically active (hh) and inactive (hhi) variants of the hammerhead ribozyme were measured as control. The dynamic range of regulation is depicted as *x*-fold below the bars. Shown are mean values + SEM of three independent measurements, each performed in triplicates. Statistically significance is indicated as *** for $p < 0.001$.

**Table 1. Luciferase Reporter Gene Assay**

| | relative luciferase activity [%][a] | | | |
|---|---|---|---|---|
| construct | w/o tc[b] | 50 $\mu$M tc | significance | dynamic range[c] |
| psi | 100.0 ± 0.0 | 102.0 ± 4.0 | ns | 1.0 |
| hh | 0.8 ± 0.1 | 0.9 ± 0.2 | ns | 1.1 |
| hhi | 97.0 ± 1.8 | 98.1 ± 3.1 | ns | 1.0 |
| K3 | 5.4 ± 0.9 | 9.3 ± 1.3 | ns | 1.7 |
| **K4** | 21.2 ± 2.0 | 80.8 ± 5.2 | *** | **3.8** |
| **K5** | 25.5 ± 1.0 | 80.3 ± 2.7 | *** | **3.1** |
| K6 | 57.1 ± 0.9 | 86.9 ± 1.9 | *** | 1.5 |
| **K7** | 7.5 ± 0.5 | 28.7 ± 0.9 | *** | **3.8** |
| K8 | 27.6 ± 0.8 | 65.5 ± 5.8 | *** | 2.4 |
| K9 | 27.7 ± 1.3 | 37.3 ± 2.9 | ns | 1.3 |
| K10 | 32.0 ± 0.2 | 79.5 ± 5.1 | *** | 2.5 |
| K11 | 17.2 ± 1.1 | 39.1 ± 1.2 | *** | 2.3 |
| K13 | 24.0 ± 0.8 | 59.4 ± 4.1 | *** | 2.5 |
| K14 | 75.4 ± 1.1 | 131.2 ± 3.4 | *** | 1.7 |
| K15 | 26.5 ± 0.6 | 37.0 ± 1.5 | ns | 1.4 |
| K16 | 4.1 ± 0.2 | 4.1 ± 0.5 | ns | 1.0 |
| K17 | 12.7 ± 2.0 | 13.3 ± 0.6 | ns | 1.1 |
| K18 | 10.2 ± 1.4 | 20.6 ± 0.5 | ns | 2.0 |
| **K19** | 7.4 ± 0.3 | 35.4 ± 3.5 | *** | **4.8** |
| K20 | 30.1 ± 4.3 | 32.5 ± 4.0 | ns | 1.1 |

[a]Shown are mean values ± SEM of three independent experiments measured in triplicates. [b]tc = tetracycline. [c]Quotient of the luciferase activity with 50 $\mu$M tc divided by the value w/o ligand. ns = not significant, *** = $p < 0.001$.

The five base pair long stem P1 of construct K4 differs from K7 in only one nucleotide at position 3 (K4, AU; K7, GU), which leads to a lower expression level but retains the same dynamic range. Constructs K5 and K19 carry one additional Watson−Crick base pair compared to K4 and K7, respectively. Thereby, construct K5 has an AU base pair at position 3, similar to K4, and both show similar expression levels. The same is true for constructs K7 and K19, which carry a GU wobble base pair at the third position that might be responsible for the low level of background expression (Figure 2C).

Five further constructs (K8, K10, K11, K13, and K18) showed a dynamic range between 2.0- and 2.5-fold. The remaining ones have no or only marginal regulation (Table 1), despite the fact that some of them carry AU or a GU base pair at the third position (Figure 2A). Interestingly, constructs that carry UU at position 3 (K16 and K20) or CA at position 2 (K17) did not show any tetracycline response, which indicates the need of base pairing at these positions (Figure 2A and Table 1). We further analyzed if the stability of the stem P1 correlated with the observed differences in regulation, but neither the calculated free energy of stem P1 or the whole ribozyme (Supporting Information Table S1) nor the base pairing pattern shed any light on the switching characteristics of the constructs. It indicates that every exchange of a nucleotide may influence the dynamic range idiosyncratically. As a consequence, it is hard to derive general rules for constructing linker sequences via rational design. A similar observation was made by the group of Yokobayashi, when they tried to figure out the relation between switching function of their guanine-responsive HDV ribozyme, its sequence, and its folding energies.[14] Nevertheless, our approach resulted in tetracycline-dependent ribozymes that permit ligand-dependent induction of gene expression in human cells.

**Tetracycline Ribozymes Show Dose-Dependent Regulation.** With regard to biological or biomedical applications, we characterized the dose-dependency of the tetracycline-inducible gene regulation system. Therefore, HeLa cells were transfected with the vectors harboring the ribozyme constructs K4, K5, K7, or K19 and incubated with increasing concentrations of tetracycline. K8 or K18 were also included in the analysis as their sequences are similar to the others (K8 compared to K4, K7; K18 compared to K5, K19) but exhibit


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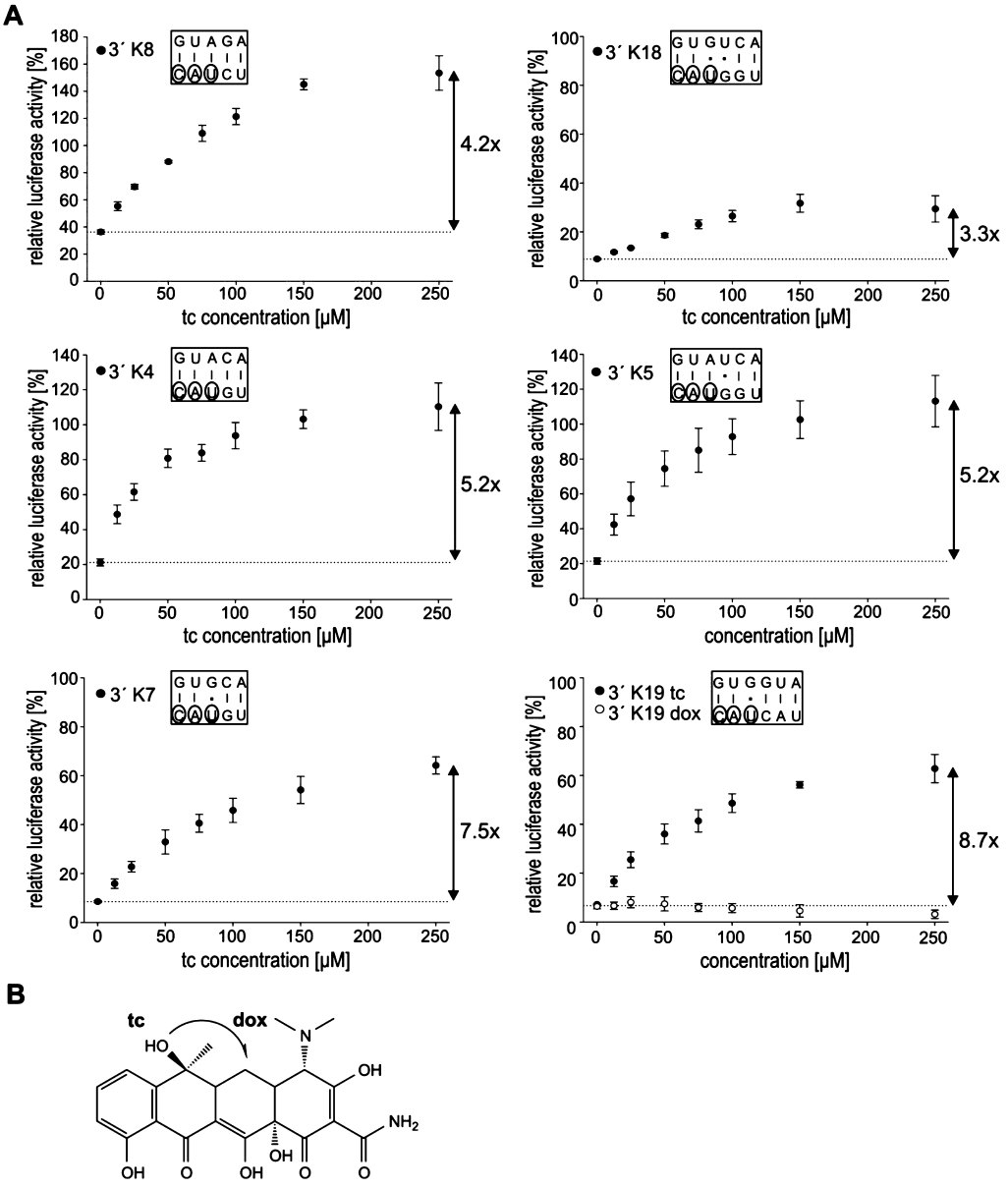


**Figure 3.** Dose-dependency of different tetracycline-dependent ribozymes. (A) Dose-dependent regulation of the indicated constructs by tetracycline (tc, black dots) or doxycycline (dox, white dots, for K19). The thin dotted lines show the activity of each reporter construct in the absence of any ligand. The dynamic range of regulation in the presence of 250 $\mu$M tc is shown on the right-hand side of each graph. *Renilla* luciferase activities are normalized to the corresponding activity measured for firefly luciferase located on the same plasmid. All values refer to psiCHECK-2 (psi) w/o tc. Shown are mean values ± SEM of three independent measurements, each performed in triplicates. Sequence of each stem P1 is indicated in the boxes. (B) Structures of tetracycline and the derivate doxycycline.

less regulation (Table 1). Interestingly, the base pair exchange from GC to CG (K8 to K4) led to a decrease of basal activity which resulted in an increase of the dynamic range from 4.2- to 5.2-fold (Figure 3A). An additional exchange from AU to GU (K4 to K7) led to a further reduction of background expression, again accompanied by an increase of the dynamic range to 7.5-fold (Figure 3A). The AU to GU exchange of the 6 bp-stem constructs K5 and K18 also reduced background activity; however, this time accompanied by a loss of regulation (maybe due to the two neighboring GU base pairs). The stabilization of one of these base pairs (in K19, UG in GC at position 4) maintained the low background activity but a considerable enhanced dynamic range of 8.7-fold resulting in the most active candidate (Figure 3A). It is conspicuous that a wobble base pair at position 3 or 4 of stem P1 mediates a low background expression, in many cases associated with good regulatory function of the ribozymes. However, it seems that the individual combination of base pairs next to each other makes the determinant rule for the design of functional linker sequences.

Despite the differences discussed above, all ribozymes show tetracycline-dependent increase of hRluc expression with a similar response curve (Figure 3A). The constructs respond to the same concentration of tetracycline with a plateau of induction reached at around 250 $\mu$M, a concentration where cell viability is still not affected by tetracycline (Supporting Information Figure S3). It indicates that binding of the ligand to the aptamer domain is not responsible for the observed differences in regulation. The addition of doxycycline, a derivative that is less-well recognized by the aptamer by more


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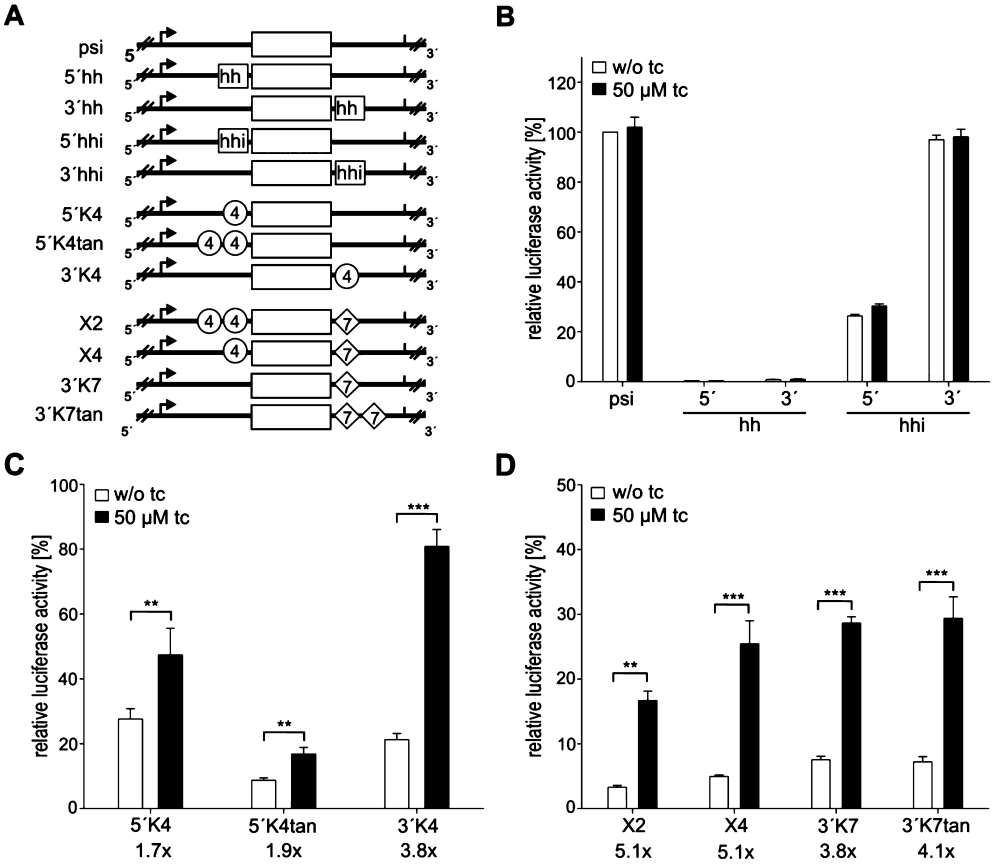


**Figure 4.** Efficiency of riboswitch regulation depends on position and copy number. (A) Schematic diagram of reporter constructs used in this experiment (white box = *hRluc* gene, angulate arrow = promoter, small box labeled with hh and hhi, respectively = active and inactive ribozyme, white circle labeled with 4 = ribozyme construct K4, white diamond labeled with 7 = ribozyme construct K7. (B−D) Cells were transfected with psiCHECK-2 (psi) and derivatives containing control constructs (B) or different ribozyme constructs in the 5′ or 3′ UTR of *Renilla* luciferase (C−D). Cells were incubated for 48 h in the absence (white bars) or presence (black bars) of 50 $\mu$M tc. Shown is the relative reporter activity of the reporter constructs indicated. The dynamic range of regulation is presented as *x*-fold below the bars. Shown are mean values +SEM of three independent experiments, each performed in triplicates. Significances are indicated as ** and *** for $p < 0.01$ and $p < 0.001$.

than 2 orders of magnitude[26] was used as negative control and did not show any effect on hRluc activity (Figure 3A, K19).

To approve that the observed regulation is indeed mediated by tetracycline-dependent inactivation of the hammerhead cleavage, we performed an *in vitro* cleavage assay (Supporting Information Figure S4). In the absence of the ligand, a cleavage rate of 1.1 $min^{-1} \pm 0.1$ was observed which is comparable to the wild type hammerhead ribozyme from *S. mansoni* (0.84 $min^{-1}$).[16] In the presence of 1 $\mu$M tetracycline, the cleavage rate was 4.6-fold diminished (0.24 $min^{-1} \pm 0.03$). This dynamic range is similar to the Theo P1−F5 ribozyme, which also shows ligand-dependent gene regulation in mammalian cells.[3] In addition, the quantification of the mRNA level of the *Renilla* luciferase was in line with the *in vitro* cleavage kinetics. qRT PCR data show a tetracycline-dependent increase of hRluc mRNA for all tested ribozyme (Supporting Information Figure S5), which provides evidence for *in vivo* cleavage mediated by the ribozyme.

**Dependence of Ribozyme Regulation on Position and Copy Number.** We tried to improve the dynamic range of regulation by inserting multiple copies of the hammerhead ribozyme. Thereby, we inserted them not only into the 3′ but also into the 5′ UTR proximal to the start codon (Figure 4A). First, we analyzed the influence of the control ribozymes (catalytically active and inactive without the aptamer domain) on gene expression. The catalytically active hammerhead ribozyme (hh) resulted in a very low hRluc expression for both the 5′ and 3′ UTR insertion (Figure 4B). The insertion of the inactive hammerhead ribozyme (hhi) has no influence on gene expression when inserted into 3′ UTR but resulted in a decrease up to 30% for the 5′ UTR insertion (Figure 4B). Already 1985, Pelletier et al. showed that structured RNA elements reduce translation efficiency when inserted in the 5′ UTR in eukaryotes[27] and 1989, Kozak figured out that inserting a base paired structure into the 5′ UTR between the 5′cap and AUG interrupts scanning of the 40S ribosomal subunit.[28] More recent data discriminate with regard to the 5′ insertion;[3,4] hence, it remains unclear to which extent 5′ insertion led to structure-dependent translation inhibition.

We designed combinations in which ribozyme constructs K4 and K7 were inserted as single copy or in tandem into the 5′ and/or 3′ UTR (Figure 4A). The data of the luciferase assay are shown in Figure 4C and D. The integration of a ribozyme into the 5′ UTR (5′K4) resulted in worse regulation compared to the 3′ insertion, accompanied by an increase in basal expression. The insertion of a second ribozyme into the 5′ UTR (5′K4tan) only slightly improved the dynamic range compared to single integration (Figure 4C). The second ribozyme further decreased basal expression down to 10%. A similar observation was made for tandem integration of two


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ribozymes into the 3′-UTR (compare 3′K7 with 3′K7tan). This was kind of expected since other groups made similar experience, when they inserted ligand-dependent ribozymes as tandem constructs in the UTR of a reporter gene in yeast[5] and human cell lines.[13] There is only one report in which a ribozyme-based regulatory system was improved by linking multiple copies of the ribozyme in the 3′ UTR of *gfp*.[29] Note, the dynamic range only increased from 1.1-fold for single copy to 1.8-fold for 4 copies. These ribozyme studies are in contrast to a tetracycline riboswitch applied in yeast. Here, the 5′ UTR insertion of several copies remarkably increased the dynamic range of regulation (one copy, 8-fold; three copies, 37-fold).[30] However, in this system the aptamers act as a roadblock for the scanning ribosome in their ligand-bound form and do not mediate mRNA degradation.

In our study, we obtained a significant increase only with the combination of a 5′ and a 3′ ribozyme (compare 1.7- and 3.8-fold for the single insertion 5′K4 and 3′K7 with 5.1-fold of X4, Figure 4). Similar results were obtained with a theophylline-dependent ribozyme.[13] Here, the combination of a 5′ with a 3′ ribozyme resulted in an increase in the dynamic range. In contrast to our data, the combination of tandem ribozyme in the 5′ and single riboswitch in the 3′ UTR decreased regulation, even worse than any single ribozyme insertion. It remains an open question how and to what extent the cellular and the sequence context of the RNA element influences the regulatory behavior. A comprehensive analysis will be necessary to decipher the multitude of factors that influence ribozyme regulation. Such a study should include not only intrinsic factors such as the sequence environment, copy number, and spacing between the ribozymes, and their regulatory capacity but also cellular factors such as the cell type or the metabolic status of the cell. Nevertheless, our data demonstrate that a combination of regulatory elements is a way to improve the efficiency of conditional gene expression system.

**Stable Integration of the Ribozymes into the Genome of HeLa Cells.** For the development of a generally applicable control system, we examined if stable integration of the engineered ribozymes into the genome retains the gene regulatory function. For an easy read out we inserted the tetracycline-dependent ribozymes into the 3′ UTR of another reporter gene, *egfp*. Making use of the Flp-In system, we stably integrated the ribozyme-containing vectors into the HeLa genome. Selection for hygromycin B resistance was carried out to isolate positive clones, and integration was controlled by genomic PCR (Supporting Information Figure S6). In this manner, we generated different cell lines that stably express *egfp* controlled by either the ribozyme controls (hh and hhi) or the constructs K4 and K5. Focusing on the regulatory capability of the integrated ribozymes, cell lines were treated for 48 h with 50 $\mu$M tetracycline and *egfp* expression was detected by confocal fluorescence microscopy (Figure 5A). Both ribozymes K4 and K5 demonstrates induction of *egfp* expression in the presence of the ligand (Figure 5A). Interestingly, almost no fluorescence could be detected for the construct K4 in the absence of tetracycline, indicating a tightly controlled system.

The quantification of the fluorescence level approves tetracycline-dependent induction of *egfp* gene expression when ribozyme construct K4 is stably integrated into the 3′-UTR (Figure 5B). Indeed, the basal level measured for HF1-3/GFP-3′K4 is only 4-fold higher than fluorescence measured for 3′hh integration. This demonstrates an improvement of the OFF state compared to the transient situation, since luciferase

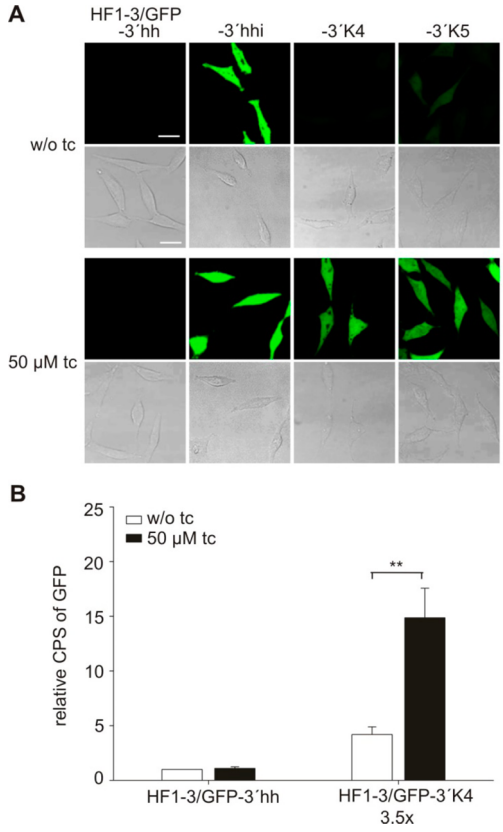


**Figure 5.** Stably integrated tetracycline-dependent ribozymes. (A) Cell lines HF1-3/GFP-3′hh, -3′hhi, -3′K4, and -3′K5 were cultivated for 48 h in the absence or presence of 50 $\mu$M tc. EGFP fluorescence was analyzed using a confocal laser scanning microscope. Transmission light was used to detect cells. A white scale bar indicates 25 $\mu$m. Shown are representative examples of $n$ = 3. (B) EGFP fluorescence measurements using fluorolog FL3-22. Shown is the relative EGFP fluorescence measured in counts per seconds at 509 nm. Cell lines HF1-3/GFP-3′hh and HF1-3/GFP-3′K4 were incubated for 48 h in the absence (white bars) and presence (black bars) of 50 $\mu$M tc. Values are normalized to control cell line HF1-3/GFP-3′hh without tc. The dynamic range of regulation by K4 is presented as $x$-fold induction below the bars and statistical significance is shown as ** for $p < 0.01$. Shown are mean values +SEM of three independent experiments, each performed in triplicates.

assay data showed a 25-fold higher background expression of 3′K4 compared to 3′hh (see Figures 2B and 5B). This may be caused by the fact, that just one copy of the *egfp*-ribozyme construct is stably integrated into the genome in contrast to presence of a high copy number per cell after transient transfection. Thus, the integration of ribozymes to the cell genome led to proven conditional gene regulation in a tetracycline-dependent manner. These data strongly improve the applicability of this gene regulation system.

**Conclusion.** Conditional control of gene expression by small molecule-dependent ribozymes has come a long way since its first inception *in vitro* with allosteric ribozymes.[8] Numerous "proof-of-principle" applications have been published since then establishing functional regulatory modules. The knowledge gained in all these experiments is currently used to design better tools that should finally allow generating highly efficient regulatory systems. The tetracycline-dependent ribozymes presented here allow to robustly flip the gene expression at a ligand concentration that does not affect cell


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viability and therefore allows a long-term application of the system. These properties turn this system into a very promising tool for conditional gene expression in mammalian cells.

## METHODS

**Vector Construction.** Luciferase measurements were performed using the bicistronic vector psiCHECK-2 (Promega), which encodes for a *Renilla* luciferase (hRluc) and a firefly luciferase gene. The ribozymes were inserted into the 3′ UTR, exactly 19 nucleotides downstream of the stop codon of hRluc using the restriction sites *Xho*I and *Not*I. The respective inserts were generated by primer overlap extension using primer pairs encoding the respective ribozyme sequence flanked by $CAAA_{(3)}$ spacers at both ends. All primers were purchased from Sigma-Aldrich, their sequences are listed in the Supporting Information Table S2. For cloning of the ribozymes into the 5′ UTR of hRluc, constructs were inserted 10 nucleotides upstream of the start codon using the restriction site for *Nhe*I. In addition, these ribozymes contain a point mutation in the hammerhead motif in order to lose a start codon (U84C, according to the ribozyme design shown in Figure 1B).

For the construction of the tandem ribozyme, we used psiCHECK-2_3′K7 as template for amplification of two ribozyme sequences flanked by either *Xho*I/*Bsi*WI or *Bsi*WI/*Not*I restriction sites. First, both ribozymes were ligated to a tandem construct using the restriction site for *Bsi*WI. Subsequently, this tandem was inserted into the 3′ UTR of hRluc using *Xho*I and *Not*I. The 5′ tandem construct was generated by the same method. The vector psiCHECK-2_5′K4 was used to amplify the ribozymes carrying restriction sites either *Nhe*I/*Bsi*WI or *Bsi*WI/*Nhe*I. For stable integration, we modified the commercially available expression vector pcDNA5/FRT (Invitrogen) by replacing the multiple cloning site with the *egfp* reporter gene (pFRT/KB, see Supporting Information S7). Ribozymes were amplified from the respective psiCHECK-2 template and further inserted into the 3′ UTR of *egfp* using restriction sites for *Sac*I and *Not*I.

**Cell Culture.** HeLa cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (PAA), 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin (following called medium) and incubated in a 5% $CO_2$ humidified atmosphere at 37 °C. In case of the HeLa "Flp-In Host Cell Line" (HF1-3), 150 μg/mL zeocin (invivogen) was added to the medium, whereas the medium of HeLa "Flp-In Reporter Cell Lines" (HF1-3/GFP clones) contained 200 μg/mL hygromycin B (invivogen). Cells were passaged every 3−4 days.

**Luciferase Assay.** One day before cell transfection with psiCHECK-2, HeLa cells were transferred to a 96-well plate (15 000 cells/well in 100 μL medium). According to the manufacturer's protocol, 0.5 μL Lipofectamine 2000 (Invitrogen) and 50 ng vector DNA per well was used for transfection of HeLa cells. After 2 h, transfection media was replaced by fresh medium supplemented with tetracycline (Sigma-Aldrich) or doxycycline (Sigma-Aldrich). The medium was again replaced 24 h later. Luminescence was measured 48 h post transfection using the Dual-Glo Luciferase Assay System according to the manufacturer's instructions (Promega). Luminescence was detected using an Infinite M200 Microplate Reader (Tecan). The ratio between *Renilla* and firefly luciferase activity was calculated for each well to normalize for transfection efficiency. Mean values and standard deviations were calculated from triplicates and normalized to the values of the vector psiCHECK-2 without ribozyme. Each experiment was repeated three times.

**Transient Transfection, RNA Isolation, cDNA Synthesis, Quantitative (q)RT PCR.** For qRT PCR experiments, $3 \times 10^5$ HeLa cells were seeded in 6-well plates and cultivated with 3 mL medium. According to manufacturer's protocol, 4 μL Lipofectamine 2000 (Invitrogen) and 2500 ng DNA per well was used for transfection. Two hours after transfection, transfection medium Opti-MEM was replaced by medium with or without 50 μM tetracycline. Another change of medium was performed 24 h later. Two days after transfection, cells were harvested for RNA extraction. Total RNA was extracted from the cells with TRIzol Reagent (Ambion) according to the manufacturer's protocol and Maxtract High Density tubes (Qiagen) were used for phase separation. RNA was resuspended in 33 μL Milli-Q and treated with 3 μL Turbo DNase (Ambion) for at least 30 min. Another purification step with 96% ethanol and 3 M NaAc pH 6.5 was performed, and purified RNA was resuspended in 40 μL Milli-Q. The RNA integrity was analyzed after DNase digest by agarose gel electrophoresis. RNA (900 ng total) was reverse transcribed using the High capacity RNA-to-cDNA Kit (Invitrogen) according to manufacturer's protocol. All qRT-PCR experiments were performed at a StepOne Plus device (Applied Biosystems) using Power Sybr Green PCR Master Mix (Applied Biosystems) according to manufacturer's protocol. Amplification of luciferase gene was used as internal control, since it is encoded at the same plasmid as hRluc which is controlled by different riboswitch constructs. Sequences of used oligonucleotides are listed in Supporting Information Table S2. Each experiment was performed in duplicates for three times.

***In Vitro* Transcription and Cleavage Kinetics.** *In vitro* transcription of hammerhead ribozyme construct K4 was performed in a 40 μL reaction in 50 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM spermidine, 5 mM DTT, 2.5 mM of each dNTP, 20 μCi [$\alpha$-$^{32}$P] UTP, and 10 μg T7-polymerase (homemade) at 37 °C for 90 min. Transcription was stopped with 40 μL LBE buffer (10 M urea, 1.5 mM EDTA). Transcribed RNA was purified by denaturating 6% PAGE (8 M urea) and eluted at 4 °C overnight in 10 mM Tris-HCl pH 7.5, 200 mM NaCl, and 1 mM EDTA, followed by ethanol precipitation and eluted in TE2 buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA). For *in vitro* cleavage, transcribed RNA was incubated in 50 mM Tris-HCl, pH 7.5 at 37 °C in the absence and presence of 1 μM tetracycline. Cleavage reaction was initiated with 0.55 mM $MgCl_2$ and stopped after several time points with LBE buffer. The amount of cleaved fractions was analyzed by denaturating 6% PAGE, detected by phosphor imaging and quantified via ImageQuant. The cleavage rate constants were analyzed by fitting data to $F_t = F_0 + F_\infty (1 - e^{-kt})$.[31]

**Generation of HeLa "Flp-In Reporter Cell Line".** To generate cell lines that stably express *egfp* under the control of riboswitch or ribozyme activity, each reporter vector based on a pFRT/KB backbone (Supporting Information Figure S7), was cotransfected with pOG44 (Invitrogen) in a 1:9 molar ratio into the "Flp-In Host Cell Line" HF1-3 using Lipofectamine 2000. The cell line HF1-3 was generated as described for the cell line HF1-1.[32] Both are independent sister cell lines that were obtained from the same selection. The integration site was described as independent from positional effects. The following selection steps are described in the manufacturer's protocol for the Flp-In system (Invitrogen). After 1−2 weeks of cultivation,


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single cell colonies were identified that were hygromycin B resistant but zeocin-sensitive, indicating stable integration of reporter vector by homologous recombination. These "Flp-In Reporter Cell Lines" were further characterized by fluorescence microscopy analysis.

**Microscopy Analysis.** For microscopy analysis, 50 000 cells were seeded on 18 mm coverslips in a 12-well plate and incubated in the absence and presence of tetracycline. After 48 h cells were washed twice with 1× PBS and fixed on microscope slides using 15 μL ProLong Gold Antifade Reagent (life technologies). After a cure time of 4−6 h in the dark at room temperature, samples were analyzed by fluorescence microscopy using a Leica SP5 confocal laser scanning microscope (Leica, Germany). EGFP was excited at 488 nm and emission recorded at 495−600 nm.

**Fluorescence Measurements.** For all measurements, 200 000 cells of stable cell lines HF1-3/GFP-3′hh and HF1-3/GFP-3′K4 were seeded per well on a 6-well plate with 3 mL medium without Phenol red but supplemented with 200 μg/mL hygromycin B. After 24 and 48 h medium was replaced by fresh hygromycin B containing medium supplemented without or with 50 μM tetracycline for treatment. 72 h after seeding, cells were harvested using trypsin-EDTA and washed with 5 mL 1× PBS via centrifugation (1300*g*, RT, 5 min). Cells were resuspended in 1× PBS to a concentration of 250 000 cells/mL. Each cell line was cultivated for each treatment in triplicates for three independent measurements.

Fluorescence measurements were carried out at room temperature on a Fluorolog FL3-22 (Horiba Jobin Yvon) with an excitation wavelength of 488 nm, Slit = 1 and an emission wavelength of 490−530 nm, Slit = 2, whereas the counts per second (CPS) measured at a wavelength of 509 nm were used for quantification analysis. 1× PBS was used as blank.

**Statistics.** GraphPad Prism 5.03 (GraphPad Sopftware) was used for statistical analysis via two-way ANOVA and Bonferroni posttest. Statistical significance was determined as $p < 0.05$, $p < 0.01$, and $p < 0.001$ and is indicated as *, **, and ***, respectively.

## ■ ASSOCIATED CONTENT

### Supporting Information

Previous design for *in vitro* selection; nucleotide sequence of the hammerhead ribozyme control; influence of tetracycline and doxycycline on cell viability; *in vitro* cleavage assay of $^{32}$P labeled ribozyme construct K4; quantification of ribozyme-controlled luciferase mRNA level; stable integration of ribozyme-controlled *egfp* into HF1-3 cell line; integration plasmid pFRT/KB; free energy of riboswitch constructs and stems P1; oligonucleotides used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Email: bsuess@bio.tu-darmstadt.de.

### Notes

The authors declare no competing financial interest.

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