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# Ribozyme-Based Aminoglycoside Switches of Gene Expression Engineered by Genetic Selection in *S. cerevisiae*

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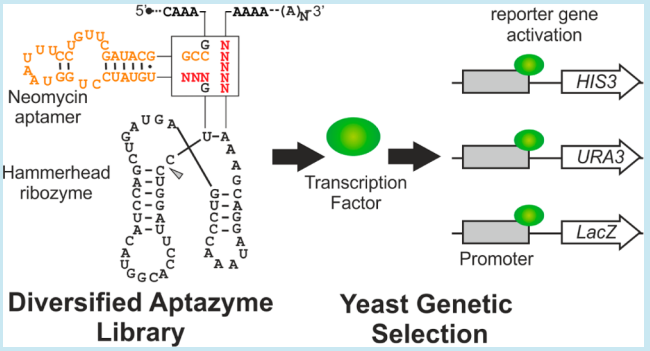
Supporting Information

**ABSTRACT:** Systems for conditional gene expression are powerful tools in basic research as well as in biotechnology. For future applications, it is of great importance to engineer orthogonal genetic switches that function reliably in diverse contexts. RNA-based switches have the advantage that effector molecules interact immediately with regulatory modules inserted into the target RNAs, getting rid of the need of transcription factors usually mediating genetic control. Artificial riboswitches are characterized by their simplicity and small size accompanied by a high degree of modularity. We have recently reported a series of hammerhead ribozyme-based artificial riboswitches that allow for post-transcriptional regulation of gene expression via switching mRNA, tRNA, or rRNA functions. A more widespread application was so far hampered by moderate switching performances and a limited set of effector molecules available. Here, we report the re-engineering of hammerhead ribozymes in order to respond efficiently to aminoglycoside antibiotics. We first established an *in vivo* selection protocol in *Saccharomyces cerevisiae* that enabled us to search large sequence spaces for optimized switches. We then envisioned and characterized a novel strategy of attaching the aptamer to the ribozyme catalytic core, increasing the design options for rendering the ribozyme ligand-dependent. These innovations enabled the development of neomycin-dependent RNA modules that switch gene expression up to 25-fold. The presented aminoglycoside-responsive riboswitches belong to the best-performing RNA-based genetic regulators reported so far. The developed *in vivo* selection protocol should allow for sampling of large sequence spaces for engineering of further optimized riboswitches.




**KEYWORDS:** *riboswitch, RNA switch, gene regulation, hammerhead ribozyme, neomycin, genetic selection*

In recent years, there has been rising interest in the creation of synthetic biological devices performing user defined tasks.[1] The functional complexity of artificial genetic networks emerges from the interplay of individual switches that can rapidly respond to environmental cues or cellular signals. Conditional gene expression systems have been created that target gene expression on the transcriptional and post-transcriptional level.[1,2] Most frequently, artificial systems are based on allosterically controlled transcription factors (TFs) that sequence-specifically bind to DNA regions in order to alter the rate of the transcription of a gene. Transcription factors are powerful protein-based regulators with important biotechnological and therapeutic applications.[3] However, TFs are hardly reprogrammable to new ligands and TFs demand massive genomic space for their encryption. In addition, the application of TFs is severely limited by the requirement for the genetic modification of two genetic sites, first the introduction of the TF itself and second the incorporation of the TF target site within the promoter of the gene of interest. Next, the TF needs to be expressed in well-defined amounts in order to fully exploit the gene-regulatory potential. This often results in the necessity to select for stable cell lines that express the TFs at appropriate levels, especially if the gene of interest is expressed and studied in eukaryotic organisms or cell cultures.[4] An additional shortcoming is related to the mentioned necessity of maintaining the TF at an optimal concentration: In expression systems that display fluctuating replication and gene expression such as in actively infecting and replicating viruses, TF-based control of gene expression often shows very limited performance.[5] This is due to changes in stoichiometry of TFs and their corresponding target sites. In these particular cases, artificial riboswitches are very well suited since the switch is physically linked to the protein-coding message and hence is not prone to failure due to the aforementioned changes in TF levels.[6,7]

Artificial riboswitches are an emerging class of genetic control elements that are small in size, respond rapidly to their ligand, and exhibit a modular and reprogrammable architec-

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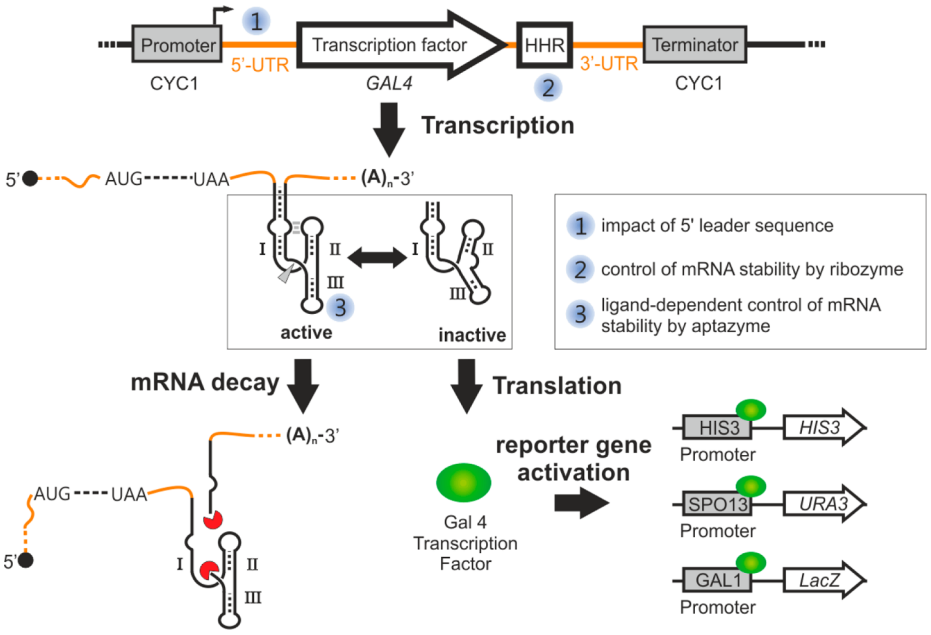


**Figure 1.** Schematic illustration of a yeast-based *in vivo* selection for the generation of synthetic ribozyme-based genetic switches. Transcription of Gal4 expression is driven by a Cyc1 promoter. Post-transcriptional control over Gal4 mRNA stability is obtained by the insertion of a hammerhead ribozyme into the 3′-UTR. Autocatalytic cleavage by the HHR results in degradation of the Gal4 mRNA and prevents expression of the transcription activator Gal4. In case of an inactive HHR, which does not cleave the RNA backbone, the Gal4 mRNA level remains unaffected and the Gal4 protein is highly expressed. The MaV203 strain encodes chromosomally encoded reporter genes *HIS3*, *URA3*, and *LacZ* each under control of a Gal4-inducible promoter. Critical parameters for the setup of the genetic system are the identity of the 5′-leader sequence and the catalytic activity of the HHR. A library of potential ligand-dependent HHRs is constructed by the fusion of an aptamer domain to the HHR via a randomized connection sequence. Potential ligand-dependent HHR-based switches are enriched by positive and negative selection based on the auxotrophy markers *HIS3* and *URA3*. *LacZ* enables the final identification of switches and is used for further characterization.

ture.[8−10] Importantly, the control of gene expression is mediated by an intramolecular (or in-cis) mechanism since the regulatory modules are part of the mRNA they control. They mimic naturally occurring riboswitches that typically exhibit a common architecture composed of an aptamer domain, which selectively binds a ligand, and an expression platform, which controls crucial genetic mechanisms such as mRNA transcription, translation, and in eukarya the splicing of pre-mRNAs.[11] For constructing artificial genetic switches, it is useful to strive for the utilization of well-understood, modular parts that can be reassembled. Natural riboswitches are perfect archetypes due to their high degree of modularity and (sometimes only apparent) simplicity. In our endeavors, we have frequently utilized the hammerhead ribozyme (HHR) as a highly modular expression platform for constructing artificial riboswitches. Ribozyme-based genetic switches are of particular interest because they can control the course of gene expression at different stages.[12−17] For example, embedding an allosteric ribozyme into either the 5′- or 3′-UTR of an mRNA constitutes a powerful tool for the conditional control of gene expression in prokaryotes and eukaryotes.[12,13,18] Eukaryotic mRNA stability is highly dependent on the presence of the 5′-cap and the 3′-poly(A)-tail. Cleaving off any of these two structures by the insertion of a HHR into either the 5′-UTR or 3′-UTR leads to an enhanced degradation of the mRNA.[19] Allosteric hammerhead ribozymes, also termed aptazymes, are engineered by the fusion of its catalytic core motif to an aptamer domain.[20] Maintenance of the tertiary interactions between stem 1 and 2 sustains efficient catalytic activity at cellular $Mg^{2+}$ concentrations.[21,22] Importantly, the modular architecture enables a simplified reprogramming of ligand selectivity.[23,24] Methods for the generation of aptamers are well established.[25] In principle, this should enable the creation of numerous allosteric ribozymes with selectivity for different small molecules. However, the development of allosteric ribozymes requires the screening of randomized libraries,[26] which is time-consuming and demands elaborate technical equipment. *In vitro*-selected allosteric ribozymes often lose their function when transferred into cellular systems.[27] An alternative and promising strategy is the generation of a randomized library, which is subsequently analyzed for ligand-dependent genetic switches by high-throughput methods within a cellular system.[9] Randomized libraries of riboswitches have been successfully screened with automated equipment[28] or fluorescence-activated cell sorting procedures.[29] In addition, *in vivo* selection schemes have been established based on a counter-selectable genetic marker in bacteria.[30] Instead of utilizing the survival, an elegant approach has used motility as selection marker for generating artificial riboswitches.[31]

Here, we present a novel *in vivo* selection approach in *S. cerevisiae* that allows for searching of large sequence spaces in a straightforward manner. Most switches reported so far display moderate responses of up to 10-fold changes of gene expression. Exogenously administered ligands (e.g., theophylline or tetracycline) require concentrations in the millimolar and hence almost toxic ranges for the induction of a cellular response, thereby limiting their application. With the *in vivo* selection protocol established in *S. cerevisiae*, we achieved the generation of aminoglycoside-responsive RNA switches that display robust and efficient (up to 25-fold) switching of gene expression. Aminoglycosides are perfectly suited ligands because they represent very potent RNA binders with well-


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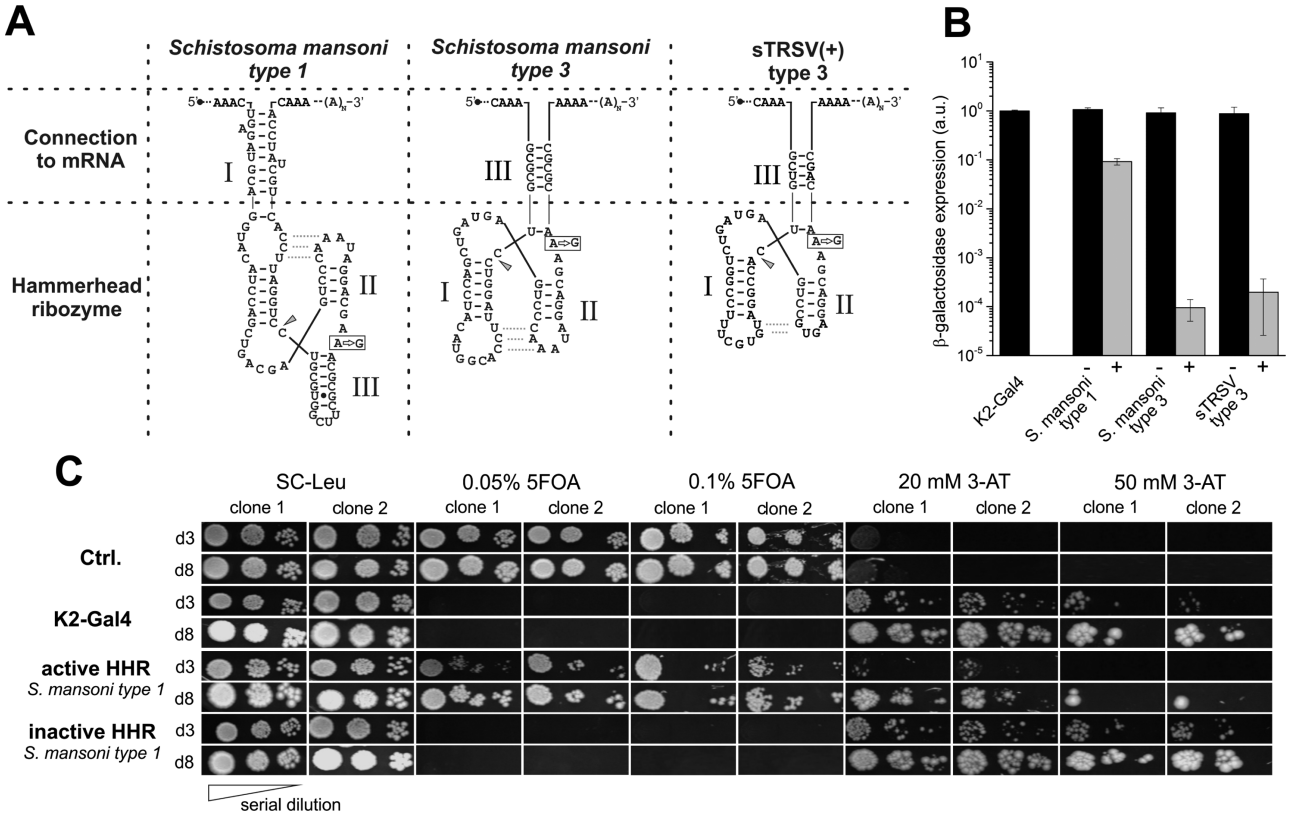


**Figure 2.** Post-transcriptional control of Gal4 expression by variants of the hammerhead ribozyme. (A) Variants of the HHRs derived from *S. mansoni* and the satellite RNA of the tobacco ringspot virus were inserted into the 3′-UTR of the transcription activator Gal4. Secondary structures of the investigated HHRs are shown. An A-to-G substitution within the catalytic core of the HHR results in a catalytically inactive HHR. The cleavage site is indicated by an arrowhead. (B) The impact of variants of the HHR on of Gal4 expression was analyzed by measuring *β*-galactosidase activity. For each HHR a catalytically inactive HHR (−) was compared to a catalytically active HHR (+). *S. cerevisiae* cultures of the *MaV203* strain were cultivated in synthetic complete medium at 30 °C and *β*-galactosidase expression of outgrown cultures was measured. Errors bars represent the standard deviation of experiments performed at least in triplicates. (C) Yeast strains expressing Gal4 protein under control of an active and inactive *S. mansoni* type 1 HHR were phenotypically assayed on selective media. Two independent clones were serially diluted and spotted on SC medium supplemented with either 3-AT or 5-FOA. Pictures of colonies were taken after 3 and 8 days.

characterized pharmacological and toxicological profiles.[32] In addition, we explored new concepts of generating allosteric ribozymes by establishing a novel way of connecting the aptamer and HHR domains, thereby increasing the design options for rendering the ribozyme ligand-dependent.

## RESULTS AND DISCUSSION

***In Vivo* Selection Method.** In this work, we aimed for the development of a reliable and straightforward technique that allows searching large sequence spaces for ribozyme-based genetic switches in *S. cerevisiae*. Our technique complements recent advances in the generation of genetic yeast-based systems for the *in vivo* study of biomolecular interactions.[33] For example, the yeast-two-hybrid system enables the analysis of protein−protein interactions, and variants of this technique enable the analysis of protein interactions with RNA, DNA, and small molecules. The power of these systems relies on the *in vivo* enrichment of cellular pools that express selection markers from libraries of up to $1 \times 10^9$ genetically diverse cells. In this work, we enlarge the toolbox of yeast-based selection systems for the analysis and generation of catalytic RNA motifs that interact with small molecule effectors.

The general strategy (Figure 1) is based on the insertion of a HHR element into the 3′-UTR of the Gal4 transcription factor in order to control its expression depending on the autocatalytic activity of the ribozyme. An active HHR self-cleaves the Gal4 mRNA, leading to mRNA decay and reduced Gal4 protein levels. In contrast, inhibition of HHR catalysis increases Gal4 mRNA levels and high levels of Gal4 transcription factor are expressed. We intended to perform both positive and negative selection steps in a single cell. The commercially available *MaV203* strain seemed to be well suited, because it is deficient in the *GAL4* gene and features the chromosomally encoded reporter genes *HIS3*, *URA3*, and *LacZ*, each under control of a Gal4-inducible promoter. Of particular benefit is the complementation of the positive selection marker *HIS3* because its gene product imidazole glycerol phosphate dehydratase can be competitively inhibited by 3-amino-1,2,4-triazol (3-AT). Titration of 3-AT concentrations enables the enrichment of cells exceeding a minimum threshold value of Gal4 expression. Negative selection is based on the expression of the *URA3* gene product, orotidine 5-phosphate decarboxylase (ODCase), and the addition of 5-fluoroorotic acid (5-FOA) to the culture medium. The higher the cellular Gal4 expression, the more 5-FOA is converted into the toxic compound 5-fluorouracil by ODCase. The third reporter gene, *LacZ*, facilitates quantitative analysis of Gal4 expression by measuring *β*-galactosidase activity. The enrichment of switches involves a negative and positive selection step of which one or the other is performed in the presence of the ligand. For


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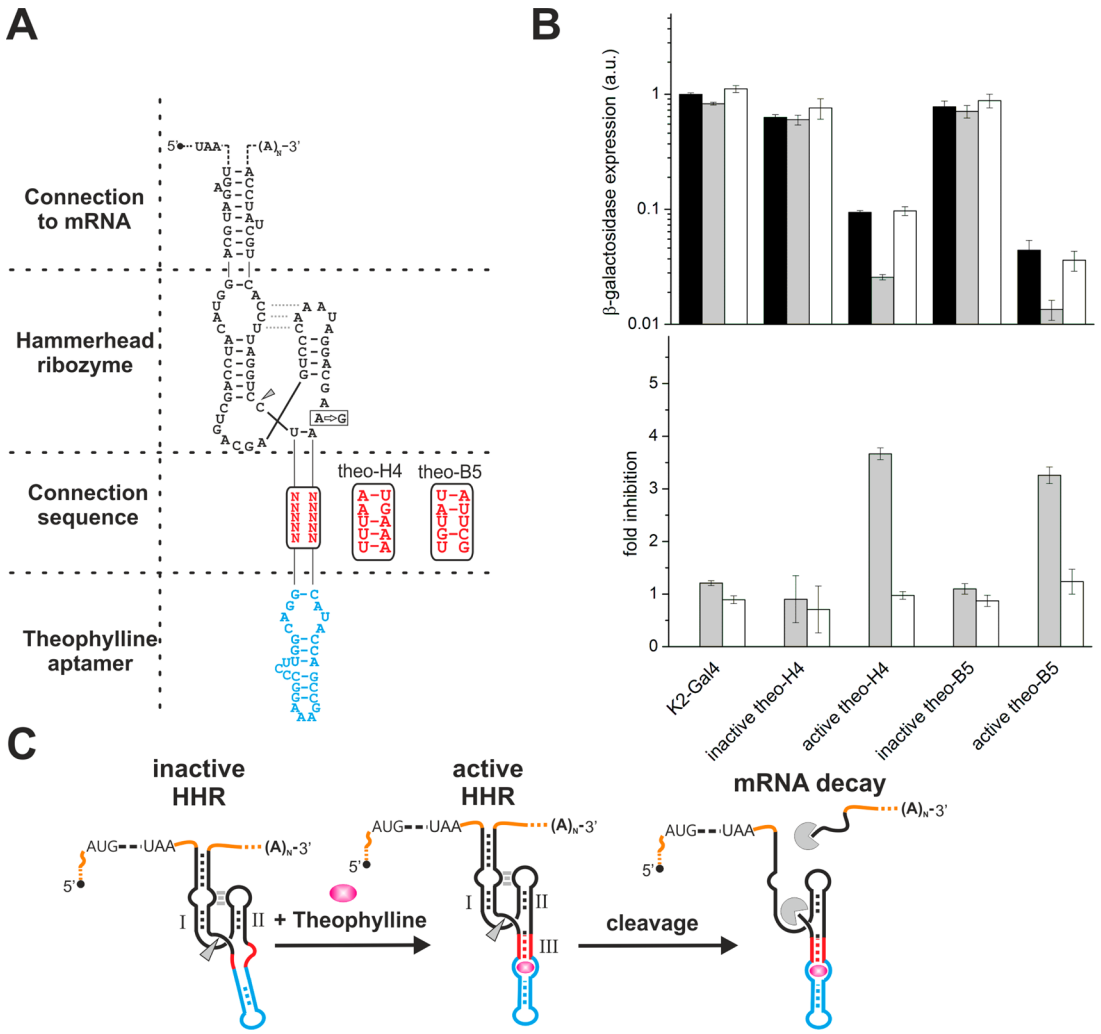


**Figure 3.** *In vivo* selection identifies theophylline-dependent genetic switches. (A) Secondary structure of a randomized pool of potential theophylline switches is shown. The theophylline aptamer is attached to stem 3 of a type 1 *S. mansoni* HHR via a connection sequence of 10 randomized nucleotides. Potential switches were enriched on medium supplemented with 3-AT and 5-FOA by genetic selection. Single upgrowing colonies were screened by measuring *β*-galactosidase expression. The nucleotide identity of two identified switches theo-H4 and theo-B5 is shown. An A-to-G substitution within the catalytic core of the HHR results in a catalytically inactive HHR. The cleavage site is indicated by an arrowhead. (B) Characterization of the identified theophylline-dependent switches theo-H4 and theo-B5. Gal4 expression was analyzed by measuring *β*-galactosidase activity of cultures grown in the absence of inducer (black bars), 2.5 mM theophylline (gray), and 2.5 mM caffeine (white). *S. cerevisiae* cultures of the *MaV203* strain were cultivated in synthetic complete medium at 30 °C and *β*-galactosidase expression of outgrown cultures was measured. Error bars represent the standard deviation of experiments performed at least in triplicates. (C) Schematic illustration of the proposed mechanism. Binding of theophylline to the aptamer domain stabilizes the catalytically active conformation resulting in the cleavage of the RNA backbone and degradation of the Gal4 mRNA.

example, addition of the ligand in the negative selection should yield OFF-switches with respect to gene expression, mediated by induction of ribozyme catalysis.

**Regulation of Gal4 Expression with 3′-UTR Hammerhead Ribozymes.** We expressed Gal4 under the control of the constitutive Cyc1 promoter from a low-copy *S. cerevisiae* plasmid. The impact of three different 5′-leader sequences on the expression of the transcription activator Gal4 were compared by analysis of *β*-galactosidase activity and of phenotypic growth (Supporting Information Figure S1). We concluded that the K2-Gal4 construct matched best the requirements for the insertion of a HHR because no background growth was observed in the negative selection and colonies resisted 50 mM 3-AT in a time-dependent fashion, for details see the Methods section in Supporting Information. Eukaryotic gene expression can be regulated by the insertion of a ribozyme into either the 5′- or 3′-UTR.[6,16] However, gene expression of *S. cerevisiae* is highly influenced by secondary structures in the 5′-UTR.[34] Therefore, we inserted the self-cleaving HHR into the 3′-UTR of the K2-Gal4 construct. In order to identify HHR motifs that are best suited for controlling gene expression in *S. cerevisiae*, we tested the influence of several ribozyme variants on gene expression (Figure 2A). The catalytic activity of a type 1 *Schistosoma mansoni* HHR[16] was compared to a type 3 HHR of *S. mansoni*[35] and a type 3 HHR derived from the satellite RNA of the tobacco ringspot virus.[36] These three HHRs have been used for the generation of artificial ribozyme-based switches before.[12,18,27] As reference, we constructed a catalytically inactive version of each HHR variant by an A-to-G mutation within the catalytic core. All inactive HHR variants did not impair LacZ expression (Figure 2B), demonstrating that the insertion of the


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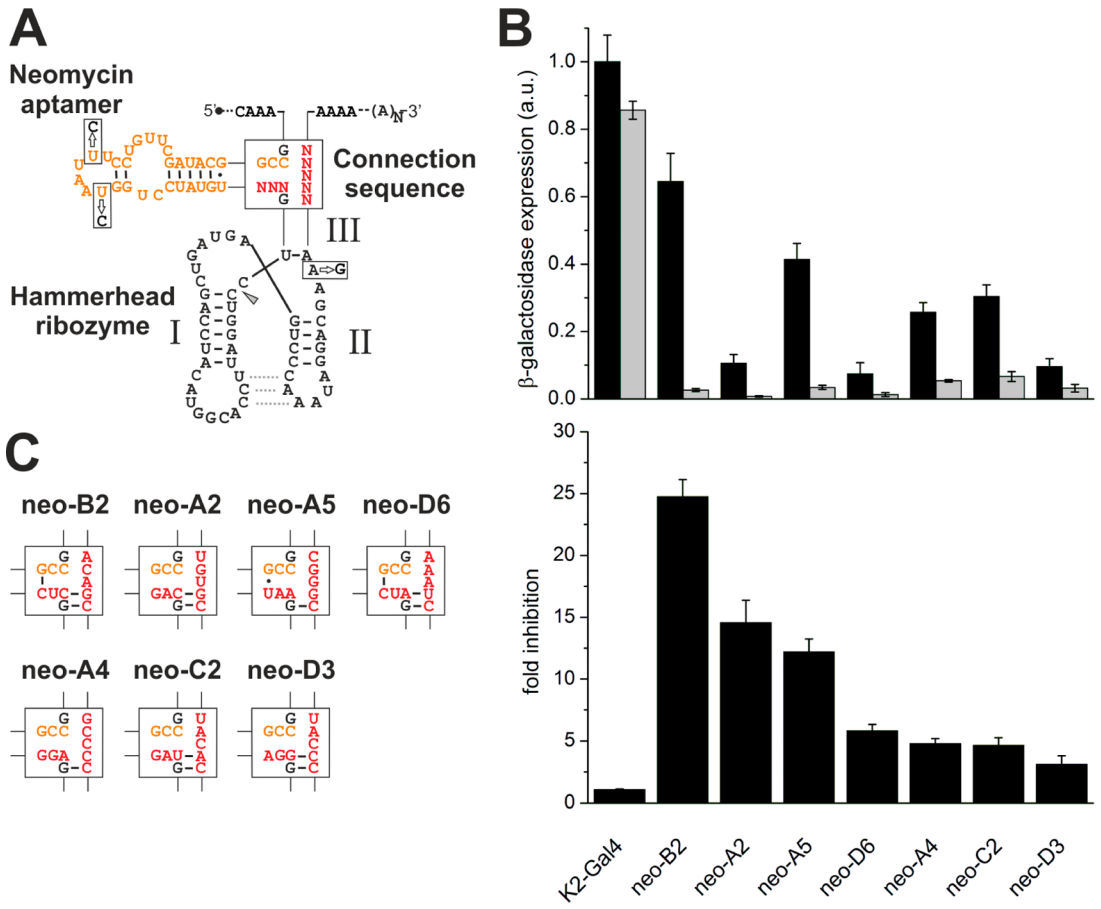


**Figure 4.** Novel aptazyme design and *in vivo* selection enables identification of neomycin-dependent genetic switches. (A) The secondary structure of a reengineered library of neomycin-dependent HHRs is shown. The aptamer domain (orange) was attached to the 5′-end of a type 3 HHR adopted from *S. mansoni*. Nucleotides that connect the aptamer to the HHR domain as well as nucleotides at the 3′-end of the HHR domain were randomized (red). Potential switches were enriched on medium supplemented with 3-AT and 5-FOA by genetic selection. Single upgrowing colonies were screened by measuring *β*-galactosidase expression. An A-to-G substitution within the catalytic core of the HHR results in a catalytically inactive HHR. Two U-to-C mutations within the terminal loop of the neomycin aptamer, termed M10 mutant, prevent binding of neomycin. The cleavage site is shown by an arrowhead. (B) Characterization of the identified neomycin-dependent HHR switches. Gal4 expression was analyzed by measuring *β*-galactosidase activity of cultures grown in the absence of effector (black bars) and with 100 *μ*g/mL neomycin (gray). *S. cerevisiae* cultures of the *MaV203* strain were cultivated in synthetic complete medium at 30 °C and *β*-galactosidase expression of outgrown cultures was measured. Error bars represent the standard deviation of experiments performed at least in triplicates; except for K2-Gal4 ($n$ = 2). (C) The nucleotide identity of the connection sequence is shown for each switch.

additional HHR motif into the Gal4 mRNA did not influence gene expression. In contrast, the active type 1 HHR displayed a 10-fold down-regulation of reporter gene expression in comparison to their catalytically inactive counterpart. Surprisingly, both type 3 HHRs inhibited reporter gene expression greater than 1000-fold. Previous studies demonstrated a high catalytic activity of these type 3 HHRs in *E. coli* and mammalian cells and our findings indicate that the type 3-format is well-suited for utilization in *S. cerevisiae* as well.[12,13,16]

Next, we investigated the phenotypic behavior of cells expressing K2-Gal4 under control of a catalytically active and inactive *S. mansoni* type 1 HHR (Figure 2C). Cells expressing Gal4 under control of the catalytically inactive type 1 HHR displayed the same behavior on 5-FOA and 3-AT as cells expressing the K2-Gal4 construct. However, cells expressing Gal4 under control of the catalytically active type 1 HHR displayed a phenotype after 3 days that resembled the one of Gal4-deficient yeast cells. Yeast cells were resistant to 0.1% (w/v) 5-FOA, and growth on 3-AT was only observed on 20 mM 3-AT after 8 days and completely inhibited on 50 mM 3-AT. Already modest differences in reporter gene expression, as reflected in case of the type 1 *S. mansoni* HHR, produce strong difference in the growth pattern of yeast cells on selective media. These results demonstrate that the system should be well suited for *in vivo* selection of randomized aptazyme libraries.

***In Vivo* Selection of Theophylline-Dependent Type 1 HHRs.** As proof-of-principle, we performed an *in vivo* selection of a randomized aptazyme library. Previously, we reported the construction of theophylline-dependent type 1 HHRs in *E. coli* and mammalian cells.[12,37,38] The theophylline aptamer domain[39] was attached via randomized connection sequences to stem 3 of the HHR. Manual screening of clonal libraries identified allosteric HHRs. In this study, we *in vivo*-selected a library of potential theophylline-dependent switches in which 10 nucleotides of the connection sequence were randomized (Figure 3A). Hence, the maximum pool diversity or sequence space of the aptazyme library was $1 \times 10^6$. A plasmid library was assembled and propagated in *E. coli*, and finally transformed into the *S. cerevisiae* MaV203 strain. Transformation yielded


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approximately $1 \times 10^7$ single colonies, which accounts for a greater than 95% coverage of the total sequence space.[40] We enriched potential theophylline-dependent switches on selective media supplemented first with 40 mM 3-AT, and within a second selection step with 0.075% (w/v) 5-FOA and 2.5 mM theophylline. Growing colonies of the negative selection step were screened by analyzing $\beta$-galactosidase activity. We identified two theophylline-dependent aptazymes, theo-H4 and theo-B5, down-regulating reporter gene expression by a factor of 3.6- and 3.2-fold, respectively (Figure 3B and Supporting Information Methods). We also aimed for the generation of switches that up-regulate gene expression upon addition of theophylline in yeast. We observed in previous screening attempts in *E. coli* and mammalian cells that the identification of sequences that inhibit the ribozyme self-cleavage activity upon theophylline binding is a surprisingly rare event.[12,13] Similarly, the screened library of this study did not yield any switches that inhibit ribozyme catalysis upon ligand binding.

In order to demonstrate that the changes in gene expression are indeed caused by a ligand-dependent change of cleavage activity of the aptazyme, we performed two important control experiments (Figure 3C). First, the catalytic activity of both identified theophylline-dependent HHRs was inactivated by an A-to-G mutation in the catalytic core. The inactive aptazyme variants restored $\beta$-galactosidase expression to the levels of the K2-Gal4 construct and the inactivated switches did not respond to theophylline anymore. Second, the addition of caffeine instead of theophylline did not induce any of the switches or their catalytically inactive variants, demonstrating that the high binding specificity reported for the theophylline aptamer is also found in the aptazyme.[39]

One of the advantages of utilizing HHRs as genetic switches is their high degree of modularity. We wondered whether the aptazymes theo-H4 and theo-B5 would sustain their switching capability when they were transferred to a bacterial expression system (Supporting Information Figure S2). We previously reported ligand-dependent RNA switches in *E. coli*, which were based on the sequestration of the ribosomal binding site by an extended stem 1 of the HHR.[13] Only autocatalytic cleavage of the HHR frees the RBS and induces gene expression. Indeed, both switches now identified in yeast selectively activated reporter gene expression about 4-fold, whereas their catalytically inactive variants did not respond to theophylline and caffeine. Taken together, the described proof-of-principle selection demonstrates that the developed protocol facilitates enriching and identification of synthetic HHR-based riboswitches.

**Development of Neomycin-Dependent Hammerhead Ribozymes.** Improving the performance of aptazyme-based genetic switches is an important step toward a more widespread application of post-transcriptional gene regulators. We anticipated that an alternative architecture of HHR-aptamer fusions (Figure 4A), not yet reported in the literature, might result in better switching performances. This line of thoughts was also motivated by the rather disappointing switching performances obtained in the proof-of-concept experiment with theophylline described above since a rather large sequence space of $1 \times 10^6$ variants was searched, indicating that the general design might limit the identification of better switches. A characteristic of all previously reported ligand-dependent HHR switches is that the HHR is split into two parts at one of the three stems, and the aptamer domain is inserted in between.[13,18,20,27] We reasoned that it might be beneficial to preserve the HHR and the aptamer domains as native as possible. Here, we introduce a new design that is inspired by the architecture of natural riboswitches, in which the aptamer domain often precedes the expression platform.[11] Similarly, we designed a library where the aptamer domain is located immediately upstream (5′) to a type 3 HHR domain. This arrangement positions the aptamer domain in close proximity to the catalytic core of the HHR.

To investigate whether our envisioned design is suitable for generating efficient RNA switches, we aimed at the generation of aminoglycoside-dependent HHRs. We speculated that the neomycin aptamer N1, which has been used for regulating gene expression before, is well suited for the purpose. It was developed by Schroeder, Süß, and co-workers utilizing a combined procedure of *in vitro* selection and subsequent *in vivo* screening of enriched pools in *S. cerevisiae*.[9,41] It is well characterized and binds to its ligand neomycin with high affinity *in vivo*.[42−45] In our novel design strategy, the neomycin aptamer domain was attached to the 5′-end of the type 3 *S. mansoni* HHR (Figure 4A). We anticipated that the region of stem 3 of the HHR would be a promising platform of influencing the integrity of the catalytic core. In order to search for ligand-dependent sequences, we randomized five nucleotides at the 3′-end of the HHR domain and three nucleotides at the bridge between the aptamer and the HHR domain. Hence, the diversity of our library amounted to a total of 65 536 variants, a sequence space that we are able to search completely with the introduced selection protocol. Enrichment of neomycin-dependent switches was carried out on selective media supplemented with 30 mM 3-AT and within a second selection step with 0.03% (w/v) 5-FOA and 100 $\mu$g/mL neomycin. Following the two steps, we screened individual colonies for neomycin-induced changes in $\beta$-galactosidase expression (see Supporting Information Methods). Seven switches that down-regulated reporter gene expression up to 25-fold in response to neomycin were identified (Figure 4B). The selection with neomycin added in the first selection step aimed at gene-activating reactivity upon aminoglycoside addition did not enable identification of efficient (>2-fold) switches. In principal, one should expect the occurrence of ON- as well as OFF-switches from respective selection strategies. However, we already experienced in other endeavors that under some circumstances not always ON- as well as OFF-switches are identified. Possible factors contributing to the observed phenomenon could be intrinsic properties of the aptamer that become limiting in certain designs. For example, as described below, the neomycin aptamer is intrinsically flexible and unfolded with ligand binding inducing the observed structure. Such a property might result in much more solutions for activating gene expression upon ligand binding via general stabilization of the ribozyme core fold, resulting in predominantly observed OFF-switches of gene expression.

The identified OFF-switches of gene expression imply a switching mechanism based on a stabilization of the catalytic core upon ligand binding. When we analyzed the sequence identity of the selected nucleotide positions of the individual switches (Figure 4C), we were not able to identify a consensus motif but found very heterogeneous sequences. $\beta$-Galactosidase expression levels in the absence of neomycin propose a weak stabilization of stem 3 for the switch neo-B2 which is increasingly stabilized for neo-A5, neo-C2, neo-A4 with the strongest stabilization seen for the switches neo-A2, neo-D3,

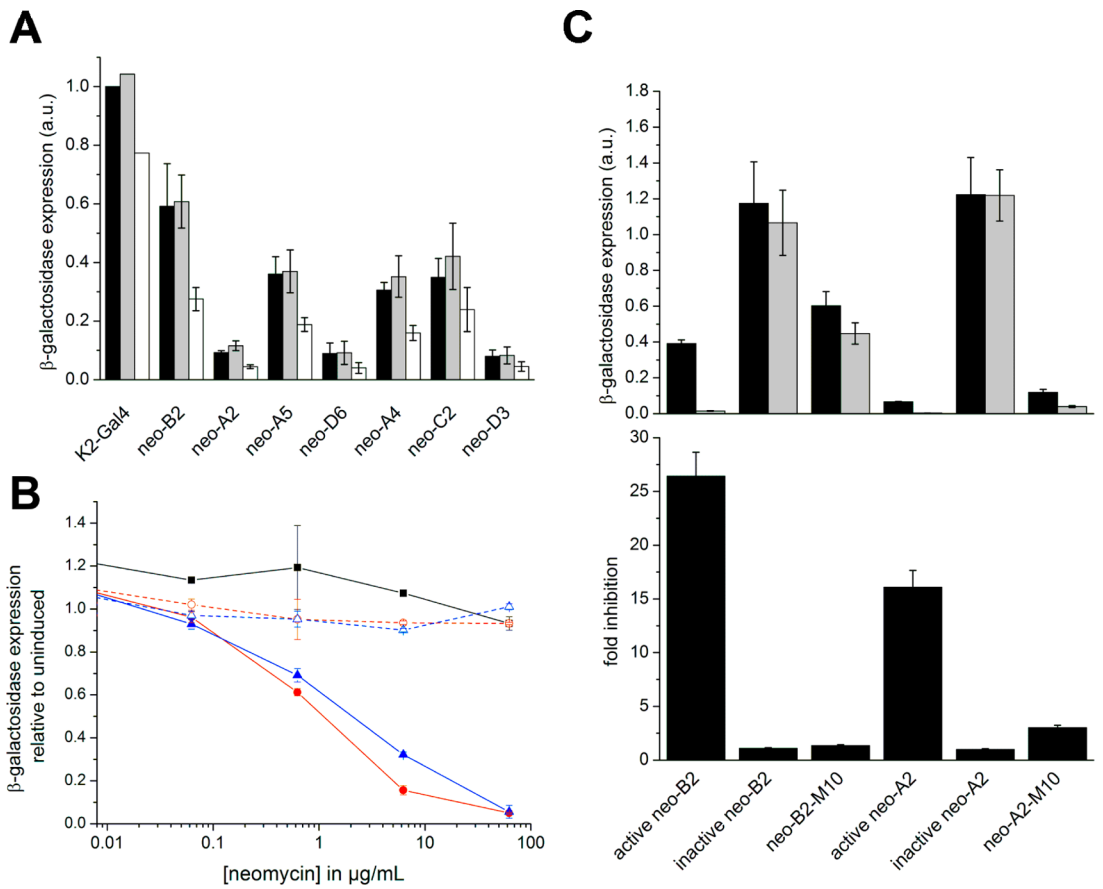


**Figure 5.** Characterization of neomycin-dependent switches. (A) Investigation of the selectivity of identified switches. The impact of 100 $\mu$g/mL amikacin (gray bars) and 100 $\mu$g/mL paromomycin (white bars) was investigated in comparison to absence of aminoglycosides (black bars). (B) Neomycin-dependent switches are induced at neomycin concentrations in the sub- and low-micromolar range. Yeast cultures expressing K2-Gal4 (black squares), Neo-B2 (red circles), and Neo-A2 (blue triangles) were incubated with increasing concentrations of neomycin and Gal4 expression was analyzed. Open symbols indicate cultures expressing a catalytically inactive version of Neo-B2 and Neo-A2 sequences. (C) Validation of the proposed switching mechanism. Catalytically inactive variants of the switches neo-A2 and neo-B2 were constructed by A-to-G mutations within the catalytic core of the HHRs. Binding of neomycin to the aptamer domain was prevented by the insertion of the M10 mutation, which is characterized by two U-to-C mutations in the terminal loop of the neomycin aptamer. Gal4 expression was analyzed by measuring $\beta$-galactosidase activity of cultures grown in the absence of effector (black bars) and with 100 $\mu$g/mL neomycin (gray). (A, B, C) *S. cerevisiae* cultures of the *MaV203* strain were cultivated in synthetic complete medium at 30 °C and $\beta$-galactosidase expression of outgrown cultures was measured. Errors bars represent the standard deviation of experiments performed at least in triplicates, except for part A K2-Gal4 ($n$ = 1).

and neo-D6. We conducted an Mfold analysis to get further insight into a possible switching mechanism.[46] Mfold analysis supports the observed pattern observed in gene expression experiments. A small number of "active HHRs folds" out of the "absolute number of predicted structures" suggests a reduced chance to adopt an active HHR structure for neo-B2, neo-A5, and neo-C2, whereas the chance to adopt an active configuration is highly increased for the other switches. Importantly, for all switches except neo-C2, an "active HHR" structure is calculated as at least second most stable structure. Previous studies reported a conformation capture mechanism by the neomycin aptamer.[44] Similarly, Mfold energy dot blot analysis predicts a highly unstructured aptamer region for our reported switches.

In order to further analyze the obtained RNA switches, we characterized the selectivity of neomycin in comparison to amikacin and paromomycin (Figure 5A). Paromomycin and neomycin both are 4,5-substituted deoxystreptamine derivatives that differ only in paromomycin having a hydroxyl functionality instead of an amine group at position 6 of ring 1. Amikacin belongs to the class of 4,6-substituted deoxystreptamine derivatives. In our setup, the switching performance was not affected by amikacin and only weakly by paromomycin. Our results are in accordance with a previous study that reported a high selectivity of the neomycin aptamer for neomycin.[9] The high discriminatory potential of the neomycin aptamer is exemplified by weakly binding to ribostamycin, an intermediate in neomycin biosynthesis that lacks ring 4, and full discrimination of paromomycin. Hence, the identified neomycin ribozyme switches displayed selectivities that match those reported for the original aptamer.

The two best-performing switches, neo-B2 and neo-A2, were further characterized. The aptazyme neo-B2 repressed gene expression 25-fold from 60% in the absence of neomycin to 2% of K2-Gal4 expression at 100 $\mu$g/mL neomycin, whereas neo-A2 inhibited gene expression from 12% to less than 1% of K2-Gal4 expression. An important question for further applications is the sensitivity of the neomycin switches with respect to ligand concentrations. For this purpose, we analyzed reporter gene expression in dependence to varying neomycin concentrations (Figure 5B). The identified switches showed half-maximum inhibition of gene expression at neomycin


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concentrations of 0.7 μg/mL for neo-B2 and 2.6 μg/mL for neo-A2. In order to be widely applicable even for future therapeutic strategies, synthetic gene switches should be triggered at effector concentrations that are principally achievable in humans. Most RNA switches developed so far (such as often-realized theophylline-responsive systems) operate at ligand concentrations far above nontoxic concentrations that could be reached in humans, hence being not useful for clinical purposes. In order to judge such applicatory potential, the concentrations of half-maximum effect of neomycin need to be compared to levels that can, in principle, be reached in clinical setups. Pharmacological studies in humans reported maximum neomycin serum concentrations of 4.3 μg/mL.[47] The neomycin switch neo-B2 displays a half-maximum response at 0.7 μg/mL and the neo-A2 sequence at 2.6 μg/mL, both well below the concentrations that are reachable in human sera. Hence, we report very sensitive switches that might be useful as effectors for regulating mammalian transgene expression.

In order to further validate the proposed ribozyme-based mechanism, we next performed important control experiments (Figure 5C). As shown before in context of the theophylline-dependent switches, we inactivated the ribozyme catalysis by an A-to-G mutation within the HHR core. As expected in case the ribozyme catalysis is responsible for controlling gene expression, we observed restored levels in both inactivated neo-B2 and neo-A2 switches to levels comparable to the parental K2-Gal4 construct. Importantly, increasing neomycin concentrations did not impact gene expression of the catalytically inactive aptazymes, proofing that the neomycin-mediated gene-regulatory effect observed with the active switches is ribozyme-dependent and not due to an unspecific inhibitory effect on gene expression caused by the addition of aminoglycosides. Along these lines, we next addressed whether neomycin targets the aptamer domain or just some other part of the RNA elements. Aminoglycosides generally display certain RNA binding affinities and *in vitro* neomycin has been demonstrated to inhibit slow-cleaving variants of the HHR with $IC_{50}$s around 20 μg/mL.[48] Although this concentration is much higher than utilized here for RNA switching *in vivo*, it is necessary to investigate whether the observed effects indeed originate from specific recognition of neomycin via the aptamer domain. Schroeder, Süß, and co-workers reported that mutations within the terminal and internal loops drastically affect the binding of neomycin to the aptamer domain.[9,45] For example two U-to-C substitutions in the terminal loop (Figure 4A), referred to as M10 mutant, resulted in loss of binding.[9] Hence, we modified the terminal loop of neo-B2 and neo-A2 by introducing the respective M10 mutations. Indeed, neo-B2-M10 did not show efficient inhibition of gene expression upon neomycin addition. The variant neo-A2-M10 showed a general decrease in gene expression even in the absence of neomycin but also a much reduced response upon neomycin addition (Figure 5C). Taken together, these results demonstrate that the neomycin-dependent gene regulation acts specifically via targeting the aptamer domain within the mRNA construct.

In conclusion, we reported a new method for searching large sequence spaces for RNA switches in yeast. The introduced *in vivo* selection protocol was established and applied to search genetic libraries with diversities up to $1 \times 10^7$. In our hands, the potential sequence space that can be sampled was limited by the actual transformation efficiency in yeast; however, optimization and up-scaling should allow for searching pools up to $1 \times 10^9$ variants. In addition, we explored a novel format of aptazyme design that more closely resembles the architecture of naturally occurring riboswitches. In this setup, the aptamer domain precedes the ribozyme expression platform, with the potential to form interactions that modulate expression platform folding and function in response to ligand binding to the aptamer domain. We demonstrated the suitability of our design by identifying powerful genetic switches triggered by theophylline and neomycin. Remarkably, the reported neomycin switches belong to the best-performing HHR-based switches known so far and respond to their ligand with high sensitivity and selectivity. The question whether the aptamer, the selection protocol, or the novel design was enabling this result is rather difficult to answer since the influence of an individual factor on the overall performance seems to be dependent on the other factors as well. It seems that the specific design utilized (with the design performance in turn often depending on the nature of the aptamer) is the most important factor. The first description of applying the neomycin aptamer by Suess and Schroeder resulted in moderate to good switching rates.[9] However, we achieved better results, pointing at the conclusion that the design is very important and (at least in this case) the aptamer was not limiting the performance in the earlier study. We have seen similar behavior with other aptamers as well: The widely employed theophylline aptamer usually results in switching performances of 2- to 10-fold in our and several other groups' results. The moderate to good performances are not a cause of sampling limited sequence space, since we have sampled a rather large sequence space of 1 000 000 together in combination with an often-utilized design in the present study, without identifying significantly improved switches. However, with the proper design much higher switching rates are possible by screening small libraries as demonstrated by >100-fold activation rates utilizing a theophylline aptamer and a design composed of a fourth stem attached to stem I of the hammerhead ribozyme.[49]

## METHODS

**General Considerations.** Standard molecular cloning procedures were performed as described in literature. Phusion Hot Start 2 Polymerase (NEB) was used for PCR amplification of DNA, endonucleases were purchased from NEB, and ligations were performed with the Quick ligation kit (NEB) or T4 DNA ligase (NEB). All constructs were verified by DNA sequencing (GATC Biotech).Theophylline and caffeine were purchased from Fluka. Neomycin trisulfate was purchased from Carl Roth. *E. coli* were grown aerobically at 37 °C in Luria–Bertani media supplemented with the respective antibiotic (carbenecillin, 100 μg/mL; kanamycin, 30 μg/mL). All plasmids were introduced by electroporation into *E. coli*. Yeast experiments were conducted with the *S. cerevisiae* MaV203 strain (Invitrogen; *MATα*; *leu2−3,112*; *trp1−901*; *his3Δ200*; *ade2−101*; *cyh2*$^R$; *can1*$^R$; *gal4Δ*; *gal80Δ*; *GAL1::-lacZ*; *HIS3*$_{UASGAL1}$*::HIS3@LYS2*; *SPAL10::URA3*) at 30 °C in Synthetic Complete medium containing 2% (w/v) glucose.

**Plasmid Construction.** All yeast plasmids are based on the pBT3-C plasmid (MoBiTec GmbH) that features a kanamycin resistance gene and a LEU2 auxotrophic marker for selection in *E. coli* and yeast. K2-Gal4 and K4-Gal4 were constructed by PCR amplification of the Gal4 ORF from *S. cerevisiae* genomic DNA and insertion via Hind3 and Apa1 into the pBT3-C vector. The Kozak sequence of K4-Gal4 was obtained from part


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BBa_J63003 of the Registry of Standard Biological Parts. In the case of the K5-Gal4 construct, Xba1 and Apa1 were used. Hammerhead ribozymes were inserted into the 3′-UTR via Spe1 and Not1 restriction sites. Sequences are given in the Supporting Information. The *E. coli XL10 gold* strain (Stratagene; *Tet$^r$*Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacI$^q$ZΔM15 Tn10 (Tet$^r$) Amy Cm$^r$]* was used for molecular cloning and plasmids were introduced into yeast via a standard lithium acetate procedure.

**Construction and Screening of Aptazyme Libraries.** Aptazyme libraries were created by PCR with primers that inserted the aptamer domain and the randomized nucleotides. Sequences of the theophylline and neomycin aptazyme libraries, which are flanked by Spe1 and Not1 restriction sites, are shown in the supplementary. The aptazyme library was molecularly cloned into the pBT3-K2-Gal4 plasmid. Transformation of the plasmid pool into the *E. coli XL10 gold* strain was performed by electroporation and yielded up to $10^7$ transformants. The plasmid pool was recovered from bacteria and used for the transformation of the *S. cerevisiae* MaV203 strain via electroporation.[50] Up to $10^7$ transformants were obtained that were grown on SC media lacking leucine. Positive selection of the theophylline aptazyme library was performed on SC media lacking leucine, uracil, and histidine supplemented with 40 mM 3-AT. Upgrowing colonies were collected and plated on negative selection media that contained 0.075% (w/v) 5-FOA and 2.5 mM theophylline. In the case of the neomycin library, positive selection media contained 30 mM 3-AT and negative selection media was supplemented with 0.03% (w/v) 5FOA and 100 μg/mL neomycin. β-Galactosidase expression levels of single colonies that were grown in the absence and presence of either 2.5 mM theophylline or 100 μg/mL neomycin were measured with the Gal-Screen β-galactosidase Reporter Gene Assay System (Life Technologies). Plasmids of yeast cultures that showed switch-like behavior were recovered and sequenced. To exclude accumulated mutations of the plasmid backbone during the selection procedure potential switches were recloned into the pBT3-K2-Gal4 plasmid using Spe1 and Not1 restriction sites.

**Reporter Gene Assays in *S. cerevisiae*.** A phenotypic growth analysis was used for measuring the *HIS3* and *URA3* gene product expression. A yeast suspension of $OD_{600}$ = 0.1 was serially diluted 1:10 with 0.9% (w/v) aqueous NaCl solution. Ten microliters of each dilution were spotted on solid medium supplemented with either 3-AT or 5FOA. Pictures were taken after 3 and 8 days.

For β-galactosidase expression measurement three single colonies were first outgrown to stationary phase in SC-Leu medium. The next day a 1% yeast suspension, supplemented with the respective concentration of inducer as indicated, was regrown with shaking at 200 rpm and 30 °C in an Infors HT Ecotron shaker. Then, 100 μL of each culture were transferred into a 96-well microtiter plate and $OD_{600}$ was measured with a Tecan Infinite M200 plate reader. LacZ expression was determined with the Gal-Screen β-galactosidase Reporter Gene Assay System (Life Technologies) and luminescence was measured with a Tecan Infinite M200 plate reader. Luminescence values were corrected by dividing with the $OD_{600}$ values. An equally treated culture, which did not express any Gal4, was used for subtraction of background luminescence. All experiments were performed in triplicates, and error bars represent standard deviations if not otherwise indicated.

## ■ ASSOCIATED CONTENT

### Supporting Information

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## ■ AUTHOR INFORMATION


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### Author Contributions

B.K. and J.S.H. designed the experiments; B.K., J.A., and L.K.S. carried out the experiments; B.K., J.A., L.K.S., and J.S.H. interpreted the results; B.K. and J.S.H. prepared the manuscript.

### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

HHR, hammerhead ribozyme; UTR, untranslated region; 3-AT, 3-amino-1,2,4-triazol; 5FOA, 5-fluoroorotic acid

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