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Exploring the modular nature of riboswitches and RNA thermometers

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ABSTRACT

Natural regulatory RNAs like riboswitches and RNA thermometers (RNAT) have considerable potential in synthetic biology. They are located in the 5′ untranslated region (UTR) of bacterial mRNAs and sense small molecules or changes in temperature, respectively. While riboswitches act on the level of transcription, translation or mRNA stability, all currently known RNATs regulate translation initiation. In this study, we explored the modularity of riboswitches and RNATs and obtained regulatory devices with novel functionalities. In a first approach, we established three riboswitch-RNAT systems conferring dual regulation of transcription and translation depending on the two triggers ligand binding and temperature sensing. These consecutive fusions control gene expression *in vivo* and can even orchestrate complex cellular behavior. In another approach, we designed two temperature-controlled riboswitches by the integration of an RNAT into a riboswitch aptamer domain. These 'thermoswitches' respond to the cognate ligand at low temperatures and are turned into a continuous on-state by a temperature upshift. They represent the first RNATs taking control of transcription. Overall, this study demonstrates that riboswitches and RNATs are ideal for engineering synthetic RNA regulators due to their modular behavior.

INTRODUCTION

Synthetic biology uses building blocks from nature to generate new regulatory devices with novel functionality or new genetic circuits. The use of RNAs as regulators has many benefits. RNA-mediated regulation is independent of protein factors and less cost-intensive for the cell. It is faster than protein-based regulation because the mRNA directly controls gene expression without the need of translation (1). Apart from canonical Watson–Crick base pairs various other interactions contribute to the geometry of RNA structures (2,3) making RNA molecules attractive as versatile regulators for applications in synthetic biology.

Riboswitches and RNA thermometers (RNATs) are naturally occurring regulatory RNAs typically located in 5′ untranslated regions (5′ UTRs), which form complex secondary structures that enable them to recognize small molecules or register changes in temperature, respectively (4,5). The riboswitch aptamer domain forms a highly specific ligand-binding pocket, which is able to bind small molecules like amino acids, purines or cofactors with high specificity and affinity (6). Ligand binding to the aptamer domain induces conformational changes in the expression platform resulting in regulation of the downstream gene at the level of transcription termination, translation initiation or RNA processing. Aptamers can be highly structured like the cobalamin aptamer (7,8) or rather simple like the purine aptamers (9,10). The specificity of binding and the broad variety of potential regulator molecules makes riboswitches interesting for applications in synthetic biology. Riboswitches have been used to control processes like ribozyme-induced cleavage (11) or splicing (12) in eukaryotes. Moreover, synthetic aptamers like the theophylline (13) or the tetracycline (14) aptamer have been designed and improved by SELEX (systematic evolution of ligands by exponential enrichment) even before the first natural riboswitches were discovered.

RNATs regulate translation of certain mRNAs in response to temperature changes. The ribosome binding site is sequestered by a stable RNA secondary structure at low temperatures (4). With increasing temperatures the RNAT structure unfolds in a zipper-like manner and translation can be initiated (15). Many RNATs fold into extended and complex structures as described for the ROSE (repression of heat shock gene expression) element, which is composed of up to four hairpins (16,17) or the *rpoH* RNAT, whose structure includes parts of the coding sequence (18). Over the years simpler RNATs were discovered, which consist of short stem–loop structures sufficient to control translation in a temperature-dependent manner. Among them are the 4U element from the *Salmonella agsA* gene (19), the *hsp17* RNAT of the cyanobacterium *Synechocystis* (20) and RNATs regulating the small heat shock proteins *hspX* and *hspY* in *Pseudomonas putida* (21). Due to their simple archi-

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tecture and molecular mechanism, RNATs are promising as regulators for synthetic biology. Different design strategies resulted in synthetic RNATs that induce expression at increasing temperature (22,23) or in heat-repressible thermosensors (24). Recently, an RNAT was applied to control ribozyme cleavage in response to temperature (25).

A prerequisite for the design of synthetic expression tools from natural regulators is that the combined building blocks are sufficiently modular and functional in a different genetic context. In this study, we investigated whether riboswitches and RNATs can be used to combine sensing of small molecules and temperature. We used well-characterized riboswitches and RNATs as building blocks to demonstrate their modular nature.

Consecutive riboswitch-RNAT fusions were able to control transcription and translation in response to ligand molecules and temperature, respectively. Integration of an RNAT into the aptamer domain of two different riboswitches resulted in temperature-controlled riboswitches. We provide a comprehensive set of *in vivo* and *in vitro* experiments exploring the functionality and molecular mechanism of RNAT-controlled riboswitches.

MATERIALS AND METHODS

Bacterial growth conditions

Bacterial strains used in this study are listed in Supplementary Table S1. *Escherichia coli* strains were cultivated in LB medium or M9 minimal salt medium supplemented with 0.4% (w/v) glucose at indicated temperatures. Media were supplemented with ampicillin (Amp, 150 μg/ml) or kanamycin (Km, 50 μg/ml) if required. For induction of the P_{BAD} promoter in strains carrying translational reporter gene fusions, L-arabinose was added to a final concentration of 0.01% (w/v) in LB medium or 0.1% (w/v) in M9 minimal medium.

Plasmid construction

Oligonucleotides and plasmids used in this study are summarized in Supplementary Tables S2 and S3, respectively. Recombinant DNA work was performed according to standard protocols (26). The correct nucleotide sequences of all constructs were confirmed by automated sequencing (Eurofins, Martinsried, Germany). Enzymes were obtained from Thermo Scientific (St. Leon-Rot, Germany).

For translational reporter gene fusions, the *tenA* 5′ UTR (TPP) comprising nucleotides +1 to +196 or the *lysC* 5′ UTR (lys) comprising nucleotides +1 to +316 with respect to the transcriptional start site was amplified by PCR as an NheI and XhoI fragment from *Bacillus subtilis* 168 chromosomal DNA using the corresponding primer pairs listed in Supplementary Table S2. Translational reporter gene fusions containing the RS10 5′ UTR (theo) were amplified from plasmid pBAD2-*bgaB*-RS10 (27).

Translational fusions to *bgaB* or *cheZ* were constructed by cloning via primer derived NheI/XhoI sites into the corresponding sites of pBO3390 or pBO3980. For construction of consecutive riboswitch-RNAT fusions both UTRs were amplified by PCR, cut with XhoI and ligated blunt end into the SmaI site of pUC18. Riboswitch-RNAT fusions were afterwards transferred into pBO3390 or pBO3980 as described above.

To generate integrative riboswitch-RNAT fusions a three-step overlap PCR was used. PCR fragments were cloned in pUC18 and then transferred into pBO3390 via NheI/XhoI restriction sites. Site-directed mutagenesis was performed on the pBAD2-*bgaB*-plasmids using mutagenic primers (listed in Supplementary Table S2).

Translational fusions to *gfp* were obtained by exchanging the reporter gene *bgaB* in pBO3393, pBO3947, pBO3968, pBO3995 and pBO3996 against PCR amplified *gfp* from pBS-U0 (22) via EcoRI/XbaI restriction sites resulting in plasmids pBO4345, pBO4346, pBO4347, pBO4342 and pBO4343, respectively.

Run-off plasmids used for *in vitro* transcription were constructed by blunt end cloning of the 5′ UTRs amplified with primers adding the T7-promoter sequence at the 5′ end (GAAATTAATACGACTCACTATAGGG) and an EcoRV site at the 3′ end into SmaI site of pK18. For structure probing the *tenA* 5′ UTR (TPP) containing nucleotides 27–124 (with respect to the transcriptional start site) was cloned. Desired variations were introduced via site-directed mutagenesis. The sequences and structures for all constructed riboswitch-RNAT fusions are provided in Supplementary Table S4.

β-Galactosidase activity assay

E. coli DH5α or ER2566 cells carrying the *bgaB* plasmids were grown overnight in 5 ml LB or M9 minimal medium at 25, 30 or 37°C. 15 ml LB or M9 minimal medium with ampicillin and L-arabinose (0.01% in LB and 0.1% in M9) were pre-warmed to 25, 30 or 37°C and inoculated with the overnight culture to an optical density (OD_{600}) of 0.05. The cultures were grown either with or without added theophylline (2 mM), thiamine (100 μM) or lysine (10 mM). After growth to an optical density (OD_{600}) of 0.5 samples were taken for β-galactosidase measurements as described previously (28). Calculated values (Miller Units) are provided in Supplementary Tables S5–S12.

Migration on semisolid media

To perform migration experiments full medium (LB medium with 0.25% agar, 150 μg/ml ampicillin, 0.01% L-arabinose and 2 mM theophylline) or selective medium (M9 minimal medium with 0.25% agar, 150 μg/ml ampicillin, 0.1% L-arabinose and 100 μM thiamine or 10 mM lysine) was prepared in petri dishes (90 mm diameter). *E. coli* BW25113 wild type (wt) and Δ*cheZ* cells harboring pBAD2-*cheZ* plasmids were grown to an optical density (OD_{600}) of 0.5. Five microliters of the cell suspension were applied to the center of the plates, which were incubated at 25, 30 and 37°C. Incubation at each temperature was stopped when wt cells reached the exterior of the petri dish and migration radius was measured.

RNA preparation

Total RNA of cultured bacteria was isolated using the RNA preparation method described in (29) with minor modifications. 0.5 ml stop buffer (100 mM Tris–HCl, pH8, 200

mM β-mercaptoethanol, 5 mM EDTA) was added to 2 ml bacterial culture. Cells were harvested and washed with 1 ml washing buffer (10 mM Tris–HCl, pH8, 100 mM NaCl, 1 mM EDTA). The following steps of the RNA preparation were performed as described. After the precipitation RNA concentrations were measured with a NanoDrop spectrophotometer ND-1000 (peqlab, Erlangen, Germany).

Northern blot analysis

E. coli cells with translational fusions to *cheZ* or *gfp* were grown at the indicated temperatures in the presence or absence of the ligand until OD_{600} of 0.5. Northern blot analysis was performed as described previously (30). For detection of *cheZ* transcript a 287 bp fragment of the *E. coli cheZ* gene was amplified. To construct the probe against the *tenA* 5′ UTR a 105 bp fragment of the *B. subtilis tenA* 5′ UTR was amplified. RNA probes were derived from *in vitro* transcription with DIG (digoxigenin) labeled nucleotides (Roche, Mannheim, Germany).

Preparation of protein extracts and Western blot analysis

E. coli cells harboring the pBAD2-*gfp* plasmids were grown overnight in 5 ml M9 minimal medium at 25, 30 or 37°C. Fifteen milliliters M9 minimal medium with ampicillin and L-arabinose were pre-warmed to 25, 30 or 37°C and inoculated with the overnight culture to an optical density (OD_{600}) of 0.05. After growth to an optical density (OD_{600}) of 0.5 1 ml of the culture was harvested (1 min, 13 000 rpm). Pellets were resuspended in TE buffer (10 mM Tris, pH 8, 1 mM EDTA; 100 μl TE buffer per optical density OD_{600} of 0.5) and mixed with protein sample buffer (final concentrations of 2% SDS (w/v), 0.1% (w/v) bromophenol blue, 10% glycerol (v/v), 1% β-mercaptoethanol, 50 mM Tris–HCl, pH 6.8). After incubation for 5 min at 95°C, samples were centrifuged and separated via SDS-PAGE. Western transfer was performed by tank blotting onto a nitrocellulose membrane (Hybond™-C Extra, GE Healthcare, Munich, Germany). GFP antibody (ABIN129570, antibodies-online GmbH, Aachen) was used in an 1:10 000 dilution, secondary antibody goat anti rabbit-HRP conjugate (Bio-Rad, Munich, Germany) in 1:3000 dilution. Luminescence signals were detected using Luminata Forte Western HRP (Merck, Darmstadt, Germany) substrate and the ChemiImager Ready (Alpha Innotec, Kasendorf, Germany).

In vitro transcription

RNAs for structure probing experiments were synthesized *in vitro* by run-off transcription with T7 RNA polymerase from EcoRV linearized plasmids (listed in Supplementary Table S3).

Enzymatic RNA structure probing

The TPP riboswitch, the P3a::4U* RNA and its variants rep (A6C) and derep (G12A/C23U) were obtained by *in vitro* transcription. The RNA was purified and dephosphorylated with CIP (Calf intestinal phosphatase, Thermo Scientific, Waltham, USA). RNA was labeled at the 5′ end as described (31). Partial digestions with ribonuclease T1 (0.00125 U) (Ambion, Austin, USA) and nuclease S1 (0.125 U) (Thermo Scientific, Waltham, USA) were performed according to (19) at indicated temperatures in absence or presence of 100 μM TPP. For digestion with RNase T1, 5x TN buffer (100 mM Tris acetate, pH 7, 500 mM NaCl) was used. Digestion with nuclease S1 was performed using the supplied 5× reaction buffer. An alkaline hydrolysis ladder (31) and a T1-ladder were prepared. For the T1-ladder 30,000 cpm labeled RNA was incubated with 1 μl sequencing buffer (provided with RNase T1) at 90°C followed by incubation with the enzyme at 45°C for 5 min.

RESULTS

Design of consecutive riboswitch-RNAT fusions

In order to construct RNA regulators, which mediate dual control on transcriptional and translational level, a selection of transcriptional riboswitches was fused to an RNAT that controls translation. In this set-up, ligand binding to the aptamer domain of the riboswitch determines whether mRNA synthesis is elongated or terminated prematurely and subsequent translation of the mRNA is controlled by the downstream located RNAT. Three previously characterized riboswitches were fused to the well-studied 4U RNAT from *Salmonella* (19). As riboswitch modules, we chose the synthetic theophylline on-switch RS10 (henceforth referred to as theo) (27) and the two natural off-switches *tenA* (referred to as TPP) sensing thiamine pyrophosphate (32,33) and *lysC* (referred to as lys) sensing lysine (34–36), both from *B. subtilis*.

Since riboswitches and RNATs are posttranscriptional modulators that do not confer a complete on- or off-response, a combination of both regulatory elements should result in tighter control of gene expression compared to either individual module. Furthermore, the combination of a ligand-binding transcriptional regulator and a temperature-sensing translational regulator should provide regulation on two levels triggered by a chemical and a physical parameter. We expected the following regulatory outcomes for our consecutive fusions. Riboswitch-RNAT fusions consisting of an on-switch prevent transcription in the absence of ligand. In addition, translation of residual read-through transcripts is repressed by the closed RNAT at 25°C resulting in poor expression (Figure 1A). At 37°C in the absence of ligand, only the residual not-terminated transcripts can be translated because repression by the RNAT is relieved. The net result again is low expression (Figure 1B). In the presence of ligand, the elongated transcript can only be translated efficiently at elevated temperature (Figure 1C and D). An off-riboswitch upstream of the RNAT results in a completely different scenario. It facilitates transcription in the absence of ligand. However, translation is blocked at 25°C (Figure 1E). Highest expression due to positive regulation of transcription and translation is expected in the minus ligand state at 37°C (Figure 1F). Formation of a terminator structure upon ligand binding and a closed RNAT at low temperature impairs gene expression (Figure 1G). At 37°C, translation is possible but full-length transcripts are rare in the presence of ligand (Figure 1H).

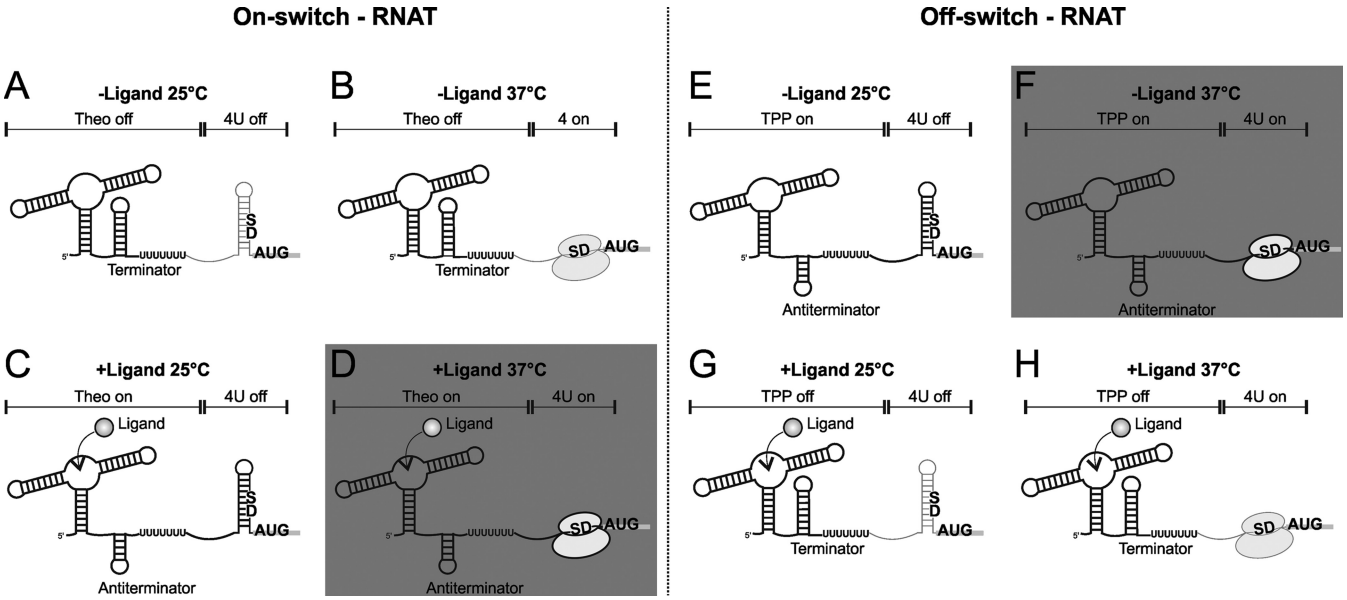

Figure 1. Possible states of riboswitch-RNAT fusions. (**A**) In absence of the ligand a terminator is formed in the expression platform of the theo on-switch, blocking transcription. Translation is inhibited at 25°C by the 4U RNAT. (**B**) Transcription is impaired in the minus-ligand state although transcribed RNA would be translated at 37°C. (**C**) In the plus-ligand state, an antiterminator is formed instead and transcription is enabled. At 25°C translation of the transcribed mRNA is prevented by the 4U RNAT, which forms a hairpin thereby masking the SD sequence. (**D**) Transcription and translation are permitted in the plus-ligand state at 37°C. (**E**) The TPP off-switch turns on transcription in the minus-ligand state but translation is impeded at 25°C (**F**) Transcription and translation are promoted in absence of ligand at high temperature. (**G**) Ligand binding and low temperature result in poor transcription and translation, respectively. (**H**) Although translation would be allowed at 37°C, presence of the ligand prevents mRNA synthesis. Transcribed elements are labeled with thick black lines. Elements that are part of the constructed riboswitch-RNAT fusion but not transcribed are marked with thin grey lines. Conditions leading to full gene expression are highlighted by grey boxes. SD: Shine-Dalgarno sequence, AUG: translational start codon.

To investigate the regulatory behavior of consecutive riboswitch-RNAT fusions, we constructed translational fusions to the *bgaB* gene encoding a heat-stable β-galactosidase, which is under control of the arabinose-inducible P_{BAD} promoter (30). To validate the contribution of the RNAT module to translational control, stabilizing and destabilizing mutations were introduced into the 4U RNAT (Figure 2A). An AG mismatch was turned into a GC pair leading to a stable hairpin structure (rep) known to prevent translation of the mRNA (37,38). To destabilize the RNAT, the GC pair G12/C23 was replaced by a weaker AU pair (derep). Reporter gene activity was measured at 25 and 37°C in *E. coli*.

The theo riboswitch clearly acts as an on-switch producing higher β-galactosidase activity in the presence of ligand at both 25 or 37°C (Figure 2B). The 4U RNAT alone repressed reporter gene activity at 25°C irrespective of the absence or presence of ligand. A direct fusion of the theo on-switch to the 4U RNAT was not able to repress *bgaB* activity at 25°C in the plus ligand condition (Supplementary Figure S1) presumably because both modules were too close to fold properly. To facilitate correct folding, we inserted a 20-nt spacer between riboswitch and RNAT. This so-called theo-4U* construct almost completely abrogated gene expression in the absence of ligand at 25°C and permitted only poor expression in the presence of ligand at 25°C suggesting that transcriptional and translational control are operational (Figure 2B). The consecutive fusion is superior to both individual control elements in conferring tight control (about four times and two times lower expression than by the riboswitch or RNAT alone, respectively). Ligand-dependent induction at 37°C is fully consistent with the anticipated scenario illustrated in Figure 1D. The stabilized 4U thermometer prevented *bgaB* expression at all four conditions suggesting that the transcript present in the plus ligand state cannot be translated even at high temperature (Figure 2B). The derep RNAT was unable to confer translational control resulting in expression values almost identical to the theo switch alone.

Dual control was also achieved by the TPP-4U fusion containing the TPP off-switch upstream of the 4U thermosensor (Figure 2C). The TPP riboswitch alone repressed β-galactosidase expression effectively in the presence of ligand at both temperatures. The 4U RNAT was unresponsive to the ligand and solely responded to temperature. When both RNA modules were fused, reporter gene activity was clearly reduced at 25°C in the minus ligand state although the riboswitch is in a transcription-permissive state. Similar to the theo-4U* fusion (Figure 2B), the TPP-4U fusion conferred tighter control than either regulatory element alone (Figure 2C). The relevance of RNAT-mediated translational control to this expression pattern was confirmed by the fusion containing the stable thermometer, which blocked expression under all conditions. The derep variant was unable to interfere with translation. Comparable results were obtained with a third riboswitch-RNAT pair consisting of the *lysC* off-switch (lys) from *B. subtilis* (36) and the 4U RNAT (Supplementary Figure S2A).

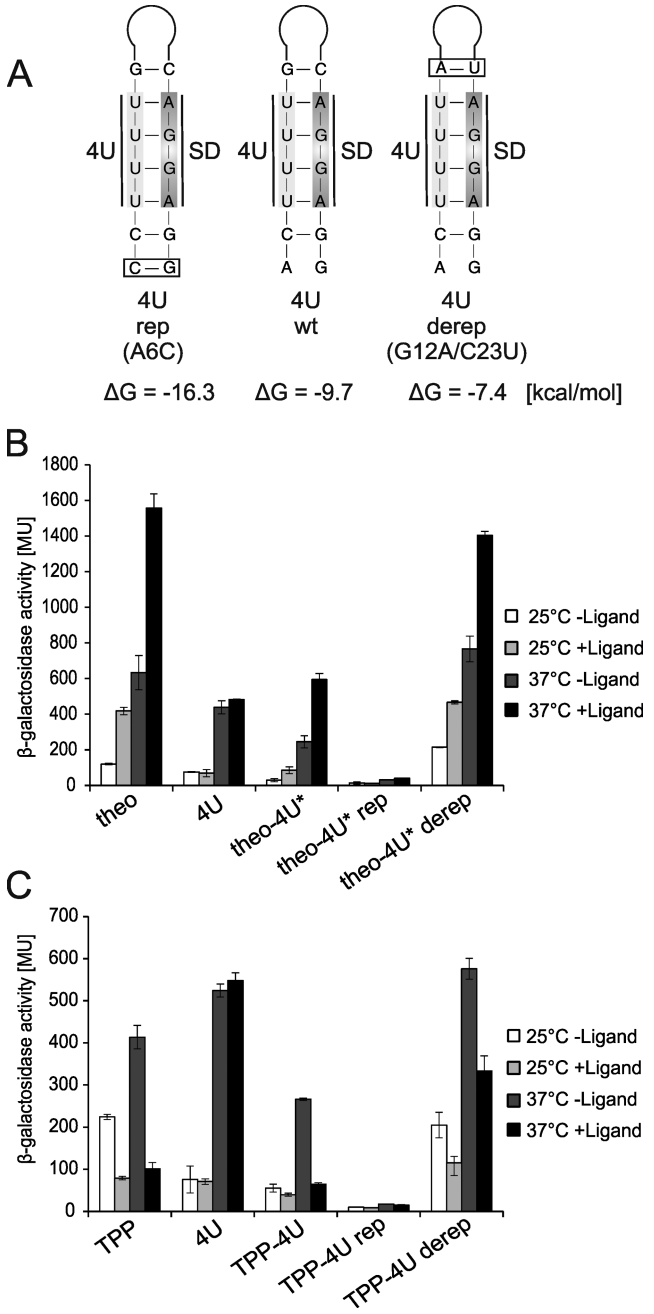

Figure 2. Riboswitch-RNAT fusions bearing on- or off-riboswitches. (**A**) Site-directed mutagenesis generated a stabilized (A6C, rep) and a destabilized (G12A/C23U, derep) variant of the 4U RNAT in the riboswitch-RNAT fusion. (**B** and **C**) Measurements of β-galactosidase activity [Miller units (MU)]. Cells were grown in presence (light grey and black bars) or absence (white and dark grey bars) of 2 mM theophylline or 100 μM thiamine at the indicated temperatures. Transcription from the P_{BAD} promoter was induced by 0.01% L-arabinose in rich medium or by 0.1% L-arabinose in M9 medium, respectively. The experiment was performed in triplicate with three replicates each. Mean standard deviation is indicated.

Synthetic regulators are able to coordinate complex cellular behavior

Given the ability of riboswitch-RNAT fusions to control reporter gene expression in response to independent signals, we asked whether such fusions can regulate complex cellular processes, like chemotaxis and motility. Therefore, we constructed a series of translational fusions to *cheZ*. CheZ is one of six chemotaxis proteins in *E. coli* (CheA, B, R, W, Y and Z), which regulate the rotational direction of the flagellar motor and thereby cell motility (39). *E. coli* cells lacking *cheZ* are non-motile (40). Translational *cheZ* fusions to the 4U RNAT, the theo and TPP riboswitches and the corresponding riboswitch-RNAT fusions were transformed into an *E. coli* Δ*cheZ* mutant. The wild-type (wt) strain migrated equally well on semisolid agar plates at all tested temperatures and the Δ*cheZ* mutant was unable to move. Swimming of Δ*cheZ* cells was successfully restored by the 4U RNAT fusion to *cheZ* only at 37°C (Figure 3A and B). Cells harboring the theo on-riboswitch were motile when ligand was present at all tested temperatures (Figure 3A; quantification in Supplementary Figure S3A). Conversely, the TPP off-switch conferred motility only in the absence of ligand (Figure 3B and Supplementary Figure S3B).

As expected (Figure 1D), cells carrying the theo-4U*-*cheZ* construct were most motile in the presence of ligand at 37°C (Figure 3A). The theo-4U* rep fusion was unable to confer motility to the Δ*cheZ* mutant and motility of the strain carrying the derep variant depended on the presence of ligand but not on temperature (Figure 3A and Supplementary Figure S3A). Also as expected, the TPP-4U fusion strain displayed highest motility in the absence of ligand at 37°C (Figure 3B). The rep variant did not complement the Δ*cheZ* mutant under any conditions whereas the derep variant of TPP-4U restored motility in the absence of ligand at all temperatures. Similar results were obtained with the lys-4U fusions (Supplementary Figure S2B and S3C) suggesting that riboswitches from different classes retain functionality in riboswitch-RNAT fusions.

Visual inspection of the swimming plates was complemented by Northern blot experiments probing for the *cheZ* transcript. Generally higher or lower transcript levels in the presence of ligand for the theo on-switch and TPP off-switch, respectively, suggest functionality of the transcriptional riboswitches (Figure 3C and D; quantification in Supplementary Figure S4). Despite equally high amounts of transcript originating from the theo-4U* fusion at all three temperatures in the presence of theophylline, the cells were motile only at 37°C establishing that translational control by the thermosensor operates in this context (Figure 3C and A). Cumulatively, these results demonstrate that dual transcriptional and translational control by the consecutive fusions in response to two input signals works in various synthetic arrangements.

Design of 'thermoswitches' by integration of a thermosensor into riboswitches

In an alternative approach to generate RNA elements responding to two independent signals, we systematically replaced individual hairpins of a riboswitch by a temperature-sensitive thermometer hairpin (Figure 4). First, we chose the well-characterized *Salmonella* 4U RNAT and the *tenA* riboswitch (TPP) from *B. subtilis* (32,33). The TPP aptamer consists of five stems (Figure 5A). Stem P1 links aptamer domain and expression platform of the TPP riboswitch and

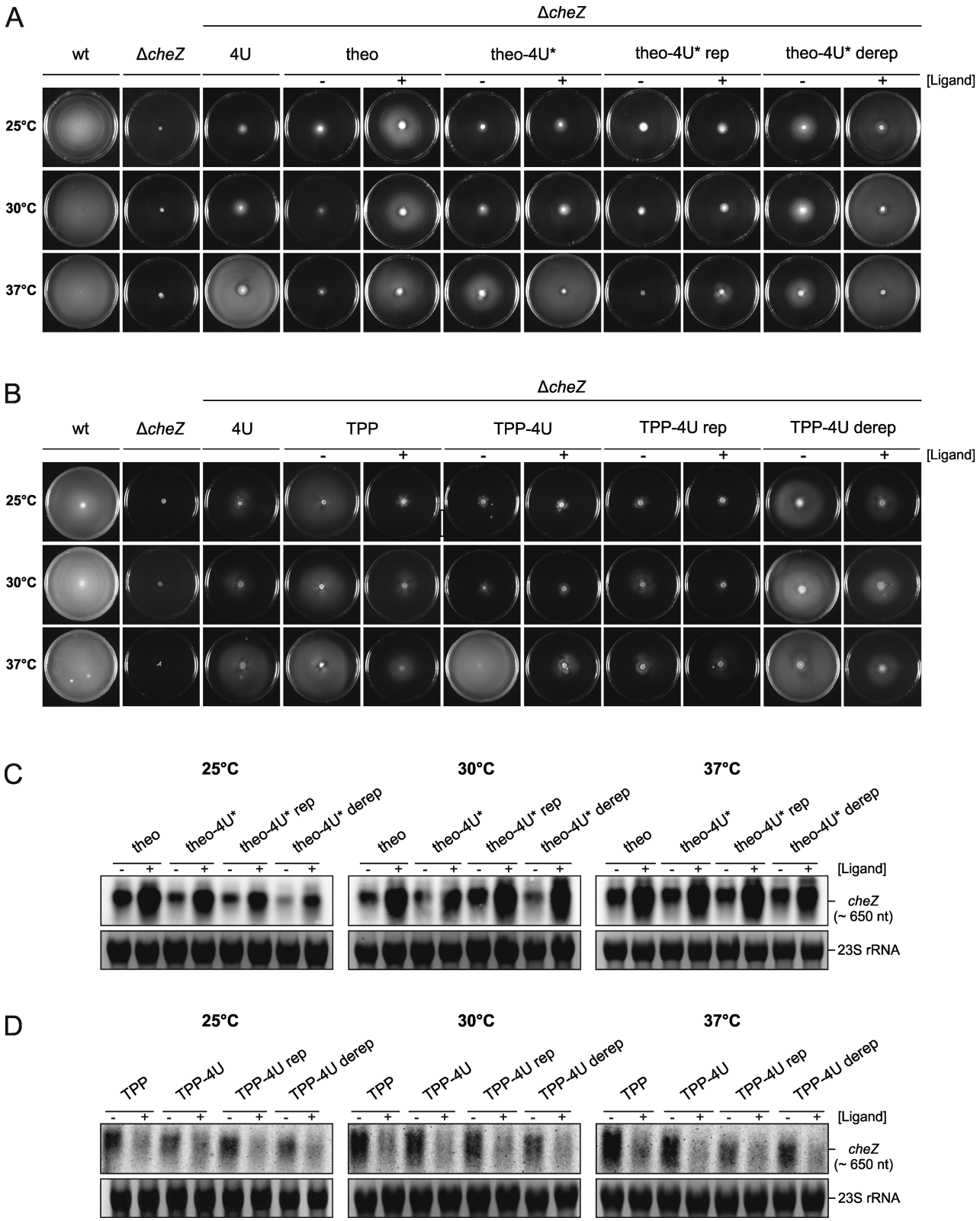

Figure 3. Motility tests on semisolid media. (**A** and **B**) *E. coli* BW25113 (wt) or BW25113 Δ*cheZ* or BW25113 Δ*cheZ* cells harboring plasmids with translational *cheZ* fusions were grown at the indicated temperatures until OD_{600} of 0.5 in the presence or absence of 2 mM theophylline or 100 μM thiamine. 5 μl cell suspensions were spotted on plates containing 0.25% agar and incubated at 25, 30 or 37°C. The experiment was performed in duplicate with five replicates each. (**C** and **D**) Northern blot analyses of *cheZ* transcript with a probe directed against the *cheZ* reporter. Ethidium bromide-stained 23S rRNA served as loading control.

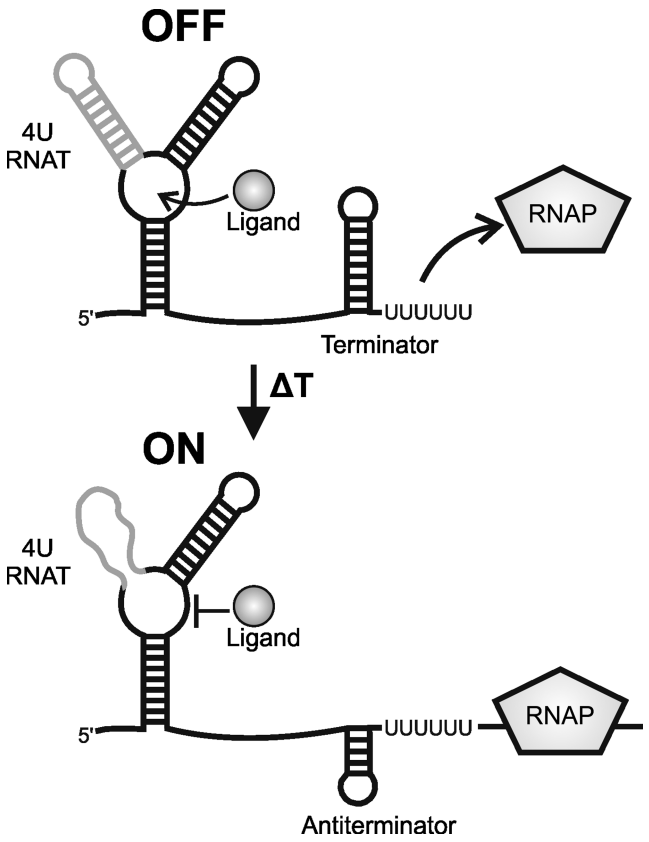

Figure 4. Predicted mechanism of RNAT-controlled riboswitches (thermoswitches). A hairpin in the secondary structure of a riboswitch aptamer domain is replaced by an RNAT. At low temperatures, the paired RNAT allows formation of the ligand binding pocket. Ligand binding leads to formation of a terminator in the expression platform and transcription is turned OFF. Melting of the RNAT at elevated temperatures destabilizes the aptamer domain, resulting in deficient ligand binding. In the expression platform an antiterminator forms and transcription is turned ON. RNAP: RNA polymerase.

contains parts of the antiterminator sequence (33). Stems P2 and P3 are involved in binding of the pyrimidine part of TPP whereas stems P4 and P5 have a function in binding the pyrophosphate group (41–44). The *B. subtilis* TPP aptamer comprises an additional not-conserved region, called stem P3a (Figure 5A and B).

The 4U thermometer was integrated into the TPP aptamer at various positions and the constructs were analyzed in the pBAD2-*bgaB* reporter gene system (30). The TPP riboswitch alone was able to repress β-galactosidase expression in the presence of ligand consistent with its being an off-switch (Figure 5C). Replacement of stem P1 by the thermosensor (termed P1::4U) abolished expression under all tested conditions. Conversely, exchange of stem P3, P3a and P5 resulted in uncontrolled high reporter gene activities that did not respond to the ligand. Apparently, manipulating riboswitch hairpins bears the risk of inactivating its regulatory function.

To optimize the constructs, we added additional base pairs at the bottom of stems P3::4U and P3a::4U (Figure 5B). Insertion of one base pair did not significantly improve ligand responsiveness (Figure 5D). Addition of a second base pair to P3::4U resulted in a ligand-induced repression to 40% but this repression was not relieved at 37°C suggesting that the thermosensor was not functional. Finally, addition of two base pairs (corresponding to the authentic nucleotides in the wt P3a hairpin) at the bottom of the RNAT in P3a::4U resulted in the desired behavior. This P3a::4U* construct was responsive to its ligand at 25°C but was barely shut-off by ligand addition at 37°C presumably because melting of the thermosensor interfered with ligand binding.

To provide evidence that the expression pattern of the optimized variant P3a::4U* depends on melting of the 4U thermometer, site-directed mutagenesis was performed in order to stabilize (rep) or loosen (derep) the RNAT structure. Variant G118A carrying a nucleotide exchange at the bottom of P1 (Figure 5B) served as a control for a riboswitch unable to turn off transcription. This variant was described to be deficient in ligand binding (33) and, as expected, produced β-galactosidase activities regardless if ligand was present or not (Figure 5E). P3a::4U* rep lost temperature control and always repressed reporter gene expression in the presence of ligand suggesting that the thermosensor remained in a stable conformation at high temperature. Conversely, P3a::4U* derep was completely unable to inhibit *bgaB* expression in the presence of ligand presumably because the 4U RNAT is in an open conformation at both temperatures.

A similar mode of action was established for the *lysC* riboswitch. Here, stem P3 and P4 could be substituted for the 4U RNAT resulting in a temperature-responsive riboswitch (Supplementary Figure S5).

The TPP thermoswitch mediates temperature-dependent transcriptional control

Northern blot experiments provided further evidence for transcriptional regulation by P3a::4U*. *E. coli* cells carrying translational fusions to *gfp* were grown at 25, 30 or 37°C in the absence or presence of 100 μM thiamine prior to RNA isolation. Using a probe directed against the TPP aptamer allowed simultaneous detection of the full-length *gfp* transcript and the short termination product. The natural TPP riboswitch reduced *gfp* levels in the presence of ligand at all tested temperatures (Figure 6A). The G118A construct produced high quantities of full-length *gfp* mRNA under all conditions consistent with its defect in ligand binding. The engineered P3a::4U* thermoswitch showed the anticipated pattern, in which *gfp* transcript levels dropped in presence of ligand at low but not at high temperature. These results and the results from the rep and derep variants are in line with the β-galactosidase dataset (Figure 5) and were further validated on protein level by Western blot analyses with an α-GFP antibody (Figure 6B).

The massive amounts of termination products detected by Northern blot analyses were unexpected. They did not change in response to the growth conditions and always exceeded the amounts of full-length *gfp* mRNA (Figure 6A). To the best of our knowledge, the amount of termination products and the relative concentrations of the truncated and full-length products have not been examined in any of the previous publications on the *tenA* TPP riboswitch

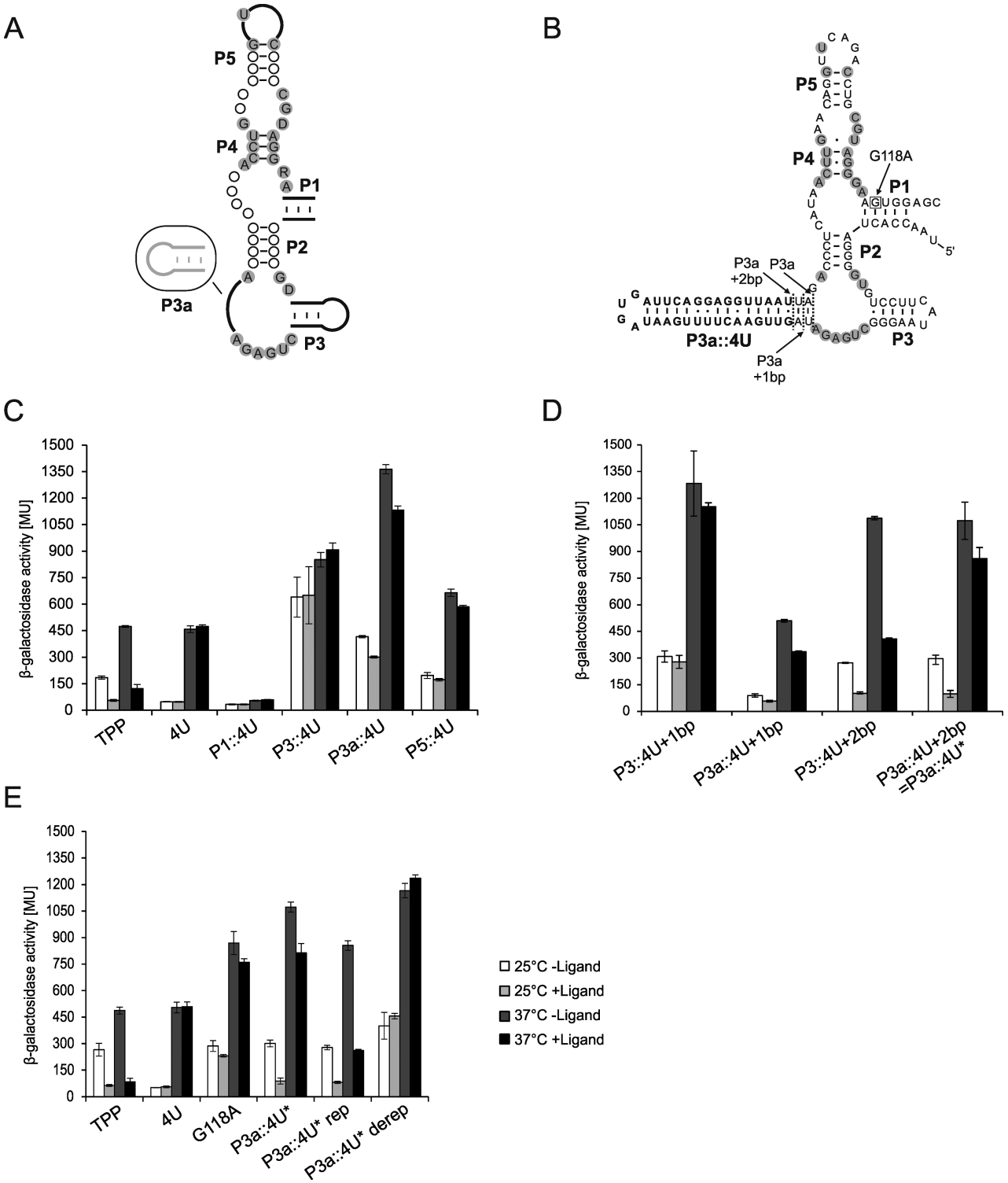

Figure 5. Optimization and functionality of the TPP thermoswitch. (**A**) Consensus structure of the TPP riboswitch class according to Mandal and Breaker (47) and Winkler and Breaker (48). Nucleotides encircled in grey are conserved in TPP riboswitches. Circles indicate a nucleotide of any base identity. Thick lines represent non-conserved regions. (**B**) Stem P3a of the *B. subtilis tenA* riboswitch (TPP) is exchanged against the temperature-sensing 4U hairpin of the *Salmonella agsA* RNAT (bold nucleotides) (19). Dotted lines indicate spacer nucleotides. (**C**–**E**) Measurements of β-galactosidase activity [Miller units (MU)]. Cells were grown in presence (light grey and black bars) or absence (white and dark grey bars) of 100 μM thiamine at indicated temperatures. Transcription from the P_{BAD} promoter was induced by 0.1% L-arabinose. The experiment was performed in triplicate with three replicates each. Mean standard deviation is indicated.

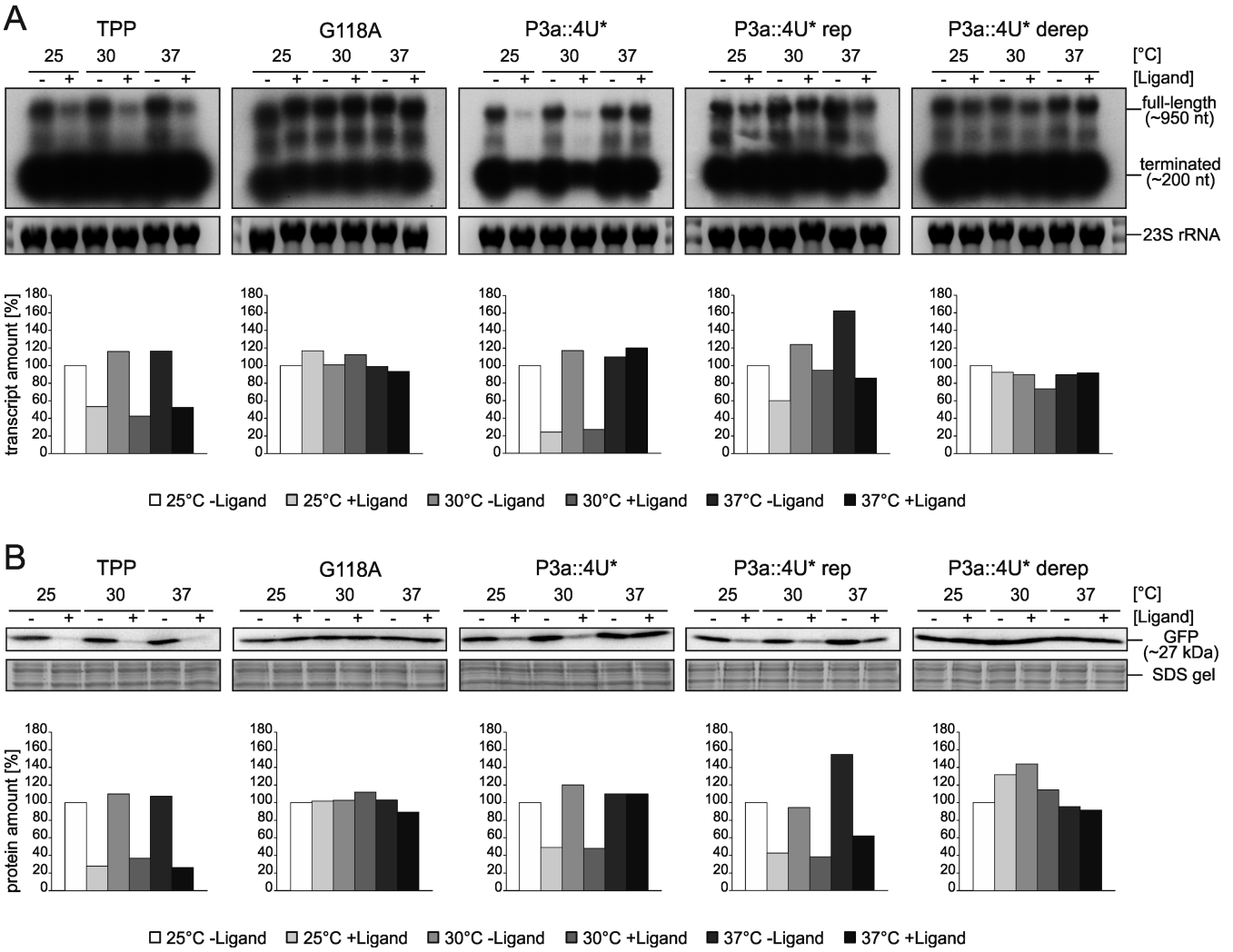

Figure 6. Comparative Northern and Western blot analyses of the thermoswitch P3a::4U* and its variants. Northern (**A**) and Western (**B**) analyses of *gfp* mRNA and GFP protein levels. *E. coli* ER2566 cells carrying translational *gfp* fusions were grown at the indicated temperatures previous to RNA or protein isolation. For detection of RNA levels a probe directed against the *B. subtilis tenA* 5′ UTR was used. Ethidium bromide stained 23S rRNA served as loading control. Synthesized GFP protein was detected with α-GFP antibody. Band intensities were quantified using the Alpha Ease software and normalized to transcript/protein detected at 25°C in the minus-ligand sample.

(32,33,45,46). Our results seem to indicate that transcription termination by the TPP riboswitch is a frequent event occurring under all conditions, and that presence of ligand only partially shifts the equilibrium toward the read-through conformation.

Probing the thermoswitch structure

Finally, we performed comparative structure probing experiments to demonstrate temperature-controlled melting of the RNAT element in the thermoswitch P3a::4U*. *In vitro*-transcribed RNA of the natural TPP riboswitch and different thermoswitch constructs were probed at 25 and 37°C in the absence or presence of 100 μM TPP, a concentration used under *in vivo* conditions. The secondary structure was mapped by RNase T1 (cuts single-stranded guanines) and nuclease S1 (cuts 3′ of unpaired nucleotides) and resulted in a cleavage pattern consistent with the known consensus structure of TPP riboswitches (Figure 7A) (47,48). Cleavage by RNase T1 confirmed melting of the 4U RNAT in the TPP aptamer domain. Guanines G61, G62, G64 and G65 (marked with asterisks in Figure 7B), which are located in and around the SD sequence of the natural 4U thermosensor, were accessible exclusively at 37°C. The structure of P3a::4U* rep was very stable and fully protected from digestion by the enzymes even at 37°C. As expected, the derep variant was accessible to both enzymes already at 25°C leading to cleavage products from nucleotide G37 to U70 especially for nuclease S1.

Fine-mapping of the temperature-responsive thermoswitch structure confirmed these results (Supplementary Figure S6). Partial digestion by RNase T1 in a temperature-range from 20 to 45°C revealed that melting of the guanines G61, G62, G64 and G65 (marked with asterisks in Supplementary Figure S6) of the 'SD' sequence (which does not act as ribosome binding site in this context and is therefore labeled as 'SD') started around 35°C,

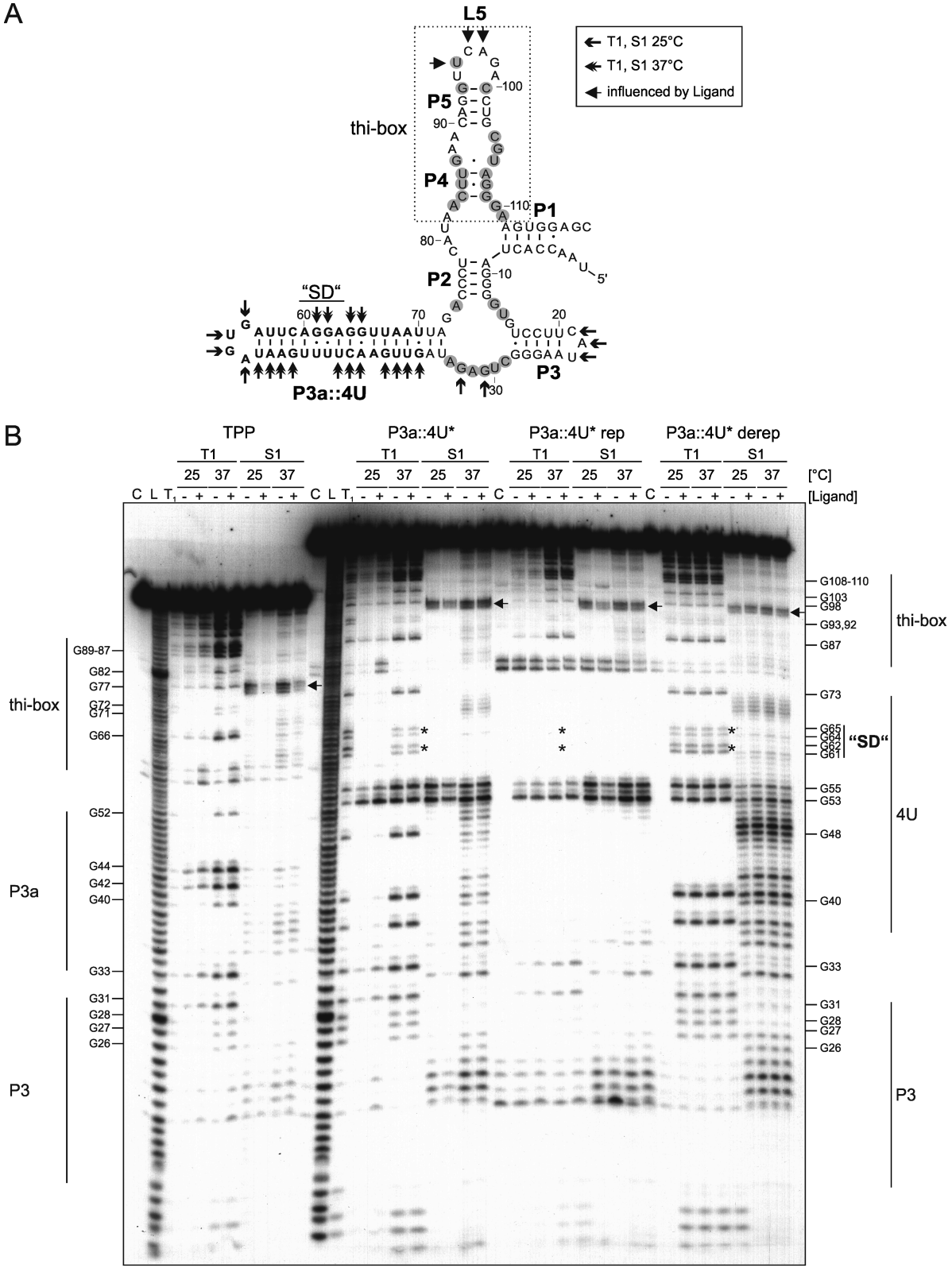

Figure 7. Structure probing of the *B. subtilis* TPP riboswitch and thermoswitch P3a::4U* and its variants. (**A**) Secondary structure of the TPP riboswitch aptamer domain from *B. subtilis*. Cleavage sites introduced by RNase T1 or nuclease S1 are indicated by arrows. (**B**) Partial digestion of radioactively labeled RNA was performed with RNase T1 (0.00125 U) and nuclease S1 (0.125 U) at 25 and 37°C. RNA samples were incubated with or without 100 μM TPP. Lane C: RNA treated with water instead of RNase served as control, lane T_1: RNase T1 cleavage in sequence buffer at 45°C, L: alkaline ladder.

whereas no melting was observed for the stabilized rep variant throughout this temperature range. The presumed thermo-labile structure of the derep RNA is supported by cuts of guanines G61, G62, G64 and G65 starting at 20°C and substantial cleavage at higher temperatures.

Less pronounced structural rearrangements were observed in the riboswitch part upon ligand binding. Ligand-dependent alterations in the TPP riboswitch structure were found at U72, C73 and A74 (marked with an arrow in Figure 7B). Cleavage of these nucleotides by nuclease S1 decreased in the presence of ligand suggesting a switch from single- to double-stranded. These nucleotides are located in the highly conserved loop L5 that is crucial for TPP binding (49). Interestingly, the structure of this region was slightly influenced by melting of the 4U RNAT. S1 cuts at nucleotides U95, C96 and A97 in the thermoswitch P3a::4U* were stronger without ligand and decreased in presence of ligand at 25°C, whereas at 37°C they were equally susceptible to the nuclease. The rep variant showed a cleavage pattern for these three nucleotides comparable to the TPP riboswitch although the cleavage decreased not as strongly as for U72, C73 and A74 of the TPP riboswitch. Nucleotides 95 to 97 of the derep variant were cleaved nearly equally by nuclease S1 suggesting that TPP has no effect on the secondary structure in this variant.

Cumulatively, the complementary set of *in vivo* and *in vitro* experiments demonstrates that the thermoswitch has a novel synthetic functionality, in which the thermometer integrated into the riboswitch controls ligand binding.

DISCUSSION

Several tandem riboswitches exist in nature. For example, the *tenA* 5′ UTR of *Bacillus anthracis* and the *metE* 5′ UTR of *Bacillus clausii* are built from two consecutive riboswitches both controlling transcription termination. Both riboswitches upstream of the *tenA* gene sense TPP (50), whereas the first riboswitch upstream of *metE* senses SAM (S-adenosyl methionine) and the second AdoCbl (51) allowing the integration of two separate signals. In both cases the riboswitches do not influence each other but function independently. Each chemical signal leads to transcription termination and each riboswitch alone is sufficient to repress gene expression. In the *bhmT* 5′ UTR of the marine α-proteobacterium *Candidatus Pelagibacter ubique*, a tandem SAM riboswitch controls two stages of gene expression, transcription and translation (52). The first SAM riboswitch forms a terminator upon ligand binding and stops transcription before the second aptamer and the downstream open reading frame are synthesized. Low ligand concentrations permit both transcription and translation. If ligand concentrations increase after the full-length mRNA has been transcribed, translation can still be repressed by the second SAM riboswitch.

All these known tandem elements respond to chemical cues. The purpose of our study was to combine transcriptional riboswitches with a translational thermosensor in different ways to generate novel RNA regulators that respond to a chemical and a physical signal. Such systems may be superior to dual riboswitches for biotechnological applications because most chemical inducers are expensive and gene expression typically is leaky. Consistent with previous observations, we found that neither riboswitches nor RNATs alone mediate a complete on- or off-response but rather fine-tune gene expression. For example, gene expression permitted by the 4U thermometer at 25°C or by the theo on-switch in the absence of ligand was still rather high but almost completely abolished by the theo-4U* construct suggesting that consecutive riboswitch-RNAT fusions have the potential to confer tight control to gene expression systems. Tight shut-off of gene expression is critical in many biotechnological processes, in particular when toxic products are synthesized. Depending on the application, various scenarios are conceivable, in which an on- or off-riboswitch is combined with an RNAT to strictly prevent heterologous expression at low temperature but turn on expression in the absence or presence of a ligand once the cultures reaches higher temperatures during fermentation.

Both types of building blocks used in our approach are wide-spread in nature and therefore promising as versatile control elements. The zipper-like melting of RNATs is simple and does not seem to depend on accessory factors (15). The *Salmonella* 4U RNAT used here is functional in quite different bacteria like *E. coli* (19) and *B. subtilis* (data not shown) suggesting that the sensory mechanism itself is largely independent of the organism. Also RNATs from cyanobacteria or from pathogens like *Yersinia pseudotuberculosis* or *Vibrio cholerae* work fine in *E. coli* (20,53,54). It is important to note, however, that some RNATs can be affected by cellular ingredients. Binding of magnesium ions to the 4U motif of the *agsA* RNAT *in vitro* stabilizes its secondary structure and thereby increases the melting temperature (38). Since the intracellular magnesium concentration is fairly robust under normal growth conditions this feature should not interfere with the applicability of the 4U elements. Otherwise, RNATs that are not affected by magnesium, like the ROSE element (55) can be used. Riboswitches can also work outside their organismic context as shown by the riboswitches from *B. subtilis* that were used in *E. coli* in our study. The sensory mechanism of riboswitches is usually more complicated than that of RNATs and other factors like ligand uptake systems and intracellular metabolism come into play. Several riboswitches are conserved among bacteria and in some cases the same gene is regulated by riboswitches in different bacteria although the mechanistic details can be very different. For example, the *lysC* genes in *E. coli* and *B. subtilis* are both downregulated in the presence of the amino acid lysine but in *E. coli* on the translational level and in *B. subtilis* on the transcriptional level (35). At least one riboswitch, the TPP riboswitch, is not only present in bacteria but also in eukaryotes like fungi and plants (56,57). Based on their RNA-only mechanisms and their transferability between organisms, riboswitches and RNATs are promising tools for synthetic applications and our study demonstrates that riboswitches from different classes (TPP and lysine) as well as synthetic riboswitches (theophylline) can be combined with an RNAT in modular ways to gain functional regulatory elements.

In our second approach, we rendered riboswitches susceptible to temperature by the replacement of an internal hairpin by an RNAT. We produced synthetic riboswitches unable to turn off expression at elevated temperatures due

to melting of the thermosensor and called them 'thermoswitches'. Recently, a natural riboswitch was described that modulates its ligand affinity in response to temperature. The adenine-sensing on-riboswitch *add* from *Vibrio vulnificus* employs a three-state mechanism (58). It exhibits two ligand-free conformations, apoA and apoB, to counterbalance the increased affinity for adenine at low temperature. Here, the binding-incompetent apoB structure is favoured, whereas an increase in temperature shifts the equilibrium towards the apoA conformation. Temperature-dependent destabilization with yet unknown biological implications was observed for the *xpt-pbuX* guanine riboswitch from *B. subtilis* by NMR spectroscopy (59). Both mechanisms are distinct from the typical zipper-like thermosensor that we installed into the TPP and lys riboswitches. The thermoswitches are off in the presence of their cognate ligand only at low temperature. This might have potential in settings when repression by an intracellular ligand needs to be overcome. This can then simply be achieved by a temperature upshift.

To gain functional thermoswitches it is essential to find a region that can be replaced against an RNAT without losing riboswitch activity. Aptamer domains of riboswitches are highly conserved and any modification can easily perturb ligand binding. We found that only the exchange of helix P3a retained riboswitch function. As stem P1 contains parts of the antiterminator sequence and has a stabilizing function for the whole riboswitch structure (60), stem P3 binds the pyrimidine moiety of TPP (44) and P5 is part of the conserved thi-box (61), they were unlikely candidates for a functional thermoswitch. Even though the non-conserved stem P3a has ideal prerequisites for genetic manipulation, generation of a functional riboswitch-RNAT fusion still required optimization. The insertion of spacer sequences is common in synthetic biology when different elements are combined or when their performance needs to be improved. For instance, a communication module consisting of six randomized nucleotides was used to fuse a hammerhead ribozyme with an RNAT in order to produce a variant able to confer cleavage of the mRNA in response to temperature (25). The same strategy was applied when a hammerhead ribozyme was combined with the theophylline aptamer (62). Selection procedures for suitable communication modules from random sequences entail a time-consuming screening process. A more straight-forward strategy uses spacers of defined length and sequence, for example a 19-nt spacer reduced background activity and increased the activation ratio of a *de novo* designed theophylline riboswitch (27). We used a linker of 20 nt to ensure sterically correct folding of the theo riboswitch and the 4U RNAT in the theo-4U* fusion. However, examples from our study (TPP-4U, lys P3::4U and lys P4::4U) and from the literature show that occasionally RNA modules can be combined directly without the requirement of any spacer. The aptamer domain of the guanine-sensing *xpt* riboswitch was fused with the expression platform of the *metE* SAM riboswitch resulting in a functional *xpt*/*metE* chimera (63). All these studies demonstrate that *cis*-acting RNA regulators can be mixed and matched in various combinations but often require careful design.

One goal of synthetic biology is to generate new functionalities. By using two different design strategies, we obtained riboregulators with novel activities that have not been found in nature. In our consecutive chimera of transcriptional riboswitches and a translational RNAT, the thermosensor had superordinate control over the riboswitches, regardless of whether they were on or off switches. The integrative chimera can be regarded as entirely novel regulatory devices. In contrast to the more versatile riboswitches, all presently known RNATs control translation by masking the ribosome binding site. The thermoswitches are the first examples of temperature-sensors that do not regulate translation but transcription by controlling the formation of terminator structures.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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