

Directional co-transcriptional folding and pausing create kinetic checkpoints for riboswitch-controlled gene expression

Nils Walter

nwalter@umich.edu

University of Michigan <https://orcid.org/0000-0002-7301-1275>

Adrien Chauvier

University of Michigan-Ann Arbor <https://orcid.org/0000-0003-4473-6194>

Javier Cabello-Villegas

University of Michigan <https://orcid.org/0000-0001-8726-433X>

Edward Nikonowicz

Rice University

Article

Keywords: Co-transcriptional RNA folding, ligand-sensing riboswitch, RNA polymerase, single molecule fluorescence microscopy, transcription

Posted Date: March 13th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8735691/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: There is **NO** Competing Interest.

1Directional co-transcriptional folding and pausing create
2kinetic checkpoints for riboswitch-controlled gene
3expression

4

5Adrien Chauvier¹, Javier Cabello-Villegas¹, Edward P. Nikonowicz² and Nils G. Walter^{1,*}

6

7¹Single Molecule Analysis Group and Center for RNA Biomedicine, Department of Chemistry,
8University of Michigan, Ann Arbor, Michigan, USA.

9²Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005, USA.

10* e-mail: nwalter@umich.edu

11

12

13

14ABSTRACT

15Transcriptional riboswitches typically regulate gene expression by sensing small metabolites
16or ions that cannot be fluorophore-labeled without perturbing recognition, limiting direct
17observation of ligand binding during transcription. The *GlyQS* T-box riboswitch instead
18senses the uncharged 3' end of a macromolecular ligand, tRNA^{Gly}, enabling visualization of
19co-transcriptional ligand binding. Using single-molecule fluorescence microscopy, we show
20that tRNA^{Gly} dynamically samples discrete structural domains of the nascent riboswitch as
21transcription proceeds. Productive recognition depends on a hierarchical 5'-to-3' folding
22pathway shaped by transcription rate and pausing. Pausing enhances tRNA anchoring,
23stabilizes the riboswitch–tRNA complex, and selectively promotes RNA polymerase
24readthrough when an uncharged 3' end is presented. These results support a kinetic
25checkpoint model in which early recruitment increases local ligand concentration, while
26pause-mediated stabilization converts transient encounters into committed regulatory
27decisions. More broadly, our findings identify directional co-transcriptional folding and
28transcriptional pausing as principles synergistically enabling selective and robust riboswitch
29function.

30

31KEYWORDS: Co-transcriptional RNA folding, ligand-sensing riboswitch, RNA polymerase,
32single molecule fluorescence microscopy, transcription

33Introduction

34A defining feature of gene regulation mediated by non-coding RNAs is that it is intimately
35coupled to the progress of transcription, with the nascent RNA beginning to fold and adopt
36functional conformations as soon as it emerges from the RNA polymerase (RNAP)¹. Co-
37transcriptional 5'-to-3' folding imposes a dominant directional hierarchy to which RNAs have
38evolutionarily adapted, ensuring proper function in gene regulation^{2,3}. In parallel, intrinsic
39pausing of RNAP at DNA-encoded sites and interactions with transcription factors have emerged
40as critical modulators of functional RNA folding, but the connections between co-transcriptional
41RNA folding and RNAP pausing remain underexplored^{2,4}.

42 In bacteria, riboswitches are ubiquitous genetic elements, commonly found in the 5'-
43untranslated regions (UTRs) of messenger RNAs (mRNAs), where they respond to cellular
44metabolites or elemental ions to regulate gene expression at the level of transcription or
45translation⁵. Because they change conformation “on-the-fly,” riboswitches serve as tractable
46models for studying the role of transcription in shaping nascent RNA folding. Ligand sensing by
47the riboswitch aptamer domain has often been viewed as straightforward due to the presence of a
48well-conserved, robust ligand binding pocket⁶. However, recent studies on both transcriptional
49and translational riboswitches have revealed the coexistence of metastable structural
50intermediates that exchange on the timescale of transcription, defining a kinetic window for
51ligand binding and downstream expression platform manipulation⁷⁻¹². This concept of a
52kinetically controlled structural partitioning is particularly evident in transcriptional
53riboswitches, where ligand recognition and any regulatory effects must occur before the RNAP
54reaches the termination point just downstream of the regulating RNA motif¹²⁻¹⁵.

55 T-box riboswitches are critical riboregulators, predominantly found in Gram-positive
56bacteria, where they control the expression of genes involved in amino acid synthesis, transport,
57and tRNA aminoacylation. This includes the archetypical *Bacillus subtilis* (*Bsu*-)*GlyQS* T-box
58riboswitch, which regulates the *GlyQS* operon encoding the glycyl-tRNA synthetase enzyme¹⁶.
59Each T-box binds a specific tRNA, sensing the uncharged 3'-aminoacylation state as a metabolic
60signal that guides gene expression¹⁷. A typical transcriptional T-box riboswitch is modular and
61consists of two main domains: a 5'-decoding or aptamer domain (including Stems I, II, and
62IIA/B) and a 3'-discriminator domain (including Stem III and the competing transcriptional
63Antiterminator, AT, and Terminator, T, hairpins), connected by a single-stranded linker¹⁷. The
64decoding domain binds the tRNA through its Stem I, while the discriminator domain senses the
65charging state of the tRNA 3'-NCCA end, shifting its conformation typically from the T to the AT
66hairpin to allow downstream transcription. While the decoding region exhibits variability, the
67overall domain architecture is highly conserved across bacterial species¹⁸.

68 Recent structural advances through X-ray crystallography^{19–21} and cryo-electron
69microscopy²² have provided detailed, yet static views of the T-box riboswitch core in complex
70with tRNA ligands, highlighting three interaction sites for the anticodon, elbow and 3'-NCCA
71end (Fig. 1a). These studies have clarified how specific contacts stabilize high-affinity tRNA
72binding and uncharged 3'-end recognition. Nevertheless, the dynamic conformational changes
73underlying tRNA sensing—particularly during the continuous release of 3'-sequence by the
74transcribing RNAP—have remain enigmatic. Recent single-molecule fluorescence resonance
75energy transfer (smFRET) studies of defined RNA-only complexes^{23–26} suggest that tRNA
76engagement occurs via a hierarchical two-step mechanism that adjusts the riboswitch

77conformation, with implications for gene regulation²⁷. Despite these advances, the real-time
78dynamics of ligand binding during transcription have remained elusive.

79 Beyond their mechanistic intrigue, and given their prevalence in pathogenic bacteria, T-
80box riboswitches have garnered attention as potential antimicrobial targets^{18,28,29}. Elucidating the
81detailed mechanisms by which T-box riboswitches sense and respond to tRNA binding thus
82promises not only to advance our understanding of bacterial gene regulation but also to inform
83the development of novel antimicrobial strategies^{30,31}. Together, these motivations call for the
84development of tools that reveal the temporal coordination of ligand recognition and structural
85rearrangements of the elongating T-box sequence.

86 In this study, we exploited the substantial size of the tRNA ligand to site-specifically
87introduce a Cy5 label at U46 with minimal functional disruption, generating U46^{Cy5}-tRNA, and
88used single-molecule fluorescence microscopy to dissect the dynamic co-transcriptional
89mechanism by which the *Bsu*-GlyQS T-box riboswitch activates gene expression. Our findings
90highlight the critical role of 5'-to-3' directional T-box RNA folding, which we find modulated by
91intrinsic RNAP pausing, for efficient tRNA^{Gly} sensing during transcription. Using the
92corresponding paused elongation complexes (PECs) together with *in situ* single-molecule
93probing approaches, we demonstrate that RNAP proximity enhances tRNA recruitment,
94facilitated by strategic positioning of the pauses. AT folding was found to expose the T-box
95motif, promoting a stabilized co-transcriptional capture of tRNA^{Gly}. Kinetic analysis of ligand
96binding further supports a hierarchical mechanism in which early, transient tRNA^{Gly} interactions
97with the first-transcribed Stem I (“sensing”) are crucial for subsequent 3'-NCCA end capture
98(“anchoring”) by the emerging AT. Moreover, by modulating transcription rates, we establish that
99RNAP speed constrains ligand recognition within a variable, but narrow kinetic window. Finally,

100deploying a double-PIFE (protein-induced fluorescence enhancement) approach, we link
101stabilized tRNA^{Gly} anchoring to transcriptional readthrough under near-physiological conditions,
102unmasking a temporally orchestrated regulatory mechanism. Together, these findings provide a
103comprehensive understanding of how the *Bsu-GlyQS* T-box riboswitch integrates dynamic,
104directional RNA folding and RNAP pausing with a hierarchical ligand sensing mechanism to
105regulate essential metabolic pathways—as a paradigm for riboswitch mechanisms more broadly
106and a steppingstone for antimicrobial interference with T-box function.

107

108Results

109**Probing co-transcriptional *Bsu-GlyQS* T-box function using fluorescently labeled tRNA.** The
110*Bsu-GlyQS* T-box riboswitch senses the uncharged tRNA^{Gly} to promote the transcription of the
111downstream *GlyQS* operon encoding glycyl-tRNA synthetase^{29,30}. As expected, single-round *in*
112*vitro* transcription of the *Bsu-GlyQS* T-box riboswitch displays efficient transcription readthrough
113in the presence of uncharged tRNA (Fig. 1b). In accordance with previous studies³⁰, we obtained
114an RT₅₀ (i.e., the concentration of tRNA^{Gly} needed to induce 50% of transcription readthrough
115regulation) of 4.9 ± 0.6 nM, showing efficient co-transcriptional ligand sensing and gene regulation
116upon tRNA^{Gly} binding (Fig. 1b).

117 To monitor how the *Bsu-GlyQS* T-box riboswitch senses its cognate ligand during
118transcription, we engineered a fluorescently labeled tRNA^{Gly} (Supplementary Fig. 1a,b)²⁵,
119assembled by ligating two synthetic RNA strands to incorporate a Cy5 fluorophore at position
120U46, within the tRNA elbow (U46^{Cy5}-tRNA)—far from the functionally critical 3'-NCCA end.
121Using this labeled uncharged tRNA during single-round *in vitro* transcription assays, we obtained

122an RT_{50} of 7.4 ± 2.5 nM (Fig. 1c). This only modestly elevated value may reflect a minor
123perturbation of the tertiary interaction between the dual T-loop motif (DTM) in Stem I and the
124elbow region of the labeled tRNA^{Gly}. By comparison, a Δ DTM mutant, which entirely disrupts
125this tertiary contact²⁵, showed a substantially (~16-fold) higher RT_{50} of 80 ± 7.6 nM
126(Supplementary Fig. 1c), highlighting the full extent of the DTM's contribution to tRNA binding
127while underscoring that perturbation by fluorophore labeling is minor.

128 Together, our results demonstrate that U46^{Cy5}-tRNA has robust functionality in promoting
129transcriptional readthrough, validating its suitability for single-molecule fluorescence studies.
130Unlike typical riboswitches that encapsulate their small-molecule or elemental ion effector
131within a tight binding pocket so that they cannot be fluorophore labeled, the T-box riboswitch
132model system thus offers a unique opportunity to introduce a fluorophore label and directly
133interrogate the dynamics of co-transcriptional ligand interactions at the single-molecule level.
134

135**Proximal RNAP stabilizes tRNA anchoring to two programmed paused elongation**
136**complexes.** Given the indirect evidence available for other transcriptional riboswitches for which
137ligand binding could not be observed directly^{13,14,32,33}, co-transcriptional tRNA sensing is
138expected to be required to efficiently promote transcription readthrough before the RNAP
139reaches the termination point. To measure the kinetics of ligand binding at functionally defined
140stages of transcription, we first performed transcription assays at limiting rNTPs concentration,
141confirming previously reported^{34,35} RNAP pause sites along the *Bsu-GlyQS* T-box transcript at
142positions 138, 178 and 207 (Supplementary Fig. 1d,e). We then assembled Paused Elongation
143Complexes (PECs) from the downstream biotinylated, streptavidin-capped DNA template,
144RNAP, and Cy3-labeled *Bsu-GlyQS* T-box RNA that was generated and folded by Cy3-primed

transcription in situ¹¹. We immobilized this complex on a biotin-PEG coated microscope slide¹⁴, and monitored U46^{Cy5}-tRNA binding events by colocalizing the Cy5 and Cy3 fluorescence signals in a total internal reflection fluorescence (TIRF) microscope (Fig. 2a-c). Consistent with earlier findings that used polymerase-free, isolated T-box RNA fragments of varying length²⁵, U46^{Cy5}-tRNA binding in the presence of RNAP is transient and dynamic, emphasizing the need for real-time single-molecule measurements to capture these events during the co-transcriptional sensing process.

For the early transcription intermediate PEC-138, which presents only Stem I outside the RNAP's exit channel, most U46^{Cy5}-tRNA^{Gly}-T-box interactions were short-lived: 90% dissociated within ~1 s and only a few persisting beyond 5 s (Fig. 2a and d). As *Bsu-GlyQS* transcription advanced to PEC-178 (adding the 5' segment of the T-box motif) and PEC-207 (adding the full T-box motif), the fraction of longer-lived binding events increased to ~12% and 25%, respectively, resulting in overall (weighted-average) decreasing dissociation rate constants of 0.40 (PEC-138), 0.28 (PEC-178) and 0.17 s⁻¹ (PEC-207; Fig. 2c,d and Supplementary Table 2). Concurrently, the overall binding rate constant (k_{on}) increased from $0.75 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at PEC-138 to $0.94 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at PEC-178 to $1.25 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at PEC-207 (Supplementary Table 2). These results support a model in which emergence of the downstream expression platform both accelerates local ligand recruitment and stabilizes the riboswitch-tRNA complex once formed.

To test whether the tRNA 3' end affects recognition, we compared PEC-207 binding of unmodified U46^{Cy5}-tRNA^{Gly} with tRNAs bearing two 3'-blocking modifications—either glycyl aminoacylation or a 3' Cy5 label. These modified tRNAs bound much less efficiently, with the overall association rate constant decreasing to $0.48 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (glycyl) and $0.32 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Cy5; Supplementary Fig. 4a–d). However, once complexes were formed, their lifetimes and the

fraction of long-lived events remained essentially unchanged (0.14 s^{-1} for glycyl and 0.21 s^{-1} for Cy5) relative to uncharged U46^{Cy5}-tRNA^{Gly} (0.17 s^{-1} ; Supplementary Fig. 4e), indicating that aminoacylation impairs association but does not destabilize already formed complexes. Furthermore, pre-incubation of PEC-207 with a high uncharged tRNA concentration (250 nM) revealed the capacity to form ultra-stable complexes (mean bound time $\sim 355\text{ s}$; Supplementary Fig. 3a–b), comparable to a previously report for an RNAP-free, isolated T-box construct of similar size²⁵. Together, these data indicate that steric obstruction of the 3' end (by tRNA charging or labeling) reduces sensing by lowering association efficiency rather than by shortening the complex's lifetime.

To quantify how the presence of RNAP alters ligand-sensing kinetics, we compared U46^{Cy5}-tRNA^{Gly} binding to PECs with binding to the corresponding released transcripts (RTs)—transcripts that were allowed to fold co-transcriptionally, but then released from the transcription bubble, and immobilized directly onto the TIRF surface (Fig. 2d). For RT-125, which contains only Stem I and lacks RNAP, tRNA binding closely resembles that observed for the corresponding PEC-138, indicating a minimal RNAP effect at this early pause. In contrast, the weighted-average dissociation rate constant is substantially higher for RT-125 (1.08 s^{-1}) compared to PEC-138 (0.40 s^{-1}), suggesting a stabilizing effect of RNAP during early transcription (Fig. 2c, d and Supplementary Table 2). Similarly, RT-207, which encompasses the full T-box motif but again is free of RNAP, showed a marked drop in the fraction of long-lived U46^{Cy5}-tRNA events ($\sim 7\%$) compared with PEC-207 ($\sim 25\%$), leading to a substantial ~ 3 -fold decrease of the weighted-average dissociation rate constant in the presence of RNAP (0.53 s^{-1} for RT-207 versus 0.17 s^{-1} for PEC-207) (Fig. 2c, d and Supplementary Table 2). Finally, RT-178 exhibited the highest tRNA complex

190stability among the released transcripts (Fig. 2d), presumably due to the absence of the
191complementary 3' segment of the AT hairpin.

192 These differences are consistent with a model in which RNAP sterically restricts folding
193of the 3'-most ~10-14 nucleotides that reside in the RNA–DNA hybrid and RNAP exit channel³⁶,
194thereby delaying formation of the AT and T hairpins in PEC-178 and PEC-207 and, as a
195consequence, promoting the T-box:tRNA 3'-end interaction (Fig. 2d). Supporting this idea, RT-207
196forms ultra-stable complexes with a mean bound time (~256 s) that is ~28% shorter than that
197measured for PEC-207 (Supplementary Fig. 3c,d), further indicating that completion of T-hairpin
198folding (permitted only when RNAP is absent) competes with and weakens tRNA 3'-end
199engagement by the Bsu-GlyQS T-box.

200 These data indicate that transcriptional pausing actively guides co-transcriptional folding
201and ligand recognition by delaying or preventing premature formation of competing 3'-end
202structures. Co-transcriptionally folded RTs recruit U46^{Cy5}-tRNA efficiently (Supplementary Fig.
2032e,f), and their binding rate constants mirror the trend observed for paused elongation complexes.
204Conversely, the proximal RNAP—specifically at pauses programmed at positions 178 and 207—
205enhances tRNA-anchoring stability during transcription by suppressing competing intrinsic T-box
206structures.

207

208**Folding of the antiterminator promotes stable tRNA anchoring.** To identify the key structural
209determinants required for stable co-transcriptional tRNA anchoring, we introduced mutations in
210the T-box motif (mutant Mbox) and the AT hairpin (MAT) (Supplementary Fig. 1a). These
211mutations were incorporated into PEC-207, which binds U46^{Cy5}-tRNA most efficiently and

212 features the fully transcribed AT domain, while minimizing potential interference from the
213 thermodynamically more stable T hairpin (Fig. 3).

214 As expected, disrupting the tRNA 3'-end recognition site (Mbox) substantially impaired
215 U46^{Cy5}-tRNA anchoring, yielding only short-lived binding events and an overall dissociation rate
216 constant comparable to that observed for Stem I only (1.16 s⁻¹ for PEC-207-Mbox) (Fig. 3a, d and
217 Supplementary Table 2). Accordingly, *in vitro* transcription assays using the Mbox mutation
218 showed no substantial effect of tRNA^{Gly} on transcription termination (Supplementary Fig. 1f),
219 reinforcing the essential role of this interaction in ligand-mediated gene regulation.

220 Strikingly, the PEC-207-MAT mutant exhibited binding behavior similar to PEC-207-
221 MBox, displaying only transient U46^{Cy5}-tRNA binding with short bound times (Fig. 3b, d and f).
222 This finding suggests that proper AT folding is essential for suitably presenting the T-box motif,
223 thereby enabling stable co-transcriptional tRNA anchoring. Similarly, both mutants showed
224 U46^{Cy5}-tRNA unbound times comparable to the Stem I-only RNA (PEC-138; Fig. 3e and
225 Supplementary Table 2), underscoring the importance of both T-box motif and AT hairpin also in
226 initial tRNA^{Gly} recognition. Notably, the introduced mutations reside within the switching region
227 that participates in both AT and T structures in the full-length transcript^{20,34} (Supplementary Fig.
228 1a), indicating that alternating structures in this region could affect tRNA^{Gly} anchoring during
229 transcription.

230 To further test this folding mechanism, we introduced compensatory mutations to restore
231 the base pairing at the bottom of the AT stem, stabilizing its predicted secondary structure (MATcp;
232 Supplementary Fig. 1a). In the context of the resulting PEC-207-MATcp, both short- and long-
233 lived U46^{Cy5}-tRNA binding events were recovered, further supporting the idea that AT folding
234 promotes T-box motif display for stable co-transcriptional tRNA anchoring (Fig. 3c, d and f)..

235 Together, our kinetic and mutational analyses reveal that AT folding promotes efficient co-
236transcriptional tRNA^{Gly} sensing by presenting the T-box motif for stable tRNA 3'-end recognition.
237

238**Real-time monitoring reveals sequential co-transcriptional recruitment and anchoring of**
239**tRNA.** Next, to understand when tRNA sensing and capture occur during active transcription, we
240employed a single-molecule assay that monitors both transcription and U46^{Cy5}-tRNA sensing in
241real-time^{14,37,38}. To this end, DNA templates encoding either Stem I only (*GlyQS*_125) or the full-
242length riboswitch prior to the termination point (*GlyQS*_213) were attached to the microscope slide
243through the 5'-biotinylated nascent RNA transcript¹⁴ (Fig. 4a and b). Upon simultaneous injection
244of rNTPs and U46^{Cy5}-tRNA, ligand sensing was visualized co-transcriptionally in real-time until
245the RNAP reached the end of the 5'-Cy3-labeled DNA template strand, as indicated by the
246occurrence of a PIFE signal (Protein Induced Fluorescence Enhancement)³⁹. For kinetic analysis,
247active transcription elongation complex traces were selected based on the occurrence of the PIFE
248signal and display of at least one U46^{Cy5}-tRNA binding event either co- or post-transcriptionally.

249 Consistent with our data from the stalled PEC-138 and released corresponding RT-125,
250PIFE-monitored real-time transcription of the *GlyQS*_125 template yielded few and short-lived
251U46^{Cy5}-tRNA binding events both during and after transcription (Fig. 4c and d). Supporting the
252functionality of the on-slide transcribed *Bsu-GlyQS* T-box RNAs, the post-transcriptional
253U46^{Cy5}-tRNA bound time (~1 s) closely matches observations with pre-transcribed RNAs of the
254same length (Supplementary Fig. 5a)²⁵. Given the average transcription time of 103 ± 11 sec
255(Fig. 4d), we expected to observe 1-2 tRNA binding events per molecule, based on the average
256unbound time of U46^{Cy5}-tRNA^{Gly} at this concentration (Supplementary Fig. 2a). By comparison,
257transcription of the longer *GlyQS*_213 template took 182 ± 6 s (Fig. 4f), which correlates with

the higher number of co-transcriptional U46^{Cy5}-tRNA binding events—1.4 (253/181) binding events per molecule for *GlyQS*_213 compared to 0.28 (29/101) events per RT-125 molecule (Fig. 4e,f). Importantly, this longer template also displayed a substantial increase in long-lived tRNA binding events (~11% among the total number of binding events), indicating that stable tRNA^{Gly} anchoring can occur during ongoing transcription (Fig. 4f and g). Notably, this fraction of long-lived events further increased post-transcriptionally (~30% - Fig. 4f, Post-Trx window), suggesting an antagonism between active transcription and stabilized tRNA anchoring that is overcome after the RNAP has passed on, reminiscent of S4 protein recruitment to the 16S rRNA dynamics during 16S rRNA synthesis³⁸.

To further elucidate the determinants leading to efficient co-transcriptional tRNA sensing, we performed PIFE-monitored real-time transcription of our *GlyQS*_213 templates bearing mutations that disrupt either tRNA binding to Stem I (Δ DTM) or recognition of the tRNA 3'-end (Mbox; Supplementary Fig. 4g and 5d,e). The overall co-transcriptional lifetime of U46^{Cy5}-tRNA on the *GlyQS*_213-Mbox transcript was comparable to that of the WT transcript, suggesting that the tRNA can still be sensed efficiently even in the absence of a functional T-box motif. However, kinetic analysis revealed that the fraction of long-lived U46^{Cy5}-tRNA:*GlyQS*_Tbox RNA complexes was substantially reduced for the Mbox mutant compared to WT, dropping to 1.2 (118/96) binding events per molecule, supporting an essential role of the T-box motif in stable tRNA^{Gly} anchoring during transcription (Supplementary Fig. 5d). Consistent with this conclusion, observation of only few (11/270, or 4%, relative to the WT's ~30%) long tRNA bound times after transcription completion (Post-Trx window) of the *GlyQS*_213-Mbox transcript similarly demonstrated that the T-box motif contributes substantially to tRNA anchoring under thermodynamic equilibrium conditions (Supplementary Fig. 5b).

281 In contrast, the *GlyQS*_213-ΔDTM transcript produced few and dominantly short-lived
282 tRNA^{Gly} binding events (Fig. 4g and Supplementary Fig. 5c), with only 0.47 (41/87) long-lived
283 binding events per molecule remaining, suggesting that early co-transcriptional recruitment via
284 Stem I is critical for effective downstream ligand anchoring during transcription elongation; that
285 is, anchoring engages both the tRNA elbow and 3'-end interaction points.

286 To further test this model, we examined the dwell times of the first U46^{Cys}-tRNA binding
287 interaction during active transcription (Fig. 4g, right panel). For the WT, we observed both short-
288 and long-lived binding events, consistent with prior models in which initial tRNA association
289 occurs independently of anchoring²⁴⁻²⁶, which is established only after AT transcription and
290 formation of Stems I and III, and most efficiently when both Stem I and the T-box motif are
291 displayed outside the RNAP exit channel (Fig. 4g). In contrast, both the Mbox and ΔDTM
292 mutants exhibited a pronounced reduction in long-lived binding events, with the effect being
293 strongest for the ΔDTM mutant (Fig. 4g). Together, these results support a model in which early
294 initial ligand recruitment via Stem I is a prerequisite for subsequent, stable tRNA anchoring
295 during transcription.

296 Kinetic analysis of U46^{Cys}-tRNA binding to the co-transcriptionally folded, RNAP-free
297 RT-125 RNA revealed that Stem I alone senses tRNA^{Gly} via two kinetic regimes (Fig. 4h). One
298 component is a comparably short unbound time (~94 s), while the other is substantially longer
299 (~300 s), likely to represent a conformation with tRNA binding events too sparse to be functional
300 under cellular conditions. Notably, the RT-125-ΔDTM transcript displayed only the slower
301 kinetic regime (230 s unbound time; Fig. 4h and i), underscoring the importance of Stem I
302 folding for early tRNA^{Gly} recruitment during transcription, as previously suggested^{24,25}.

303 When PIFE-monitored real-time transcription was performed with the tRNA 3' end
304 blocked in Gly-tRNA-3'-Cy5, very few co-transcriptional binding events were observed, further
305 supporting the notion that discrimination of the aminoacylation state is actively performed during
306 transcription (Supplementary Fig. 5d). Together, our real-time analysis of co-transcriptional
307 U46^{Cy5}-tRNA binding uncovers a hierarchical mechanism of ligand recognition, in which early
308 recruitment to a properly folded Stem I (sensing) enables efficient downstream tRNA^{Gly} capture
309 (anchoring) to the T-box motif.

310

311 **Transcription rate and pausing set a kinetic checkpoint for efficient tRNA sensing.** Co-
312 transcriptional processes such as RNA folding, protein recruitment and ligand recognition are
313 tightly and rapidly coordinated to precisely tune gene regulatory responses to ever-changing
314 environmental cues^{2,40}. In riboswitch systems, the transcription rate is an additional key
315 determinant of ligand recognition and the resulting gene regulation, by enforcing a kinetic window
316 during which sensing has to occur^{3,7,11,13,14}.

317 To assess the impact of the transcription rate on tRNA^{Gly} sensing, we increased the
318 concentration of rNTPs from 25 μ M to 250 μ M, accelerating T-box RNA elongation during
319 PIFE-monitored real-time transcription of the *GlyQS*_213 template (Fig. 5a). This 10-fold
320 increase led to an $\sim$ 4-fold rise in transcription rate⁴¹, taking only 52 ± 1 s of mean transcription
321 time (Fig. 5a) compared to 182 ± 6 s at 25 μ M rNTPs (Fig. 4f). This shortened time markedly
322 reduced the number of co-transcriptional U46^{Cy5}-tRNA binding events, consistent with the
323 narrower kinetic window available for ligand sensing. Conversely, an RNAP S1105A mutant,
324 which elongates 2-fold more slowly at 250 μ M rNTPs than WT RNAP, partially restored U46^{Cy5}-
325 tRNA sensing opportunity due to a longer mean transcription time (104 ± 2 s, Fig. 5b). We note

326that increased transcription rates generally were accompanied by a narrowing of the transcription
327time distribution across single transcription complexes (compare Figs. 4f and 5a-d). Together,
328these observations indicate that rNTP availability—and the resulting transcription rate as a
329sensor of cellular energy status—sets the kinetic window for co-transcriptional tRNA^{Gly}
330recognition.

331 Strikingly, despite these rate-dependent changes in overall sensing opportunity, the
332fraction of long-lived U46^{Cy5}-tRNA:*GlyQS* T-box interactions remained essentially constant
333(~15%) across all transcription rates tested (Fig. 5e and Supplementary Fig. 6c,d). This finding
334suggests that, although fast transcription narrows the sensing window, stable tRNA^{Gly} anchoring
335still occurs with unchanged probability under these more physiological elongation conditions,
336where intracellular rNTP concentrations are estimated to be in the low-millimolar range⁴².
337Consistent with this observation, transcription termination assays conducted under both fast and
338slow transcription regimes yielded similar termination and tRNA^{Gly} binding efficiencies
339(Supplementary Fig. 6a,b).

340 Next, to examine how transcriptional pausing shapes co-transcriptional riboswitch
341folding and ligand sensing, we performed PIFE-monitored real-time transcription of DNA
342templates truncated at the 178 and 207 pause sites (Fig. 5c and d). Consistent with our prior
343assays using similarly truncated, co-transcriptionally folded RTs (Supplementary Fig. 2e,f),
344nascent RNAs produced from these templates displayed mostly sparse, short-lived U46^{Cy5}-tRNA
345binding events during transcription (Fig. 5e). Closer analysis of the bound times revealed that
346tRNA^{Gly} binding slightly increases both co- and post-transcriptionally as the transcript extends
347from Stem I (178 pause) to completion of the AT/T switching region (207 pause, Fig. 5e).

348 Strikingly, the full-length *GlyQS*_213 real-time transcript, which includes a complete T
349 sequence, displayed substantially more long-lived U46^{Cy5}-tRNA binding events than the only
350 slightly shorter *GlyQS*_207 transcript (Supplementary Fig. 6e). This dependence on the final
351 nucleotides is consistent with an off-pathway fold involving nucleotides 202-206 within the
352 nascent T hairpin that compete with formation of the productive AT hairpin (nucleotides 178-
353 182) required to engage the tRNA 3' end, until additional T-hairpin sequence is transcribed.
354 Consistent with this model, dwell-time analysis revealed a substantially higher fraction of co-
355 transcriptional U46^{Cy5}-tRNA:T-box complexes for *GlyQS*_213 than for *GlyQS*_207 (Fig. 5f),
356 delineating the transcriptional window available for stable tRNA anchoring. Accordingly, in a
357 purely post-transcriptional binding context, the fraction of long-lived complexes was
358 substantially reduced from 41% for *GlyQS*_213 to 24% for *GlyQS*_207 (Supplementary Fig. 6e).

359 Together, these findings demonstrate that tRNA^{Gly} recruitment and full (stable) anchoring
360 occur within a narrow transcriptional window—a kinetic checkpoint governed by RNAP
361 elongation rate and pause-site positioning—and depend on timely formation of the
362 thermodynamically less favored AT hairpin immediately before the termination point.

363

364 **Real-time correlation of ligand binding with transcription unveils the readthrough**

365 **mechanism.** Ligand binding is central to riboswitch-mediated gene regulation in response to
366 cellular cues⁴³. To directly link tRNA^{Gly} binding to downstream transcriptional output, we aimed
367 to extend our real-time transcription assay to monitoring regulation at the single-molecule level
368 (Fig. 6). Because both intrinsic termination and readthrough followed by run-off result in RNAP
369 and DNA dissociation from the surface-tethered transcript—rendering these outcomes
370 indistinguishable in our standard format—we implemented a double-PIFE strategy⁴⁴ to resolve

371them. We designed a doubly labeled DNA template (*GlyQS*_207^{Cy3}_247^{Cy3}) bearing Cy3
372fluorophores at two positions: an internal label at position 207 to report RNAP passage through
373the final pause site, and a second label at the downstream template end to report transcription
374beyond the poly-U tract and subsequent run-off (Fig. 6a,b).

375 Real-time transcription of this template—initiated by simultaneous rNTP and U46^{Cy5}-
376tRNA injection—yielded two classes of single molecule fluorescence traces. Termination events
377were characterized by a single PIFE signal originating from position 207, followed by loss of the
378signal upon RNAP and (likely) DNA dissociation at the termination point poly-U (Fig. 6a). In
379contrast, readthrough events engendered two distinct PIFE signals, indicating transcription
380through both the pause site 207 and beyond the termination point to the end of the template (Fig.
3816b). This design enables direct visualization of transcription termination regulation at single-
382molecule resolution.

383 To validate that this DNA template produces functional *Bsu-GlyQS* T-box riboswitches,
384we compared termination efficiency on-slide with that measured in bulk transcription assays
385analyzed by gel electrophoresis. Real-time transcription of the *GlyQS*_207^{Cy3}_247^{Cy3} template
386resulted in $49 \pm 11\%$ and $58 \pm 1\%$ termination at low (25 μ M) and high (250 μ M) rNTP
387concentrations—i.e., slow and fast transcription rates), respectively. The close match with bulk
388results (Fig. 6c) confirms the suitability of our double-PIFE assay in assessing gene regulation
389outcomes.

390 Next, we asked how initial tRNA^{Gly} recruitment and subsequent anchoring relate to
391transcriptional fate. Readthrough traces exhibited ~4-fold more U46^{Cy5}-tRNA binding events
392than prematurely terminated traces (Fig. 6d), consistent with ligand engagement promoting
393antitermination. This relationship held at both slow and fast transcription rates: Although fast

transcription (high rNTP) reduced the total number of binding events, the ~4-fold enrichment in readthrough versus termination was preserved (Fig. 6d). Notably, U46^{Cy5}-tRNA:T-box complexes associated with terminated transcripts were uniformly short-lived (<1 s; Fig. 6e), whereas long-lived (>5 s) complexes were observed only in readthrough events—most prominently under fast transcription (Fig. 6d)—indicating that stable tRNA^{Gly} anchoring is required for productive antitermination under near-physiological conditions.

Together with the data in Figures 4 and 5, these results support a model in which early tRNA^{Gly} recruitment to Stem I, followed by AT-hairpin-mediated 3'-end anchoring, actively promotes transcriptional readthrough, directly illuminating how this riboswitch executes gene regulation under cellular conditions.

Discussion

Riboswitches are co-transcriptional molecular switches that modulate gene expression—most commonly by controlling transcription termination or translation initiation—through recognition of cognate ligands². Here, we directly couple ligand binding dynamics to transcriptional outcome at the single-molecule level, providing a real-time mechanistic view of how the *Bsu-GlyQS* T-box riboswitch senses uncharged tRNA^{Gly} and drives antitermination. These observations—shaped by strategically positioned pause sites—reveal how the riboswitch exploits directional, hierarchical 5'-to-3' directional, folding to create a defined kinetic window for ligand engagement and efficient regulation of downstream gene expression (Fig. 7).

The *Bsu-GlyQS* T-box riboswitch follows a hierarchical folding program that enables efficient recruitment and stable anchoring of uncharged tRNA^{Gly}, thereby biasing transcription toward readthrough rather than termination (Fig. 1). Our results support a dual mechanism in

417which maturation of the riboswitch scaffold is tightly coupled to dynamic tRNA recognition,
418with Stem I and the downstream T-box motif playing distinct, sequential roles. Stem I is essential
419for establishing the global architecture of the aptamer and for initiating a two-stage, co-
420transcriptional binding pathway previously inferred for translational T-box systems²⁴: Early ,
421transient interactions mediated by Stem I—captured here as U46^{Cy5}-tRNA binding events—pre-
422organize the complex and promote the structural register required for subsequent 3'-end
423anchoring later in transcription (Fig. 7). Within Stem I, the DTM strengthens specifier-anticodon
424recognition and stabilizes adoption of the functional conformation, endowing Stem I with a
425chaperone-like role. In this respect, Stem I parallels the bacterial RNA-binding protein Hfq,
426which promotes productive RNA-RNA interactions by stabilizing and coordinating RNA
427conformational transitions^{45,46}. By stabilizing early tRNA engagement (Figs. 2 and 4), Stem I
428primes the riboswitch for downstream T-box-mediated 3'end anchoring during a key RNAP
429pausing event, enabling a seamless transition to the fully bound, antiterminating state (Fig. 7).

430 Our kinetic analyses uncover tight coupling between riboswitch folding and RNAP-
431mediated pausing, which extends the time available for AT-region rearrangements and for tRNA
432sensing. Formation of the AT hairpin exposes the T-box motif and enables recognition of the
433tRNA 3' end—an essential step in committing the complex to antitermination and transcriptional
434readthrough. Consistent with this model, mutagenesis establishes that both Stem I and the T-box
435motif are required to support stable tRNA binding, underscoring the functional importance of
436hierarchical folding in gene regulation²⁵. More broadly, the ordered assembly pathway we
437observe mirrors principles of ribosomal RNA folding and RNP complex formation⁴, where
438temporally staged interactions enforce specificity and robustness. In this view, RNAP pausing
439operates as a kinetic checkpoint that coordinates successive structural transitions, analogous to

440promoter-proximal pausing in developmental gene control⁴⁷. By gating progression until
441upstream steps are completed, such checkpoints help preserve the fidelity, timing, and efficiency
442of gene-expression decisions.

443 T-box riboswitches are highly adaptive regulators that couple amino acid availability to
444gene expression by sensing changes in the abundance of uncharged tRNA. Our results show that
445conformational plasticity during elongation enables the riboswitch to tune transcriptional output
446as a function of both RNAP elongation rate and tRNA availability (Figs. 4,5). In particular,
447competition between charged and uncharged tRNA species provides a dynamic input that can
448shift riboswitch behavior and thereby shape the transcriptional response to nutrient stress (Fig.
4497). In cells, metabolic state strongly influences the fraction of charged versus uncharged tRNA⁴⁸,
450a key determinant of T-box function. Under nutrient-starved conditions, ~70–90% of tRNAs are
451typically aminoacylated, leaving only a small pool of uncharged Gly-tRNA (estimated at ~0.2–1
452μM)^{49–52}. In this regime, abundant charged Gly-tRNA can occupy early binding contacts yet fail
453to support the critical 3'-NCCA:T-box anchoring interaction required for AT stabilization and
454terminator destabilization, effectively biasing the switch toward termination (Fig. 7). During
455glycine starvation, by contrast, rising levels of uncharged tRNA^{Gly} promote 3'-end anchoring and
456antitermination, upregulating genes involved in amino acid biosynthesis, transport, and tRNA
457charging. Given glycine's roles in protein synthesis, one-carbon metabolism, and broader
458intermediary metabolism⁵³, fluctuations in its availability position the T-box riboswitch as an
459integrative sensor of cellular nutritional state.

460 In summary, the *Bsu-GlyQS* T-box riboswitch illustrates how hierarchical folding,
461strategically positioned transcriptional pauses, and ligand availability converge to drive efficient,

462 responsive control of gene expression. The mechanistic parallels to other RNA-guided pathways,
463 including Hfq-facilitated sRNA–mRNA pairing, underscore broader principles of RNA-based
464 regulation. Notably, the T-box RNA itself exhibits chaperone-like activity, promoting productive
465 hybridization between the tRNA 3' end and the T-box motif. By directly linking
466 co-transcriptional tRNA binding kinetics to transcriptional fate in real-time, the current study
467 establishes a first quantitative framework and experimental blueprint for atomic-resolution
468 dissection of these transitions, for testing how shared design principles extend across riboswitch
469 and RNA regulatory networks, and for ultimately informing strategies to therapeutically perturb
470 T-box-controlled pathways in bacterial pathogens.

471

472 **Materials and Methods**

473 **DNA templates and Oligonucleotides.** A 349-nucleotide DNA template including the *GlyQS*-
474 TboxT-box riboswitch from *B. subtilis* under the control of the T7A1 promoter was cloned into
475 pUC-GW plasmid (Azena Genewiz). Primers were purchased from Integrated DNA
476 technologies, Inc. (IDT) with HPLC purification when containing modifications. Transcription
477 templates for *in vitro* transcription were generated by PCR using the “T7A1-PCR” forward
478 oligonucleotide and the according reverse oligonucleotides. For mutant DNA templates, the
479 mutation was inserted in two PCR steps using overlapping oligonucleotides containing the
480 corresponding mutation.

481 Fluorescent DNA templates for transcription were generated by PCR using Phusion high
482 fidelity polymerase (NEB). Following PCR, fluorescently labeled DNA templates were separated
483 on 1% agarose, column purified and eluted in 40 μ L of water.

484 For the DNA template containing two Cy3 fluorophores, reverse primer (see key resources
485table) containing an internal amino-alkyl modified nucleotide (IDT; see Supplementary Table 2)
486was fluorescently labeled with Cy3-NHS dye (Lumiprobe) as follows: 0.5 mg of Cy3 dye was
487dissolved in 33 μ L of DMSO and added to a reaction mixture containing 5 nmol modified primer
488adjusted to 100 μ L final volume with 100 mM sodium bicarbonate pH 8.5. Reactions were
489incubated overnight at room temperature and then free dye was removed using a Chroma TE-10
490spin column (Takara Bio) followed by clean-up on a Monarch DNA column using the
491oligonucleotide clean-up protocol.

492 Oligonucleotides used in this study are listed in Supplementary Table 1.

493

494***In vitro* transcription assays.** Halted complexes (EC-13) were prepared in transcription buffer
495(20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 20 mM MgCl₂, 14 mM 2-mercaptoethanol, 0.1 mM
496EDTA) containing 25 μ M ATP/UTP mix, 50 nM α^{32} P-GTP (3000 Ci/mmol), 10 μ M ApC
497dinucleotide primer (Trilink), and 50 nM DNA template. *E. coli* RNAP holoenzyme (New England
498Biolabs) was added to 100 nM, and the mixture was incubated for 10 min at 37 °C. The sample
499was passed through G50 column to remove any free nucleotides. To complete the transcription
500reaction all four rNTPs were added concomitantly with heparin (450 μ g/mL) to prevent the re-
501initiation of transcription. The mixture was incubated at 37 °C, and reaction aliquots were
502quenched at the desired times into an equal volume of loading buffer (95% formamide, 1 mM
503EDTA, 0.1% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol). Reaction aliquots were
504denatured before loading onto a denaturing 8 M urea, 6% polyacrylamide sequencing gel. The gel
505was dried and exposed to a phosphor screen (typically overnight), which was then scanned on a
506Typhoon Phosphor Imager (GE Healthcare).

507

508**Transcription data analysis.** To determine the RT_{50} for regulation of termination by Gly-tRNA,
509the relative intensity of the full-length product was divided by the total amount of RNA transcript
510(full-length + terminated product) for each Gly-tRNA concentration. The resulted percentages of
511readthrough were plotted against the Gly-tRNA concentration using equation: $Y=Y_0 +$
512 $((M1 \times X)/(T_{50}+X))$, where Y_0 is the value of Y at $X_{\min}$ (no ligand condition) and $M1 = Y_{\max} -$
513 $Y_{\min}$. Error bars in transcription quantification represent the standard deviation of the mean from
514independent replicates.

515

516**Aminoacylation of Gly-tRNA and 3'-end labeling.** Aminoacylation of Cy5-labeled Gly-tRNA
517was achieved using *E. coli* S100 extracts (tRNA probes, College Station, TX, USA) in a 25 μ l
518reaction volume following a protocol previously described⁵⁴. Briefly, $\sim 2 \mu$ M of Cy5-tRNA^{Gly} was
519charged with 3 μ l of *E. coli* S100 extract in a 50 μ l reaction containing 100 mM HEPES-KOH, pH
5207.6, 200 μ M glycine, 10 mM ATP, 1 mM dithiothreitol, 20 mM KCl, 20 mM MgCl₂, and incubated
521at 37 °C for 30 min. The reaction was stopped by adding 3 M sodium acetate, pH 5.2, to a final
522concentration of 100 mM in 100 μ l volume. Aminoacylated tRNA was purified by performing
523phenol–chloroform extraction to remove the proteins. The concentration of the Gly-tRNA^{Gly} was
524measured using a nanodrop 2000 and multiple aliquots of it were made and stored at -80 °C. An
525acid-urea PAGE was used for checking the aminoacylation efficiency of the reaction. Under our
526reaction conditions, the aminoacylation efficiency was close to $\sim 90\%$ ²⁵. For the single-molecule
527binding experiments, Cy5-labeled Gly-tRNA^{Gly} was diluted to the required concentration in
528standard buffer.

529 For 3'-end labeling of the Gly-tRNA with Cy5 fluorophore, we employed RNA ligation with pCp-
530 Cy5 (Jena Bioscience). Briefly, in a 30 μ L reaction volume, 3 pmol of unlabeled Gly-tRNA were
531 incubated with 30 pmol pCp-Cy5, 1 mM ATP, 10% DMSO (v/v) and 1 U of T4 RNA ligase I
532 (NEB) overnight at 16 °C in the reaction buffer provided by the supplier. Labeling reaction was
533 run on a 20% denaturing urea PAGE and gel-excised to purify the labeled from the unlabeled
534 product followed by ethanol precipitation.

535

536 **Preparation of fluorescently labeled nascent transcripts.** *In vitro* transcription reactions were
537 performed in two steps to allow the specific incorporation of Cy3 at the 5'-end of the RNA
538 sequence. Transcription reactions were performed in the same transcription buffer described
539 above. Transcription reactions were initiated by adding 100 μ M 5'-Cy3-ApC and 25 μ M
540 ATP/UTP/GTP nucleotides at 37 °C for 10 min, thus yielding a fluorescent halted complex. The
541 sample was next passed through a G50 column to remove any free nucleotides, and the
542 transcription was resumed upon addition of all four rNTPs at the indicated concentration and
543 heparin (450 μ g/mL) to prevent the re-initiation of transcription. The resulting nascent transcript
544 was hybridized to the 5'-biotinylated CP (Anchor Bio oligonucleotide) complementary to the 3'-
545 end capture sequence, allowing immobilization of the complex to the microscope slide. The CP
546 was mixed to a ratio of 10:1 with RNA transcript and added for 5 min before flowing the whole
547 complex to the microscope slide. In the case of PEC transcription, the DNA templates contain a
548 biotin at the 5'-end of the template DNA strand. Streptavidin was mixed to a ratio of 5:1 with the
549 DNA template for 5 min prior to starting the transcription reaction.

550 For real-time transcription assays, transcription reactions were initiated by adding 100
551 μ M 5'-Biotin-ApC (Horizon Discovery) and 25 μ M ATP/UTP/GTP nucleotides at 37 °C for 10

552min, thus yielding a fluorescent halted complex. The sample was next passed through a G50
553column and directly flowed on microscope slide pre-incubated with streptavidin.

554

555**Single molecule fluorescence microscopy experiments.** All single-molecule fluorescence
556microscopy experiments were performed using an Oxford Nanoimager (ONI) S with a 100x 1.4
557NA oil immersion super apochromatic objective, 405, 473, 532, 640 nm lasers with appropriate
558dual-band emission filters, and a Hamamatsu sCMOS Orca flash 4 V3 camera, run in TIRF mode.
559All movies were collected at 100 ms time resolution. PEG-passivated, biotin-modified slides with
560a chamber or a microfluidic channel containing inlet and outlet ports for buffer exchange were
561assembled as described in previous works⁵⁵. The surface of the microfluidic channel was coated
562with streptavidin (0.2 mg/mL) for 10 to 15 min prior to flowing the nascent transcripts hybridized
563to the CP. In the case of fluorescent PECs, the complexes were directly injected into the channel
564surface using the biotin-streptavidin roadblock for immobilization.

565 For all experiments, the nascent RNA transcripts were diluted in in imaging buffer (40
566mM Tris-HCl, pH 8.0, 330 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1
567mg/mL bovine serum albumin). U46^{Cy5}-tRNA (6 nM for immobilized complexes and 12 nM for
568real-time transcriptions) was injected into the channel in the corresponding imaging buffer along
569with an enzymatic oxygen scavenging system consisting of 44 mM glucose, 165 U/mL glucose
570oxidase from *Aspergillus niger*, 2,170 U/mL catalase from *Corynebacterium glutamicum*, and 5
571mM Trolox to extend the lifetime of the fluorophores and to prevent photo-blinking of the dyes.
572In the case of aminoacylated U46^{Cy5}-tRNA and 3'-Cy5-tRNA, 12.5 nM and 25 nM were used for
573immobilized complexes and real-time transcription respectively. The raw movies were collected
574for 10-15 min with direct green (532 nm) and red (638 nm) laser excitation.

575

576**Single molecule data acquisition and analysis.** Locations of molecules and fluorophore intensity
577traces for each molecule were extracted from raw movie files using custom-built MATLAB codes.
578Traces were manually selected for further analysis using the following criteria: single-step
579photobleaching of Cy3, ≥ 2 spikes of Cy5 fluorescence of more than 2-fold the background
580intensity. Traces showing binding events were idealized using a two-states (bound and unbound)
581model using a segmental k -means algorithm in QuB⁴⁸. From the idealized traces, dwell times of
582U46^{Cy5}-tRNA in the bound (τ_{bound}) and the unbound (τ_{unbound}) states were obtained. Cumulative
583plots of bound and unbound dwell-time distributions were plotted and fitted in Origin lab with
584single-exponential or double-exponential functions to obtain the lifetimes in the bound and
585unbound states. The dissociation rate constants (k_{off}) were calculated as the inverse of the τ_{bound} ,
586whereas the association rate constants (k_{on}) were calculated by dividing the inverse of the τ_{unbound}
587by the concentration of U46^{Cy5}-tRNA used during the data collection.

588 tRNA binding traces that showed PIFE were plotted as 2D plots of horizontal vectors
589corresponding to each time trace, color coded for bound times and sorted by their time for the PIFE
590effect on the vertical dimension using an ad-hoc Python script.

591

592

593**SUPPLEMENTAL INFORMATION**

594Supplemental information can be found online at...

595

596**ACKNOWLEDGMENTS**

597We thank Yun Zhang for help with the Python code for real-time transcription analysis. This work
598was supported by NIH grants R01 GM118524 and MIRA R35 GM131922 to NGW.

599**AUTHOR CONTRIBUTIONS**

600Conceptualization, A.C. J.C-V and N.G.W.; Methodology, A.C.; Investigation, A.C. Writing -
601Original Draft, A.C.; Writing - Review & Editing, A.C., J.C-V., N.G.W.; Supervision, N.G.W;
602Funding Acquisition, N.G.W.

603

604**DECLARATION OF INTERESTS**

605The authors declare no competing interests.

606

607

608**REFERENCES**

6091. Shine, M. *et al.* Co-transcriptional gene regulation in eukaryotes and prokaryotes. *Nature Reviews*
610 *Molecular Cell Biology* **25**, 534–554 (2024).
6112. Chauvier, A. & Walter, N. G. Regulation of bacterial gene expression by non-coding RNA: It is all
612 about time! *Cell Chem Biol* **31**, 71–85 (2024).
6133. Chauvier, A. & Walter, N. G. Beyond ligand binding: Single molecule observation reveals how
614 riboswitches integrate multiple signals to balance bacterial gene regulation. *Curr Opin Struct Biol* **88**,
615 102893 (2024).

6164. Rodgers, M. L. & Woodson, S. A. A roadmap for rRNA folding and assembly during transcription.
617 *Trends Biochem Sci* **46**, 889–901 (2021).
6185. Kavita, K. & Breaker, R. R. Discovering riboswitches: the past and the future. *Trends Biochem Sci* **48**,
619 119–141 (2023).
6206. Olenginski, L. T., Spradlin, S. F. & Batey, R. T. Flipping the script: Understanding riboswitches from an
621 alternative perspective. *Journal of Biological Chemistry* **300**, 105730 (2024).
6227. Chauvier, A. *et al.* Transcriptional pausing at the translation start site operates as a critical
623 checkpoint for riboswitch regulation. *Nat Commun* **8**, 13892 (2017).
6248. Steinert, H. *et al.* Pausing guides RNA folding to populate transiently stable RNA structures for
625 riboswitch-based transcription regulation. *Elife* **6**, (2017).
6269. Chauvier, A., Dandpat, S. S., Romero, R. & Walter, N. G. A nascent riboswitch helix orchestrates
627 robust transcriptional regulation through signal integration. *Nat Commun* **15**, 3955 (2024).
62810. Lou, Y. & Woodson, S. A. Co-transcriptional folding of the glmS ribozyme enables a rapid response to
629 metabolite. *Nucleic Acids Research* gkad1120 (2023) doi:10.1093/nar/gkad1120.
63011. Chauvier, A. *et al.* Monitoring RNA dynamics in native transcriptional complexes. *Proc Natl Acad Sci*
631 *U S A* **118**, e2106564118 (2021).
63212. Strobel, E. J., Cheng, L., Berman, K. E., Carlson, P. D. & Lucks, J. B. A ligand-gated strand displacement
633 mechanism for ZTP riboswitch transcription control. *Nat. Chem. Biol.* **15**, 1067–1076 (2019).
63413. Wickiser, J. K., Winkler, W. C., Breaker, R. R. & Crothers, D. M. The speed of RNA transcription and
635 metabolite binding kinetics operate an FMN riboswitch. *Mol. Cell* **18**, 49–60 (2005).
63614. Chauvier, A., Ajmera, P., Yadav, R. & Walter, N. G. Dynamic competition between a ligand and
637 transcription factor NusA governs riboswitch-mediated transcription regulation. *Proc Natl Acad Sci U*
638 *S A* **118**, e2109026118 (2021).
63915. Helmling, C. *et al.* Life times of metastable states guide regulatory signaling in transcriptional
640 riboswitches. *Nat Commun* **9**, 944 (2018).
64116. Kreuzer, K. D. & Henkin, T. M. The T-Box Riboswitch: tRNA as an Effector to Modulate Gene
642 Regulation. *Microbiol Spectr* **6**, (2018).

64317. Zhang, J. & Ferré-D'Amaré, A. R. Structure and mechanism of the T-box riboswitches. *Wiley*
644 *Interdiscip Rev RNA* **6**, 419–433 (2015).

64518. Giarimoglou, N., Kouvela, A., Zhang, J., Stamatopoulou, V. & Stathopoulos, C. Structural
646 idiosyncrasies of glycyl T-box riboswitches among pathogenic bacteria. *RNA* rna.080071.124 (2024)
647 doi:10.1261/rna.080071.124.

64819. Suddala, K. C. & Zhang, J. High-affinity recognition of specific tRNAs by an mRNA anticodon-binding
649 groove. *Nat Struct Mol Biol* **26**, 1114–1122 (2019).

65020. Battaglia, R. A., Grigg, J. C. & Ke, A. Structural basis for tRNA decoding and aminoacylation sensing by
651 T-box riboregulators. *Nat Struct Mol Biol* **26**, 1106–1113 (2019).

65221. Li, S. *et al.* Structural basis of amino acid surveillance by higher-order tRNA-mRNA interactions. *Nat*
653 *Struct Mol Biol* **26**, 1094–1105 (2019).

65422. Jia, X. *et al.* Cryo-EM-guided engineering of T-box-tRNA modules with enhanced selectivity and
655 sensitivity in translational regulation. *bioRxiv* 2023.02.28.530422 (2023)
656 doi:10.1101/2023.02.28.530422.

65723. Niu, X. *et al.* Structural and dynamic mechanisms for coupled folding and tRNA recognition of a
658 translational T-box riboswitch. *Nat Commun* **14**, 7394 (2023).

65924. Campos-Chavez, E. *et al.* Translational T-box riboswitches bind tRNA by modulating conformational
660 flexibility. *Nat Commun* **15**, 6592 (2024).

66125. Suddala, K. C. *et al.* Hierarchical mechanism of amino acid sensing by the T-box riboswitch. *Nature*
662 *Communications* **9**, 1896 (2018).

66326. Niu, X., Cai, S., Wang, J., Chen, C. & Fang, X. tRNA-dependent conformational dynamics and folding
664 coordinate translational regulation by a full-length T-box riboswitch. *Nature Communications* **16**,
665 10438 (2025).

66627. Zhang, J. *et al.* Specific structural elements of the T-box riboswitch drive the two-step binding of the
667 tRNA ligand. *Elife* **7**, (2018).

66828. Warriar, I. *et al.* RNA cis-regulators are important for *Streptococcus pneumoniae* in vivo success.
669 *PLoS Genet* **20**, e1011188 (2024).

67029. Giarimoglou, N. *et al.* Specific targeting of transcriptional T-box riboswitches leads to effective
671 inhibition of *S. aureus*. *RNA* **080644.125** (2025) doi:10.1261/rna.080644.125.

67230. Giarimoglou, N. *et al.* A Riboswitch-Driven Era of New Antibacterials. *Antibiotics (Basel)* **11**, 1243
673 (2022).

67431. Ellinger, E. *et al.* Riboswitches as therapeutic targets: promise of a new era of antibiotics. *Expert Opin*
675 *Ther Targets* **27**, 433–445 (2023).

67632. Grundy, F. J., Yousef, M. R. & Henkin, T. M. Monitoring uncharged tRNA during transcription of the
677 *Bacillus subtilis* *glyQS* gene. *J Mol Biol* **346**, 73–81 (2005).

67833. Widom, J. R. *et al.* Ligand Modulates Cross-Coupling between Riboswitch Folding and Transcriptional
679 Pausing. *Mol. Cell* **72**, 541–552.e6 (2018).

68034. Grundy, F. J. & Henkin, T. M. Kinetic analysis of tRNA-directed transcription antitermination of the
681 *Bacillus subtilis* *glyQS* gene in vitro. *J. Bacteriol.* **186**, 5392–5399 (2004).

68235. Larson, M. H. *et al.* A pause sequence enriched at translation start sites drives transcription
683 dynamics in vivo. *Science* **344**, 1042–1047 (2014).

68436. Komissarova, N. & Kashlev, M. Functional topography of nascent RNA in elongation intermediates of
685 RNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14699–14704 (1998).

68637. Chatterjee, S., Chauvier, A., Dandpat, S. S., Artsimovitch, I. & Walter, N. G. A translational riboswitch
687 coordinates nascent transcription-translation coupling. *Proc Natl Acad Sci U S A* **118**, e2023426118
688 (2021).

68938. Rodgers, M. L. & Woodson, S. A. Transcription Increases the Cooperativity of Ribonucleoprotein
690 Assembly. *Cell* **179**, 1370–1381.e12 (2019).

69139. Hwang, H. & Myong, S. Protein induced fluorescence enhancement (PIFE) for probing protein-
692 nucleic acid interactions. *Chem Soc Rev* **43**, 1221–1229 (2014).

69340. Landick, R. Transcriptional Pausing as a Mediator of Bacterial Gene Regulation. *Annu Rev Microbiol*
694 **75**, 291–314 (2021).

69541. Duss, O. *et al.* Real-time assembly of ribonucleoprotein complexes on nascent RNA transcripts. *Nat*
696 *Commun* **9**, 5087 (2018).

69742. Buckstein, M. H., He, J. & Rubin, H. Characterization of nucleotide pools as a function of
698 physiological state in Escherichia coli. *J Bacteriol* **190**, 718–726 (2008).

69943. Bastet, L., Turcotte, P., Wade, J. T. & Lafontaine, D. A. Maestro of regulation: Riboswitches
700 orchestrate gene expression at the levels of translation, transcription and mRNA decay. *RNA Biology*
701 **0**, 01–34 (2018).

70244. Rodgers, M. L., O'Brien, B. & Woodson, S. A. Small RNAs and Hfq capture unfolded RNA target sites
703 during transcription. *Molecular Cell* **83**, 1489-1501.e5 (2023).

70445. Małacka, E. M. & Woodson, S. A. RNA compaction and iterative scanning for small RNA targets by
705 the Hfq chaperone. *Nat Commun* **15**, 2069 (2024).

70646. Park, S. *et al.* Dynamic interactions between the RNA chaperone Hfq, small regulatory RNAs, and
707 mRNAs in live bacterial cells. *eLife* **10**, e64207 (2021).

70847. Levine, M. Paused RNA Polymerase II as a Developmental Checkpoint. *Cell* **145**, 502–511 (2011).

70948. Raina, M. & Ibba, M. tRNAs as regulators of biological processes. *Front Genet* **5**, 171 (2014).

71049. Dittmar, K. A., Sørensen, M. A., Elf, J., Ehrenberg, M. & Pan, T. Selective charging of tRNA
711 isoacceptors induced by amino-acid starvation. *EMBO Rep* **6**, 151–157 (2005).

71250. Pavlova, N. N. *et al.* Translation in amino-acid-poor environments is limited by tRNA^{Gln} charging.
713 *Elife* **9**, e62307 (2020).

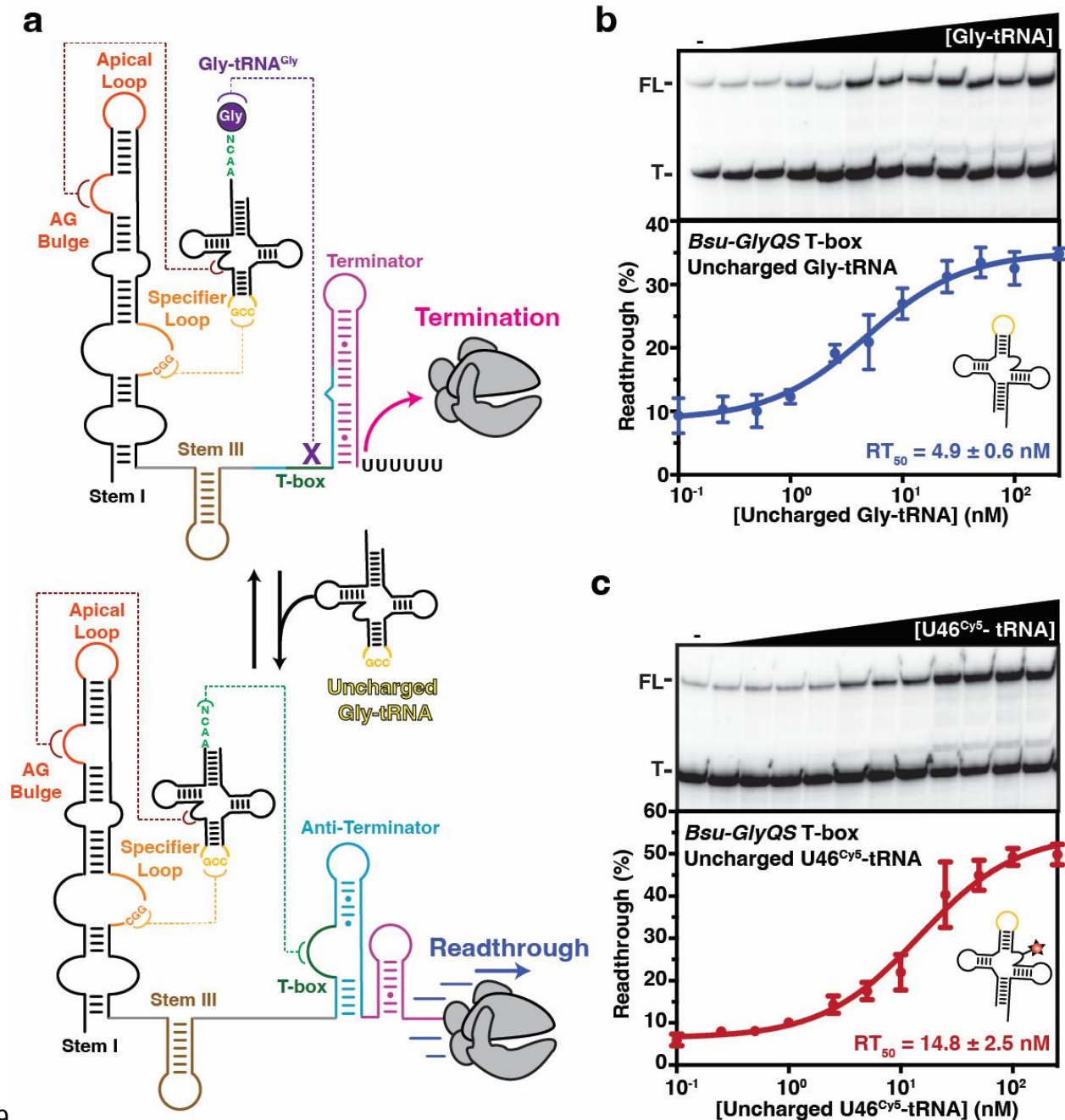
71451. Elf, J. & Ehrenberg, M. Near-critical behavior of aminoacyl-tRNA pools in E. coli at rate-limiting
715 supply of amino acids. *Biophys J* **88**, 132–146 (2005).

71652. Ferro, I., Liebeton, K. & Ignatova, Z. Growth-rate dependent regulation of tRNA level and charging in
717 *Bacillus licheniformis*. *J. Mol. Biol.* <https://doi.org/10.1016/j.jmb.2017.09.010> (2017)
718 doi:10.1016/j.jmb.2017.09.010.

71953. Hong, Y., Ren, J., Zhang, X., Wang, W. & Zeng, A.-P. Quantitative analysis of glycine related metabolic
720 pathways for one-carbon synthetic biology. *Curr Opin Biotechnol* **64**, 70–78 (2020).

72154. Köhrer, C. & Rajbhandary, U. L. The many applications of acid urea polyacrylamide gel
722 electrophoresis to studies of tRNAs and aminoacyl-tRNA synthetases. *Methods* **44**, 129–138 (2008).

72355. Chauvier, A., Cabello-Villegas, J. & Walter, N. G. Probing RNA structure and interaction dynamics at
724 the single molecule level. *Methods* **162–163**, 3–11 (2019).
72556. Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*
726 **31**, 3406–3415 (2003).
727



729

730Fig. 1 | Transcriptional Regulation Induced by the *Bsu-GlyQS* T-box Riboswitch. a, The

731*GlyQS* T-box riboswitch from *B. subtilis* regulates gene expression at the transcriptional level.

732Stabilization of the Antiterminator (AT) upon binding of the uncharged tRNA^{Gly} prevents the

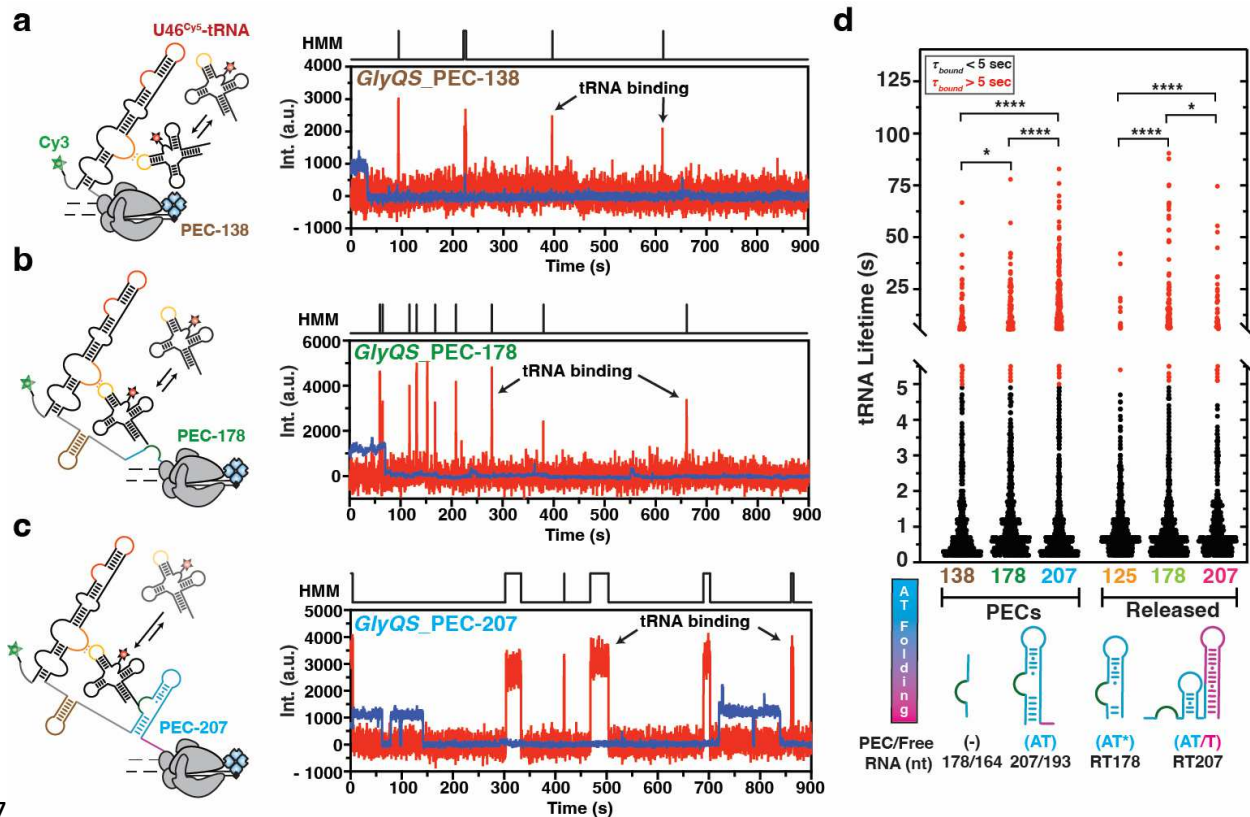
733formation of an intrinsic Terminator (T) hairpin leading to the transcription of the downstream

734gene. tRNA selection is performed through interaction between the Specifier Loop within Stem I

735and the anticodon of the tRNA. Steric hindrance caused by the presence of glycine amino acid
736(charged tRNA – Gly-tRNA^{Gly}) prevents the interaction between the tRNA 3'-end and the T-box
737motif which would otherwise promotes the formation of the AT. **b-c**, Gly-tRNA (**b**) and U46^{Cys}-
738tRNA (**c**) dependent single round transcription assay of a DNA template containing the 5'-UTR of
739the *GlyQS* operon from *B. subtilis*. Transcription reactions were performed using the *E. coli* RNAP
740with 10 μ M rNTPs. ³²P-labeled products were resolved on 6% polyacrylamide denaturing gel
741separating the full-length (FL) and terminated (T) RNA products. Plot of the fraction of
742transcriptional readthrough versus the concentration of tRNA ligand are displayed below the
743representative gels. RT₅₀ are indicated. Error bars represent the Standard Deviation (SD) of the
744mean from independent replicates.

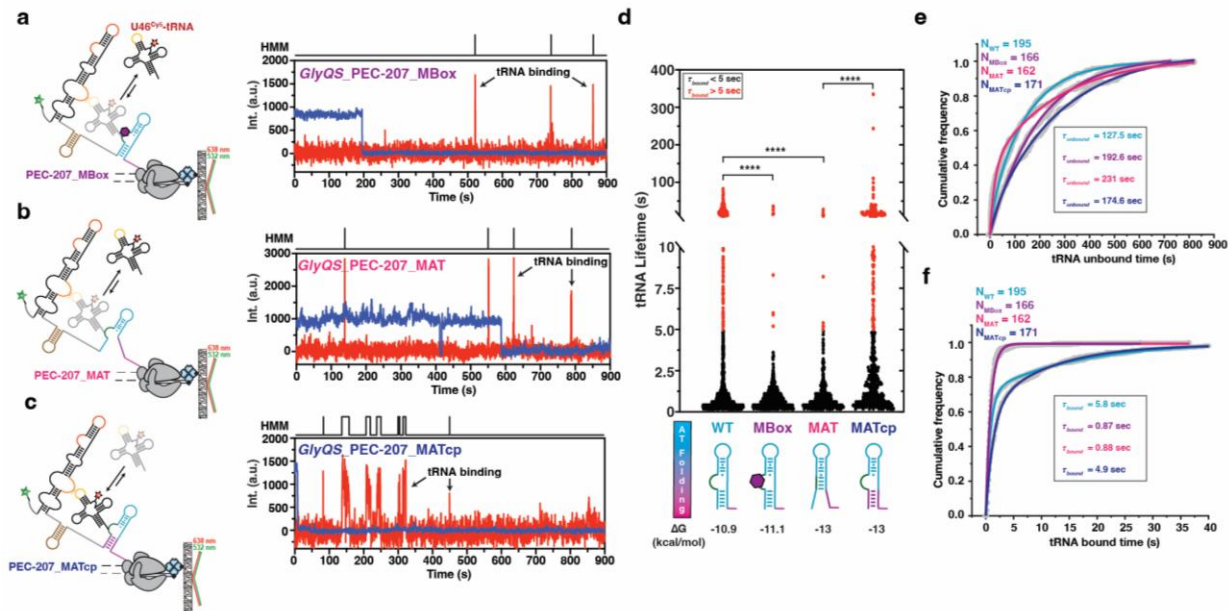
745

746



747

748**Fig. 2 | Monitoring tRNA^{Gly} Binding Kinetics at Key Transcriptional Pause Sites.** a-c,
749Secondary structure of the *Bsu*-GlyQS T-box riboswitch in the context of the Paused Elongation
750Complexes (PEC) at the 138 (a), 178 (b) and 207 (c) positions, alongside representative single-
751molecule trajectories showing U46^{Cys}-tRNA binding (red) to the corresponding PEC (blue).
752Hidden Markov Modeling (HMM) is indicated on the top of each trace. d, Plot displaying the
753overall distribution of tRNA lifetimes for the different constructs tested. Short binding events
754($\tau_{bound} < 5$ s) are indicated in black and long-lived binding events ($\tau_{bound} > 5$ s) are indicated in red.
755Predicted secondary structures of the AT is shown at the bottom with the corresponding length of
756the construct and the free RNA emerging from the RNA exit channel in the case of PECs. The total
757number of molecules analyzed is as follows: PEC-138 = 204; PEC-178 = 208; PEC-207 = 195;
758RT-125 = 236; RT-178 = 237; RT-207 = 203. (****P < 0.001, ***P < 0.005, **P < 0.01, *P < 0.05)



759

760**Fig. 3 | Folding of the Antiterminator Promotes Ligand Sensing and 3'-end anchoring. a-c,**

761Secondary structure of the *Bsu-GlyQS* T-box riboswitch in the context of the PEC-207_MBox (a),

762PEC-207_MAT (b) and PEC-207_MATcp (c), alongside representative single-molecule

763trajectories showing U46^{Cy5}-tRNA binding (red) to the corresponding PEC (blue). Hidden Markov

764Modeling (HMM) is indicated on the top of each trace. **d**, Plot displaying the overall distribution

765of tRNA lifetimes for the different constructs tested. Short binding events ($\tau_{\text{bound}} < 5$ s) are indicated

766in black and long-lived binding events ($\tau_{\text{bound}} > 5$ s) are indicated in red.. Predicted secondary

767structure of the AT is shown at the bottom with the calculated ΔG from UNAFold⁵⁶. The total number

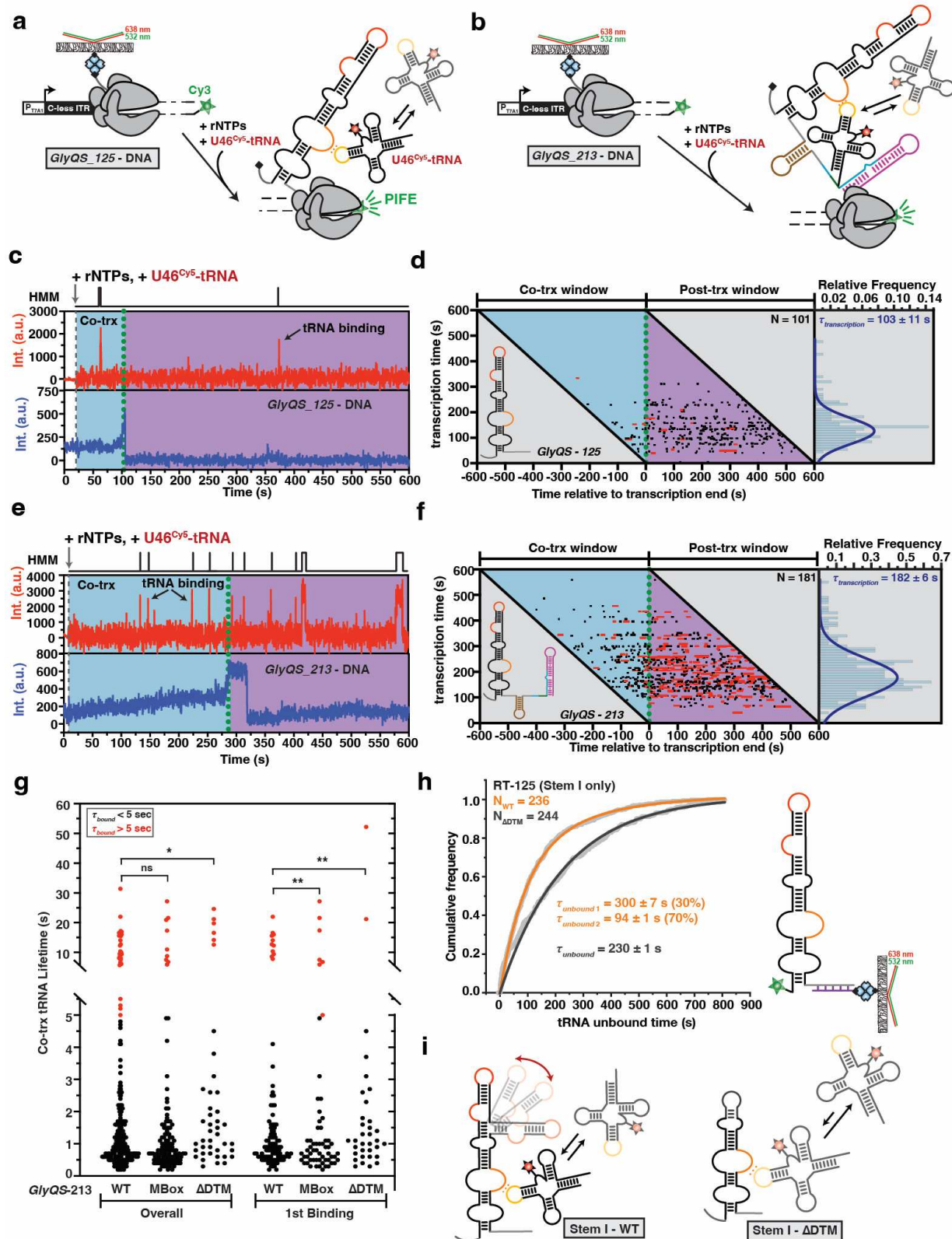
768of molecules analyzed is as follow: PEC-207_WT = 195; PEC-207_Mbox = 166; PEC-207_MAT = 162;

769PEC-207_MATcp = 171; (****P < 0.001, ***P < 0.005, **P < 0.01, *P < 0.05). **e-f**, Plots displaying the

770cumulative unbound (e) and bound (f) dwell times of U46^{Cy5}-tRNA in the different PEC-207

771constructs. The overall unbound (τ_{unbound}) and bound (τ_{bound}) times are indicated.

772



773

774Fig. 4 | Real-time Monitoring of Ligand Binding during Transcription. a-b, Transcription of

775the DNA templates labeled with Cy3 at the 3'-end using the *E. coli* RNAP allows the formation
 776and detection of a fluorescent Halted Complex (EC-13) attached to the microscope slide through
 777the nascent RNA transcript. DNA templates encoding either Stem I only (**a**) or the full-length
 778riboswitch prior to the termination point (**b**) were used in this study. Transcription is re-started
 779upon addition of all rNTPs and addition of U46^{Cy5}-tRNA in the transcription mix allows surveying
 780co-transcriptional ligand binding in real-time. When the RNAP reaches the end of the DNA
 781template, the occurrence of the PIFE signal (Protein Induced Fluorescence Enhancement) delimits
 782the co-transcriptional window. **c**, Representative single molecule trajectory showing the real-time
 783transcription of the *GlyQS*_125 DNA transcribed in the presence of 25 μ M rNTPs and 25 nM
 784U46^{Cy5}-tRNA. Transcription restart is indicated by rNTPs injection, and the end of transcription is
 785indicated by PIFE (green dotted line). Repeated co-transcriptional bindings of U46^{Cy5}-tRNA are
 786monitored upon direct excitation of the Cy5 fluorescent dye. Hidden Markov Modeling (HMM) is
 787indicated on the top of the trace. **d**, Rastergram of U46^{Cy5}-tRNA binding (black and red bars)
 788during (cyan background) and just after transcription (magenta background) of the *GlyQS*_125
 789DNA template; each horizontal line represents a single transcribed molecule. The lengths and
 790colors of horizontal bars indicate the lifetimes of individual complexes. On the x axis, $t = 0$
 791represents the time relative to the transcription end marked by occurrence of the PIFE signal.
 792Histogram of transcription time is indicated on the right. The indicated error is the error of the
 793gaussian fit. **e**, Representative single molecule trajectory showing the real-time transcription of the
 794*GlyQS*_213 DNA transcribed in the presence of 25 μ M rNTPs and 25 nM U46^{Cy5}-tRNA.
 795Transcription restart is indicated by rNTPs injection, and the end of transcription is indicated by
 796PIFE (green dotted line). Repeated co-transcriptional bindings of U46^{Cy5}-tRNA are monitored
 797upon direct excitation of the Cy5 fluorescent dye. Hidden Markov Modeling (HMM) is indicated

798 on the top of the trace. **f**, Rastergram of U46^{Cy5}-tRNA binding (black and red bars) during (cyan
799 background) and just after transcription (magenta background) of the *GlyQS_213* DNA template;
800 each horizontal line represents a single transcribed molecule. The lengths and colors of horizontal
801 bars indicate the lifetimes of individual complexes. On the x axis, $t = 0$ represents the time relative
802 to the transcription end marked by occurrence of the PIFE signal. Histogram of transcription time
803 is indicated on the right. The indicated error is the error of the gaussian fit. **g**, Plot displaying the
804 distribution of tRNA lifetimes during the transcription (co-trx) of the *GlyQS_213* DNA templates
805 WT, Mbox and Δ DTM. Short binding events ($\tau_{bound} < 5$ s) are indicated in black and long-lived
806 binding events ($\tau_{bound} > 5$ s) are indicated in red. The total number of molecules analyzed is as
807 follows: WT = 181; Mbox = 61; Δ DTM = 35. (****P < 0.001, ***P < 0.005, **P < 0.01, *P <
808 0.05). **h**, Plots displaying the cumulative unbound dwell times of U46^{Cy5}-tRNA for the released
809 transcript comprising Stem I only (RT-125) in the WT and Δ DTM context with 12.5 nM U46^{Cy5}-
810 tRNA. Unbound ($\tau_{unbound}$) times are indicated with the error of the fit. **i**, Secondary structural model
811 of Stem I folding in the WT and Δ DTM context.

812

813

814

824 indicated error is the error of the gaussian fit. **c-d**, Rastergrams of U46^{Cy5}-tRNA binding (black
 825 and red bars) during (cyan background) and just after transcription (magenta background) using
 826 250 μ M rNTPs for the transcription of the *GlyQS*_178 (**c**) and *GlyQS*_207 (**d**) DNA templates with
 827 WT RNAP; each horizontal line represents a single transcribed molecule. The lengths and colors
 828 of horizontal bars indicate the lifetimes of individual complexes. On the x axis, $t = 0$ represents
 829 the time relative to the transcription end marked by occurrence of the PIFE signal. Histogram of
 830 transcription time is indicated on the right. The indicated error is the error of the gaussian fit.
 831 **(e)** Plot displaying the distribution of tRNA lifetimes during (cyan) and after the transcription
 832 (magenta) of the *GlyQS*_213, *GlyQS*_178 and *GlyQS*_207 DNA templates using slow (S) and fast
 833 (F) transcription rates. Short binding events ($\tau_{bound} < 5$ s) are indicated in black and long-lived
 834 binding events ($\tau_{bound} > 5$ s) are indicated in red. (**** $P < 0.001$, *** $P < 0.005$, ** $P < 0.01$, * $P <$
 835 0.05). **f**, Plots displaying the cumulative bound dwell times of U46^{Cy5}-tRNA after transcription
 836 completion of the *GlyQS*_213, *GlyQS*_178 and *GlyQS*_207 DNA templates. The proportions of
 837 the long-lived bound times ($\tau_{bound} > 5$ s) relative to the overall bound time are indicated.

838

839

840

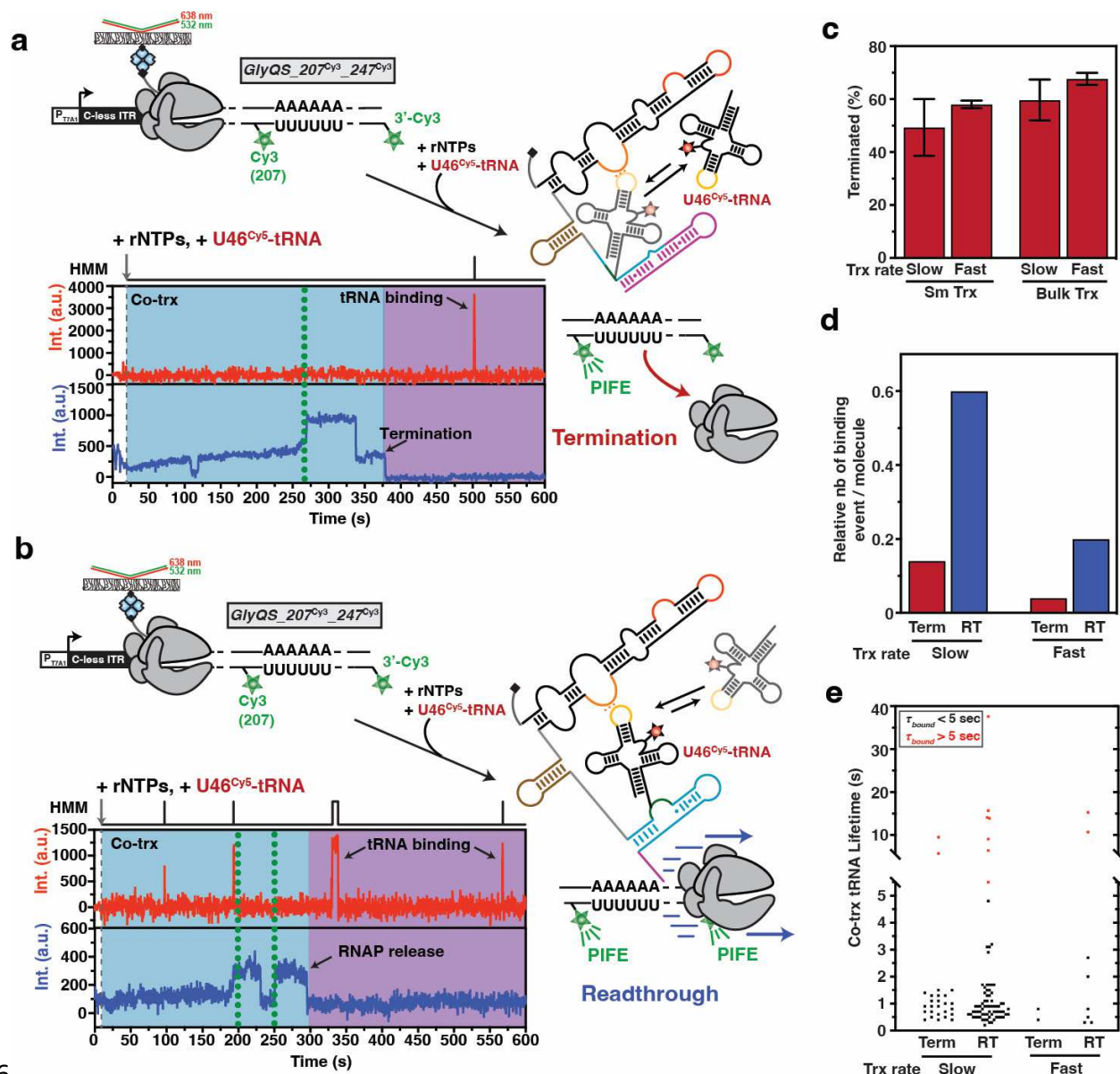
841

842

843

844

845



846

Fig. 6 | Monitoring Transcription Regulation at the Single-molecule Level. a-b, Transcription of the DNA template labeled with two Cy3 fluorophores at the 207 position and downstream of the Poly-U sequence allows for the detection of transcription termination (a) and transcriptional readthrough (b) at the single molecule level. Transcription is re-started upon addition of all rNTPs and addition of U46^{Cy5}-tRNA in the transcription mix allows surveying co-transcriptional ligand binding in real-time. **c,** Transcription termination efficiencies determined during real-time transcription (Sm Trx) and during bulk transcription performed *in vitro* (Bulk Trx). Transcriptions

854 were performed using 25 μM (Slow) or 250 μM (Fast) rNTPs with 25 nM U46^{Cy5}-tRNA. The total
855 number of molecules analyzed is as follows: Terminated_{slow} = 176; Readthrough_{slow} = 119;
856 Terminated_{fast} = 50; Readthrough_{fast} = 37. **d**, Relative number of U46^{Cy5}-tRNA binding events
857 detected per molecule transcribed in real-time in the context of terminated (Red) and readthrough
858 (Blue) transcripts. Transcriptions were performed using 25 μM (Slow) or 250 μM (Fast) rNTPs
859 with 25 nM U46^{Cy5}-tRNA. **e**, Plot displaying the distribution of tRNA lifetimes during the
860 transcription of the *GlyQS_207^{Cy3}_247^{Cy3}* DNA template during slow and fast transcription rates.
861 Short binding events ($\tau_{\text{bound}} < 5$ s) are indicated in black and long-lived binding events ($\tau_{\text{bound}} > 5$ s)
862 are indicated in red. The total number of molecules analyzed is as follows: Terminated_{slow} = 176;
863 Readthrough_{slow} = 119; Terminated_{fast} = 50; Readthrough_{fast} = 37.

864

865

866

867

868

869

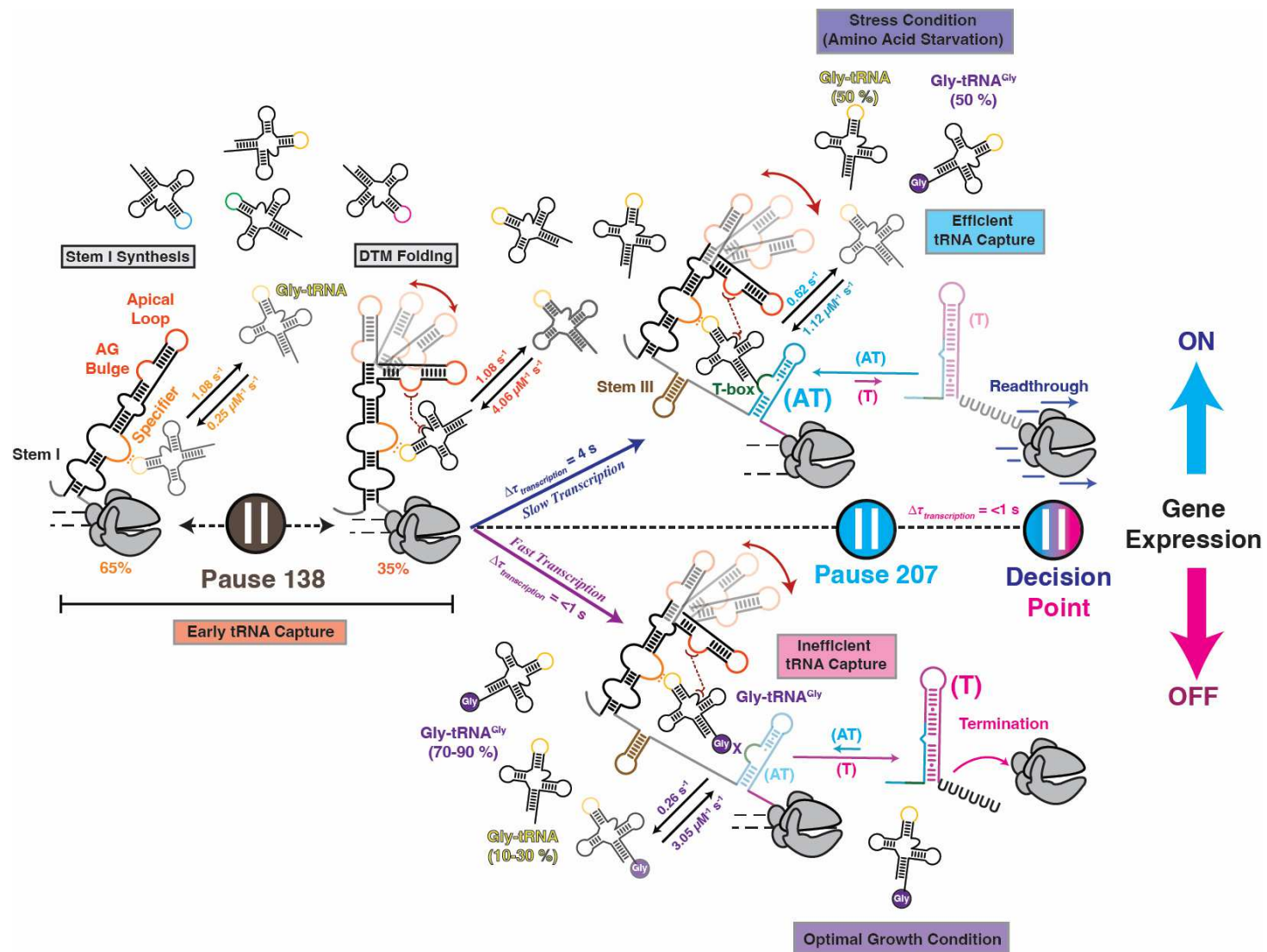
870

871

872

873

874



875

876Fig. 7 | Co-transcriptional Model of Gly-tRNA Sensing to the *Bsu-GlyQS* T-box Riboswitch. Recruitment of tRNA^{Gly} begins once
877Stem-I, with the DTM and the Specifier sequence, emerges from the RNA polymerase (RNAP). The first transcriptional pause site (138)

878allows more time for proper folding of the DTM and early tRNA^{Gly} engagement. Stem-I binds both the charged and uncharged tRNAs
879with similar k_{on} and k_{off} ³¹. Under stress condition (top panel), transcription is slowed down ($\tau_{transcription} \sim 4$ s), favoring the anchoring of
880uncharged tRNA^{Gly}. Moreover, a longer-lasting pause 207 prior to the decision point allows additional time for AT folding and efficient
881anchoring of the tRNA^{Gly} 3'-end. In the absence of stress (bottom panel), transcription progress is fast ($\tau_{transcription} < 1$ s), directly
882competing with ligand anchoring (bound time ~ 3 s). In addition, under this cellular condition the majority of tRNA^{Gly} molecules will be
883charged, therefore creating an additional competition barrier that suppresses uncharged tRNA^{Gly} anchoring, thereby down-regulate the
884expression of downstream genes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ChauvieretalDirectionalTBoxRiboswitchFoldingXPausingDriveRegulationSIFinal.docx](#)