

tRNA-dependent conformational dynamics and folding coordinate translational regulation by a full-length T-box riboswitch

Corresponding Author: Dr Xianyang Fang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Niu et al. 2025 – Nature Communications

Reviewer Comments/Questions

Overview:

In this manuscript, Niu et al. investigate the conformational dynamics of the ileS tRNA-sensing (T-box) riboswitch in *Mycobacterium tuberculosis*. Using single-molecule fluorescence resonance energy transfer (smFRET), they track the conformational changes induced by tRNA binding across different riboswitch constructs and tRNA variants. Their assays reveal that tRNA sensing occurs in two distinct steps, with the two domains responsible for efficient tRNA binding folding independently. Additionally, they identify the "linker" region as a critical determinant for distinguishing between charged and uncharged tRNA, adding another layer of regulation mediated by riboswitch folding. This study builds on well-established techniques previously described by the same group (PMID: 37968328) and employs methodologies widely used in the field, including for studies on other T-box riboswitches. While it confirms a previously proposed two-step binding mechanism, it also provides a more comprehensive mechanistic understanding of the full-length riboswitch, particularly its genetic regulatory region (expression platform). Moreover, it enhances our understanding of RNA folding dynamics during transcription. As such, the study offers valuable insights into RNA biology and biophysics. Nevertheless, the manuscript has some weaknesses in the organization and interpretation of the data. The authors should consider the following suggestions for improvement.

Major Comments:

1) The references to "co-transcriptional" folding starting with the titles are arguably an overstatement. While the findings here are strong and provide new insights into T-box riboswitch folding and tRNA sensing and discrimination, the authors do not perform their experiments in a way that would be close to a co-transcriptionally folded RNA. In particular, the consistent use of high Mg²⁺ concentrations (20 mM) is far from being close to the *in vivo* concentration, especially in this particular bacterial species. In addition, the authors only use arbitrarily truncated lengths of the ileS T-box riboswitch (i.e., not based on known or experimentally determined transcriptional pause sites) that, after transcription, are heat-denatured and refolded *in vitro*. In fact, it has been shown that the presence of bacterial RNA polymerase and additional cellular proteins such as transcription factors plays a significant role in modulating the co-transcriptional folding of RNAs and particularly riboswitches (e.g., PMID: 30388413; 35017489; 34782462), which are not included in the present study. In another example, the authors use the T7 phage RNA polymerase to generate their RNA, which has been shown in numeral studies to not behave similarly to the bacterial enzyme in terms of the transcription rate, transcriptional pausing behavior, and RNA-protein interactions. Finally, the only mention and consideration of "co-transcriptional folding" comes very late in the main text (Figure 6) and is displayed as only a small panel.

Therefore, while the notion of co-transcriptional folding is well-suited for the Discussion, the authors ought to temper their statements throughout the Results section since the current work does not elucidate the co-transcriptional folding of this riboswitch.

2) The authors present their smFRET data using TDPs (Transition Density Plots). While this representation is often used in this field, this methodology is not well-suited for datasets containing mostly static molecules, as this obscures this population entirely and risks poor statistics and relevance of the quantified rate constants from only a low fraction of molecules. This is especially true for the data presented in Figures 4 and 5, where the presence of the AntiS stabilizes the overall dynamics of

the T-box riboswitch. The authors to utilize the previously described TODPs (Transition Occupancy Density Plots; PMID 20580964) to mitigate this problem and further highlight the inherent behavior of a majority of molecules in these datasets.

Minor Comments:

- 1) There are numerous spelling and grammatical errors in the text, which should ideally be proofread by a native speaker. The following lists a few examples. In addition, some of the references are incomplete, e.g., Ref. 18.
- 2) Page 2, Line 24: Please correct the spelling of "mechanism".
- 3) Please define the term "AntiS" in the Abstract.
- 4) Page 2, Line 34: Please correct the spelling of "sequestrator". Please check through the entire manuscript, since this error was found several times, for example: Page 4, Lines 117 and 119.
- 5) Page 3, Line 54: Please correct the spelling of "existence".
- 6) Page 3, Line 56-57: The statement "[...] found to be widely spread in Gram-positive bacteria including some pathogenic bacteria but less in humans,..." should be corrected because it implies that such riboswitches exist in human, which is not correct.
- 7) Page 9, Line 161: "[...] even the first contact site is formed" misses a word.
- 8) Page 9, Line 163: Please avoid the contraction (it's), here and throughout the manuscript.
- 9) Figure 2a: The complete description of the panel figure is presented only late into the text (page 21). It would be beneficial from a reader's perspective to either fully describe the figure or to present the results in a separate figure later in the narrative focusing on the mutant constructs.
- 10) In Figure 2a, it would be greatly appreciated if the authors could comment on the differences in migration between the different mutants relative to the WT. For example, why does the G96C construct migrate much higher on the native gel than the WT in the absence of tRNA?
- 11) Page 9, Line 179: please provide a description of the PDDF acronym.
- 12) Page 10, Line 185: Please correct the notation for the tRNA constructs to follow the 5' to 3' orientation. For example, tRNA Δ -5' Cy3 should be: 5'-Cy3-tRNA Δ
- 13) Supplemental Figure 3: Could the authors provide a lane-by-lane quantification (and a titration plot to yield an apparent KD) for their EMSA in order to corroborate that the presence of the fluorophore does not impact the binding affinity as stated in Page 10, Lines 191-192? In addition, when using the labeled construct T166-6Cy3/46Cy5 (Supplementary figure 3), an additional band is visible below the T-Box/tRNA complex. Could the authors please comment on this band? Does it signify a substantial fraction of misfolded RNA in this case, but not others, perhaps because of interference by the fluorophores?
- 14) Figure 2 panels d-g: The color scale for the TDPs should probably not have units of s⁻¹, but instead reflect a probability distribution.
- 15) Page 11, Lines 225-226: Where do the rate constants come from? There is no reference to them anywhere in a panel.
- 16) Similarly to the EMSA in panel a, the description in the text of the results obtained for the different mutants in the Figure 2, namely G96C, G94U and Δ 88-93, only appears later in the text. Please revise the text or figure organization to present the data in similar order in text and figures.
- 17) The results section is ending abruptly without a clear or concise conclusion.
- 18) Page 13, Line 247: What does N. far stand for?
- 19) Page 13, Line 247: Please correct "dynamcis".
- 20) Page 13, Line 250: Replace "firstly" by "first" and throughout the manuscript.
- 21) Page 14, Line 263: Please correct "labeleling".
- 22) Page 14, Lines 264-266: The authors mentioned that the high Mg²⁺ concentration (20 mM) could account for the presence of the mid-FRET population, representing the docking state in the apo form. It would be beneficial to perform experiments at a lower Mg²⁺ concentration (e.g., 5 mM), at least for the apo condition, to corroborate this statement. The authors may want to consider this suggestion throughout the manuscript and for all the labeling schemes tested.
- 23) Page 14, Lines 283-284: The sentence is not grammatically correct, as a word seems to be missing.
- 24) Figure 3: To characterize the contribution of the second-step binding to the overall dissociation kinetics, the authors used a lower concentration of tRNA for the two constructs tested (panels d and e). Therefore, the conclusion about cooperative binding is not strongly backed up by the experiments. It would be more convincing to present a titration with tRNA and/or using a mutant construct that does not allow for the second step.
- 25) Page 18, Line 353: Remove "becomes".
- 26) Page 24, Lines 428-429: What do the authors mean with "T-box induction assay".
- 27) Page 26, Line 493: Please correct "nasent".
- 28) Page 29, Line 538: Please correct "attatched".
- 29) Figure 7: As a summary, it would be useful for the authors to incorporate into the model the different rate constants determined in the present study.
- 30) Page 30, Line 568: Please correct "discriminitor".
- 31) Could the authors comment on how their findings relate to the regulation of gene expression mediated by tRNA sensing and T-Box riboswitch folding? In other words, how do the presented results and findings align with regulation of translation initiation (ribosome binding) and/or transcription-translation coupling? The Discussion could be more detailed in light of the presented model.
- 32) The manuscript would benefit from the consideration of more work related to co-transcriptional folding and regulation by riboswitches. Currently, only 41 citations are used with some being relatively outdated. For example, reference 27 has long been a reference review for co-transcriptional RNA folding but is now almost 20 years old. It is suggested that the authors consider more recent work and reviews such as PMIDs: 33850018; 38211587; 34740970; 39067113; 34348029; 28134517; 35736222 to cite just a few.

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The study by Niu et al. investigates the dynamic conformational landscape and regulatory mechanism of a full-length translational ileS T-box riboswitch from *Mycobacterium tuberculosis*, using cutting-edge single-molecule FRET (smFRET) and SAXS analyses. The authors implemented an unnatural base pair (UBP)-based internal labeling strategy they had previously developed to track the conformational shifts induced by tRNA binding in real time. They reveal a stepwise, conformational selection model in which both charged and uncharged tRNAs guide distinct structural transitions in the T-box, modulating translation initiation. The authors propose a two-step sequential binding mechanism: first, T-box stem I recruits tRNA through the specifier-anticodon interaction, followed by recognition and binding of the 3' NCCA end via its discriminator domain, similar to the transcriptional *B. subtilis* glyQS T-box riboswitch. Specifically, they show that the aptamer domain of the ileS T-box exhibits significant conformational heterogeneity in the absence of the tRNA ligand. Upon tRNA binding, this heterogeneity diminishes and most T-box molecules adopt a closed, more stable conformation, consistent with prior findings (Campos-Chavez et al., Nature Comms 2024) in which a smFRET approach was used to examine the core of the translational ileS T-box riboswitch from *M. tuberculosis*. However, that study lacked the RBS region and used two tRNA constructs (one non-aminoacylated containing the NCCA end, and another lacking this 3' terminus) to study T-box–tRNA complex dynamics. The current study confirms that tRNA anticodon binding to the specifier enhances complex formation independently of the aminoacylation status of the tRNA, as previously shown for the transcriptional glyQS T-box riboswitch from *B. subtilis*. Moreover, this work extends beyond prior knowledge: unlike earlier studies based on truncated constructs, it examines the entire T-box, including the regulatory AntiS/Sequestrator domain. This enables insights into actual translation initiation regulation and demonstrates that T-box folding occurs co-transcriptionally and is regulated by binding of the tRNA's 3' NCCA end. The study advances our understanding of RNA-based translational regulation and co-transcriptional folding of full-length T-box riboswitches and provides a more physiologically relevant model compared to those based on truncated constructs. However, several points must be addressed.

Major Comments

1. The authors describe co-transcriptional dynamics effectively. However, an experiment comparing folding trajectories under post-transcriptional conditions (with and without tRNA) would more explicitly reveal the influence of transcriptional context.
2. Mechanistic claims regarding the roles of base triples in pre-docking or the S-turn in stabilization could be strengthened by rescue mutations (e.g., compensatory base swaps) that restore folding or function.
3. The impact of aminoacylation on AntiS vs. Sequestrator conformations should be addressed in greater detail. Since competition with translation affects the availability of tRNA ligands (e.g., competition with elongation factor Tu), analyzing chemically aminoacylated tRNA or mimics, even partially, would bolster the conclusions about charged vs uncharged tRNA regulation.
4. The authors should clarify whether anticodon pairing is reversible or locked once formed. This distinction would sharpen the mechanistic difference between conformational selection and induced fit.
5. The two-step conformational selection model is supported by EMSA, SAXS, and smFRET data. However, the discussion could better contrast this model with traditional “induced-fit” paradigms in other T-box systems, emphasizing how this model aligns with real-time data and improves regulatory efficiency.
6. The comparison between *M. tuberculosis* and *N. farcinica* T-boxes offers insight into species-specific adaptations. However, the influence of stem II length on the mechanism remains speculative. Further elaboration would help clarify this point.

Minor Comments

1. Since UBP labeling is central, the authors should briefly discuss whether this strategy may influence global folding or domain interactions near the label sites.
2. In several smFRET experiments, transition densities are described qualitatively. Quantitative transition rates and confidence intervals should be reported more explicitly to substantiate statistical claims.
3. Some figure legends (particularly for smFRET data) are overly dense. While the supplementary material is robust, the main text should more clearly reference specific figures or tables (e.g., Supplementary Table 1) when discussing SAXS parameters or mutant constructs.
4. A schematic folding timeline illustrating how transcriptional elongation aligns with structural transitions (i.e., formation of AntiS and Sequestrator) would be a helpful addition to the supplementary material.
5. Use consistent terminology for domains throughout the text (e.g., “AntiS” vs “AntiSequestrator”) to avoid confusion.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have performed a major revision of the manuscript that addresses all points my team and I raised. Of note, the utility of TODPs lies in that they allow to suitably represent static smFRET time traces as on-diagonal spots on the heatmap, properly calibrated to the number of molecules that show this behavior. Instead, the authors noted the fraction of static versus dynamic molecules, which serves a similar purpose and is acceptable.

Reviewer #2

(Remarks to the Author)

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Reviewer #3

(Remarks to the Author)

The revised version of manuscript #NCOMMS-25-09151A by Niu et al appears improved. The authors addressed all the points raised convincingly and supported their conclusions with additional experimentation. Overall, it is a significant work that provides novel additional insights into the transcriptional folding dynamics of elaborate regulatory RNA macromolecules, such as riboswitches, and its results are sound and merit publication.

Reviewer #4

(Remarks to the Author)

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Reviewer #1:

In this manuscript, Niu et al. investigate the conformational dynamics of the ileS tRNA-sensing (T-box) riboswitch in Mycobacterium tuberculosis. Using single-molecule fluorescence resonance energy transfer (smFRET), they track the conformational changes induced by tRNA binding across different riboswitch constructs and tRNA variants.

Their assays reveal that tRNA sensing occurs in two distinct steps, with the two domains responsible for efficient tRNA binding folding independently. Additionally, they identify the "linker" region as a critical determinant for distinguishing between charged and uncharged tRNA, adding another layer of regulation mediated by riboswitch folding.

This study builds on well-established techniques previously described by the same group (PMID: 37968328) and employs methodologies widely used in the field, including for studies on other T-box riboswitches. While it confirms a previously proposed two-step binding mechanism, it also provides a more comprehensive mechanistic understanding of the full-length riboswitch, particularly its genetic regulatory region (expression platform). Moreover, it enhances our understanding of RNA folding dynamics during transcription. As such, the study offers valuable insights into RNA biology and biophysics.

Nevertheless, the manuscript has some weaknesses in the organization and interpretation of the data. The authors should consider the following suggestions for improvement.

Response #1: We thanks the reviewers for their nice comments and constructive suggestions on our work. We have addressed all the concerns point by point as below.

Major Comments:

1) *The references to “co-transcriptional” folding starting with the titles are arguably*



an overstatement. While the findings here are strong and provide new insights into T-box riboswitch folding and tRNA sensing and discrimination, the authors do not perform their experiments in a way that would be close to a co-transcriptionally folded RNA. In particular, the consistent use of high Mg^{2+} concentrations (20 mM) is far from being close to the in vivo concentration, especially in this particular bacterial species. In addition, the authors only use arbitrarily truncated lengths of the ileS T-box riboswitch (i.e., not based on known or experimentally determined transcriptional pause sites) that, after transcription, are heat-denatured and refolded in vitro. In fact, it has been shown that the presence of bacterial RNA polymerase and additional cellular proteins such as transcription factors plays a significant role in modulating the co-transcriptional folding of RNAs and particularly riboswitches (e.g., PMID: 30388413; 35017489; 34782462), which are not included in the present study. In another example, the authors use the T7 phage RNA polymerase to generate their RNA, which has been shown in numeral studies to not behave similarly to the bacterial enzyme in terms of the transcription rate, transcriptional pausing behavior, and RNA-protein interactions.

Finally, the only mention and consideration of “co-transcriptional folding” comes very late in the main text (Figure 6) and is displayed as only a small panel.

Therefore, while the notion of co-transcriptional folding is well-suited for the Discussion, the authors ought to temper their statements throughout the Results section since the current work does not elucidate the co-transcriptional folding of this riboswitch.

Response #2: We appreciate the reviewers’ comments that the notion of co-transcriptional folding is well-suited for discussion, and agree with the reviewers that our claimed “co-transcriptional folding” differs from the cellular co-transcriptional folding in several aspects. We have then revised the title of manuscript and the discussion section.



In this study, we used constructs with different lengths to mimic the co-transcriptional folding of T-box riboswitch (Figure 7c-d). As the reviewers mentioned, the transcriptional pause sites of translational *ileS* T-box riboswitch have not been reported so far. We then determined the lengths of truncated constructs used in this study based on the predicted secondary structure of T-229. Since SD sequence is complementary to the upstream sequence in the AntiS region (Figure 1) and will be base paired with this region to form the thermodynamically stable Sequestrator structure, indicating that SD sequence plays an essential role in the structural transition between AntiS and Sequestrator conformations. Therefore we used truncations before and after the SD sequence (T209 and T215) together with full-length T229 and structurally well-defined T166 to mimic the transcription intermediates of T-box riboswitch. We agree with the reviewer that these *in vitro* transcribed constructs are different from the naturally transcribed RNAs in cells, still, these data provide some insights into the potential role of co-transcriptional folding on AntiS/Sequestrator transition and tRNA NCCA end recognition of *ileS* T-box riboswitch. Additionally, we also prepared co-transcriptionally synthesized and dual-fluorophore labeled T-box/tRNA complex and our data further supported the conclusion that the recognition of tRNA NCCA end is facilitated by the transcription process. In this study, the site-specific co-transcriptional fluorophore labeling was achieved by adding Cy5-labeled TPT3TP and tRNA variants into the transcription system. The limitation of this method is that only one fluorophore can be labeled. Future advances in the site-specific co-transcriptional dual-labeling methods for RNAs will enable a more comprehensive investigation of the co-transcriptional structural dynamics of T-box riboswitch.

2) The authors present their smFRET data using TDPs (Transition Density Plots). While this representation is often used in this field, this methodology is not well-suited for datasets containing mostly static molecules, as this obscures this population



entirely and risks poor statistics and relevance of the quantified rate constants from only a low fraction of molecules. This is especially true for the data presented in Figures 4 and 5, where the presence of the AntiS stabilizes the overall dynamics of the T-box riboswitch. The authors need to utilize the previously described TODPs (Transition Occupancy Density Plots; PMID 20580964) to mitigate this problem and further highlight the inherent behavior of a majority of molecules in these datasets.

Response #3: Thank you for the comments and suggestions. We have performed TODP analysis for T-box constructs in Figure 4 and Figure 5 (Figure 6 in revised manuscript) and the TODPs were summarized in Supplementary Figures 6 and 7. In TODPs, all of the labeling constructs in each condition showed similar transitions between two FRET states as that we observed in the corresponding TDPs. The fractions of dynamic traces over the total number of molecules in each condition are very close, ranging from 40% to 60% (Supplementary Figures 6 and 7).

Minor Comments:

1) There are numerous spelling and grammatical errors in the text, which should ideally be proofread by a native speaker. The following lists a few examples. In addition, some of the references are incomplete, e.g., Ref. 18.

Response #4: Thanks for the reviewers' careful review. We have corrected all of the spelling and grammatical errors throughout the text.

2) Page 2, Line 24: Please correct the spelling of "mechanism".

Response #5: Corrected.

3) Please define the term "AntiS" in the Abstract.



Response #6: Yes. We have defined the term “Antisequestrator” with “AntiS” in the Abstract.

4) *Page 2, Line 34: Please correct the spelling of “sequestrator”. Please check through the entire manuscript, since this error was found several times, for example: Page 4, Lines 117 and 119.*

Response #7: Corrected.

5) *Page 3, Line 54: Please correct the spelling of “existence”.*

Response #8: Corrected.

6) *Page 3, Line 56-57: The statement “[...] found to be widely spread in Gram-positive bacteria including some pathogenic bacteria but less in humans,...” should be corrected because it implies that such riboswitches exist in human, which is not correct.*

Response #9: Yes. This sentence has been corrected in the revised manuscript.

7) *Page 9, Line 161: “[...] even the first contact site is formed” misses a word.*

Response #10: Yes. We have corrected this sentence in the revised manuscript. “...even though the first contact site is formed.”

8) *Page 9, Line 163: Please avoid the contraction (it’s), here and throughout the manuscript.*

Response #11: Thank you for pointing this out. We have corrected these errors throughout the manuscript.



9) Figure 2a: The complete description of the panel figure is presented only late into the text (page 21). It would be beneficial from a reader's perspective to either fully describe the figure or to present the results in a separate figure later in the narrative focusing on the mutant constructs.

Response #12: Thank you for the suggestions. We have moved the panels of mutants in Figure 2 to Figure 5 (a new figure) in the revised manuscript.

10) In Figure 2a, it would be greatly appreciated if the authors could comment on the differences in migration between the different mutants relative to the WT. For example, why does the G96C construct migrate much higher on the native gel than the WT in the absence of tRNA?

Response #13: Thank you for the suggestions. It is likely that G96C mutation leads to the folding of T-box into a less compact structure than WT, which will migrate slower on the native gel. We have included this explanation in the revised manuscript (Page 21).

11) Page 9, Line 179: please provide a description of the PDDF acronym.

Response #14: PDDF is the acronym for paired distance distribution function. We have described it in the revised manuscript (Page 9).

12) Page 10, Line 185: Please correct the notation for the tRNA constructs to follow the 5' to 3' orientation. For example, tRNA Δ -5'Cy3 should be: 5'-Cy3-tRNA Δ

Response #15: Thanks for the suggestions. We have replaced "tRNA Δ -5'Cy3" with "5'-Cy3-tRNA Δ " in the revised manuscript.



13) *Supplemental Figure 3: Could the authors provide a lane-by-lane quantification (and a titration plot to yield an apparent K_D) for their EMSA in order to corroborate that the presence of the fluorophore does not impact the binding affinity as stated in Page 10, Lines 191-192? In addition, when using the labeled construct T166-6Cy3/46Cy5 (Supplementary figure 3), an additional band is visible below the T-Box/tRNA complex. Could the authors please comment on this band? Does it signify a substantial fraction of misfolded RNA in this case, but not others, perhaps because of interference by the fluorophores?*

Response #16: Thank you for your suggestions. We have quantified the EMSA results and plotted the bound fractions as a function of [T-box]:[tRNA] molar ratios in Supplementary Figure 3b.

For the labeled construct T166-6^{Cy3}/46^{Cy5}, the lower band should be the unbound T-box riboswitch, which shifted to a higher position in the presence of tRNA. As the reviewer pointed out, this band is not clear or smear in other unlabeled or labeled constructs, which might be due to the minor interference of fluorophores. However, as all the labeling sites are located at the loop regions, this labeling scheme has a minor effect on the tRNA binding affinity by T-box riboswitch.

14) *Figure 2 panels d-g: The color scale for the TDPs should probably not have units of s^{-1} , but instead reflect a probability distribution.*

Response #17: The TDPs in the current manuscript are represented as the number of transitions per seconds, so the color scale for the TDPs have units of s^{-1} . To avoid confusion, in the figure legend, we stated ‘Transition density plots illustrate number of transition events per second.’



15) Page 11, Lines 225-226: *Where do the rate constants come from? There is no reference to them anywhere in a panel.*

Response #18: Sorry for the confusion. The rate constants were calculated by fitting the single-molecule traces in Figure 2d with a two-state HMM model and then the dwell time was extracted to calculate the k_{on} and k_{off} . We have revised the text and also summarized the dwell time and transition rates in Supplementary Table 4.

16) *Similarly to the EMSA in panel a, the description in the text of the results obtained for the different mutants in the Figure 2, namely G96C, G94U and Δ 88-93, only appears later in the text. Please revise the text or figure organization to present the data in similar order in text and figures.*

Response #19: Thanks for the suggestions. We have reorganized Figure 2 and moved the mutants in Figure 2 to Figure 5 (a new Figure) in the revised manuscript.

17) *The results section is ending abruptly without a clear or concise conclusion.*

Response #20: Thanks for the suggestions. We have revised the results section.

18) Page 13, Line 247: *What does N. far stand for?*

Response #21: *N. far* is the acronym of *Nocardia farcinica*, we have described it in the revised manuscript.

19) Page 13, Line 247: *Please correct “dynamcis”.*

Response #22: We have corrected it in the revised text.



20) Page 13, Line 250: Replace “firstly” by “first” and throughout the manuscript.

Response #23: We have corrected it in the revised text.

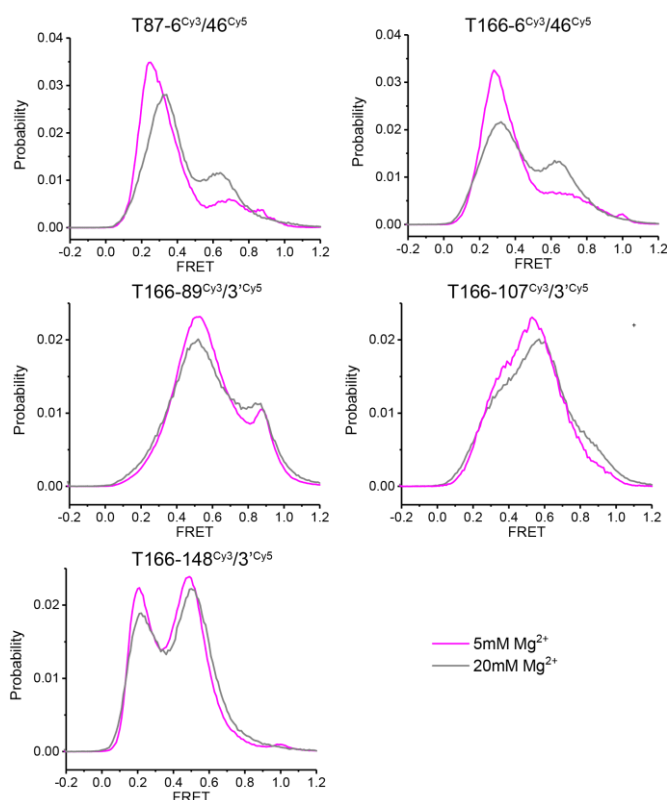
21) Page 14, Line 263: Please correct “labeleling”.

Response #24: We have corrected it in the revised text.

22) Page 14, Lines 264-266: The authors mentioned that the high Mg^{2+} concentration (20 mM) could account for the presence of the mid-FRET population, representing the docking state in the apo form. It would be beneficial to perform experiments at a lower Mg^{2+} concentration (e.g., 5 mM), at least for the apo condition, to corroborate this statement. The authors may want to consider this suggestion throughout the manuscript and for all the labeling schemes tested.

Response #25: Thanks for the suggestions. We have performed smFRET experiments for all the labeling constructs in the presence of 5 mM Mg^{2+} (Response figure 1). For T87-6^{Cy3}/46^{Cy5} and T166-6^{Cy3}/46^{Cy5}, the low-FRET value in 5 mM Mg^{2+} is slightly lower than that in 20 mM Mg^{2+} ($E \sim 0.27$ vs 0.33) and the middle-FRET occupation decreases from $\sim 30\%$ to $\sim 10\%$. For another three labeling constructs, the FRET histograms are very close in both conditions.

In our previous work on *N. farcinica* T99 (PMID: 37968328), Mg^{2+} titration (1-20 mM) experiments suggested that Mg^{2+} promote the pre-folding of T-box stem I and II, which is essential for the high-affinity binding between T-box and tRNA. Considering the highly conservation of primary sequence and secondary structure between T-boxes from these two species, performing smFRET experiments for *M. tub* T-box in 20 mM Mg^{2+} was used to ensure the high-affinity binding of T-box to its cognate tRNA.



Response figure 1. Comparison of FRET histograms for each T-box labeling construct *in apo* form, under 5 mM Mg^{2+} and 20 mM Mg^{2+} conditions.

23) Page 14, Lines 283-284: The sentence is not grammatically correct, as a word seems to be missing.

Response #26: We have corrected the sentence as “These results suggest that the first-step binding may not be thermo-dynamically affected by the second-step binding.” in the revised text.

24) Figure 3: To characterize the contribution of the second-step binding to the overall dissociation kinetics, the authors used a lower concentration of tRNA for the two constructs tested (panels d and e). Therefore, the conclusion about cooperative binding is not strongly backed up by the experiments. It would be more convincing to



present a titration with tRNA and/or using a mutant construct that does not allow for the second step.

Response #27: Thank you for your suggestions. We have performed smFRET experiments for T87-6^{Cy3}/46^{Cy5} in the presence of different concentrations of tRNA^{Ile} (from 0.1 nM to 2 μ M). The occupancy of middle-FRET fraction increases until the tRNA^{Ile} concentration reaches 100nM (Supplementary Figure 5). However, the middle-FRET fraction for T166-6^{Cy3}/46^{Cy5} in the presence of only 10 nM tRNA^{Ile} is close to that in the presence of saturating tRNA^{Ile}. These results support the conclusion that the second-step binding contributes to the stability of T-box/tRNA complex.

25) Page 18, Line 353: Remove “becomes”.

Response #28: We have corrected the sentence as “stem III moves away from the bottom helix of AntiS upon binding to the aminoacylated tRNA.” in the revised manuscript.

26) Page 24, Lines 428-429: What do the authors mean with “T-box induction assay”.

Response #29: In Robert A. Battaglia’s study (PMID: 31740853), a T-box induction assay was employed to assess the translational regulation activity of *ileS* T-box by measuring the β -galactosidase activity in bacterial cells after tyrosine starvation.

27) Page 26, Line 493: Please correct “nasent”.

Response #30: Yes. We have corrected it in the revised text.

28) Page 29, Line 538: Please correct “attatched”.



Response #31: Yes. We have corrected it in the revised text.

29) Figure 7: As a summary, it would be useful for the authors to incorporate into the model the different rate constants determined in the present study.

Response #32: Thanks for the suggestions. The rate constants determined in this study have been incorporated into the model in Figure 7 (Figure 8 in the revised manuscript).

30) Page 30, Line 568: Please correct “discriminator”.

Response #33: Yes. We have corrected it in the revised text.

31) Could the authors comment on how their findings relate to the regulation of gene expression mediated by tRNA sensing and T-Box riboswitch folding? In other words, how do the presented results and findings align with regulation of translation initiation (ribosome binding) and/or transcription-translation coupling? The Discussion could be more detailed in light of the presented model.

Response #34: Thanks for the suggestions. We have revised the discussion section.

32) The manuscript would benefit from the consideration of more work related to co-transcriptional folding and regulation by riboswitches. Currently, only 41 citations are used with some being relatively outdated. For example, reference 27 has long been a reference review for co-transcriptional RNA folding but is now almost 20 years old. It is suggested that the authors consider more recent work and reviews such as PMIDs: 33850018; 38211587; 34740970; 39067113; 34348029; 28134517; 35736222 to cite just a few.



Response #35: Thank you for your suggestions. We have updated the citations in the revised manuscript.

Reviewer #2:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3:

The study by Niu et al. investigates the dynamic conformational landscape and regulatory mechanism of a full-length translational ileS T-box riboswitch from Mycobacterium tuberculosis, using cutting-edge single-molecule FRET (smFRET) and SAXS analyses. The authors implemented an unnatural base pair (UBP)-based internal labeling strategy they had previously developed to track the conformational shifts induced by tRNA binding in real time. They reveal a stepwise, conformational selection model in which both charged and uncharged tRNAs guide distinct structural transitions in the T-box, modulating translation initiation. The authors propose a two-step sequential binding mechanism: first, T-box stem I recruits tRNA through the specifier-anticodon interaction, followed by recognition and binding of the 3' NCCA end via its discriminator domain, similar to the transcriptional B. subtilis glyQS T-box riboswitch. Specifically, they show that the aptamer domain of the ileS T-box exhibits significant conformational heterogeneity in the absence of the tRNA ligand. Upon tRNA binding, this heterogeneity diminishes and most T-box molecules adopt a closed, more stable conformation, consistent with prior findings (Campos-Chavez et al., Nature Comms 2024) in which a smFRET approach was used to examine the core of the translational ileS T-box riboswitch from M. tuberculosis. However, that study



lacked the RBS region and used two tRNA constructs (one non-aminoacylated containing the NCCA end, and another lacking this 3' terminus) to study T-box-tRNA complex dynamics. The current study confirms that tRNA anticodon binding to the specifier enhances complex formation independently of the aminoacylation status of the tRNA, as previously shown for the transcriptional glyQS T-box riboswitch from B. subtilis. Moreover, this work extends beyond prior knowledge: unlike earlier studies based on truncated constructs, it examines the entire T-box, including the regulatory AntiS/Sequestrator domain. This enables insights into actual translation initiation regulation and demonstrates that T-box folding occurs co-transcriptionally and is regulated by binding of the tRNA's 3' NCCA end. The study advances our understanding of RNA-based translational regulation and co-transcriptional folding of full-length T-box riboswitches and provides a more physiologically relevant model compared to those based on truncated constructs. However, several points must be addressed.

Response #36: Many thanks for the reviewer's comments and suggestions. We have addressed all the concerns point-by-point as below.

Major Comments

1. The authors describe co-transcriptional dynamics effectively. However, an experiment comparing folding trajectories under post-transcriptional conditions (with and without tRNA) would more explicitly reveal the influence of transcriptional context.

Response #37: Thanks for the reviewer's comments and suggestions. We actually have performed smFRET experiments for T229-139^{Cy5}/166^{Cy3} and T229-148^{Cy5}/166^{Cy3} under post-transcriptional conditions (Figure 7a). The FRET histograms for T229 in the presence of tRNA^{Ile} are very close to that *in apo* form, rather than showing a major high-FRET ($E \sim 0.8$) or middle-FRET ($E \sim 0.6$) state as that for T166. Therefore, we speculated that *in vitro* synthesized T229 mainly adopts



the Sequestrator conformation *in apo* form, which presumably impairs the recognition of tRNA NCCA 3'-end. We then use T229-139^{Cy5}/tRNA-5'^{Cy3} instead of T229-139^{Cy5}/166^{Cy3} or T229-148^{Cy5}/166^{Cy3} to study the co-transcriptional folding and tRNA recognition of T-box due to the limitations in the current dual fluorescent co-transcriptional labeling methods (Figure 7c). Although the fluorophores were labeled in tRNA and T-box, respectively, this labeling scheme still provide some insights into the conformational dynamics of full-length T-box induced by tRNA binding. As shown in Figure 7 d and f, binding of tRNA^{Ile} induced a middle-FRET ($E \sim 0.6$), which becomes more significant and homogeneity in the co-transcriptional context. Future development of dual fluorescent co-transcriptional labeling techniques will enable a more comprehensive understanding about the co-transcriptional T-box conformational dynamics.

2. Mechanistic claims regarding the roles of base triples in pre-docking or the S-turn in stabilization could be strengthened by rescue mutations (e.g., compensatory base swaps) that restore folding or function.

Response #38: Thanks for the reviewer's suggestions. We have constructed a G96C-C131G rescue mutant and performed smFRET experiments for this mutant (G96C-C131G) *in apo* form, in the presence of tRNA^Δ, tRNA^{Ile} and tRNA⁷⁸ (Supplementary Figure 8). As expected, this mutant mainly samples high-FRET state in the presence of tRNA^{Ile} and transits to middle-FRET upon addition of tRNA⁷⁸, suggesting that this rescue mutation restores the tRNA NCCA 3'-end discrimination function of T-box riboswitch.

3. The impact of aminoacylation on AntiS vs. Sequestrator conformations should be addressed in greater detail. Since competition with translation affects the availability of tRNA ligands (e.g., competition with elongation factor Tu), analyzing chemically



aminoacylated tRNA or mimics, even partially, would bolster the conclusions about charged vs uncharged tRNA regulation.

Response #39: Thanks for the reviewer's suggestions. Since aminoacylated tRNAs are reported to be unstable and prone to be hydrolyzed under experimental conditions and the aminoacylation efficiency is usually not high enough *in vitro*, we finally used tRNA⁷⁸ with an extra nucleotide C at the 3'-end of tRNA^{Ile} to mimick the aminoacylation state. This allowed us to capture the tRNA aminoacylation induced structural dynamics of T-box. Previous structural and biochemistry studies also used tRNA variants with a smaller terminal function group like cyclic phosphate, azido, et al to mimick the aminoacylation state, which would not completely disrupt the T-box/tRNA complex but also capable of capture conformational changes (PMID: 31740854, 31740853).

We have performed smFRET experiments for full-length T229 construct in the presence of tRNA⁷⁸ under both post-transcriptional and co-transcriptional conditions (**Figure 7f**) to further clarify the impact of aminoacylation on AntiS/Sequestrator conformations. Under both conditions, binding of tRNA⁷⁸ induced a significant high-FRET ($E \sim 0.8$) population, coexisting with the low-FRET ($E \sim 0.35$) and middle-FRET ($E \sim 0.6$) state. This is quite different from the tRNA^Δ bound state ($E \sim 0.35$) and tRNA^{Ile} bound state ($E \sim 0.6$). These data suggested that NCCA end of tRNA⁷⁸ interacts with T-Box but form a structure different from T-box/tRNA^{Ile} ($E \sim 0.8$ vs. 0.6). Additionally, our smFRET data on T166 in the presence of different tRNA constructs also showed that tRNA⁷⁸ binding stabilizes a structural intermediate distinct from that in the T-box/tRNA^{Ile} complex, in which the single-stranded linker region dynamically transits to a conformational state away from the discriminator domain and subsequently leads to structural rearrangement of stem III.



4. *The authors should clarify whether anticodon pairing is reversible or locked once formed. This distinction would sharpen the mechanistic difference between conformational selection and induced fit.*

Response #40: Thank you for the suggestions. According to our smFRET data on T87-6^{Cy3}/46^{Cy5} and T166-6^{Cy3}/46^{Cy5} (Figure 3), the decoding domain of T-box in the absence of tRNA samples both of low-FRET and middle-FRET state, which corresponds to the stem I/II undocking and docking conformation, respectively. Upon addition of tRNA, the middle-FRET population becomes much more prominent. In addition, transition density plots also showed that decoding domain of T-box transits frequently between low- and middle-FRET state under each condition. These data indicated that decoding domain of T-box is in a dynamic undocking and docking equilibrium *in apo* form and binding of tRNA anticodon to the specifier loop shifted the equilibrium toward the docking conformation, which is consistent with the mechanism of conformation selection model.

5. *The two-step conformational selection model is supported by EMSA, SAXS, and smFRET data. However, the discussion could better contrast this model with traditional “induced-fit” paradigms in other T-box systems, emphasizing how this model aligns with real-time data and improves regulatory efficiency.*

Response #41: Thank you for the suggestions. We have revised the discussion sections. Briefly, the conformational selection mechanism may facilitate faster and more efficient ligand recognition, as the bound-like conformation is already pre-existing in the apo state. This is especially critical for riboswitches that must respond within a restricted time window. Moreover, the binding energy requirement and its corresponding energy barrier is lower in the conformational selection mechanism, as the induced-fit pathway may need additional energy penalty to drive RNA conformational changes. Therefore, the conformational selection mechanism



adopted by *M. tuberculosis* T-box could enable a faster and more efficient translation regulation.

5. *The comparison between M. tuberculosis and N. farcinica T-boxes offers insight into species-specific adaptations. However, the influence of stem II length on the mechanism remains speculative. Further elaboration would help clarify this point.*

Response #42: Thank you for the comments and suggestions. We propose that the impact of stem II length on the induced-fit versus conformational selection mechanism can be explained based on a structural perspective. According to the structure of T-box/tRNA complex (PDB: 6UFG, 6UFM), stem II coaxially stacks with stem IIA/B pseudoknot and further assembles with stem I to recognize the tRNA anticodon. Longer stem II in *N. farcinica* T-box may contribute to a more stable stacking interaction with stem IIA/B and the orientation of stem I relative to stem IIA/B is fixed in high Mg^{2+} , thus results in an undocked conformation *in apo* form and only tRNA binding induced the transition from undocked state to docked state. However, shorter stem II in *M. tuberculosis* T-box may have a weaker stacking interaction with stem IIA/B, therefore the conformation *in apo* form is relatively flexible and transits between undock and docking-like conformations. This hypothesis requires further experiments (smFRET assay on T-box constructs with different stem II lengths) to validate, which is beyond the scope of this manuscript.

6. Minor Comments

1. *Since UBP labeling is central, the authors should briefly discuss whether this strategy may influence global folding or domain interactions near the label sites.*

Response #43: Thanks for the suggestions. Since the labeling sites used in our study are mostly located in the single-stranded regions of T-box riboswitch, it should have a minor effect on the global folding of RNA. Additionally, EMSA data on all the



labeling constructs used in this study suggested that the UBP-based labeling has a minor effect on the tRNA binding affinity of T-box riboswitch (Supplementary Figure 3). We have also revised the discussion section and briefly discussed the potential effect of UBP-based labeling strategy on the folding and functions of RNAs (Page 32).

2. In several smFRET experiments, transition densities are described qualitatively. Quantitative transition rates and confidence intervals should be reported more explicitly to substantiate statistical claims.

Response #44: Thanks for the suggestions. We have summarized the dwell time and transition rates of different FRET states for each construct in different conditions into the Supplementary Table 4.

3. Some figure legends (particularly for smFRET data) are overly dense. While the supplementary material is robust, the main text should more clearly reference specific figures or tables (e.g., Supplementary Table 1) when discussing SAXS parameters or mutant constructs.

Response #45: Thanks for the suggestions. We have revised the figure legends and the main text, so the Supplementary material is clearly referenced.

4. A schematic folding timeline illustrating how transcriptional elongation aligns with structural transitions (i.e., formation of AntiS and Sequestrator) would be a helpful addition to the supplementary material.

Response #46: Thank you for the suggestions. We have modified the Figure 8 for a better illustration on how transcriptional elongation aligns with structural transitions.



5. Use consistent terminology for domains throughout the text (e.g., “AntiS” vs “AntiSequestrator”) to avoid confusion.

Response #47: Thanks for the suggestions. We have defined AntiS in the revised manuscript (line 47-48, Page. 3) and used AntiS throughout the main text.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.