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Peer Review File

Translational T-box riboswitches bind tRNA by modulating conformational flexibility

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this paper, the authors investigate the intrinsic and tRNA-dependent conformational dynamics of a translational T-box riboswitch. They find that this T-box riboswitch undergoes spontaneous transitions between conformational states in the absence of the tRNA indicating that binding of the tRNA is likely mediated through conformational selection. Using mutations in the tRNAs and riboswitch to isolate interactions with the discriminator and decoding domains, the authors demonstrate that the tRNA is recognized through both domains independently and that anticodon interaction is recognized first followed by the aminoacylation status. These results are convincing and provide important new insight into how RNA structural dynamics drive the molecular function of the riboswitch. I think the authors have done a fantastic job applying single-molecule methods to characterize this T-box riboswitch's structural dynamics. I have a few comments on the author's conclusions:

Major comments:

1. The authors do not include the complete RBS/antiRBS stem in their RNA constructs possibly for technical reasons and therefore, a major question is left unanswered: how do the structural dynamics for recognition of the tRNA lead to rearrangement of the RBS/antiRBS stem? If the riboswitch is sampling the closed conformation, does that mean that the RBS/antiRBS stem is also conformationally dynamic and if so, how does regulation occur post-transcription? Would the authors explain in more detail what they think the structural relationship is between the intrinsic riboswitch dynamics and the regulatory outcome of revealing the RBS for translation initiation?

2. The authors suggest that "translation regulation is not limited to cotranscriptional regulation, but could also potentially occur on fully transcribed mRNAs." Is there evidence that the translational T-box riboswitches undergo conformational rearrangement post-transcription upon tRNA binding? In the Sherwood, Grundy, and Henkin PNAS paper that the authors cite, the folding path of the riboswitch is measured co-transcriptionally and indicated that tRNA binding influences accessibility of the RBS, but it is not clear to me if it has been established that binding of the tRNA to a fully transcribed 5'UTR would facilitate rearrangement of the RBS/antiRBS stem post-transcription and enable additional rounds of

regulation as the authors suggest. Further, the authors note that their constructs fold heterogeneously when refolded and only natively fold vectorially. I wonder if this observation supports that the folding path of the riboswitch is established during transcription and therefore, regulation must also be. Would the authors explain in more detail why they think that regulation is not occurring during transcription?

3. Along similar lines, translation initiation in Actinobacteria is likely functionally coupled to transcription similar to E. coli such that the activity of the riboswitch could still be under kinetic control at the very least in the pioneering round of translation. Would the authors include a commentary on this in the discussion?

Minor comments:

1. In Fig 3A, please indicate and label the mutant on the figure so that it is differentiated from previous figures.

2. It is confusing that Figure 3H-K is not mentioned until after Figure 4 is discussed. For example, the largely diminished 0.7 state is mentioned but is not discussed in detail until later in the text making it hard to follow the logic. Please consider revising so that the flow of the figures is not disrupted in the text.

3. I am confused by the statement that the mutant 0.5 state with a lifetime of 18.8 s is more transiently sampled than the WT 0.7 state with a lifetime of 21 s. What does this mean?

4. The authors report some of the rates in the model figure that correspond to the mutant riboswitch or tRNA but their assignments are not explained in the text. For example, the transition from the 0.4 state to the 0 state is reported as 0.0533 s-1 for both lifetimes of the 0.4 state, but this number is the transition rate for the 0.5 state to 0. Please explain the reasoning.

5. The kinetic model is hard to follow because the authors intersperse rates with the mutants which had different FRET efficiencies. It would be helpful if the authors included a figure, perhaps in the supplement, with all the rates drawn in a scheme like Figure 8 containing all the mutants analyzed.

6. In the model figure, the structure of the open state looks more closed than the closed state and is different than the structures shown in Figure 5. What are the states that the authors are trying to depict? Perhaps “open” and “closed” labels could be modified to indicate that the states are similar to tRNA bound and unbound?

Reviewer #2 (Remarks to the Author):

The manuscript by Campos-Chavez et al. describes the results of single-molecule FRET characterization of a T-box riboswitch. These are widespread genetic regulators in bacteria, where they sense the aminoacylation state of cognate tRNAs and regulate gene expression accordingly. As regards their locus of action, T-boxes are known that function during transcription or translation. Previously the kinetics of a transcriptional riboswitch were characterized by smFRET. The new study examines a translational riboswitch. Overall the results appear technically sound. As T-boxes are important regulators in many bacteria, I think the results would be of interest beyond the narrow confines of T-box biology, and thus I think in principle suitable for publication. I have some issues with presentation of data that the authors may wish to consider.

1. While the SEC chromatograms in fig S1 are interesting, denaturing PAGE analysis of the purified material needs to be shown for each species. In addition, it would be valuable to show SEC of the purified material, and re-chromatography after some time under the storage conditions the authors use, to establish how well resolved their material is in relation to the first chromatography, and if there is conformational homogeneity (at this gross level, at least) of the RNAs being studied by smFRET.

2. In figure 1C, the dashed lines indicating what distances are measured are very hard to see. The panel should be modified in some way so that the 29 and 40 A lines are more visible.

3. A major concern tis that for a number of derived parameters, no statistical analysis is present or p-values presented when statements are made that changes occurred. Thus, for instance, in supplementary table 1, what are the uncertainties in the kinetic constants and the Kd's? When the text states that, for instance the Kd for tRNA ice increased from 0.474 to 1.48 uM (in the RAG section; incidentally, manuscript pages ought to be numbered), first, what is the statistical basis for quoting Kd's to three significant digits and (2) are these two numbers actually different? At a minimum a t-test should be quoted; I am open to more sophisticated analysis, if that is forthcoming.

4. Finally, throughout the manuscript, the authors insist that this study shows that translational riboswitches functon(in various ways) differently from transcriptional ones. This is a fallacy. What they show is that this particular T-box is different from the specific glycine T-box studied previously. How general the finding is remains to be established (and I think is immaterial to the merit of the present work).

Reviewer #2 (Remarks on code availability):
I didn't see code as part of the supplemental file I was able to download.

Reviewer #3 (Remarks to the Author):
The authors use single molecule FRET to study the conformation of T-box riboswitch in response to the related tRNA ligand. Besides the direct kinetic measurement of conformational transitions, the authors utilize mutants/variants and alternative labeling strategies to identify crucial intermediates in riboswitch ligand recognition and folding. Most interestingly, the authors are able to distinguish the apo closed conformation of the riboswitch, suggesting the riboswitch may pre-fold, and then capture the ligand to become fully folded. Such conformational selection folding pathway is distinct from the induced-fit mechanism found previously adopted by a transcriptional T-box riboswitch.
Overall, the manuscript is well written and organized, while figures are mostly clear—the reviewer finds the kinetic network summarized in figure 8 particularly confusing, and thus in need for clarification/modification (see comments below). It's worth noting that the two-step folding of another T-box riboswitch has been characterized in previous work by the same group(s) (ref. 19). It is possible that part of the current data interpretation may be affected by the pre-established kinetic model. Such examples can be found in the reviewer comments below.
This manuscript may be considered for publication after major revision. The authors need to clarify their kinetic model with unbiased data analysis.

Major comments:
1. Figure 2b shows three types of trajectories and the figure caption states: "Type I trajectories only show FRET efficiency around 0.7, Type II trajectories only show FRET values

around 0.4, and Type III trajectories sample the 0.4 and 0.7 states." The author seems to neglect the overall > 20% of direct transitions between 0.0 and 0.7 (according to the supplementary info), which apparently contradicts to their two-step folding model.

2. The reviewer would also like to point out the 0.0 to 0.7 transition is missing in the density plot (Figure 2d), or perhaps color coded in plain white. The probability threshold to selectively show data points seems arbitrary, and possibly misleading. The authors should know the 0.0 to 0.7 transitions are real, and also necessary for detailed balance.

3. According to the manuscript, photobleaching of Cy5 may accelerate the 0.4/0.7 dwell time decay (Figure 2e), resulting underestimated lifetimes for each state. The reviewer is also curious about the dwell time plot for the 0.0 state (not shown), where photobleaching and Cy5-unlabeled tRNA could potentially disrupt the single exponential decay.

4. Figure 5b indicates the intra-FRET construct responds to 5/13 μM tRNA, while the tRNA concentration in the inter-FRET study is only 100 nM. Although it is unambiguous that some structure is spontaneously formed in the absence of tRNA ligand, the authors should provide more evidence to show that the conformational changes in the intra-FRET is actually relevant to the overall folding of the T-box riboswitch studied by inter-FRET.

5. The parameter A appears to fluctuate a lot in each dwell time fit—e.g., A = 1 in Figure 6d and 6e; A = 0.398 in Figure 7d. Since the data are fit to the presumed single exponential decay model in HMM analysis, the significant deviation may suggest a different kinetics. Moreover, the reviewer is concerned that some of the data are fit with fixed A (i.e., A set to exactly 1 in Figure 2e, 6d and 6e), while the rest are not. If so, the authors should explain the reason to analyze the data differently.

6. To follow up., fitting uncertainties (for both k and A) would be helpful to indicate the fitting qualities, and to determine suitable significant figures to report.

7. (page 7) "While the nature of the 0.4 FRET state is not clear from the data above, there are more frequent transitions from the zero to the 0.4 state than from the zero to the 0.7

state, suggesting that it is more likely for tRNAIle to bind this unknown intermediate conformation associated with the 0.4 state than the fully bound state."
There is less/no tRNA binding to the fully bound state because it is fully bound. Moreover, according to the provided kinetic model, binding to the 0.0 state (not 0.4) promotes 0.0 to 0.4 transition. The quoted statement doesn't seem to make sense.

8. (page 8) "The lifetimes of the partially bound states in these two cases were two to three times longer compared to the lifetime of the 0.4 FRET state observed in the case of the WT MT ileS T-box with tRNAIle (Supplementary Table VI). This difference is expected however, given the fact that the 0.4 FRET state in WT MTBC ileS T-box with tRNAIle is capable of transitioning to the fully bound state."
It is peculiar that the authors choose to compare the lifetimes of the 0.4 state, instead of the 0.4 to 0.0 transition rates that are readily provided by HMM. It would be a nice opportunity for the authors to make a meaningful comparison to relate different FRET states in WT/mutant experiments.

9. The reviewer has multiple questions/concerns about Figure 8.
a. 1.91 x 105 s-1 M-1 should be the sum of the rates for the low FRET state to each of the 0.4 (0.5) states. It's also unclear where the reverse rates 0.0533 s-1 comes from—unfolding with or without ligand is unlikely to share the same rate.
b. For the 0.4 and 0.7 states/transitions, the authors mix the results obtained from WT/ΔNCCA experiments, which is extremely confusing. For instance, the transition rates are inconsistent with lifetimes. The inclusion of ΔNCCA data seems only to overcomplicate the mechanism.

Minor points:
1. Figure 2b is too small to read.
2. Figure 3a and 4a are extremely similar. It's hard to tell if the tiny displacement of tRNA is related to each of the mutations.
3. Figure 3g, the authors label the population transient, while its lifetime is > 6s, one of the longest in this study.

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Campos-Chavez *et al.*

We thank all three reviewers for their overall positive and constructive comments. As one critique expressed by more than one reviewer relates to the final model (Figure 8) and associated rate constants (#4-6 of Reviewer 1 and #9 of Reviewer 3), we would like to address this concern first, followed by point-by-point responses to each reviewer. **In the revised version, this longer explanation on the choice of rate constants used in the model is also included as Supplementary Information together with an annotated version of Figure 8.**

1. The final model is built based on a combination of data from the WT T-box with WT tRNA complex, and the WT T-box with $tRNA^{-\Delta NCCA}$ complex, as these combinations give a better representation of the fully and partially bound states. In the presence of WT tRNA, the majority of the traces only show a stable FRET state at 0.7 (Type I-1, 71.0%, Supplementary Table III), suggesting that when both the anticodon and uncharged NCCA end are present, the T-box/tRNA complex is very stable. As this stable population is already formed prior to the start of the imaging experiment, it provides a good estimate of the overall stability of the fully bound state, but with limited kinetic information related to reaching that state. Within the remaining traces, 17.8% corresponds to ones only sampling the 0.4 FRET state (Type II, Supplementary Table III) and 4.9% sampling both the 0.4 and 0.7 FRET state (Type III, Supplementary Table III), which represent either the population that fails to reach the stable fully bound state, or a slow population in the binding process. Therefore, while Type II and III traces are indicative of the presence of the 0.4 FRET state, they do not represent the majority of the population and are not ideal to reveal accurately or confidently the binding rates. $tRNA^{Ile\text{-}\Delta NCCA}$, on the other hand, is uncapable of forming the fully bound state but nonetheless attempts futilely to reach a stable 0.7 state from the 0.4 state, as shown clearly in Figure 4. For this reason, the $tRNA^{Ile\text{-}\Delta NCCA}$ data gives a better estimation of the first binding step, from 0 to 0.4. In addition, $tRNA^{Ile\text{-}\Delta NCCA}$ does sample the 0.7FRET state, but without establishing a stable interaction with the discriminator domain due to the absence of the NCCA sequence to base pair with the T-box sequence. Thus, the $tRNA^{Ile\text{-}\Delta NCCA}$ data also provide kinetic information for the conformational sampling step before the formation of the fully bound state. For these reasons, we mainly use the $tRNA^{Ile\text{-}\Delta NCCA}$ data to estimate the kinetic parameters, except for the stability of the fully bound state, where the use of the WT tRNA is needed.

2. Based on the above explanation, we have completely revised Figure 8 to help explain better the kinetic model.
   (a) In this model, the partially bound state (middle panel in Figure 8) refers to a state after anticodon recognition but before NCCA binding. The partially bound state can sample both the 0.5 and 0.7 FRET states. The fully bound state refers to the state after both anticodon and NCCA binding (right panel in Figure 8) and corresponds to the 0.7 FRET state.
   (b) Due to the reasons explained in 1) above, the binding and dissociation rate constants for the anticodon recognition step are calculated using $tRNA^{Ile\text{-}\Delta NCCA}$ data.
   (c) The partially bound state has two observed type of traces, which we termed One-state and Two-state populations (Figure 4 and Supplementary Table III). The One-state population visits the only 0.5 FRET state transiently from the zero state (Figure 4a), whereas the Two-state population largely remains in the 0.5 FRET state but rapidly samples the 0.7 FRET state (Figure 4e). $\tau^{(0.5)}_{partially\_1}$

corresponds to the lifetime of the 0.5 FRET in the One-state population of the partially bound state, whereas $\tau(0.5)_{partially\_2}$ corresponds to the lifetime of the 0.5 FRET in the Two-state population of the partially-bound state. Considering the relative percentage of these two populations (27% Two-State, 73% One-state, Supplementary Table III), we can estimate the average lifetime of the 0.5 FRET state ($\tau(0.5)_{partially}$) to be 4.8 s. $\tau(0.7)_{partially}$ corresponds to the lifetime of the unstable 0.7 FRET in the partially bound state, which is very short lived as evident in Figure 4e. As explained in (1), all the transitions rates and lifetimes are calculated from the $tRNA^{Ile\text{-}\Delta NCCA}$ data.

(d) The $tRNA^{Ile\text{-}\Delta NCCA}$ data show that the partially bound state can transiently and reversibly sample the 0.7 FRET, which probably mimics structurally the fully bound state. In the case of WT tRNA, with an intact NCCA, the 0.7 FRET state is stably locked. However, since the data with WT tRNA mostly report the features of an already formed complex, without capturing the actual transition to form it, we do not know how many attempts to sample the 0.7 FRET state are made before securing the interaction. For this reason, we leave as an unknown ("?") rate constant for the second binding step. We can still estimate the lifetime of the fully bound 0.7 FRET state from the WT tRNA data. This value is probably an underestimate due to i) the complex already being formed when measurements start and ii) the bleaching of the fluorophores.

(e) In the intra-T-box FRET experiments, not many reversible transitions between the zero and 0.3 FRET are observed, suggesting that the transition rate between the open and closed conformation may be slower than the imaging time window. We cannot conclude whether tRNA binds preferentially to one state or equally to both. This would require, for example, 3-color FRET experiments, which are technically challenging given the difficulty on working with this particular T-box. Since binding of tRNA shifts the equilibrium to the closed conformation, it is most likely that the close conformation corresponds to the tRNA bound state. In addition, the 0.3 FRET is expected from the distance between the labeled positions in the tRNA-bound T-box. As we cannot estimate the rates between the 0.3 and zero FRET states, we leave them as unknowns.

(f) As discussed in c), we observe two populations for the partially bound state. The data do not provide sufficient information to measure the interconversion between the two populations. However, given that the Two-state population represents 27% of the total in the partially bound state with $tRNA^{Ile\text{-}\Delta NCCA}$ (Supplementary Table III), but with WT tRNA ultimately 71% of the population (91.8% of the 77.3% Type I traces, Supplementary Table III) are able to reach the stable fully bound state, we reason that the One-state population is able to transit into the Two-state population.

3. There is consistency when using rates calculated from the WT tRNA and $tRNA^{Ile\text{-}\Delta NCCA}$ data.

a) Binding rate constant of anticodon recognition step: assuming the zero to 0.4 and zero to 0.7 transitions in the Type I-2, Type II, and Type III traces report the binding rate of WT tRNA (Type I-1 traces are stable at 0.7 and do not transition from zero), the transition rate from zero to any non-zero state is 0.0094 s^{-1} at 100nM WT tRNA (Supplementary Table V), which is 51% slower than the transition rate from zero to 0.5 FRET state at 100nM tRNA-NCCA (0.019 s^{-1}, Supplementary Table V).

b) Transitions from the 0.4/0.5 state. The transition rate out of the 0.4 FRET state to any other FRET state (zero or 0.7) is 0.144 s^{-1} in the presence of WT tRNA (Supplementary Table V), whereas the transition out of 0.5 FRET to zero (dissociate) or 0.7 (attempt to sample the 0.7 FRET state) in the

Stable population is 0.21 s^{-1} in the presence of $tRNA^{Ile\text{-}\Delta NCCA}$ (see point 1c above), the former being 31% slower than the latter rate.

These consistencies support the notion that the minor population that has not reached the stable 0.7 FRET with WT tRNA represents a population that either fails to reach the stable full bound state or is a slow population in the kinetic process.

Reviewer #1 (Remarks to the Author):

In this paper, the authors investigate the intrinsic and tRNA-dependent conformational dynamics of a translational T-box riboswitch. They find that this T-box riboswitch undergoes spontaneous transitions between conformational states in the absence of the tRNA indicating that binding of the tRNA is likely mediated through conformational selection. Using mutations in the tRNAs and riboswitch to isolate interactions with the discriminator and decoding domains, the authors demonstrate that the tRNA is recognized through both domains independently and that anticodon interaction is recognized first followed by the aminoacylation status. These results are convincing and provide important new insight into how RNA structural dynamics drive the molecular function of the riboswitch. I think the authors have done a fantastic job applying single-molecule methods to characterize this T-box riboswitch's structural dynamics. I have a few comments on the author's conclusions:

We thank the reviewer for the positive comments.

Major comments:

1. The authors do not include the complete RBS/antiRBS stem in their RNA constructs possibly for technical reasons and therefore, a major question is left unanswered: how do the structural dynamics for recognition of the tRNA lead to rearrangement of the RBS/antiRBS stem? If the riboswitch is sampling the closed conformation, does that mean that the RBS/antiRBS stem is also conformationally dynamic and if so, how does regulation occur post-transcription? Would the authors explain in more detail what they think the structural relationship is between the intrinsic riboswitch dynamics and the regulatory outcome of revealing the RBS for translation initiation?

2. The authors suggest that "translation regulation is not limited to cotranscriptional regulation, but could also potentially occur on fully transcribed mRNAs." Is there evidence that the translational T-box riboswitches undergo conformational rearrangement post-transcription upon tRNA binding? In the Sherwood, Grundy, and Henkin PNAS paper that the authors cite, the folding path of the riboswitch is measured co-transcriptionally and indicated that tRNA binding influences accessibility of the RBS, but it is not clear to me if it has been established that binding of the tRNA to a fully transcribed 5'UTR would facilitate rearrangement of the RBS/antiRBS stem post-transcription and enable additional rounds of regulation as the authors suggest. Further, the authors note that their constructs fold heterogeneously when refolded and only natively fold vectorially. I wonder if this observation supports that the folding path of the riboswitch is established during transcription and therefore, regulation must also be. Would the authors explain in more detail why they think that regulation is not occurring during transcription?

3. Along similar lines, translation initiation in Actinobacteria is likely functionally coupled to transcription similar to E. coli such that the activity of the riboswitch could still be under kinetic control at the very least in the pioneering round of translation. Would the authors include a commentary on this in the discussion?

We thank the reviewer for the inspiring questions. Since questions 1-3 are all related to the timing of translational regulation by this particular T-box and how switching from sequestrator to antisequestrator can be achieved post-transcriptionally, we would like to address them together.

(1) The Sherwood, Grundy, and Henkin PNAS (2015) manuscript did the toeprinting assay on 30S binding, where transcription of the full-length T-box was performed in the presence or absence of tRNA. Therefore, it is reflecting the *in vivo* setting, i.e. the tRNA ligand is already there during transcription. However, the experiment itself did not provide discrimination between co- and post-transcriptional folding or ligand binding. While it is highly likely that some tRNAs bind during transcription, the possibility of tRNA binding after transcription persists. Overall, no previous studies have discerned whether tRNA binds co- and post-transcriptionally.

(2) Fundamentally, the probability of cotranscriptional *versus* post-transcriptional binding depends on the accessibility of the binding site and the binding rate, which may not exclusively happen in one stage. We would like to refer the reviewer to one of our early works on bacterial trans-acting small RNA (sRNA) (Reyer *et al*., *Cell Reports*, 2021), where we showed that previously characterized post-transcriptionally regulating sRNAs can actually regulate co-transcriptionally. Later, it was also demonstrated by the Woodson Lab (Rodgers, *et al.*, *Molecular Cell*, 2023) that, for sRNA binding sites that are hard to access after RNA folding, co-transcriptional binding is beneficial, as the binding site is more accessible co-transcriptionally.

(3) Because of the argument in (2), the question then revolves around whether tRNA binding in *ileS* T-box (leading to the formation of the antisequestrator) is possible only co-transcriptionally. This argument largely holds true for the *glyQS* T-box because in this case the terminator structure is much more stable than the antiterminator one (see new Supplementary Figure S9). Interestingly, we noticed that the difference in calculated minimum free energy between the sequestrator and the antisequestrator conformations is much smaller compared to the difference between the *B. subtilis glyQS* T-box terminator and antiterminator conformations (Supplementary Figure S9 shows the minimum free energy for the predicted structures using the RNAFold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi)). In addition, the *ileS* T-box antisequestrator structure has lower energy than the *glyQS* T-box antiterminator structure. This observation suggests that in the absence of tRNA it may be possible that i) in translation-regulating T-box riboswitches, the bias towards folding into the sequestrator over the antisequestrator conformation co-transcriptionally is weaker compared to the terminator over antiterminator conformations in a transcription-regulating T-box; ii) that the antisequestrator conformation, if formed during transcription, can be maintained stably throughout transcription; and iii) that due to the small energy difference, switching between antisequestrator and sequestrator conformations may occur post-transcriptionally in the absence of tRNA ligand. Given these possibilities, we suggest without proving it, that translational regulation by the *Mtb ileS* T-box may not be limited to co-transcriptional regulation, but could also occur post-transcriptionally.

(4) The reviewer raised an interesting question “If the riboswitch is sampling the closed conformation, does that mean that the RBS/antiRBS stem is also conformationally dynamic”. Based on the observations we outline in (3), we think that there is indeed a possibility that the *ileS* T-box is intrinsically more “dynamic” and heterogeneous. These intrinsic dynamic and heterogeneous nature was also observed in the recent smFRET studies of the decoding domain of translation regulating riboswitches. However, since we do have the RBS included in the measurement, we cannot observe any correlations between different conformations. It would certainly inspire future investigations.

We added one paragraph in the Discussion to address these points. We have also revised the Introduction to emphasize post-transcriptional regulation is just one possibility.

Minor comments:
1. In Fig 3A, please indicate and label the mutant on the figure so that it is differentiated from previous figures.

We have reorganized Figures 3 and 4 for added clarity and also added labels in the cartoons to make it clear which molecules are used.

2. It is confusing that Figure 3H-K is not mentioned until after Figure 4 is discussed. For example, the largely diminished 0.7 state is mentioned but is not discussed in detail until later in the text making it hard to follow the logic. Please consider revising so that the flow of the figures is not disrupted in the text.

We have rearranged Figure 3 and 4 by moving the old Figure 4 to the new Figure 3 and making the old Figure 3a-c into new Figure 4.

3. I am confused by the statement that the mutant 0.5 state with a lifetime of 18.8 s is more transiently sampled than the WT 0.7 state with a lifetime of 21 s. What does this mean?

We apologize for the confusion. Indeed, the average lifetime of 18.8 S for the partially bound state with $tRNA^{Ile\text{-}\Delta NCCA}$ should not be considered as "transient". This transient binding is only true for the One-state subpopulation with the lifetime of ~6 s. We have removed this sentence from the text. Please note that to avoid any confusion we have now changed terms, the "transient population" is now the "One-state population" and the "stable population" is now the "Two-state population".

4. The authors report some of the rates in the model figure that correspond to the mutant riboswitch or tRNA but their assignments are not explained in the text. For example, the transition from the 0.4 state to the 0 state is reported as 0.0533 s-1 for both lifetimes of the 0.4 state, but this number is the transition rate for the 0.5 state to 0. Please explain the reasoning.

We addressed this point at the beginning as it relates to the proposed model. We have added a figure in the Supplementary Information (Supplementary Figure 8) together with an extensive figure caption to explain our assignments.

5. The kinetic model is hard to follow because the authors intersperse rates with the mutants which had different FRET efficiencies. It would be helpful if the authors included a figure, perhaps in the supplement, with all the rates drawn in a scheme like Figure 8 containing all the mutants analyzed.

Thank you for this suggestion. We did this. See our responses to 4 above.

6. In the model figure, the structure of the open state looks more closed than the closed state and is different than the structures shown in Figure 5. What are the states that the authors are trying to depict? Perhaps "open" and "closed" labels could be modified to indicate that the states are similar to tRNA bound and unbound?

We have changed the Figure to try to emphasize the difference between the open and closed conformations.

Reviewer #2 (Remarks to the Author):

The manuscript by Campos-Chavez et al. describes the results of single-molecule FRET characterization of a T-box riboswitch. These are widespread genetic regulators in bacteria, where they sense the aminoacylation state of cognate tRNAs and regulate gene expression accordingly. As regards their locus of action, T-boxes are known that function during transcription or translation. Previously the kinetics of a transcriptional riboswitch were characterized by smFRET. The new study examines a translational riboswitch. Overall, the results appear technically sound. As T-boxes are important regulators in many bacteria, I think the results would be of interest beyond the narrow confines of T-box biology, and thus I think in principle suitable for publication. I have some issues with presentation of data that the authors may wish to consider.

We thank the reviewer for the positive comments.

1. While the SEC chromatograms in fig S1 are interesting, denaturing PAGE analysis of the purified material needs to be shown for each species. In addition, it would be valuable to show SEC of the purified material, and re-chromatography after some time under the storage conditions the authors use, to establish how well resolved their material is in relation to the first chromatography, and if there is conformational homogeneity (at this gross level, at least) of the RNAs being studied by smFRET.

We have added data to show that the T-box does remain intact and folded after storing at -80 ^{o}C. Supplementary Figure 1 now has two extra panels, one showing a gel of stored material and one of re-chromatographed samples, to illustrate the integrity of the frozen samples. Due to time constraints with the review, we could only store the material for a bit over a week before doing the experiments.

2. In figure 1C, the dashed lines indicating what distances are measured are very hard to see. The panel should be modified in some way so that the 29 and 40 A lines are more visible.

We have now separated Figure 1c into two panels. The top panel shows smFRET labels for T-box-tRNA FRET and intra-T-box FRET in the WT T-box-tRNA complex, with two views. The bottom panel shows smFRET labels for T-box-tRNA FRET in the Δ-Discriminator/tRNA complex.

3. A major concern is that for a number of derived parameters, no statistical analysis is present or p-values presented when statements are made that changes occurred. Thus, for instance, in supplementary table 1, what are the uncertainties in the kinetic constants and the Kd's? When the text states that, for instance the Kd for tRNA ice increased from 0.474 to 1.48 uM (in the RAG section; incidentally, manuscript pages ought to be numbered), first, what is the statistical basis for quoting Kd's to three significant digits and (2) are these two numbers actually different? At a minimum a t-test should be quoted; I am open to more sophisticated analysis, if that is forthcoming.

We agree that quoting Kd to three significant figures is not warranted. We have changed to one significant figure, which is in agreement with our error estimates. To address the uncertainties in the kinetic constants, we have estimated the errors in our estimates by using a bootstrap method. Using a 10,000 step residuals bootstrap algorithm, we can get estimates of the errors. The bootstrap method shows the errors to be small and that comparing Kd's is within our errors. The errors have been added to the relevant table and a note on the bootstrapping method is now in the Methods section.

In addition, we have added error estimates for the smFRET rate parameters in Supplementary Table V. The errors associated with transition rates are estimated from variance of the transition probability generated by tMAVEN. Overall, we added error estimates for the BLI parameters, the Kd's, the rate constants, and lifetimes. The details in the error estimation are now included in the Methods section.

4. Finally, throughout the manuscript, the authors insist that this study shows that translational riboswitches function (in various ways) differently from transcriptional ones. This is a fallacy. What they show is that this particular T-box is different from the specific glycine T-box studied previously. How general the finding is remains to be established (and I think is immaterial to the merit of the present work).

We have changed the text to make it clear that our results and comparisons are specific to the *B. subtilis glyQS* and *Mtb ileS* T-box riboswitches. We also emphasize in the discussion now that these are "one representative transcription/translation-regulating T-box riboswitch".

Reviewer #2 (Remarks on code availability):
I didn't see code as part of the supplemental file I was able to download.

The code is available along with the Source Data Files, which are now updated to reflect the changes made during revision.

Reviewer #3 (Remarks to the Author):
The authors use single molecule FRET to study the conformation of T-box riboswitch in response to the related tRNA ligand. Besides the direct kinetic measurement of conformational transitions, the authors utilize mutants/variants and alternative labeling strategies to identify crucial intermediates in riboswitch ligand recognition and folding. Most interestingly, the authors are able to distinguish the apo closed conformation of the riboswitch, suggesting the riboswitch may pre-fold, and then capture the ligand to become fully folded. Such conformational selection folding pathway is distinct from the induced-fit mechanism found previously adopted by a transcriptional T-box riboswitch.
Overall, the manuscript is well written and organized, while figures are mostly clear—the reviewer finds the kinetic network summarized in figure 8 particularly confusing, and thus in need for clarification/modification (see comments below). It's worth noting that the two-step folding of another T-box riboswitch has been characterized in previous work by the same group(s) (ref. 19). It is possible that part of the current data interpretation may be affected by the pre-established kinetic model. Such examples can be found in the reviewer comments below.
This manuscript may be considered for publication after major revision. The authors need to clarify their kinetic model with unbiased data analysis.

We thank the reviewer for the overall positive comments.

Major comments:

1. Figure 2b shows three types of trajectories and the figure caption states: "Type I trajectories only show FRET efficiency around 0.7, Type II trajectories only show FRET values around 0.4, and Type III trajectories sample the 0.4 and 0.7 states." The author seems to neglect the overall > 20% of direct transitions between 0.0 and 0.7 (according to the supplementary info), which apparently contradicts to their two-step folding model.

Our initial wording may have led to a confusion regarding the percentage that shows zero to 0.7 transitions. This number is 2.7%, well below 20% (Supplementary Table IV) as explained below. For this reason, we do not think the trace percentage or transition percentage support a significant transition directly from zero to 0.7, which would indeed contradict our model.

More explicitly, the percentage of traces showing only 0.7 FRET state as a non-zero state is 77.3%, among them 91.8% show the single-step transition from 0.7 to zero. Based on this, the fraction of traces that contain the zero to 0.7 transition is only 6.3% (0.773*0.082 from above and Supplementary Table III). In terms of the transitions, 22.6% transitions are from 0.7 to zero (Supplementary Table IV), which is consistent with the fact that the WT tRNA binds stably to the T-box. The 0.7-to-zero transition is largely due to the slow dissociation of the tRNA or photobleaching of the fluorophore. Only 2.7% of the transitions are from zero to 0.7 (Supplementary Table IV), which further supports the notion that it is very unlikely for the tRNA to directly bind to the 0.7 FRET state from zero (unbound) state, compared to the 12.8% of transitions from zero to 0.4 and 16.7% of transitions from 0.4 to 0.7. To contradict the two-step binding model one would expect a significant population of zero to 0.7 FRET transitions and more traces showing a zero to 0.7 FRET transition event.

In summary, we do think that the percentage of traces and percentage of transitions support the idea that a direct transition from zero to 0.7 is unlikely. There is also the possibility that with the intact NCCA end in the WT tRNA, the 2.7% of zero to 0.7 transitions is actually going through the 0.4 FRET state, but due to the time resolution in our experiments (100ms) it may be impossible to see the 0.4 FRET state if the transition is fast. Moreover, our proposed two-step binding model does not only rely on the transition frequencies. We performed two independent experiments, with a noncognate tRNA and with a T-box with the specifier mutated, where we did not observe any binding for either of them, supporting that the binding to the anticodon needs to occur first. To provide more clarity to the readers, we have now added some further descriptions to the main text to explain the expected asymmetry for the percentage of 0.7-to-zero and zero-to-0.7 transitions.

2. The reviewer would also like to point out the 0.0 to 0.7 transition is missing in the density plot (Figure 2d), or perhaps color coded in plain white. The probability threshold to selectively show data points seems arbitrary, and possibly misleading. The authors should know the 0.0 to 0.7 transitions are real, and also necessary for detailed balance.

As explained above, the zero-to-0.7 transition represents only 2.7% of the total transitions. As we had used a lower threshold for the white color, the low percentage transitions did not show up in the transition density plots. The higher density for 0.7-to-zero transition is expected because the T-box/tRNA complex is stable, and the 0.7-to-zero transition accounts for 22.6% of all transitions. It is likely that the 0.7-to-zero transitions are mostly due to photobleaching. To avoid confusion, we have now remade all the TDPs with

a 5% lower threshold. With this lower threshold, many of the less frequent transitions are visible, including the zero-to-0.7 transition.

3. According to the manuscript, photobleaching of Cy5 may accelerate the 0.4/0.7 dwell time decay (Figure 2e), resulting underestimated lifetimes for each state. The reviewer is also curious about the dwell time plot for the 0.0 state (not shown), where photobleaching and Cy5-unlabeled tRNA could potentially disrupt the single exponential decay.

We agree with the reviewer that the photobleaching time would limit the observation time and impact the lifetime analysis in some cases. Particularly, the lifetime estimation for the fully bound state would be most affected because Cy5 photobleaching is faster in higher FRET states, and the fully bound state is long-lived. We therefore emphasized in the manuscript that this lifetime is likely to be underestimated. However, this does not affect our conclusion that the fully bound state is stable. Considering the photobleaching impact, as the apparent lifetime of the fully bound state (0.7 FRET) is over 20 s, we expect the photobleaching time of the 0.4/0.5 FRET state would be even longer than this. Given that the lifetime of the 0.4 and 0.5 FRET state is significantly shorter (6-7 s), we do not expect the photobleaching time causes large misestimation of the lifetime.

We do not expect the dwell time of the zero FRET would be affected by the photobleaching as data points after photobleaching are not included in lifetime/rate analysis. However, as the reviewer suggested, we still included the dwell time analysis for the zero state (see the revised Supplementary Table VI). The dwell time of zero FRET state is well described with single-exponential decay when repetitive tRNA binding is observed, but best described with double-exponential decay in these cases: WT Tbox/tRNA^{Ile} combination, the Two-state subpopulation of WT Tbox/tRNA$^{Ile\text{-}\Delta NCCA}$ combination, and RAG Mutant/tRNA^{Ile} combination. In these three cases, the complexes are either already formed before imaging acquisition and/or tRNAs mostly remains bound, therefore the unbound state is incompletely sampled, which can lead to apparent heterogeneity in the population and inaccuracy of dwell time analysis. We included the discussion of single-exponential and double-exponential decay fitting in the caption of revised Supplementary Table VI.

In addition, in the revised manuscript, we now include lifetime analysis in two different ways, using transition matrix and dwell time analysis (Supplementary Table V and VI). It is possible to see that the lifetime for each non-zero FRET state is highly consistent between the two methods. However, the zero FRET state lifetime has a larger variation between the two analyses, particularly for the above three cases, where the estimation of zero FRET lifetime can be off due to incomplete sampling. Due to these reasons, we do not draw any conclusion from the lifetime analysis of the zero FRET state in the manuscript.

4. Figure 5b indicates the intra-FRET construct responds to 5/13 μM tRNA, while the tRNA concentration in the inter-FRET study is only 100 nM. Although it is unambiguous that some structure is spontaneously formed in the absence of tRNA ligand, the authors should provide more evidence to show that the conformational changes in the intra-FRET is actually relevant to the overall folding of the T-box riboswitch studied by inter-FRET.

The use 100 nM in the inter-FRET experiments is purely due to technical reasons, as high concentrations of labeled tRNA will cause too much background even with TIRF imaging. We chose to use higher concentrations of unlabeled tRNA in the intra-FRET experiment so that the shift in the equilibrium from

the open to the close configuration is more evident given that the K_D of tRNA bound to this T-box is ~0.5-1 μM. Unfortunately, we cannot directly couple tRNA binding to the conformational changes of the T-box without performing 3-color FRET experiments, which are technically challenging given the difficulty on working with this particular T-box. In other words, we cannot directly measure whether tRNA binds preferentially to one state or equally to both. However, since binding of tRNA shifts the equilibrium to the closed conformation, it is most likely that the closed conformation corresponds to the tRNA bound state. While further investigation is required, this data, together with two recently published smFRET studies, show that the *ileS* T-box has different levels of structural heterogeneity, which we added to the Discussion in the revised manuscript, along with the limitations of the current study.

5. The parameter A appears to fluctuate a lot in each dwell time fit—e.g., A = 1 in Figure 6d and 6e; A = 0.398 in Figure 7d. Since the data are fit to the presumed single exponential decay model in HMM analysis, the significant deviation may suggest a different kinetics. Moreover, the reviewer is concerned that some of the data are fit with fixed A (i.e., A set to exactly 1 in Figure 2e, 6d and 6e), while the rest are not. If so, the authors should explain the reason to analyze the data differently.

6. To follow up., fitting uncertainties (for both k and A) would be helpful to indicate the fitting qualities, and to determine suitable significant figures to report.

We thank the reviewer for pointing out the error in the dwell time fitting. Since #5 and #6 are both related to the fitting, we are addressing them together. Indeed, we were not consistently using the exact same fitting throughout. In some cases, the parameter A was constrained to be 1, but not in other cases, which was a mistake. We have now redone all the fitting consistently with unconstrained A. The normalized amplitude A reflects the percentage of the population captured by the fitting. In other words, a small A value in a single-exponential decay fitting suggests that the fitting fails to describe the major population. In most of the cases, the single-exponential decay fitting is sufficient to describe the dwell time description (with A > 0.75), suggesting of a homogenous population. For the cases that A < 0.75, we provide the double-exponential decay fitting. For the example the reviewer mentioned, as the dwell time was not described well with single-exponential decay, we have now included the double-exponential fitting.

For the few cases where double double-exponential decay fitting is needed, the presence of a heterogeneous population is expected. When analyzing all trajectories of the WT T-box/$tRNA^{Ile\text{-}\Delta NCCA}$ complex, the dwell time of the 0.5 FRET state is best fit with a double-exponential decay function, which is consistent with the presence of two subpopulations: One-state and Two-state population. In contrast, the dwell time of the 0.5 FRET state in each of the subpopulation is well described by a single-exponential decay function. Consistent with the presence of two subpopulations in the partially bound state, for the WT T-box/$tRNA^{Ile}$ and RAG Mutant/$tRNA^{Ile}$ constructs, the dwell times of the 0.4 FRET are best described by double-exponential decay functions. We included this discussion in the footnote of the new Supplementary Table VI.

7. (page 7) "While the nature of the 0.4 FRET state is not clear from the data above, there are more frequent transitions from the zero to the 0.4 state than from the zero to the 0.7 state, suggesting that it is more likely for tRNAIle to bind this unknown intermediate conformation associated with the 0.4 state than the fully bound state."

There is less/no tRNA binding to the fully bound state because it is fully bound. Moreover, according to the provided kinetic model, binding to the 0.0 state (not 0.4) promotes 0.0 to 0.4 transition. The quoted statement doesn't seem to make sense.

Thank you for pointing this out. We agree. We revised the sentence.

8. (page 8) "The lifetimes of the partially bound states in these two cases were two to three times longer compared to the lifetime of the 0.4 FRET state observed in the case of the WT MT ileS T-box with tRNAIle (Supplementary Table VI). This difference is expected however, given the fact that the 0.4 FRET state in WT MTBC ileS T-box with tRNAIle is capable of transitioning to the fully bound state." It is peculiar that the authors choose to compare the lifetimes of the 0.4 state, instead of the 0.4 to 0.0 transition rates that are readily provided by HMM. It would be a nice opportunity for the authors to make a meaningful comparison to relate different FRET states in WT/mutant experiments.

We agree with the reviewer that comparing both rates and lifetimes is applicable in our analysis. Specifically, the lifetime of a particular state is the reciprocal of the transition rate from that state to **all other** states. We indeed have compared both lifetimes and transition rates in most cases, particularly when we compared the linker mutant and RAG mutant with the WT T-box. For this particular example mentioned by the reviewer, we actually think our original comparison is confusing due to the presence of both 0.5 FRET and 0.7 FRET states in the partially bound state in the $tRNA^{Ile\text{-}\Delta NCCA}$ experiments. In this case, the lifetime of the partially bound state is actually the average lifetime of tRNA bound without establishing the NCCA-discriminator interactions, which is not equivalent to the lifetime of the partially bound state when using WT tRNA, which can form stable NCCA-discriminator interactions. Therefore, the comparison is not meaningful, and we have removed this sentence from the revised manuscript.

In our point #3(b) of responses to the comments on Figure 8 at the beginning, we compared the transition rate from the 0.4 FRET state, as the reviewer suggested. The transition rate out of the 0.4 FRET state to any other FRET state (zero or 0.7) is 0.144 s^{-1} in the presence of WT tRNA (Supplementary Table V), whereas the transition out of 0.5 FRET to zero (dissociate) or 0.7 (attempt to sample the 0.7 FRET state) in the Two-state population is 0.21 s^{-1} in the presence of $tRNA^{Ile\text{-}\Delta NCCA}$. The rates are consistent with the former being 31% slower than the latter rate. We have now included such comparison in the caption of new Supplementary Figure **S8**.

In addition, we have added a general description of the transition rate and lifetime at the beginning of the revised manuscript, quoted here:

"In most cases —unless specified otherwise—, Global Hidden Markov Modeling (HMM) was used to simultaneously model all trajectories of each particular experimental condition 26. Transition frequencies (Supplementary Tables IV), as well as transition rates from an initial state to a final state (Supplementary Tables V), and state dwell times (Supplementary Tables VI), were revealed by this HMM modeling approach. Throughout this work, we emphasize the transition rates between two FRET states as described by transition matrices (Supplementary Tables V). We also highlight the lifetime of a state before transitioning out of that state —to any other states— from dwell time analysis (Supplementary Tables VI). In addition, the lifetime of a given state was also estimated using the reciprocal of the sum of transition rates out of that state from the transition matrices (Supplementary Tables V). This calculation is largely consistent with the dwell time analyses in the instances hereby presented."

9. The reviewer has multiple questions/concerns about Figure 8.
a. 1.91 x 105 s-1 M-1 should be the sum of the rates for the low FRET state to each of the 0.4 (0.5) states. It's also unclear where the reverse rates 0.0533 s-1 comes from—unfolding with or without ligand is unlikely to share the same rate.
b. For the 0.4 and 0.7 states/transitions, the authors mix the results obtained from WT/ΔNCCA experiments, which is extremely confusing. For instance, the transition rates are inconsistent with lifetimes. The inclusion of ΔNCCA data seems only to overcomplicate the mechanism.

We apologize for the confusion in Figure 8. We have now remade Figure 8 with detailed explanation on the transition rates and the lifetime associated with each state, including the discussion of limitations in Supplementary Figure S8. Please refer to our detailed response at the beginning.

Minor points:

1. *Figure 2b is too small to read.*

We have changed Figure 2 to make the panels larger and easier to read.

2. Figure 3a and 4a are extremely similar. It's hard to tell if the tiny displacement of tRNA is related to each of the mutations.

We have labeled the panels to make it easier to distinguish them.

3. Figure 3g, the authors label the population transient, while its lifetime is > 6s, one of the longest in this study.

We agree that calling it a transient population is confusing. We have renamed the two populations as One-state and Two-state to avoid confusion.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed my concerns.

Reviewer #2 (Remarks to the Author):

I think the revised manuscript is appropriate for publication

Reviewer #3 (Remarks to the Author):

The authors have addressed all the issues raised by the reviewer.