

Deciphering tRNA Repertoires and Translation Coordination during Mouse Early Embryogenesis by ORACLE-tRNAseq

Corresponding Author: Professor Yunfang Zhang

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications. Mentions of prior referee reports have been redacted.

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)

The authors have addressed all of the concerns I raised during the initial review of the manuscript **[REDACTED]**

In the thorough revision the author have incorporated additional controls, cross-validations and analyses were included. I recommend the manuscript for publication.

Reviewer #2

(Remarks to the Author)

Regarding Comment 5, the authors state the following:

[REDACTED]

m²G6 is known to be introduced by Thumpd3–Trmt112. However, no activity converting m²G to m²,²G has been reported. On the basis of RT-signature data alone, the hypothesis that m²,²G is present at this position is completely unacceptable. RT misincorporation patterns are expected to be influenced by the type of tRNA modification as well as by the surrounding sequence context. To make such a claim, it would be necessary to experimentally verify whether mouse Thumpd3–Trmt112 possesses the ability to biosynthesize m²,²G.

Reviewer #3

(Remarks to the Author)

It is difficult to evaluate the author's responses. There are no indications in the response file specifically where each response is incorporated into the revised manuscript, so one cannot easily evaluate the context of the incorporated changes.

As for comment 1, it is not appropriate to compare non-templated RT products of unstructured and unmodified miRNAs with tRNA, as the non-template addition likely depends on the secondary structure and modification in the tRNA (RT can be sensitive to the context of multiple nucleotides in the template). The authors should use a mixture of template switching oligos (not just those ending with GGG; do this at reduced temperature to maximize coverage), or use low-biased ligation (literature methods, not just commercial kits) to sequence their tRNA samples, and then evaluate the proportion of the sequencing products.

I am fine with responses as written here for comments 2-5. Again, the authors should indicate how these responses are incorporated into the text, not just "We have incorporated these benchmark comparison data into the revised manuscript."

Version 1:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The authors adequately addressed my comments.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Point-by-point response for NCOMMS-26-003288-T

Reviewer #1 (Remarks to the Author):

The authors have addressed all of the concerns I raised during the initial review of the manuscript [REDACTED] In the thorough revision the author have incorporated additional controls, cross-validations and analyses were included. I recommend the manuscript for publication.

Response: We appreciate the reviewer's positive assessment of our revised manuscript and recommendation for publication. The reviewer's constructive comments have significantly enhanced the quality and clarity of our work.

Reviewer #2 (Remarks to the Author):

Regarding Comment 5, the authors state the following:

[REDACTED]

m2G6 is known to be introduced by Thumpd3–Trmt112. However, no activity converting m2G to m2,2G has been reported. On the basis of RT-signature data alone, the hypothesis that m2,2G is present at this position is completely unacceptable. RT misincorporation patterns are expected to be influenced by the type of tRNA modification as well as by the surrounding sequence context. To make such a claim, it would be necessary to experimentally verify whether mouse Thumpd3–Trmt112 possesses the ability to biosynthesize m2,2G.

Response: We thank the reviewer for this important and insightful comment. We concur that RT misincorporation signatures alone are insufficient to definitively identify the presence of m2,2G at position 6 of Asp-GTC-1. As noted, while the Thumpd3–Trmt112 complex is known to catalyze m2G6, there is currently no experimental evidence demonstrating an enzymatic conversion from m2G6 to m2,2G6 in mammals. Additionally, it is worth noting that m2G10 generally does not induce misincorporation during reverse transcription (Hao-Tian Lei *et al.*, *Nucleic Acids Research*, 2022). We also acknowledge that RT-derived mismatch patterns may be influenced not only by the chemical identity of the modification but also by local sequence context. Therefore, we have revised the manuscript to avoid presenting m2,2G6 as a definitive modification assignment. Instead, we now describe the observed mismatch pattern as being consistent with, but not conclusive for, the presence of a modification with properties similar to m2,2G ([Results section 8, page 11, lines 438-441](#)).

We agree that experimentally confirming whether mouse Thumpd3–Trmt112 is capable of biosynthesizing m2,2G is necessary to substantiate such a mechanistic claim. We would like to note, however, that a detailed biochemical characterization of the enzymatic activity in question is beyond the scope of current study. The example was included primarily to illustrate the dynamic and potentially coordinated changes in tRNA modifications during embryonic development, which can be effectively captured by ORACLE-tRNAseq. Accordingly, we have revised the text to clarify this intention and to prevent any overinterpretation of the data ([Results section 8, page 11, lines 446-448](#)).

We appreciate the reviewer highlighting this point, which has helped us refine both the interpretation and presentation of these results.

Reviewer #3 (Remarks to the Author):

It is difficult to evaluate the author's responses. There are no indications in the response file specifically where each response is incorporated into the revised manuscript, so one cannot easily evaluate the context of the incorporated changes.

Response: We apologize for the lack of clear cross-referencing in our previous response file. In this revised submission, we have carefully annotated both the current and previous round of response with specific page and line numbers indicating where each change has been incorporated into the manuscript. The previous response with detailed cross-referencing for your part is included at the end of this file for your convenience. We also uploaded the revised version of our previous response file to the submission system, which including clear incorporation marks for all of the reviewers' comments.

As for comment 1, it is not appropriate to compare non-templated RT products of unstructured and unmodified miRNAs with tRNA, as the non-template addition likely depends on the secondary structure and modification in the tRNA (RT can be sensitive to the context of multiple nucleotides in the template). The authors should use a mixture of template switching oligos (not just those ending with GGG; do this at reduced temperature to maximize coverage), or use low-biased ligation (literature methods, not just commercial kits) to sequence their tRNA samples, and then evaluate the proportion of the sequencing products.

Response: We thank the reviewer for this thoughtful comment and agree that non-templated RT additions observed in miRNAs may not fully recapitulate those occurring during tRNA reverse transcription. As the reviewer accurately point out, template switching behavior may be influenced by the secondary structure and various chemical modifications of the RNA. In light of this, we have refrained from extrapolating the previously presented miRNA data to tRNAs within the manuscript.

To evaluate the reliability of our sequencing strategy, we conducted a spike-in test (Figure R1-A, [Results section 1, page 4, lines 148–152](#)) and benchmarked our tRNA sequencing workflow against several established, widely-used tRNA sequencing methods, such as mim-tRNAseq, Induro-tRNAseq, ALL-tRNAseq, Hydro-tRNAseq (Figure R1-B, [Results section 1, page 4, lines 144–148](#)). This comparison at readout-level, rather than focusing on individual library preparation variables, demonstrates strong concordance with other methods, including those incorporating low-bias ligation or related bias-reduction strategies (e.g., mim-tRNAseq, Induro-tRNAseq), thereby supporting the reproducibility of our workflow and the reliability of the comparative analyses presented in this study.

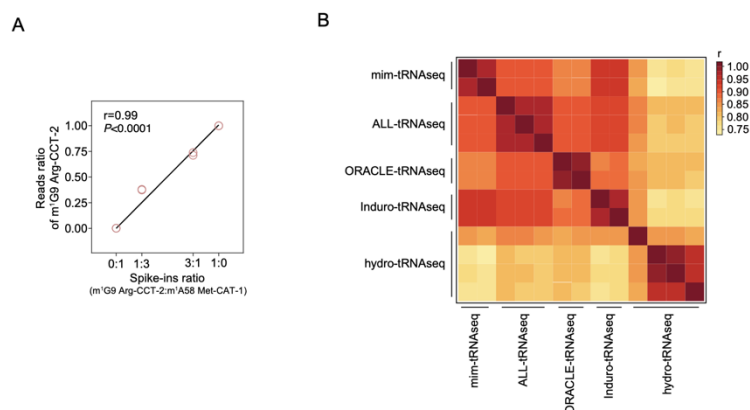


Figure R1. Quantitative assessment of ORACLE-tRNAseq. (A) Correlation between the input ratio of m¹G9 Arg-CCT-2 and m¹A58 Met-CAT-1 oligos and the misincorporation ratio detected by ORACLE-tRNAseq. Pearson correlation coefficient is shown. (B) Heatmap shows Spearman correlation comparison of HEK-293T datasets generated by five tRNA-seq methods (at the tRNA isodecoder level).

We fully acknowledge that reverse transcription and library preparation biases cannot be completely excluded in tRNA sequencing. To address this, we have added statements in the Discussion section explicitly acknowledging these potential limitations ([Discussion section, page 11, lines 459–461](#)).

I am fine with responses as written here for comments 2-5. Again, the authors should indicate how these responses are incorporated into the text, not just “We have incorporated these benchmark comparison data into the revised manuscript.”

Response: We thank the reviewer for the careful evaluation of our responses to comments 2–5 and for indicating that the responses are satisfactory. We apologize for the lack of clarity in the previous response file. In the revised response (this round and a previous round), we have now explicitly indicated where each revision has been incorporated into the manuscript by providing page and line numbers for all changes.

The previous round of point-by-point response with incorporation markers:

Editorial Note: comments here, and originally on the subsequent pages have been omitted from this file in line with our policy to publish only those comments from the Peer Review Process while at Nature Communications