nature portfolio

Peer Review File

Single-Molecule Direct RNA Sequencing Reveals the Shaping of Epitranscriptome Across Multiple Species

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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)
This is a transferred paper from Nature Genetics to Nature communication. According to my previous comments, there are two primary contributions of this manuscript to the field: 1) A new computational method to detect m6A from Nanopore sequencing, which has been challenged by other review. 2) Insights into the interplay between m6A and RNA processing mechanisms, particularly alternative polyadenylation (APA) and alternative splicing (AS), at a single-molecule level. In this revised version, the authors have chosen to remove the second part, which, in my opinion, significantly diminishes the scientific interest and novelty of the manuscript. Moreover, the other findings, such as the relationship between m6A and RNA stability, as well as RNA heterogeneity, have already been well characterized at the bulk level. As a result, the overall novelty and impact of the manuscript are compromised.
Additionally, the current conclusions need to be strengthened through further analysis leveraging the single-molecule m6A detection capability. For example:
1) In Figure 4E, the authors aim to demonstrate that "the correlation between MPKM and RNA stability persists regardless of the specific positions of m6A modifications." However, the analysis lacks statistical significance testing between the boxplots. Notably, when MPKM values fall within the range [3,4], there appears to be a marked difference in RNA stability across m6A modification positions, with the t1 group exhibiting significantly lower stability compared to other groups. Given the absence of rigorous statistical comparisons (e.g., pairwise significance tests), the claim that "m6A-mediated RNA stability is position-independent" seems insufficiently supported.
2) To substantiate the claim of "m6A-mediated RNA decay regulation at the single-molecule level," the authors need to provide stronger evidence. For instance: A direct comparison of RNA stability between molecular isoforms of the *same gene* with *distinct m6A modification stoichiometry* (e.g., high vs. low m6A levels) would help isolate the specific effect of m6A from confounding genetic or transcriptional variables, which is the advantage of detected m6A at single-molecular.

3) What's the exact exon numbers used for the comparative analysis are shown in Fig. 5f?

4) What are the differences in gene structure among the three species? How many genes in C. reinhardtii and A. thaliana require splicing? If a significant portion of their genes do not require splicing, the EJC (Exon Junction Complex) may indeed be unnecessary.

5) Regarding the three defined patterns defined in Fig.5, can they be detected in bulk level or only in single-molecular level?

Minor comments:

1) Several figures, such as Fig. 5a, have very small font sizes, which may affect readability.

2) The upper panel and the bottom panel in Fig. 4c should be displayed in the same format to facilitate comparison.

3) Is the methylation rate shown in Fig. 5e, 5g, 5i, and 5k an average value? If so, how was this value calculated?

Reviewer #3

(Remarks to the Author)
The authors have addressed the key concerns raised by the reviewers, especially by improving their use of Dorado for their comparison studies

Point-by-point response to reviewer comments

Summary

We sincerely appreciate the constructive feedback from both reviewers. We have revised the manuscript and updated the figures accordingly. We believe that all concerns and questions raised by the reviewers have now been thoroughly addressed. Please check the following point-by-point response. Our replies are highlighted, and the modified text/figures in the manuscript are also marked.

Reviewer #1 (Remarks to the Author):

This is a transferred paper from Nature Genetics to Nature communication. According to my previous comments, there are two primary contributions of this manuscript to the field: 1) A new computational method to detect m6A from Nanopore sequencing, which has been challenged by other review. 2) Insights into the interplay between m6A and RNA processing mechanisms, particularly alternative polyadenylation (APA) and alternative splicing (AS), at a single-molecule level. In this revised version, the authors have chosen to remove the second part, which, in my opinion, significantly diminishes the scientific interest and novelty of the manuscript. Moreover, the other findings, such as the relationship between m6A and RNA stability, as well as RNA heterogeneity, have already been well characterized at the bulk level. As a result, the overall novelty and impact of the manuscript are compromised.

Respond: Thank you for your valuable comments. The primary focus of this manuscript is the development and application of SingleMod, the first open-source tool capable of accurate single-molecule m6A detection. This represents a significant methodological advancement in the field, opening new research opportunities into m6A heterogeneity, dynamics, and its functional roles at the single-molecule level. While bulk studies have indicated trends in m6A stability and heterogeneity, SingleMod allows, for the first time, direct quantification of these phenomena at the level of individual molecules within endogenous transcripts, revealing nuances and distributions inaccessible through aggregated data. We believe this granular view, enabled by our methodological advance, provides significant new insights. The comprehensive biological analyses presented—such as the single-molecule m6A profiles in human cell lines, the exploration of m6A heterogeneity within and across transcripts, the quantitative comparison of m6A profiles across multiple species, and the identification of distinct m6A distribution patterns—have made a substantial contribution to the field.

Additionally, the current conclusions need to be strengthened through further analysis leveraging the single-molecule m6A detection capability. For example:

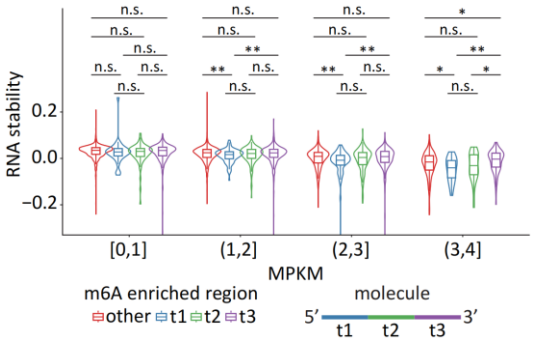
1) In Figure 4E, the authors aim to demonstrate that "the correlation between MPKM and RNA stability persists regardless of the specific positions of m6A modifications." However, the analysis lacks statistical significance testing between the boxplots. Notably, when

MPKM values fall within the range [3,4], there appears to be a marked difference in RNA stability across m6A modification positions, with the t1 group exhibiting significantly lower stability compared to other groups. Given the absence of rigorous statistical comparisons (e.g., pairwise significance tests), the claim that "m6A-mediated RNA stability is position-independent" seems insufficiently supported.

Respond: Thank you for pointing this out. As you suggested, we conducted pairwise significance tests for groups (other, t1, t2, t3) within the same MPKM range. The majority of these tests (16 out of 24) yielded non-significant p-values (Fig. 4e). However, as you noted, certain groups did exhibit statistically significant differences ($p < 0.05$), such as t1 and t3 in the MPKM range of [3,4]. Nevertheless, the observed differences between groups within the same MPKM range were relatively small compared to the differences between groups in different MPKM ranges (Fig. 4e).

To further evaluate the contributions of MPKM and m6A enriched region in explaining RNA stability variance, we quantified their effect sizes using eta-squared (η^2), which measures the proportion of total variance explained. The results indicate that, compared to MPKM, the contribution of m6A enriched region to the variance is minimal (Supplementary Fig. 11h).

We have also revised our wording to more accurately reflect these data.



（Fig. 4e）

2) To substantiate the claim of "m6A-mediated RNA decay regulation at the single-molecule level," the authors need to provide stronger evidence. For instance: A direct comparison of RNA stability between molecular isoforms of the *same gene* with *distinct m6A modification stoichiometry* (e.g., high vs. low m6A levels) would help isolate the specific effect of m6A from confounding genetic or transcriptional variables, which is the advantage of detected m6A at single-molecular.

Respond: We agree that analyzing the relationship between isoform-level RNA stability and m6A stoichiometry could provide valuable insights into how m6A mediates RNA degradation regulation. However, rigorously addressing this requires dedicated experimental designs and significantly deeper datasets, representing a substantial research topic on its own. Given that the primary focus of our current manuscript is the development and application of SingleMod, we believe this comprehensive investigation would be better suited for future dedicated studies rather than our present work. We sincerely appreciate this insightful suggestion and will explicitly acknowledge its scientific

merit in the Discussion section as an important direction for subsequent research.

3) What's the exact exon numbers used for the comparative analysis are shown in Fig. 5f?

Respond: Exon numbers are shown below:

	H. sapiens	*A. thaliana*	*C. reinhardtii*
>=1500	56037	12317	4368
1000-1500	79229	30904	20017
800-1000	73025	25500	29452
600-800	102001	103692	62977
400-600	208460	274226	90620
200-400	499266	567074	113198
<200	619307	64133	78551

4) What are the differences in gene structure among the three species? How many genes in C. reinhardtii and A. thaliana require splicing? If a significant portion of their genes do not require splicing, the EJC (Exon Junction Complex) may indeed be unnecessary.

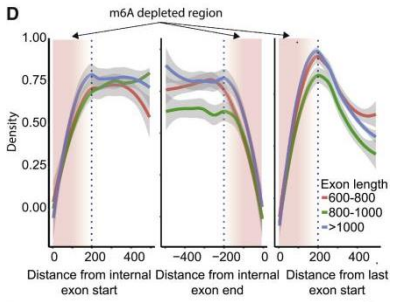
Respond: We analyzed the proportion of single-exon genes (which do not require splicing) and multiple-exon genes (which require splicing), to all detected genes (coverage >= 5) in our DRS results, across the three species. Our findings indicate that the majority of genes are multiple-exon genes across all species. Additionally, we specifically referenced EJC in the context of vertebrate-related results, while it was not discussed in the analyses of other species.

	single-exon gene	multiple-exon gene
H. sapiens	0.044	0.955
A. thaliana	0.105	0.895
C. reinhardtii	0.036	0.964

5) Regarding the three defined patterns defined in Fig.5, can they be detected in bulk level or only in single-molecular level?

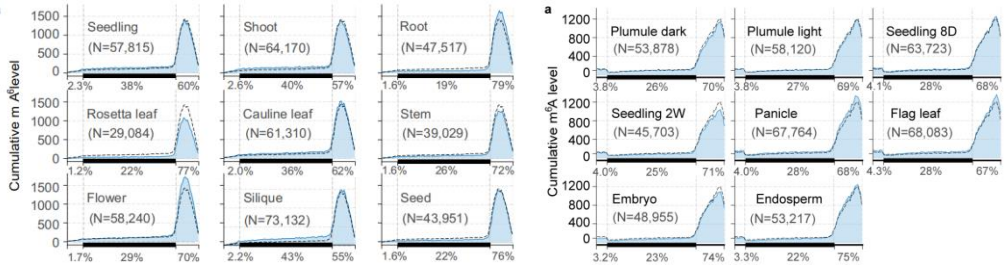
Respond: These three defined patterns are indeed aligned with previous bulk-level (e.g., using MeRIP-seq and m6A-SAC-seq) observations of m6A distribution in these species, as shown below. However, our work advances this understanding by leveraging the single-molecule resolution of DRS technology and SingleMod. While bulk-level studies can broadly identify enrichment trends, our works allows for precise delineation of enrichment regions. For example, Guanqun Wang et al. (https://doi.org/10.1038/s41467-024-48941-7) found that in *Arabidopsis* and Rice, m6A is enriched at the 3' end of the last exon, while in our works precisely identifies the specific enrichment region, that is within 300 bp of the 3' end of the last exon.

For human and mouse (vertebrate) pattern:



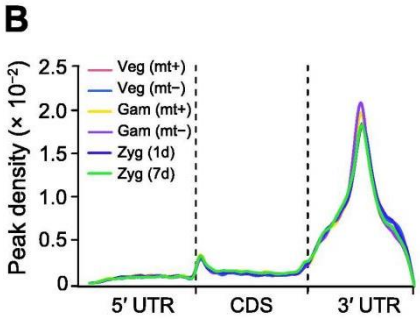
(Anna Uzonyi et al., 2023, https://doi.org/10.1016/j.molcel.2022.12.026)

For Arabidopsis and rice (plants) pattern:



(Guanqun Wang et al., 2024, https://doi.org/10.1038/s41467-024-48941-7)

For C. reinhardtii pattern:



(Ying Lv et al., 2022, https://doi.org/10.1016/j.gpb.2022.04.004)

Minor comments:

1) Several figures, such as Fig. 5a, have very small font sizes, which may affect readability.

Respond: Thank you for pointing this out. We have checked and revised all the figures to enhance their readability.

2) The upper panel and the bottom panel in Fig. 4c should be displayed in the same format to facilitate comparison.

Respond: Thank you for pointing this out. This figure aims to show how the molecular heterogeneity of a group of genes, within a specific range of m6A-induced heterogeneity

(e.g., 0.9–1), is distributed when considering only SS and PAS differences. In other words, we control for m6A heterogeneity and examine the distribution of SS and PAS heterogeneity, rather than directly comparing the distribution of m6A heterogeneity and SS/PAS heterogeneity. Therefore, we believe the current presentation is appropriate. Nevertheless, we revised the figure legend to improve clarity.

3) Is the methylation rate shown in Fig. 5e, 5g, 5i, and 5k an average value? If so, how was this value calculated?

Respond: Thank you for pointing this out. These values represent the overall methylation rate. We pooled all molecules together, and calculated the proportion of m6A-modified A bases (or m6A-modified motifs) among all A bases (or motifs) that share the same distance (grouped into 20 bp bins) from the exon edge.

Reviewer #3 (Remarks to the Author):

The authors have addressed the key concerns raised by the reviewers, especially by improving their use of Dorado for their comparison studies

Respond: We sincerely appreciate your positive comments.