Nucleocytoplasmic shuttling of Gle1 impacts DDX1 at transcription termination sites

Manisha Sharma and Susan Wente

Corresponding author(s): Susan Wente, Vanderbilt University School of Medicine

Review Timeline:

Submission Date:	2020-04-01
Editorial Decision:	2020-05-01
Revision Received:	2020-07-24
Accepted:	2020-07-27

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-03-0215
TITLE: Nucleocytoplasmic shuttling of Gle1 impacts DDX1 at transcription termination sites

Dear Dr. Wente:

Your manuscript, entitled "Nucleocytoplasmic shuttling of Gle1 impacts DDX1 at transcription termination sites" has been seen by two referees, whose verbatim comments are attached. Both referees were generally positive, and felt that your findings would be of interest to our MBC readership. However, both asked for clarification and quantitation of existing data, and suggested a few additional experiments that could strengthen your conclusions. Thus, we would be happy to consider a revised manuscript that satisfies the major concerns of the referees. We look forward to receiving your revised manuscript, together with a letter indicating the changes you've made and your responses to the issues raised by the referees.

Best regards,
Sandra Wolin

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Wente,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this manuscript, Sharma and Wente present a compelling narrative linking the shuttling of a nucleoporin (Gle1) to messenger (m)RNA transcription termination, specifically to the potential resolution of DNA:RNA hybrids (R-loops). The work is presented logically beginning with an RNA-seq and RT-qPCR analysis demonstrating the specific accumulation of a subset of ~70 transcripts in the nucleus upon treating cells with a previously described peptide (Gle1-SD) that inhibits Gle1 shuttling. The authors further demonstrate by complementary approaches (a click-chemistry strategy is a strength) that this nuclear accumulation is due to an accumulation of nascent transcripts. They then go on to demonstrate an association between Gle1, DDX1, and R-loops (using an antibody that detects DNA:RNA hybrids) via a series of proximity ligation assay (PLA) experiments. Overall, the work convincingly demonstrates a new role for Gle1 in transcription termination and I support its publication pending addressing a few considerations to strengthen the manuscript.

1) Figure 1A: The RNA-Seq data presented here is not well described and could use additional details in text, figure legends and in the figure itself to help the reader navigate. Are these two biological replicates presented for each condition, and if so, there seems to be substantial differences between the replicates, how is this explained? Is each condition (i.e. Scr and Gle1-SD) normalized to untreated cells?
2) Figure 2A: Reads are plotted across transcripts here and there is an indication that there is an enrichment of reads around the TTS. This dataset is also difficult to interpret as the peaks nicely overlap between the Scr and Gle1-SD samples but there are simply more reads overall in the Gle1-SD samples. In order to conclude that there is a specific enrichment of reads after the TTS, the authors should better convey how their RNA-seq data support this point, perhaps by adding some kind of additional normalization or a ratiometric quantitative metric of peaks before and after the TTS.
3) Figure 4. The PLA presented seems interesting but the quantification of "nuclear intensity" is a bit superficial as there are additional data that could be mined here. First, as the PLA is in discrete

foci, one would want to have a quantitative assessment of the localization of these foci (i.e. intranuclear or extranuclear), their number and their average intensity. In addition, is the quantification from a single confocal plane or is it derived from entire 3D volumes? This should be indicated, as should whether the images shown are a mid plane section. Additionally, the authors note that there is significant PLA signal above background in untreated cells, but this control should be shown somewhere (as well as the controls for DDX1 and CstF-64 PLA in untreated cells). Finally, the titular claim of this figure (and the Results sub-header on page 7) that the transcription termination defect observed following treatment with Gle1-SD is mediated through DDX1 is not justified based solely on the data presented. Orthogonal approaches, such as knockdown or other inhibition of DDX1 to demonstrate that a similar accumulation of uncleaved transcripts occurs would strengthen this claim. Otherwise, the authors may want to temper their conclusion from these data.
4) Figure 5 B,C. These data are interesting but could benefit from a clarification of why there is so much staining in the cytosol with the S9.6 antibody. See point above for quantification as well. The use of "nuclear intensity" seems to be an inaccurate metric for what appears to be an increase in S9.6 foci in the nucleus. A comment on whether there are also additional nucleolin foci would also be helpful.
5) Figure 6A. An explanation for why there is so much PLA in the cytosol between S9.6 and Gle1 is essential to be able to interpret this data. Ideally, a specificity control here would either be the expression of the RNAaseH or knockdown of Gle1.
6) Supplementary Figure S1A: I would appreciate some comment on the apparent accumulation of distinct rRNA species, as this seems to be a striking and specific effect of the Gle1-SD peptide.
7) The figure legend for Supplementary Figure S1 repeats (C).
8) In Supplementary Figure S3, unlike the rest of the quantifications, no statistics are shown. This should be added, even for NS comparisons.
9) Supplementary Figure S3C is incorrectly referenced as Supplementary Figure S3B on page 9, line 14.
10) Supplementary Figure S3D is not discussed in the Results and is only referenced in the Discussion.
11) Scale bars are present only in some micrographs and are rarely defined in the figure legends.

Reviewer #2 (Remarks to the Author):

In this manuscript, Sharma and Wente uncovered a novel nuclear function for the Gle1 protein on transcription termination. Gle1 has been previously shown to also have a role in mRNA export. The authors determined some of the players and mechanism involved in this novel Gle1 function on transcription termination. They showed that Gle1 activity involves the RNA helicase DDX1 and the pre-mRNA cleavage stimulation factor CstF-64 at transcription termination sites. Impaired Gle1 activity increases R-loops in the nucleus, indicating abnormal transcription termination as these RNA-DNA loops persist and the mRNAs present an extended 3'UTR. The findings are novel, the data is convincing, and the manuscript is well written. The authors used sensitive techniques that clearly demonstrate the spatial and functional interactions between the players. In sum, the manuscript is strong therefore there are only 4 minor points to be addressed:

1. In Supplementary Figure 1A, it appears that that the SD lane in the nuclear fraction has more 28S and 18S rRNA than in the scr lane. Is this gel quantitative? If there is an increase, the authors should comment on it.

2. I think it would be clearer to show total levels of the mRNAs and their N/C ratios in Figure 1 instead of only their enrichment in the nuclear fraction. This is because an increase in nuclear mRNA level (without showing the total and/or cytoplasmic levels) may, for example, simply mean increase in the overall mRNA level (increase in both nuclear and cytoplasmic levels), which would not indicate nuclear retention. Additionally, changes in both N/C ratios and total mRNA levels may complicate the interpretation since this could indicate effect in more than one step within the mRNA processing and export pathways. The authors do show the N/C data in supplement but I think it would be better to show this information together with the total levels of the mRNAs in the main figure to clearly demonstrate the effect.

3. The authors keep using "Fold Change" throughout the paper in the Y axis of several graphs but do not write in the plot fold change of what? One has to look for the information elsewhere. Please add this information directly in the plots to facilitate the reading.

4. Is there any evidence that Gle1 interacts, directly or indirectly, with some of these mRNAs identified by RNAseq?

Dear Dr. Wolin,

Thank you for giving us the opportunity to submit a revision of the manuscript titled "*Nucleocytoplasmic shuttling of Gle1 impacts DDX1 at transcription termination sites*" by M. Sharma and S. R. Wente for consideration as a Research Article publication in *Molecular Biology of the Cell.*

We appreciate the comments and concerns raised by the reviewers. We carefully addressed each issue, and in doing so have significantly strengthened the manuscript. We added eight new data panels to the submitted figures (three panels within the main figures and five in the supplementary figures), and we revised the text accordingly. The revised manuscript now consists of 7 main figures and 5 supplementary figures. Below are the specific changes we've made to the manuscript and our point by point response to the reviewers' suggestions (review comments in italics).

Response to reviewer #1

1a. *The RNA-Seq data presented here is not well described and could use additional details in text, figure legends and in the figure itself to help the reader navigate*.
We appreciate this suggestion and have included more details in the results and methods section and in the legend to Figure 1A. We also added a volcano plot showing the changes in nuclear accumulation of all transcripts as quantified by edgeR (Supplementary Figure 1B).

1b*. Are these two biological replicates presented for each condition, and if so, there seems to be substantial differences between the replicates, how is this explained?*
We have revised the text to better explain the data. The heat map presented in Figure 1A represents all the genes that showed greater than or equal to 2 fold change (total of 3090 genes) in two biological repeats. Some variability was observed. Thus, we focused on the top 70 genes with greater than or equal to 5 fold change for consistent accumulation in the nucleus.

1c. *Is each condition (i.e. Scr and Gle1-SD) normalized to untreated cells*?
No, each condition is not normalized to untreated cells. We have clarified this in the method section of the text.

2. *Figure 2A: Reads are plotted across transcripts here and there is an indication that there is an enrichment of reads around the TTS. This dataset is also difficult to interpret as the peaks nicely overlap between the Scr and Gle1-SD samples but there are simply more reads overall in the Gle1-SD samples. In order to conclude that there is a specific enrichment of reads after the TTS, the authors should better convey how their RNA-seq data support this point.*
We agree with the reviewer's comment that there is an overall increase in the transcript levels. We revised the text to better convey this message.

3. *Figure 4: The PLA presented seems interesting but the quantification of "nuclear intensity" is a bit superficial as there are additional data that could be mined here.*
We have addressed each suggestion as described below.

3*a. First, as the PLA is in discrete foci, one would want to have a quantitative assessment of the localization of these foci (i.e. intranuclear or extranuclear), their number and their average intensity.*
To address suggestion, we re-analyzed the images to quantify the number of PLA dots for DDX1/Gle1 and DDX1/CstF-64. This data is now included in the revised Figure 5B and 5E. We also clarified nuclear intensity as Integrated density (defined as product of area and mean gray value) in the revised Figure 5C

and 5F. Since the focus of this study is to determine whether Gle1 performs a nuclear role, PLA analysis was focused on signal localized to the nucleus.

3b*. In addition, is the quantification from a single confocal plane or is it derived from entire 3D volumes? This should be indicated, as should whether the images shown are a mid plane section*.
As suggested, we revised figure legends to include this specific information more clearly.

3c. *Additionally, the authors note that there is significant PLA signal above background in untreated cells, but this control should be shown somewhere (as well as the controls for DDX1 and CstF-64 PLA in untreated cells).*
As suggested, we have now included this data in Supplementary Figure 3B and 3C for DDX1 and CstF-64 respectively.

3d. *Finally, the titular claim of this figure (and the Results sub-header on page 7) that the transcription termination defect observed following treatment with Gle1-SD is mediated through DDX1 is not justified based solely on the data presented. Orthogonal approaches, such as knockdown or other inhibition of DDX1 to demonstrate that a similar accumulation of uncleaved transcripts occurs would strengthen this claim*.
We thank the reviewer for this suggestion. For the revised manuscript, we silenced DDX1 using siRNA and observed nuclear accumulation of uncleaved transcripts for *Fos* and *FosB* downstream of the PAS and cleavage site. This dataset is included in the revised Figure 4D and the control for siRNA efficiency is included in Supplementary Figure 3A. We believe this data significantly strengthens our conclusions.

4. *Figure 5 B,C: These data are interesting but could benefit from a clarification of why there is so much staining in the cytosol with the S9.6 antibody.*
As suggested, we clarified in the text and included additional references to support the results with the S9.6 antibody. The cytosolic signal observed with S9.6 is a well-documented complication of targeting RNA:DNA hybrids, which has been attributed to mitochondrial material and/or RNA Pol III-derived RNA hybrids.

5. *The use of "nuclear intensity" seems to be an inaccurate metric for what appears to be an increase in S9.6 foci in the nucleus A comment on whether there are also additional nucleolin foci would also be helpful*.
In the revised results section, as suggested, we note that no changes were observed in nucleolin foci. Unfortunately, an automated image analysis measurement for the number of S9.6 foci is complicated due to the antibody's cross-reactivity with RNA hybrids in the nucleolus. In order to subtract nucleolar signal, all S9.6 foci must be counter stained for nucleolin and subtractive quantification performed. This is the only quantitative method for fluorescent images that we are aware of in the literature to address this issue. To further confirm that S9.6 foci are due to R-loops and not due to other hybridized RNA species, we also overexpressed mCherry-RNAse H and observed a reduction in S9.6 foci after Gle1-SD treatment (Figure 6D and 6E in the revised manuscript).

6. *Figure 6A:* a*. An explanation for why there is so much PLA in the cytosol between S9.6 and Gle1 is essential to be able to interpret this data*. *Ideally, a specificity control here would either be the expression of the RNAse H or knockdown of Gle1*.
We have included additional data in Supplementary Figure 4B of the revised manuscript to further address this question. Indeed, Gle1 and other types of R-loops are both present in the cytoplasm and colocalize by PLA. This co-localization is likely related to Gle1's other well-documented cytoplasmic

functions such as its role in translation or the stress response, which are beyond the scope of this manuscript. To address specificity, we silenced GLE1 and analyzed PLA signal between Gle1 and R-loops. We observed a significant reduction in PLA signal in randomly selected cells wherein GLE1 was silenced (see Supplementary Figure 4B in revised manuscript).

7. *Supplementary Figure S1A: I would appreciate some comment on the apparent accumulation of distinct rRNA species, as this seems to be a striking and specific effect of the Gle1-SD peptide*.
We thank the reviewer for pointing out this omission. We have included this observation in the revised result section. This interesting finding could not be further addressed within the scope of this study as the library for RNA-seq was generated from ribosomal depleted RNA. However, it certainly warrants further investigation in the future.

8. *The figure legend for Supplementary Figure S1 repeats (C)*.
This duplication has been corrected in the revised manuscript.

9. *Scale bars are present only in some micrographs and are rarely defined in the figure legends*.
Thank you, and we apologize for this omission. The addition of scale bars and appropriate definitions have been made in the revised manuscript.

Response to reviewer #2

1. *In Supplementary Figure 1A, it appears that that the SD lane in the nuclear fraction has more 28S and 18S rRNA than in the scr lane. Is this gel quantitative? If there is an increase, the authors should comment on it*.
We appreciate the reviewer for pointing out this omission. We have revised the text to include this observation and revised the figure legend to include quantity of RNA loaded.

2. *I think it would be clearer to show total levels of the mRNAs and their N/C ratios in Figure 1 instead of only their enrichment in the nuclear fraction*.
We have included additional data in the revised Supplementary Figure 1D, and revised the text to explain our interpretations of the results. Overall, by performing RT-qPCR on total RNA after Gle1-SD treatment, we observed an increase the levels of Gle1 target genes, albeit not as strong as nuclear levels.

3. *The authors keep using "Fold Change" throughout the paper in the Y axis of several graphs but do not write in the plot fold change of what*?
We have included this information in the revised figure legend of Figure 1.

4. *Is there any evidence that Gle1 interacts, directly or indirectly, with some of these mRNAs identified by RNAseq*?
Previous studies from our and other labs show that Gle1 does not interact with RNA directly. Gle1 binds to DEAD-box helicases which bind to RNA.

We look forward to hearing from you and thank you for consideration of our manuscript.

Sincerely,

Susan R. Wente, Ph.D.

Professor of Cell and Developmental Biology
Vanderbilt University

RE: Manuscript #E20-03-0215R
TITLE: "Nucleocytoplasmic shuttling of Gle1 impacts DDX1 at transcription termination sites"

Dear Dr. Wente,

Thank you for the revised manuscript, which we are pleased to accept for publication in Molecular Biology of the Cell. Thank you for sending this elegant study to Molecular Biology of the Cell.

Best regards,
Sandra Wolin

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Wente:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
