Distinct RNA polymerase transcripts direct the assembly of phase-separated DBC1 nuclear bodies in different cell lines

Taro Mannen, Masato Goto, Takuya Yoshizawa, Akio Yamashita, Tetsuro Hirose, and Toshiya Hayano

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Review Timeline:

Submission Date:	2021-03-16
Editorial Decision:	2021-04-02
Revision Received:	2021-08-24
Accepted:	2021-08-24

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 #E21-02-0081
TITLE: Distinct RNA polymerase transcripts direct the assembly of phase-separated DBC1 nuclear bodies in different cell lines

Dear Dr. Mannen:

You will see that both referees are generally supportive, but make various requests. They are mostly for clarification purposes and should not require extensive experimentation.

Sincerely,

Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Mannen,

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).

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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):

Mannen et al., identify a DBC1 nuclear body (DNB) in the human colon carcinoma HCT116 cell line. This is an important observation. They characterize the DNB and its distinction from Sam68 nuclear bodies (SNBs). The DBC1 interactome was defined, HNRNPL and HNRNPK are components. Knockdown of HNRNPL or DBC1 led to the disappearance of DNBs. HNRNPL knockdown affects 18S rRNA, thus it is proposed that DNB may play a role in pre-rRNA processing. The data are convincing and of high quality.

Comments
1. The uploaded version did not have abstract.
2. DBC1 interacts with Sirt1 to regulate p53. Why was it not identified in the mass spectrometry? Please add text to address this in the manuscript. What is the status of p53 activation in these HCT116 perhaps -/+ HNRNPL or DBC1 siRNA. As DBC1 regulates p53 pathway, is it altered in depleted HCT116 of HNRNPL or DBC1?
3. Does the sequestering of DBC1 in DNBs in HCT116 affect p53 response?

Reviewer #2 (Remarks to the Author):

In the present manuscript, the authors have convincingly demonstrated that DNB bodies (DBC +ve) in HCT116 cells required RNA pol I transcription and/or transcripts for its maintenance. In a previous study (Mannen et al., JCB), they demonstrated that in HeLa cells, both DNB and SNB bodies co-localized with each other, and was mediated by HnRNP L. However, in HeLa cells only RNA pol II and pol I inhibition disrupted the DBC1-containing SNB bodies, implying cell line specific changes. In the present study, the authors identified HnRNP L and K (along with DBC1) as the two other components of DNB bodies in HCT116 cells. Depletion of HnRNP L (and not HnRNP K) disrupted DNB bodies, implying that HnRNP L plays vital role in organizing this RNAse-sensitive NB. Finally, authors have provided data implying that HnRNP L forms phase-separated structures and might be partially responsible for the formation and/or maintenance of DNB in HCT116 cells.

In general, it is an interesting study, and forms a natural extension of their previous work.

Specific comments

Based on the data presented in the ms, most if not all of the HCT116 cells showed only one DNB body. Is that the case? Is the DNB is associated with some chromosomes or a chromosome allele?

Authors have performed all of the experiments in HCT116 cells. DNB is also present in NIH3T3 cells (Mannen et al., JCB). Authors should perform RNA pol I inhibition, and HNRNP L localization and KD experiments in the 3T3 cells to rule out that what was observed is unique to a particular cell line.

The authors showed phase separated structures of hnRNP L under in vitro conditions (Fig 5). Does the concentration of HnRNP L that display phase separation under in vitro conditions is comparable to the levels present within the DNB structures? In similar lines, why is HnRNP L forms only one phase separated structure in HCT116 cells, leaving a significant amount of HNRNP L homogenously distributed in the nucleoplasm?

Similar to the dPR, the dRRM1 & 2 also did not rescue the formation DNB in HnRNP L-depleted cells. Is the dRRM1 & 2 mutants form in vitro droplets comparable to WT or dPR mutants?

The main article file does not contain the abstract or summary section.

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Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

August 24, 2021

Dear Dr. Tom Misteli:

Thank you for giving us the opportunity to revise our manuscript (E21-02-0081) entitled “Distinct RNA polymerase transcripts direct the assembly of phase-separated DBC1 nuclear bodies in different cell lines” for publication in *Molecular Biology of the Cell*. We appreciate the time and effort that the editor and the reviewers dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We fundamentally agree with the reviewers’ comments, and we have incorporated the corresponding revisions into our revised manuscript. These changes have been highlighted in the revised manuscript. Please see below, in blue, for a point-by-point response to the comments and concerns raised by the editor and reviewers. All page numbers refer to the revised manuscript file with tracked changes.

We believe that the additional experiments and corrections you recommended have substantially improved our manuscript. We hope that our revised manuscript will now be acceptable for publication in *Molecular Biology of the Cell*.

Thank you for your consideration.

Yours sincerely,
Taro Mannen

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Reviewer #1

1. The uploaded version did not have abstract.

Author response: We sincerely apologize for this oversight. We have included an abstract to the revised manuscript.

2. DBC1 interacts with Sirt1 to regulate p53. Why was it not identified in the mass spectrometry? Please add text to address this in the manuscript.

Author response: SIRT1 was not identified in our IP–MS analysis of the DBC1 co-IP fraction. As a normal issue, IP–MS may not comprehensively identify interacting proteins. It is possible that SIRT1 abundance was lower in the cell line used and that the DBC1–SIRT1 interaction was weak in the prepared cell extract; alternatively, SIRT1-derived peptides might have been poorly detected under the MS condition. Our co-IP experiment detected that SIRT1 was coprecipitated with DBC1; however, our IF did not detect SIRT1 localization in DNBs. Considering these data, we argue that SIRT1-DBC1 interaction only occurs outside of DNBs. We included a new Figure S4D showing the above data and inserted our argument on page 15 (lines 308–312).

What is the status of p53 activation in these HCT116 perhaps -/+ HNRNPL or DBC1 siRNA. As DBC1 regulates p53 pathway, is it altered in depleted HCT116 of HNRNPL or DBC1?

Author response: We knocked down DBC1 and HNRNPL in HCT116 cells to monitor the status of p53 activation (see Reference data for Reviewer). The results showed that the depletion of DBC1 and HNRNPL resulted in increased p53 expression, but not p53 activation (judged by the ratio of Acetyl-p53 and total p53). These results did not support the possibility that DNB is associated with the SIRT1-dependent regulation of the p53 pathway.

3. Does the sequestering of DBC1 in DNBs in HCT116 affect p53 response?

Author response: As mentioned in our response to #2, DBC1 was not involved in the SIRT1-dependent p53 pathway in HCT116 cells. In addition, we observed that the depletion of DBC1 resulted in an increased expression of p53 and

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acetylation of p53 (see Reference data for Reviewer). As it may be related, a previous report has shown that DBC1 promotes the expression of p53 and acetylation of p53 in collaboration with androgen receptor (AR), which is likely a regulatory mechanism distinct from the SIRT1-dependent mechanism (Wagle et al., Scientific reports 2015). Herein, we observed that the depletion of HNRNPL also increased the expression of p53 and the acetylation of p53 (see Reference data for Reviewer). Taken together, it may raise an intriguing possibility that DNB is involved in a distinct mechanism in the regulation of p53. Further studies will be needed to clarify this intriguing possibility, but it is beyond the scope of this study.

Reviewer #2

Specific comments

Based on the data presented in the ms, most if not all of the HCT116 cells showed only one DNB body. Is that the case?

Author response: In many cases, DNBs were detected as a single focus per cell, whereas SNBs were detected as two foci per cell. To illustrate these features, we added Figure S1B and mentioned this observation on page 6 (lines 95–97).

Is the DNB is associated with some chromosomes or a chromosome allele?

Author response: We appreciate this important question. However, the arcRNA of DNB remains to be identified; hence, we have no clue to investigate this point at present. In addition, our observation that DNBs were detected as a single focus raised an intriguing possibility that a monoallelically expressed RNA and its chromosome locus might be involved in DNB formation. We mentioned this possibility on page 14 (lines 281–284).

The authors have performed all the experiments using HCT116 cells. DNB is also present in NIH3T3 cells (Mannen et al., JCB). Authors should perform RNA pol I inhibition, and HNRNP L localization and KD experiments in the 3T3 cells to rule out that what was observed is unique to a particular cell line.

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Author response: We thank the reviewer for this valuable suggestion. We performed the experiments using NIH3T3 cells and observed that DNB in NIH3T3 cells also abolished upon RNAPI inhibition and that DNB formation requires DBC1 and HNRNPL, as observed in HCT116 cells. These results suggest that the mechanism of DNB formation is conserved in human and mouse. We have added these data into new Figure 1A; Figure 3C and D; Figure S1A, B, and D; and Figure S5B as well as described them on page 6 (lines 95–97, 100–101, and 110), page 7 (line 122), and page 9 (lines 168, 170, 175, and 181–182).

The authors showed phase separated structures of hnRNP L under in vitro conditions (Fig 5). Does the concentration of HnRNP L that display phase separation under in vitro conditions is comparable to the levels present within the DNB structures?

Author response: We appreciate the reviewer for the thoughtful comment on this important point. The protein concentration of HNRNPL used in our *in vitro* droplet experiments was 3.3 μM; alternatively, the cellular abundance levels of proteins—measured via MS-based quantitative proteomics studies—were obtained from PaxDb (Wang et al., Proteomics 2015). The cell line integrated abundance values retrieved from PaxDb were converted into concentrations using the following formula:

$C = (k \times A) / N_A$ (Milo R, Bioessays 2013),

where $k \approx 3 \times 10^6$ proteins/fL, the Avogadro constant $N_A = 6.02 \times 10^{23}$ molecules/mol, and A is the abundance. The intracellular concentration of HNRNPL was estimated at 2.0 μM. Our cell fractionation experiment determined that the intracellular distribution of HNRNPL nuclei and cytoplasm was 4:1. In addition, the nuclear-to-cytoplasmic volume (N/C) ratio of HCT116 was approximately 0.18 (Ganguly et al., FEBS Letter 2016). Consequentially, the nuclear and cytoplasmic concentrations of HNRNPL were estimated to be 5.5 μM and 1.4 μM, respectively. The nucleoplasm and DNB volumes and

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proportion of HNRNPL protein present within the DNB were estimated from the immunofluorescence intensity profile over the different Z-positions (Z-stack) using cellSens Dimension software (Olympus). The proportion of nucleoplasm volume to DNB volume was 629:1, whereas the proportion of HNRNPL protein present within the nucleoplasm to that in the DNB was 0.4:1.0. Consequently, the nucleoplasmic and DNB concentrations of HNRNPL were estimated at 5.5 μM and 13.7 μM, respectively. These data suggested that the concentration of HNRNPL used in our *in vitro* experiments (3.3 μM) falls within the range of HNRNPL concentration in DNBs. We have added the data into new Figure S9 and described them on page 12 (lines 235–238) and page 23 (lines 495–519).

In similar lines, why is HnRNP L forms only one phase separated structure in HCT116 cells, leaving a significant amount of HNRNP L homogenously distributed in the nucleoplasm?

Author response: We appreciate this important question. It was recently shown that a type of arcRNA-dependent nuclear bodies is formed through a specific mechanism of condensation that is distinct from the liquid–liquid phase separation process, such as the typical macrophase separation, which is simply driven by the protein concentration in cells. The paraspeckle built around NEAT1 lncRNA is formed through transcription-dependent micellization of the triblock lncRNPs (Yamazaki et al. EMBO J 2021). This mechanism requires continuous supply of the locally concentrated lncRNPs from the chromosomal locus through the ongoing transcription process. It is possible that a similar mechanism is employed for DNB formation at a chromosome locus that might monoallelically express a putative arcRNA as pointed above (see the second point of Reviewer #2).

Similar to the dPR, the dRRM1 & 2 also did not rescue the formation DNB in HnRNP L-depleted cells. Is the dRRM1 & 2 mutants form in vitro droplets comparable to WT or dPR mutants?

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Author response: We appreciate this valuable suggestion. We conducted an *in vitro* droplet assay using each of the deletion mutants of HNRNPL. The results showed that in addition to ΔPR, ΔRRM1 and ΔRRM2 also prevented phase separation *in vitro*. Thus, we believe that RRM1, RRM2, and PR of HNRNPL are involved in both *in vivo* DNB formation and *in vitro* droplet formation and that phase separation via these domains is a driving force of DNB formation. We added Figure 5A, B, and C and Figure S8C, D, E, and F to illustrate these features and provided a comprehensive description of these results on page 12 (lines 243, 244–246, 249–250, 253, and 255–260) and page 13 (lines 261 and 263).

The main article file does not contain the abstract or summary section.

Author response: We sincerely apologize for this oversight. We added an abstract to the revised manuscript.

RE: Manuscript #E21-02-0081R
TITLE: "Distinct RNA polymerase transcripts direct the assembly of phase-separated DBC1 nuclear bodies in different cell lines"

Dear Dr. Mannen:

Thank you for your revised manuscript. Your revisions satisfactorily address the referees' comments and I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Tom Misteli
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Mannen:

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.

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