Super resolution light microscopy of the Drosophila Histone Locus Body reveals a core-shell organization associated with expression of replication dependent histone genes

James Kemp Jr., Xiao-Cui Yang, Zbigniew Dominski, William Marzluff, and Robert Duronio

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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.)
Dear Dr. Duronio:

Your manuscript, entitled "Super resolution light microscopy of the Drosophila Histone Locus Body reveals a core-shell organization associated with expression of replication dependent histone genes" has been seen by two referees whose verbatim comments are enclosed. Both referees felt that your findings, in principle, would be of interest to our MBC readership. However, both reviewers raised some important points that need to be addressed. Some of these are textual changes for clarity or to reduce speculation. The reviewers indicate important control experiments needed to rule out fixation or antibody labeling artifacts. I also encourage you to consider the use of a transcription inhibitor to directly test your hypothesis as suggested by Reviewer #1. We would be happy to consider a revised manuscript that satisfies the joint concerns of the referees. Therefore, we look forward to receiving your revised manuscript, together with a letter indicating the changes you've made and your responses to the referees.

Sincerely,

Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Duronio,

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 have opted 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,
In this manuscript, Kemp et al. use structured illumination microscopy to examine the internal organization of the Drosophila histone locus body, a subnuclear domain in which histone genes are transcribed. They present evidence that HLBs have a "core-shell" structure, similar to other subnuclear bodies such as nucleoli, paraspeckles, and PML bodies. The large Mxc protein is found to orient with its N-terminus pointed into the HLB and its C-terminus pointed outward. The FLASH protein is found in biochemical assays to bind the C-terminus of Mxc, consistent with its localization to the HLB shell.

This is a timely study, given the intense recent interest in how subnuclear bodies are organized. The data would be more convincing if presented in terms of radial distribution rather than colocalization, and I additionally have some substantial concerns about potential artifacts due to fixation and antibody staining that have recently been well documented. Because the results of the present study say nothing about the physical mechanism or functional consequences of this putative core-shell organization, the authors should dispense with the excessive speculation in the Discussion. I think that this manuscript could be appropriate for publication provided the additional experiments suggested here are included and the discussion is significantly toned down regarding the actual functional consequences of the structures seen in the revision.
Major points:
- To quantify the organization of proteins within the HLB, the authors measure the distribution of distances between the peaks in one fluorescence channel and an ROI boundary obtained by thresholding a second fluorescence channel. This metric can be notoriously difficult to interpret, and segmentation into peak "spots" seems somewhat artificial. It would be more persuasive to quantify the radial distribution of different proteins relative to the centroid of the HLB and show that this differs between different proteins as predicted by the "core-shell" model.
- The background pixels in the SIM images appear to be mostly black. Is there truly no fluorescence signal throughout most of the image, or are the images thresholded in some way? It is important to show the full range of signal intensities and state clearly what lookup table is used to display the images.
- The authors raise a good point that antibody staining may artifactually label the exterior of a structure due to poor antibody penetration into the structure. They do some reasonable controls in Fig. 2I-K and Fig. 3A-C to assess this possibility. However, it is not clear that the results actually adequately address this concern. In the images shown, the anti-GFP and anti-RFP signals colocalize rather poorly with GFP and mScarlet, and anti-RFP does in fact look more shell-like than mScarlet itself. It is worrisome that all of the immunofluorescence images in the manuscript look either shell-like or punctate and potentially under-labeled. One way of showing more convincingly that the N and C-termini of Mxc reside in different layers would be to image GFP-MXC/MXC-mScarlet heterozygotes. Has this experiment been tried? If not, it would be highly advisable to do so and include the data. As mentioned above, a radial distribution analysis may also help to make the case more convincingly.
- All of the experiments presented in this manuscript involved fixed specimens. However, formaldehyde fixation has been well documented to generate significant artifacts. Showing that similar results are obtained by live imaging of GFP-MXC and MXC-mScarlet embryos would greatly strengthen the conclusions and should be included in a revised Ms.
- In the Discussion, the authors suggest that the two termini of Mxc occupy different phase-separated domains and that RNA processing reactions may occur at the interface of these two domains. This model is highly speculative and is not directly supported by the results. The authors should curb their speculation and consider whether models not involving LLPS may be compatible with their observations.

Minor points:
- There is no nonspecific IgG control in Fig. 4A
- The label "F178C" in Fig. 4 is confusing, because it sounds like a phenylalanine to cysteine mutant. Perhaps "FC" or "FC178" would be less confusing.
- In Fig. 4D, it may be clearer to put the protein names (296C, 229C, etc.) above each set of three lanes and label the first lane as "input"
Reviewer #1 (Remarks to the Author):

This manuscript reveals the substructure underlying the histone locus body with this group has shown to contain both transcription factors as well as specific RNA processing machineries, consistent with the notion that the bodies form at the sites of histone gene transcription. The elucidation of shell and core domains to the HLB here indicate a partitioning in which the active genes are located at the core and the RNA processing machinery in the shell. This clearly points to a gradient of molecular activities within the HLB and will help the field to imagine how nuclear bodies built on transcription units – including nucleoli, Cajal bodies, paraspeckles and even speckles – assemble and function.

The importance of this work notwithstanding, we have several constructive criticisms, comments and questions:

Major:

1. In general, Figure 4 and its labeling is difficult to interpret and the system of abbreviations is confusing. In particular Figure 4A labeling is unclear. “F178C” sounds like a point mutation, not a region of a protein, for example. Better schematics of what proteins are bait and prey in each panel and clarifying the labeling scheme would help. The pictograph to the right of panel E does not make sense and the legend doesn’t explain its relationship to the data. Overall the experiments seem to point to where these two proteins bind one another but we can’t really be certain of what should be concluded from this analysis in terms of a binding site.

We apologize for not providing a better labeled figure. We have relabeled “F178C” as “FLASH178C” to denote that this construct is composed of the last 178 amino acids of full length FLASH protein. We have also rewritten the figure legend with the same goal and to match the new figure. The main conclusion from these in vitro interaction data is that Drosophila FLASH binds to a region in the COOH-terminus of Mxc between amino acids 1542 and 1745 of the 1876 amino acid Mxc protein. There is a sentence in the text on page 11 making this explicit statement. This conclusion is highlighted in panel E, which also provides a pictorial summary of the binding data. We also now describe the panel E pictorials in the revised figure legend.

2. The point made by Figure 6 would be significantly strengthened by the addition of live cell imaging corresponding to the RFP labeled MXC, which should form the “donut” over time at nuclear cycle 14. It is likely that the donut is difficult to see in Figure 3, because the characteristics of the dye become problematic for obtaining the best resolution. If the authors have another tag available it might be better to switch (GFP, Halo?).

As suggested by the reviewer, we performed live embryo imaging of early Drosophila embryos expressing only the Mxc-mScarlet protein using a newly acquired Leica SP8 LIGHTNING microscope capable of the necessary resolution, even though it’s lower than what we can obtained via structured illumination of fixed embryos. These data are presented in the new Figure 4 and reveal “donut” like Mxc structures in live embryos similar to what we observed in fixed embryos. Moreover, as the Mxc-mScarlet bleached from continuous illumination, the “holes” became more apparent, suggesting that the concentration of
mScarlet (i.e. the C-terminus of Mxc) is lower in the HLB center. These results clearly demonstrate that our detection of a “donut” with antibodies against the COOH-terminal region of Mxc is not solely the result of our fixation conditions. Because the Mxc-mScarlet protein bleached rapidly under the time lapse conditions necessary to visualize dynamic changes in HLB structure in cycle 14 embryos, we were unable to use live imaging to obtain temporal data as we did with fixed cycle 14 embryos. Nevertheless, our ability to order fixed cycle 14 embryos in time based on morphological criteria provides us with confidence in making the conclusion that HLB organization changes over the course of interphase of cycle 14.

3. Related to 2, an important test of the authors’ hypothesis would be to use a transcription inhibitor or a temperature sensitive mutant in Pol II to see what happens to their HLB structure at cycle 14. It would directly test their hypothesis.

As suggested by the reviewer, we obtained a previously characterized temperature sensitive allele of RNA pol II large subunit (y[1] RpII215[ts] ff[1]) (PMID: 6427038) and performed a temperature shift experiment prior to collection and fixation and SIM imaging of HLBS. However, what we learned is that this allele is not strong enough to test the hypothesis; i.e. it does not sufficiently eliminate RNA pol II function. Nevertheless, our previous work definitively demonstrates that histone gene transcription contributes to full HLB assembly. We showed conclusively in two prior publications (Salzler et al., 2013 and Hur et al., 2020) that small HLBS (which we termed “proto HLBS”) result from the inhibition of histone gene transcription. These experiments were quite different (i.e. genetic and pharmacological inhibition of histone gene transcription) yet gave the same result: very little (but not none) Mxc and FLASH are recruited to histone genes in the absence of transcription. In the former study a transgene containing histone H3 and H4 genes with mutated TATA boxes was used, and in the latter study alpha-amanitin injection into syncytial embryos was used. These results formed the basis of our interpretation that HLBS in early cycle 14 are small because of low levels of histone transcription.

Minor:

4. There appears to be a problem where the text and figure order do not match up. The section beginning on page 11 refers again to figure 4, which in the actual figures is the protein interaction data.

We apologize for this mistake which resulted from adding a figure late in the processes of assembling the manuscript. In the revision we have tried hard to make sure that all the figure call outs are accurate and in the correct order.

5. The use of the APEX labeling is unclear.

We apologize for this confusion. We did not perform APEX labeling in this manuscript. We used anti-APEX antibodies to recognize an Mxc-APEX2 fusion protein produced from a CRISPR engineered allele. Thus, in these experiments APEX2 was essentially used as an epitope tag for detecting the COOH-terminus of Mxc in addition to and distinctly from using Mxc-mScarlet and antibodies against the endogenous Mxc COOH terminal region. We added
a statement to the main text on page 9 explicitly indicating that we did not use APEX2 enzymatic activity to biotinylate proteins. Notably, all these different approaches gave the same “donut” staining of HLBs. In the future we hope to use the enzymatic activity of APEX2 in experiments aimed at identifying other HLB components by proteomics—this is what initially motivated us to construct the Mxc-APEX2 before we realized we could also use it to probe HLB organization by SIM.

6. In the discussion, the referenced “overlapping signal” from FLASH and pol II (top of page 15) may be an over-interpretation. SIM is not diffraction-limited microscopy, but it is a factor of 2-3 worse on resolution than other methods. They observe a “fair amount” of overlap, not “some”, and they would need a different technique to get a better answer from that question.

We have eliminated the reference to overlapping FLASH and RNA pol II signal upon rewriting the Discussion.

7. The authors’ conclusions center on the transcription-dependent formation of nuclear bodies and this explanation for their appearance and disappearance in embryos. In that regard, the two papers by Falahati and Wieschaus seem highly relevant to discuss; these papers pinpoint the relationship between cleavage stage, transcription activation of rDNA and nucleolar appearance in fly and furthermore pose a distinction between nucleation and growth. It would also be worth referencing the evolutionary conservation of HLB and other nuclear body nucleation during zygotic gene activation (Heyn et al 2017; Arias-Escayola and Neugebauer, 2018).

We are aware of these studies and agree entirely with the reviewer that they are highly relevant. We have cited them in other publications of ours. However, not citing them here was an oversight and we thank the reviewer for pointing this out. We have now incorporated a brief discussion of this work with citations into our newly written Discussion section.

Reviewer #2 (Remarks to the Author):

In this manuscript, Kemp et al. use structured illumination microscopy to examine the internal organization of the Drosophila histone locus body, a subnuclear domain in which histone genes are transcribed. They present evidence that HLBs have a "core-shell" structure, similar to other subnuclear bodies such as nucleoli, paraspeckles, and PML bodies. The large Mxc protein is found to orient with its N-terminus pointed into the HLB and its C-terminus pointed outward. The FLASH protein is found in biochemical assays to bind the C-terminus of Mxc, consistent with its localization to the HLB shell.

This is a timely study, given the intense recent interest in how subnuclear bodies are organized. The data would be more convincing if presented in terms of radial distribution rather than colocalization, and I additionally have some substantial concerns about potential artifacts due to fixation and antibody staining that have recently been well documented. Because the results of the present study say nothing about the physical mechanism or functional consequences of this putative core-shell organization, the authors should dispense with the excessive speculation in the Discussion. I think that this manuscript could be appropriate for publication provided the additional experiments suggested here are included and the discussion is significantly toned down regarding the actual functional consequences of the structures seen in the revision.
We appreciate the reviewer’s comments and have made all the general changes suggested, and these are described in more detail below.

Major points:
- To quantify the organization of proteins within the HLB, the authors measure the distribution of distances between the peaks in one fluorescence channel and an ROI boundary obtained by thresholding a second fluorescence channel. This metric can be notoriously difficult to interpret, and segmentation into peak "spots" seems somewhat artificial. It would be more persuasive to quantify the radial distribution of different proteins relative to the centroid of the HLB and show that this differs between different proteins as predicted by the "core-shell" model.

We are aware of the weaknesses of various ways of quantifying the data, and in fact struggled with landing on the best way to quantify our data. We are performing our analyses with Imaris software, and after confirming with the manufacturer of this software we cannot perform the type of radial distribution analysis the reviewer suggests. Moreover, we have been unsuccessful in attempts to use other available software for this purpose. With the intent of providing the reader with maximal confidence in our overall interpretation that the SIM data are consistent with a “core-shell” model of HLB organization, we note that we used two methods for quantification, orthogonal line scans (Supplemental Figure S2) and surface rendering (main text and figures), and each arrive at the same conclusion and thus support one another. Moreover, identifying the centroid of the HLB in order to quantify radial distribution of staining signal is somewhat arbitrary given that the HLB is not perfectly spherical no matter how we detect it and consequently.

- The background pixels in the SIM images appear to be mostly black. Is there truly no fluorescence signal throughout most of the image, or are the images thresholded in some way? It is important to show the full range of signal intensities and state clearly what lookup table is used to display the images.

The images are contrast adjusted using Image J software such that the background fluorescence would appear black as seems to be typically done with SIM imaging in the literature. We have now included this point in the Materials and Methods. Thank you for pointing out that we should also include an example of a non-adjusted image, which is now included in Supplemental Figure S1.

- The authors raise a good point that antibody staining may artifactually label the exterior of a structure due to poor antibody penetration into the structure. They do some reasonable controls in Fig. 2I-K and Fig. 3A-C to assess this possibility. However, it is not clear that the results actually adequately address this concern. In the images shown, the anti-GFP and anti-RFP signals colocalize rather poorly with GFP and mScarlet, and anti-RFP does in fact look more shell-like than mScarlet itself. It is worrisome that all of the immunofluorescence images in the manuscript look either shell-like or punctate and potentially under-labeled. One way of showing more convincingly that the N and C-termini of Mxc reside in different layers would be to image GFP-MXC/MXC-mScarlet heterozygotes. Has this experiment been tried? If not, it would be
highly advisable to do so and include the data. As mentioned above, a radial distribution analysis may also help to make the case more convincingly.

We performed the reviewer’s suggested experiment, which is a good one. We imaged via SIM fixed embryos obtained from GFP-Mxc/Mxc-mScarlet mothers. Because all the Mxc in the embryonic stages that we analyze is provided maternally, these HLBs should in theory be composed of a 1:1 ratio of each fluorescently labeled Mxc protein. The data are shown in new Figure 3. As the reviewer perhaps anticipated, the results are complicated. Quantifying these data using our method clearly reveals variations in intensity of GFP-Mxc and Mxc-mScarlet SIM signal within the HLB. We see non-uniform signal distribution in our live imaging data as well, thus these variations in signal are not simply the result of under labeling or the SIM algorithm. However, when present in the same HLB the GFP-Mxc and Mxc-mScarlet signals overlap substantially, unlike what we obtain when using MXC-C antibody with GFP-Mxc. In addition, when the Mxc-Scarlet molecules are present together with the GFP-Mxc, the GFP signal looks less uniform than when GFP-Mxc is present by itself. What this means is unclear, but perhaps assembling an HLB with two different types of Mxc proteins alters the organization somehow. Importantly, in the GFP-Mxc/Mxc-mScarlet genotype we observe using anti-FLASH antibodies that FLASH primarily surrounds the fluorescently labeled Mxc fusion proteins, consistent with a core/shell model.

These data highlight the fact that no single way of observation HLB structures is definitive or perfect. Moreover, using antibodies to detect a nuclear body like the HLB is different than detecting it using fluorescent fusion proteins, which by definition are not wild type even though they provide full genetic function. Thus, what we are observing is always an inference of what the true structure is. In the revised Discussion section we discuss the incongruent data regarding the N- and C-terminus of Mxc, and stress that Mxc versus FLASH staining better support the core-shell model of the HLB. We would like to emphasize that we used three different CRISPR engineered Mxc fusion proteins, which is not typically seen in the literature, plus a highly specific antibody to visualize Mxc, and two different antibodies that recognize different regions of the FLASH protein to visualize its localization within the HLB. We also observe active transcription in two different ways by using FISH to detect nascent transcripts and an antibody to detect active RNA pol II. Although the data are complicated, Thus we feel we have performed the SIM analysis of fly embryonic HLBs as thoroughly as we can currently. And although the data are complicated, we tried to address these complexities carefully in the rewritten Discussion section. In the end, we conclude that when considering the Mxc and FLASH proteins together a core/shell model best explains the configuration of the HLB when histone genes are being expressed.

- All of the experiments presented in this manuscript involved fixed specimens. However, formaldehyde fixation has been well documented to generate significant artifacts. Showing that similar results are obtained by live imaging of GFP-MXC and MXC-mScarlet embryos would greatly strengthen the conclusions and should be included in a revised Ms.

We have now included these data; see response to Reviewer #1.

- In the Discussion, the authors suggest that the two termini of Mxc occupy different phase-
separated domains and that RNA processing reactions may occur at the interface of these two domains. This model is highly speculative and is not directly supported by the results. The authors should curb their speculation and consider whether models not involving LLPS may be compatible with their observations.

We appreciate the reviewer’s point and have revamped the Discussion to focus more on a critical evaluation of how strongly our data support the core-shell model rather than any function such a structure might provide. However, our observation that the essential pre-mRNA processing factor FLASH is enriched in the HLB shell while nascent histone RNA is enriched in the core merits comment, as the observation is not intuitive. In this context, mentioning that a core-shell arrangement of factors within the nucleolus has been previously reported to be associated with transcription and processing of rRNAs seems warranted. We have tried not to be overly speculative when citing this previous work. We have removed the penultimate paragraph of the origin version which discussed possible functions of LLPS in the context of the HLB, as we agree it was overly speculative and unnecessary.

Minor points:
- There is no nonspecific IgG control in Fig. 4A

We have replaced this figure with a new one showing that MXC-C antibodies immunoprecipitate FLASH from a Kc cell extract. This experiment includes a control using an antibody that recognizes a protein not involved in histone mRNA biology, and this antibody does not IP FLASH.

- The label "F178C" in Fig. 4 is confusing, because it sounds like a phenylalanine to cysteine mutant. Perhaps "FC" or "FC178" would be less confusing.

- In Fig. 4D, it may be clearer to put the protein names (296C, 229C, etc.) above each set of three lanes and label the first lane as "input"

Reviewer #1 made similar points regarding Figure 4 and we agree. In response we have revamped the labeling of Figure 4 and its legend in hopes that the data are now more clearly presented.
RE: Manuscript #E20-10-0645R
TITLE: "Super resolution light microscopy of the Drosophila Histone Locus Body reveals a core-shell organization associated with expression of replication dependent histone genes"

Dear Dr. Duronio:

Thank you for revising your manuscript in response to the referees' recommendations. I have read the revised manuscript carefully along with your responses to the referees and it is clear that you have satisfactorily addressed their concerns. I am pleased to accept your manuscript for publication in MBoC. Congratulations to you and your colleagues.

Sincerely,
Diane Lidke
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Duronio:

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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