Heterochromatic foci and transcriptional repression by an unstructured MET-2/SETDB1 co-factor LIN-65

Colin Delaney, Stephen Methot, Micol Guidi, Iskra Katic, Susan Gasser, and Jan Padeken

Corresponding Author(s): Susan Gasser, Friedrich Miescher Institute for Biomedical Research

Review Timeline:

Submission Date:	2018-11-09
Editorial Decision:	2018-11-28
Revision Received:	2019-01-22
Editorial Decision:	2019-01-23
Revision Received:	2019-01-24
Accepted:	2019-01-25

Monitoring Editor: Maria Elena Torres-Padilla

Scientific Editor: Melina Casadio

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.)

DOI: https://doi.org/10.1083/jcb.201811038

November 29, 2018

Re: JCB manuscript #201811038

Dr. Susan M Gasser
Friedrich Miescher Institute for Biomedical Research
Maulbeerstrasse 66
Basel CH-4058
Switzerland

Dear Dr. Gasser,

Thank you for submitting your manuscript entitled "An unstructured MET-2/SETDB1 cofactor ensures H3K9me2, focus formation and perinuclear anchoring". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that our reviewers were split in their recommendations to us and in their assessments of the level of advance for JCB. Reviewer #2 is largely supportive and raised points that seemed straightforward to address via minor changes. On the other hand, although Reviewer #1 appreciated the high quality of the work, they felt that the advance was somewhat limited in depth without more insight into the mechanistic role of LIN65 - especially as its potential contribution to phase-separated droplets is not tested (but suggested) - and into how heterochromatin is methylated through MET2-LIN65-ARLE14 mechanistically.

We have discussed these comments in depth editorially. We appreciate that ablation of lin-65 resulting in decreased H3K9me2 was previously reported in adult intestinal cells in C. elegans, but the mechanism underlying this observation (e.g. direct interaction, enzymatic activity or others) was unknown. We find the conclusion that Met-2 localization is independent of 'canonical' H3K9me readers rather novel and interesting. Indeed, it points towards H3K9me/heterochromatin regulation through alternative pathways. In addition, we appreciate that the current manuscript clarifies the localization of Met-2 and provides a deeper analysis of gene expression as compared to published studies. However, other results seem consistent with prior work - for instance, based on your previous published work, the loss of anchoring to the nuclear periphery would be expected, considering the Met-2 dependency in anchoring and the fact that Lin65 reduces Met-2 levels and H3K9me2.

Given these points, we would be supportive of further consideration at the journal should you address/provide experimental information towards any of the following:

(i) That Lin-65 is an unstructured protein is irrelevant for the manuscript, as this is not addressed nor investigated at all, and therefore this cannot contribute to the appeal and novelty of the work unless you examine for example whether Lin65 can form aggregates in vitro.

(ii) Alternatively, if you could show how Lin65 can potentially mediate Met-2 localization and/or targeting, the work would increase in mechanistic definition and advance/appeal for cell biologists. It is clear from the data that Lin65 is required for the localization of Met-2 in the nucleus, the question

is: what comes 'first' for Met-2 function and repression: targeting Met-2 to chromatin regions or chromatin regions being targeted to Met-2 foci? E.g. what is Lin65 actually doing: does it target Met-2 to its target sites (e.g. heterochromatin), or does it promote 'bringing' together/clustering of heterochromatic regions for e.g. subsequent methylation by Met-2? Potentially a Lin65 ChIPseq (or ChIP-PCR) in the absence of Met-2 could address this issue.

We strongly feel that addressing these points - and thereby tackling the questions opened up by the results - will significantly strengthen the contribution of this work to the field and raise the interest for a broad audience of cell biologists. Please also address the reviewers' requests for controls (knock-down controls as per Rev#1) and edits to the figures/text from Rev#2. In addition, we feel that it would be important and scholarly for the manuscript to more rigorously cite prior work, as follows:

1. The comparison between the lin-65 single mutant and the lin-65; met-2 double mutant confirms that in lin-65 mutants a subset of the met-2 dependent genes are not significantly derepressed (this is similar to what was reported in cco-1 RNAi in Tian et al, 2016): e.g. there are dependent and independent genes regulated by Lin-65 and met-2. Please discuss this point to adequately give credit to previous work.
2. Likewise, the regulation of H3K9me2 levels by Lin-65 is not new; we encourage you to rephrase the following sentence on Page 7. ..."...where it appears to regulate the repression of stress response genes "AND OF H3K9ME2 levels" (Tian et al).
3. CAF-1 association with Setdb1: the correct reference seems to be Yang et al, from YH Loh's group in Singapore, there's no mass spectrometry data in the Cheloufi et al reference.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will

not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Maria-Elena Torres-Padilla, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, Delaney et al describe the regulation of MET2 histone methyltransferase and hetrochromatin formation in C. elegans. They first investigated the localization of a histone H3K9 methyltransferase MET2 tagged with mCherry in a knock-in line. MET2-mCherry accumulated in heterochromatic foci around nuclear periphery throughout the cell cycle. RNAi experiments suggest that the localization did not depend on the known reader proteins for H3K9 methylation, like HP1 homologs. Proteome analyses resulted in identifying two proteins (LIN65 and ARLE14) that physically interact with MET2. The heterochromatic localization and stability of MET2 were impaired by depletion of LIN65, but not ARLE14. The phenotypes of LIN65 depletion were similar to those of MET2 depletion. These observations indicate a critical role of LIN65 in MET2 function. The data are generally of high quality and convincing. Identifying the new protein involved in histone methylation and heterochromatin formation is important, but the mechanism remains unaddressed.

A weak point of this manuscript is a lack of molecular mechanism. The authors emphasize LIN65 is an unstructured protein and suggest its contribution to liquid droplet formation, but there is no biochemical analysis of this protein. Moreover, how heterochromatin region is methylated through MET2-LIN65-ARLE14 is not addressed at all.

Other points:

All RNAi experiments need Western blots or RT-PCR to show the depletion level. Particularly in Fig. 1C, no evidence is shown for the depletion of the target protein. In Fig. 4 and S4, the depletion of the target protein is shown by fluorescence microscopy, but it is still needed to show how much the (FP-tagged) proteins are depleted by Western blots.

In the results section, the manuscript under submission is often referred. This is frustrating because the readers are unable to read the manuscript. It is better to use BioRxiv if the citation is necessary.

Discussion is too long. There are lots of redundant and less relevant description.

Minor points:

Introduction, "the trimethylation of histone H3K in nucleosomes associated with. . . forming so-called facultative heterochromatin." In mammals it is generally acknowledged that facultative heterochromatin is associated with H3K27me3, and constitutive heterochromatin with H3K9me3. Please rephrase.

There are some typos (e.g., p7, l14; ...either than LIN-65...)

Reviewer #2 (Comments to the Authors (Required)):

The paper by Delaney et al is a logic continuation of a series of works from Susan Gasser's lab that systematically dissect epigenetic mechanisms of heterochromatin formation using C. elegance as a model. In particular, in the recent years, Gasser's team demonstrated the different roles of the HMTs MET-2 and SET-25 in H3K9 methylation, peripheral heterochromatin localization and transcription inhibition, as well as the anchoring role of CEC-4 and the role of HPLs and LIN-61in transcription repression.

In the current paper, the authors focus on the protein MET-2 that mediates H3K9 mono- and di-methylation. They show the predominant nuclear localization of MET-2 (an issue that previously was a matter of debate), demonstrate its enrichment and co-localization with H3K9me foci, as well as its high cell-cycle dependent dynamics and tendency for peripheral positioning. The authors showed that depletion of known H3K9me readers (HPL-1, HPL-2, LIN-61) does not influence localization of heterochromatic MET-2 foci, which prompted them to search for other interacting partners and to identify two proteins, LIN-65 and ARLE-14. They further show that depletion of LIN-65 (but not ARLE-14) leads to de-heterochromatinization, manifested by loss of peripheral heterochromatic MET-2 foci, decrease of H3K9 methylation and, as a consequence, changes in transcription, including de-repression of microsatellites and simple repeats. Moreover, the comparison of effects on transcription after depletion of one of the two proteins, MET-2 or LIN-65, with the effects of a double mutant lacking both proteins, showed that LIN-65 represses genes via interaction with MET-2 but it is not needed for MET-2 catalytic activity. The authors conclude that the role of LIN-65 is to direct and associate MET-2 with heterochromatic foci. This conclusion is reinforced by the intrinsically disordered nature of LIN-65 suggesting its possible role in phase separation of heterochromatic foci.

This work is an important contribution to the field of nuclear biology: it uncovers yet another mechanism of heterochromatin formation leading to a better understanding of spatial chromatin segregation within the nucleus and its role in genome regulation. The work is performed on a high technical level, using cutting-edge cell biology techniques. The manuscript is clearly written and very well illustrated. I can only recommend this manuscript for publication.

I have a couple of technical issues that are listed below:

(1) Fig. 1A and others show peripheral localization of MET-2 foci, a phenomenon that is further quantified in Fig.2. However in Fig.1D and in the corresponding movies the foci seem to be rather

internal. This should be explained / commented by the authors.

(2) One of the readouts of the presented experiments is an assessment of the intranuclear localization of MET-2 foci, so called "3 zone assay". As far as I could understand from this paper and from Meister et al (2010), the measurements are not 3D but 2D and performed on those optical section that have a maximum of a signal. Every focus plane of each analyzed signal is divided into 3 equivalent zones. First of all, the data presented in Fig.2E must be biased towards the internal (3d) zone, because in case that a focal plane is close to the nuclear top or bottom, signals that belong to zone 1 or 2 can be scored as belonging to zone 3. A nonbiased assessment using this type of analysis can be done only in case when a mid plane is used. Therefore, the authors have to indicate the bias in M&M and note that despite this caveat, which actually works against of the author's conclusion (!),Fig.2F still shows predominantly peripheral positioning of foci.

Second, the schematics in Fig.2E is not correct: the shortest distance from a given signal to the nuclear periphery (black line) should coincide with the diameter (red line) and comprise a fraction of the radius (as it is indeed described in M&M and in the main text).

The same comments concern scoring of FISH signals in Fig.6C

(3) A typo in Fig.4C should be corrected ("total")

(4) A reference to Figure 7 (schematics) is missing in the text.

(5) I suggest the authors to include "transcription de-repression" to the title.

January 21, 2019
Basel, Switzerland

Re: JCB manuscript #201811038, Delaney et al.

Dear Melina and Maria-Elena,

Thanks for your email of November 29. We have now totally revised the above mentioned manuscript, adding a number of new experiments and correcting points in existing figures, such that we can address all the reviewers' comments. I hope you are happy with the revised version.

In brief, we agree that it would be beyond the scope of our paper - which is based on *in vivo* results - to prove that LIN-65 is mediating liquid-liquid phase separation, and "somehow" brings MET-2 with it into the droplet phase. In fact, work in this direction would entail *in vitro* experiments with purified proteins, and LIN-65 is very difficult to express and purify, precisely because it is unstructured. Seeing this, we now understand that it would be a project of its own to characterize this protein as a "gel forming" protein *in vitro* and to characterize its interaction with MET-2. We did, however, carry out a series of experiments that make this hypothesis more likely. Namely, we show that the MET-2 is held in foci by weak interactions (rapid turnover following FRAP), we show that the foci of both LIN-65 and MET-2 are sensitive to hexanediol, which is commonly used to disrupt such phase separated globules in vivo, and we add videos showing the dynamic behavior of the foci *in vivo*. These results are included, although we agree that it is beyond the scope of our paper to prove conclusively that MET-2 foci are a result of liquid-liquid phase separation.

We further add to the ms an important analysis of the behavior of MET-2 foci (and LIN-65 foci) in response to heat shock. We know that *met-2* deficient worms are temperature sensitive sterile, and we find that loss of either MET-2 or LIN-65 renders worms unable to survive an acute heat shock (new Figure 5). Moreover, the heterochromatin foci disappear progressively upon exposure to 37°C, and then reappear upon hs recovery. Thus, we have a physiological stress response to environmental insult that correlates with LIN-65 orchestrated changes in MET-2 foci. We think this adds some relevance beyond the transcriptional changes, which could be attributed simply to a drop in H3K9me2. We hope you agree that this experiment greatly enhances the impact of the paper, enhancing its physiological importance.

Below I respond point by point to the reviewers' comments. We also upload a version where the major changes are highlighted in yellow to make it clear where the new experiments are discussed. The character count is < 40,000, not including spaces, and our article now has 8 figures, prepared according to the policies outlined in our Instructions to Authors. All original images are available if needed.

Thanks for a rapid processing of our contribution.

Best wishes,

Susan M. Gasser

Replies to editor first, then to each reviewer:

Thank you for submitting your manuscript entitled "An unstructured MET-2/SETDB1 cofactor ensures H3K9me2, focus formation and perinuclear anchoring". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that our reviewers were split in their recommendations to us and in their assessments of the level of advance for JCB. Reviewer #2 is largely supportive and raised points that seemed straightforward to address via minor changes. On the other hand, although Reviewer #1 appreciated the high quality of the work, they felt that the advance was somewhat limited in depth without more insight into the mechanistic role of LIN65 - especially as its potential contribution to phase-separated droplets is not tested (but suggested) - and into how heterochromatin is methylated through MET2-LIN65-ARLE14 mechanistically.

It is unclear what is meant by "mechanistic". MET-2 is an HMT, which can methylate to a certain degree without any co-factor. ARLE-14 has no clear function, even though it copurifies with MET-2, and the loss of the unstructured and clearly noncatalytic factor LIN-65 disperses foci, and renders MET-2 less efficient both for H3K9me2 deposition and gene/repeat repression. Our hypothesis is clear: we propose that the concentration of MET-2 in foci through interaction with LIN-65 is necessary for its efficient function. This we substantiate with additional experiments as described above. The argument for this mechanism are more clearly presented in the revised discussion.

We have discussed these comments in depth editorially. We appreciate that ablation of lin-65 resulting in decreased H3K9me2 was previously reported in adult intestinal cells in C. elegans, but the mechanism underlying this observation (e.g. direct interaction, enzymatic activity or others) was unknown. We find the conclusion that Met-2 localization is independent of 'canonical' H3K9me readers rather novel and interesting. Indeed, it points towards H3K9me/heterochromatin regulation through alternative pathways. In addition, we appreciate that the current manuscript clarifies the localization of Met-2 and provides a deeper analysis of gene expression as compared to published studies. However, other results seem consistent with prior work - for instance, based on your previous published work, the loss of anchoring to the nuclear periphery would be expected, considering the Met-2 dependency in anchoring and the fact that Lin65 reduces Met-2 levels and H3K9me2.

We present considerable new data on localization in foci and their subnuclear positioning, that no one has presented previously, particularly in a quantitative manner. The focus of the work is entirely new, and we demonstrate the key role played by MET-2 (not SET-25) in the nuclear organization of heterochromatin. Finally, linking foci to the heat shock response and recovery is new.

Given these points, we would be supportive of further consideration at the journal should you address/provide experimental information towards any of the following:

(i) That Lin-65 is an unstructured protein is irrelevant for the manuscript, as this is not addressed nor investigated at all, and therefore this cannot contribute to the appeal and novelty of the work unless you examine for example whether Lin65 can form aggregates in vitro.
Note that we have further characterized the biophysical properties of MET-2 and LIN-65 foci *in vivo*. In addition to the dynamic assembly of foci during the cell cycle, Fluorescence Recovery After Photobleaching (FRAP) experiments show that MET-2 flux into and out of foci is highly dynamic. In addition, treatment with 1,6-hexanediol, a compound commonly used to probe for liquid:liquid phase separation, dissolved MET-2 and LIN-65 foci *in vivo*. While this does not constitute definitive proof, given the limited methods established to investigate liquid:liquid phase separation, we feel our findings justify the speculation about MET-2 focus formation through phase separation.

(ii) Alternatively, if you could show how Lin65 can potentially mediate Met-2 localization and/or targeting, the work would increase in mechanistic definition and advance/appeal for cell biologists. It is clear from the data that Lin65 is required for the localization of Met-2 in the nucleus, the question is: what comes 'first' for Met-2 function and repression: targeting Met-2 to chromatin regions or chromatin regions being targeted to Met-2 foci? E.g. what is Lin65 actually doing: does it target Met-2 to its target sites (e.g. heterochromatin), or does it promote 'bringing' together/clustering of heterochromatic regions for e.g. subsequent methylation by Met-2? Potentially a Lin65 ChIPseq (or ChIP-PCR) in the absence of Met-2 could address this issue.

It is clear that LIN-65 is "upstream" of MET-2, for nuclear focus localization, because MET-2 alone does not form foci. The two are mutually dependent, however, for protein stability, suggesting that MET-2 may methylate LIN-65 or alter its structure in a manner that stabilizes it sufficiently to ensure focus formation. Indeed, they are interdependent for many phenotypes, but LIN-65 is the driver of heterochromatin focus formation. MET-2 is the driver of the methylation. This is clear from the proteins' primary structure. Importantly, HP1 proteins are not involved.

In addition, we feel that it would be important and scholarly for the manuscript to more rigorously cite prior work, as follows:

1. The comparison between the lin-65 single mutant and the lin-65; met-2 double mutant confirms that in lin-65 mutants a subset of the met-2 dependent genes are not significantly derepressed (this is similar to what was reported in cco-1 RNAi in Tian et al, 2016): e.g. there are dependent and independent genes regulated by Lin-65 and met-2. Please discuss this point to adequately give credit to previous work.
   This is now extensively cited and referred to in the Discussion and in the text
2. Likewise, the regulation of H3K9me2 levels by Lin-65 is not new; we encourage you to rephrase the following sentence on Page 7. ..."...where it appears to regulate the repression of stress response genes "AND OF H3K9ME2 levels" (Tian et al). Added.
   However please note: Tian et al (2016) performed RNAseq on WT, *met-2*, or *lin-65* mutants in the presence or absence of mitochondrial stress, but they did not compare *met-2* and *lin-65* gene targets at steady state. They also did not analyse repeat repression (only genes). Therefore, we discuss their work primarily with respect to stress response, rather than with respect to overall transcription changes.

3. CAF-1 association with Setdb1: the correct reference seems to be Yang et al, from YH Loh's group in Singapore, there's no mass spectrometry data in the Cheloufi et al reference. Corrected

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Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, Delaney et al describe the regulation of MET2 histone methyltransferase and hetrochromatin formation in C. elegans. They first investigated the localization of a histone H3K9 methyltransferase MET2 tagged with mCherry in a knock-in line. MET2-mCherry accumulated in heterochromatic foci around nuclear periphery throughout the cell cycle. RNAi experiments suggest that the localization did not depend on the known reader proteins for H3K9 methylation, like HP1 homologs. Proteome analyses resulted in identifying two proteins (LIN65 and ARLE14) that physically interact with MET2. The heterochromatic localization and stability of MET2 were impaired by depletion of LIN65, but not ARLE14. The phenotypes of LIN65 depletion were similar to those of MET2 depletion. These observations indicate a critical role of LIN65 in MET2 function. The data are generally of high quality and convincing. Identifying the new protein involved in histone methylation and heterochromatin formation is important, but the mechanism remains unaddressed.

A weak point of this manuscript is a lack of molecular mechanism. The authors emphasize LIN65 is an unstructured protein and suggest its contribution to liquid droplet formation, but there is no biochemical analysis of this protein. Moreover, how heterochromatin region is methylated through MET2-LIN65-ARLE14 is not addressed at all.
See comments above to the editor on this point.

Other points:

All RNAi experiments need Western blots or RT-PCR to show the depletion level. Particularly in Fig. 1C, no evidence is shown for the depletion of the target protein. In Fig. 4 and S4, the depletion of the target protein is shown by fluorescence microscopy, but it is still needed to show how much the (FP-tagged) proteins are depleted by Western blots.
We have included western blots showing that RNAi is effective against tagged *met-2, lin-65*, and *arle-14*. See Fig S1A and Fig S3C. qPCR was performed against untagged RNAi targets and is shown in Figure S2A. In addition, we show loss of the protein level by fluorescence - thus the downregulation events are robustly controlled.

In the results section, the manuscript under submission is often referred. This is frustrating because the readers are unable to read the manuscript. It is better to use BioRxiv if the citation is necessary.
The paper we refer to is now in press (open access) and will be on line before the JCB paper is out. Padeken et al., (2019) Genes & Dev. In press. It is now included in the reference list.

Discussion is too long. There are lots of redundant and less relevant description.
It is now significantly shorter and focuses on concrete examples of stress response and LIN-65.

Minor points:

Introduction, "the trimethylation of histone H3K in nucleosomes associated with. . . forming so-called facultative heterochromatin." In mammals it is generally acknowledged that facultative heterochromatin is associated with H3K27me3, and constitutive heterochromatin with H3K9me3. Please rephrase.
The introduction is extensively rewritten and this phrase is omitted. We cite the recent Ken Zaret paper in reference to H3K9me and tissue specific genes.

There are some typos (e.g., p7, l14; ...either than LIN-65...)
These have all been taken care of - thanks for the careful reading.

Reviewer #2 (Comments to the Authors (Required)):

The paper by Delaney et al is a logic continuation of a series of works from Susan Gasser's lab that systematically dissect epigenetic mechanisms of heterochromatin formation using C. elegance as a model. In particular, in the recent years, Gasser's team demonstrated the different roles of the HMTs MET-2 and SET-25 in H3K9 methylation, peripheral heterochromatin localization and transcription inhibition, as well as the anchoring role of CEC-4 and the role of HPLs and LIN-61 in transcription repression.

In the current paper, the authors focus on the protein MET-2 that mediates H3K9 mono- and di-methylation. They show the predominant nuclear localization of MET-2 (an issue that previously was a matter of debate), demonstrate its enrichment and co-localization with H3K9me foci, as well as its high cell-cycle dependent dynamics and tendency for peripheral positioning. The authors showed that depletion of known H3K9me readers (HPL-1, HPL-2, LIN-61) does not influence localization of heterochromatic MET-2 foci, which prompted them to search for other interacting partners and to identify two proteins, LIN-65 and ARLE-14. They further show that depletion of LIN-65 (but not ARLE-14) leads to de-heterochromatinization, manifested by loss of peripheral heterochromatic MET-2 foci, decrease of H3K9 methylation and, as a consequence, changes in transcription, including de-repression of microsatellites and simple repeats. Moreover, the comparison of effects on transcription after depletion of one of the two proteins, MET-2 or LIN-65, with the effects of a double mutant lacking both proteins, showed that LIN-65 represses genes via interaction with MET-2 but it is not needed for MET-2 catalytic activity. The authors conclude that the role of LIN-65 is to direct and associate MET-2 with heterochromatic foci. This conclusion is reinforced by the intrinsically disordered nature of LIN-65 suggesting its possible role in phase separation of heterochromatic foci.

This work is an important contribution to the field of nuclear biology: it uncovers yet another mechanism of heterochromatin formation leading to a better understanding of spatial chromatin segregation within the nucleus and its role in genome regulation. The work is performed on a high technical level, using cutting-edge cell biology techniques. The manuscript is clearly written and very well illustrated. I can only recommend this manuscript for publication.

Thank you for this kind and very positive evaluation

I have a couple of technical issues that are listed below:

(1) Fig. 1A and others show peripheral localization of MET-2 foci, a phenomenon that is further quantified in Fig.2. However in Fig.1D and in the corresponding movies the foci seem to be rather internal. This should be explained / commented by the authors.

The time lapse series were processed as maximum intensity projections to avoid artifacts due to nuclear rotation and movement of the cells. This has been indicated below the figure, in the figure legend and explained in the Material and methods.

(2) One of the readouts of the presented experiments is an assessment of the intranuclear localization of MET-2 foci, so called "3 zone assay". As far as I could understand from this paper and from Meister et al (2010), the measurements are not 3D but 2D and performed on those optical section that have a maximum of a signal. Every focus plane of each analyzed signal is divided into 3 equivalent zones. First of all, the data presented in Fig.2E must be biased towards the internal (3d) zone, because in case that a focal plane is close to the nuclear top or bottom, signals that belong to zone 1 or 2 can be scored as belonging to zone 3. A nonbiased assessment using this type of analysis can be done only in case when a mid plane is used. Therefore, the authors have to indicate the bias in M&M and note that despite this caveat, which actually works against of the author's conclusion (!). Fig.2F still shows predominantly peripheral positioning of foci.

This comment stems from an incomplete understanding of the 3 zone assay. We now explain it in detail in the Materials and methods. Whereas it is true that distance measurements are made in a 2D plane, the sum of 2D distribution (thanks to Cavalieri's principle) reflects 3D space. This is described in Meister et al. (2010a) Methods in Enzymology. We now are careful to note that the measurements are made in a single plane, but it is incorrect to think that this does not represent 3D space, as long as the nucleus is spherical. We do not score focal planes where the GFP-spot is close to the N or S pole of the sphere. Here is a detailed description:
The zoning assay, which our laboratory and others in the field of nuclear organization have used extensively, exploits two facts: first, that confocal image resolution is better in x–y than in z, and second, that a tagged locus can usually be assigned to specific plane of a through-focus stack of images. The quantitation of localization is carried out as follows: a stack of 50-70 focal planes is taken through a spherical nucleus for which the nuclear envelope is marked by mCherry and the chromatin locus of interest by GFP. In the plane of focus where the GFP spot is brightest, we measure the diameter of the nucleus (through the spot) and the distance from the spot to the nearest point on the nuclear envelope. That ratio can be compared from sample to sample no matter whether the plane is "near the equator or near either pole" of the sphere. The disc of the sphere in which the locus is found, is divided into three zones of equal surface, each containing 33% of the area. Thanks to Cavalieri's principle, we know that the sum of discs add up to the full volume of the sphere, thus loci found in a third of each planar surface, also are localized in one third of the volume of the sphere. A randomly distributed focus scored in a population of nuclei, yields 33% in each of the three zones.
Due to the so-called z-stretch, which limits resolution in z, we do not score spots that are in the upper most and lower most planes, and we generally eliminate the top and bottom 20% of the sphere with a procedure called "decapping". As long as the focal stacks capture nuclei in all

orientations, this does not bias results, and, as explained in Meister et al., Methods in Enzymology, 2010 470: 435-467, decapping improves the accuracy of the zone measurements. The method was originally developed for yeast nuclei, but was applied to spherical *C. elegans* nuclei in Meister et al., 2010 G&D 24: 766-782, and has been used and cited ~ 180 times.

Second, the schematics in Fig.2E is not correct: the shortest distance from a given signal to the nuclear periphery (black line) should coincide with the diameter (red line) and comprise a fraction of the radius (as it is indeed described in M&M and in the main text).
Sorry, for this. The black line is offset from its theoretical position for graphical clarity. We think the description of the method is sufficiently clear that the lines need not obscure each other. See Materials and Methods.

The same comments concern scoring of FISH signals in Fig.6C see above

(3) A typo in Fig.4C should be corrected ("total") done

(4) A reference to Figure 7 (schematics) is missing in the text. added

(5) I suggest the authors to include "transcription de-repression" to the title.
We have now included this in the title.

January 23, 2019

RE: JCB Manuscript #201811038R

Dr. Susan M Gasser
Friedrich Miescher Institute for Biomedical Research
Maulbeerstrasse 66
Basel CH-4058
Switzerland

Dear Dr. Gasser,

Thank you for submitting your revised manuscript entitled "Perinuclear MET-2/SETDB1 foci and transcriptional repression require an unstructured co-factor". Based on our assessment of the rebuttal and the revision, we think your responses and the new additions adequately address the points of major concern and that the work is now sufficiently developed for publication. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends. (This limit is flexible if the total count remains reasonably close to the limit.)

2) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: Heterochromatin formation and transcriptional repression through the unstructured co-factor Lin65

Running title: MET-2/LIN-65 control heterochromatinization and repress transcription
(the extended count is OK; please feel free to edit)

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
Suggested revised eTOC to match this style:
Delaney et al. show that the unstructured domain cofactor LIN-65 is essential for the stable formation of heterochromatic foci. It forms a nuclear complex with the H3K9 methyltransferase MET2/SETDB1 and modulates MET-2 localization, activation, and H3K9me-mediated silencing in C. elegans.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to S4E if possible.

Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: 1B, 4FG, S1A, S3B

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: S2D, 5D

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- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
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g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

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7) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.

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Sincerely,

Maria Elena Torres-Padilla, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

January 25, 2019

RE: JCB Manuscript #201811038RR

Dr. Susan M Gasser
Friedrich Miescher Institute for Biomedical Research
Maulbeerstrasse 66
Basel CH-4058
Switzerland

Dear Dr. Gasser,

Thank you for contributing your Article entitled "Heterochromatic foci and transcriptional repression by an unstructured MET-2/SETDB1 co-factor LIN-65". It is a pleasure to let you know that your manuscript is now accepted for publication in Journal of Cell Biology. Congratulations on this interesting work.

Your manuscript will now progress through image editing, copyediting, and proofing. It is journal policy that authors provide original data upon request. You may contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu), with any questions throughout the process.

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Melina Casadio, PhD
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