

EPIGENETIC MODULATION VIA THE C-TERMINAL TAIL OF H2A.Z

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript "Epigenetic modulation via the C-terminal tail of H2A.Z" by Imre et al., contains a wealth of – mostly – biochemical and immunofluorescence-based data on the potential role of the C-terminal tail of H2A.Z on chromatin structure and function. The findings that H2A.Zs' C-terminal region influences nucleosome stability, possibly by nucleosome interaction, and contributes to the regulation of gene expression is highly interesting. While the used assays, such as e.g. QINESIn and peptide addition to nuclei are innovative, there are some parts of the manuscript that need clarification or improvement. In general, the presentation of data in Figures is too minimal, graphs lack information. Several controls are missing or are only used in "later" experiments. This makes it difficult to interpret the data, as it is possible that H2A.Z lacking the C-terminal tail is not deposited into nucleosomes, explaining the diffuse nuclear distribution. In conclusion, this is a highly interesting and surprising story that needs some more experiments before publication:

Major concerns:

1. Characterization of antibodies: As ZAbA and ZAbB antibodies showed different nuclear staining patterns, their specificity needs to be analyzed in greater depth. Is the signal lost in DT40 DKO cells (immunofluorescence and immunoblotting)? Peptide competition experiments with histones from HeLa or DT40 cells can be applied to determine specificity of the antibodies?
2. Supplemental Figure 2D, E: This is a very interesting finding. Can the authors use these high salt fractions for mass spec, immunoblots or IPs to verify that this 15% fraction of H2A.Z is indeed associated with the nuclear lamina?
3. Figure 1F: As the protein concentration of H2A-containing dimers appear to be much higher than the one of the H2A.Z-containing ones, it is difficult to conclude that H2A.Z nucleosomes need higher salt concentrations than H2A ones. A quantitation of different replicates would enhance the validity of the interpretation.
4. Supplemental Figure 4A-C: Why is recombinant human H2A.Z running this high (40kDa?) and in different sizes (as if it is modified, which should not happen with recombinant proteins)? Is it tagged? Please indicate. Why are there several bands in the silver stained gel? What does reciprocal labelling mean? How was the efficient stripping procedure controlled? Why did the authors use shRNAs, when they have the KO cells?
5. Supplemental Figure 4F-H: These data are very interesting. However, it is difficult to conclude that "... H2A.Z silencing induced reorganization of the H3K9me3 heterochromatin and the decreased presence of H2A.Z was mainly due to the disappearance of the variant from H3K9me3-rich peripheral heterochromatin." In Suppl. Fig. 4H that authors show the co-localization coefficient between H2A.Z and H3K9me3. As H2A.Z is depleted, it is expected that the co-localization drops as there is less H2A.Z present. In order to make such statement, the authors should use markers for the different chromatin states and their localizations, such as e.g. H3K27me3 and lamin A. In addition, how does H3K9me3 localize in DT40 DKO compared to WT DT40 cells? Is the same change in pattern quantitatively observed?
6. C-terminal truncation leads to genome architectural changes: a) The authors need to show with in vitro nucleosome-assembly assays and in vivo mononucleosome-purification experiments that the C-terminal truncated H2A.Z protein is indeed incorporated into nucleosomes. It is possible that this truncated variant is not recognized/bound by the required chaperones (p400 or SRCAP) and therefore not deposited into chromatin. b) Also, the authors should show a scatter plot comparing gene expression profiles of DKO and DKO/DeltaZ with each other. Do they look very similar? Then the truncated variant might not be incorporated into chromatin. c) The ChIP-seq data in Suppl. Fig. 11 are difficult to interpret. What does the antibody recognize in untransfected DKO cells (control)? Is there an enrichment of DeltaZ at +1 and -1 nucleosomes? It does not look like it, arguing that the variant is not deposited into chromatin. As positive control, WT DT40 cells should be used to verify the ZAbA is working in ChIP assays. d) How do DKO cells survive treatment with DNA damaging agents

(Suppl. Fig. 12)? Does DeltaZ have any effect?

7. Peptide-experiments: the authors should use a scrambled C9 peptide as negative control in all experiments involving this peptide (not just in Figure 6).

8. The authors speculate that the C-terminal peptide might bind to possible molecular partners. This would indeed be one good explanation for the observed effects after peptide addition. Here it is crucial to perform peptide-binding assays (protocol by e.g. Wysocka, ScienceDirect, 2006) followed by mass spectrometry, to identify potential binding partners. These should be validated in IPs with H2A.Z WT and DeltaC, as well as with depletion studies, looking at the changes in H2A.Z localization. In case the peptide binds nucleosomes, this should be shown by peptide-binding assays with mononucleosomes followed by mass spectrometry or immunoblotting.

Minor concerns:

1. According to the histone variant nomenclature (Talbert et al., Epigenetics & Chromatin, 2012) it would be preferable to call the H2A.Z variants H2A.Z.1, H2A.Z.2.1 or H2A.Z.2.2.

2. General: the labelling of figures and graphs is extremely minimal. Without reading the legend, it is impossible to understand the results. To make it easier for readers, add info about the experiment (e.g. in y-axis and on top of graphs).

3. Please specify which H2A.Z isoform (H2A.Z.1 or H2A.Z.2.1) was used in the experiments (e.g. Figure 1).

4. Please add a graphic of H2A.Z isoform sequences and indicate the antibody epitopes. This way the reader gets a better understanding about the different regions recognized by the two antibodies.

5. In the result section, the authors refer to Suppl. Fig. 2A-C for the characterization of different H2A.Z antibodies. In this Figure, these data sets cannot be found.

6. Supplemental Figure 2F: How does H3 (which is more stable than H2B that was used as control) behaves after nickase usage? Does it show a similar slope as H2A.Z? And how does the data look when the ZAbB antibody is used in this assay?

7. Supplemental Figure 2L: Labelling of the figure is different to the legend. Were DKO cells used, expressing either WZ H2A.Z or the 5KR H2A.Z mutant or were WT DT40 cells used instead as control?

8. Supplemental Figure 3C: Did the authors use GFP-PWWP2A or PWWP2A-GFP? The tag at the C-terminus, next to the PWWP domain, might interfere with its function.

9. Supplemental Figure 3D, E): I am missing a figure showing that the N- or C-terminal localization of the Tag (GFP) does not influence the stability, as stated in the text.

10. Supplemental Figure 3F: What is PI? Propidium iodide?

11. Supplemental Figure 3H: Why is the DNA signal so strong in the nucleoli? Was Propidium iodide staining without RNase digest used and are DNA as well as RNA seen?

12. Supplemental Table 2: A heatmap (with replicates) would be better.

13. Supplemental Figure 4F: Labelling of panels is missing.

14. Supplemental Figure 4G: Why is there still H2A.Z signal present in the shRNA transfected cells? When the signal was technically amplified, it should be shown with and without enhancement.

Reviewer #2

(Remarks to the Author)

The authors investigate the role of H2A.Z isotypes, highlighting their high stability and exploring its tethering to other nuclear structures such as other histones, nucleosomes and lamin. They also investigate the effect of a peptide representing the last 9 C-terminal aminoacids (C9) by knock-out, live-cell insertion of the peptide, characterizing its role in modulating chromatin accessibility.

It is a very well-written and enjoyable paper featuring many relevant techniques and outlining a comprehensive, well-thought story of H2A.Z structural and functional role. I honestly can't fault it, the authors managed to address every remark I might have had in the next few sentences.

I strongly recommend it for publication

Reviewer #3

(Remarks to the Author)

Imre L et al argue that the localization of H2A.Z-nucleosomes to euchromatin or heterochromatin depends on the 9 amino acids of the C-terminal tail. They have established an assay system that distinguishes the majority of euchromatin from heterochromatin using antibodies with different reactivity to QINESIn, which they previously reported. They also show that the 9 amino acids of the C-terminal tail of H2A.Z are important for its localization to heterochromatin. Furthermore, they show that the addition of the 9-amino acid peptide biases the localization of H2A.Z to euchromatin. This finding of differential localization of H2A.Z-nucleosomes to euchromatin and heterochromatin as a phenomenon provides new insights into H2A.Z for chromatin and related fields. On the other hand, its mechanism has yet to be described.

Major points:

The impact of localization on heterochromatin in ΔC is presumed to be of minor magnitude. The authors should demonstrate with data to clearly state what the biological impact is. For example, in the salt elution profiles in Sup Fig6C, D, global H3K27me3 and H3K9me3 are unchanged between DKO H2A.Z1 (CTRL) and H2A.Z1 ΔC (ΔC). On the other hand, in Fig. 2D, the localization of H3K9me3 is reduced in H2A.Z ΔC . This indicates that the effect of H2A.Z ΔC in maintaining heterochromatin formation is minor. Furthermore, the fact that there are few genes with variable expression in RNA-seq

analysis also provides evidence that H2A.Z Δ C has little effect on maintaining heterochromatin formation. Since H3.3, which is present in both euchromatin and heterochromatin, is involved in the repression of transposon expression, transposon expression may also be elevated in H2A.Z1 Δ C. The C-terminal tail of H2A.Z may function during perturbation, such as in response to stress.

The authors need a clear explanation for their choice of Manders Correlation in co-localization analysis. In a co-localization analysis where the correspondence is the same (when comparing the localization of the same molecule in wt and mut), as in this case, a scatter plot of the two-color fluorescence signal should be created to visualize the distribution of the data. If there are no outliers or anomalies in the signals, the Pearson coefficient should be the first candidate for analysis. Manders Correlation can discuss whether the signals are spatially located in the same place, but not the correlation of co-localization. Therefore, we may not be able to address whether the change in H2A.Z localization affected the decrease in H3K9me3.

The -MNase-seq analysis makes no sense at all. The authors discuss the distribution of CTRL - CTRL(MN), and C9 - C9(MN) processed signals, but subtracting individually standardized data may not reflect the original data. In such an analysis, individual heatmap and aggregation plots for CTRL, CTRL(MN), C9, and C9(MN) can be created and compared to analyze differences in localization. Differences in the number of reads and fragment length after MNase treatment may also be altered as differences in susceptibility to MNase. These are also subject to discussion and should be indicated.

Minor points:

The text is long and difficult to read from Fig. 1 to Fig. 2. The author should think about the structure of the text, for example, making the Supp data the main Fig.

In Suppl. Fig. 2E, data with different salt concentrations should be shown as representative data.

Suppl. Fig. 3I Why is the salt tolerance of H2A.Z stronger than that of K9me3? It would be more convincing if there is a counterstain such as H3K4me3.

Suppl. Fig. 4A-C H2A.Z is the correct band around 14kDa, but why does it react at 40kDa? Also, two main bands are visible at different locations in the silver staining. The author should explain these logically.

In Suppl. Fig. 4D-G, the author claims that "The background signals detected in the still viable cells exhibiting near-complete silencing were minimal for all In Suppl. What is the background in this case?"

In Suppl. Fig. 3F, To clearly show the difference in localization of the C- and N-terminal tags, it is recommended to include the actual immunostaining data of the C-terminal tag for comparison, instead of subtracting it from the reference.

Reviewer #4

(Remarks to the Author)

The manuscript by Imne et al., describes the nuclear distribution and stability of H2A.Z containing nucleosomes using different antibodies and propose important roles for the C-terminal last 9 amino acids of H2A.Z protein. The manuscript is sometimes difficult to follow, contains inaccuracies and conclusions are often not supported by the data. In addition, the manuscript does not contain solid new information for the field.

Main concerns

1.- The authors describe different stability of H2A.Z in the nucleosomes and different immunofluorescence data using two different antibodies that they call ZAbA and ZAbB. They try to argue that both antibodies recognize different populations of H2A.Z. Throughout the manuscript it is not clear what the molecular basis of these differences are. However, an obvious experiment such as a comparison between the ChIP-seq signals of both antibodies is not performed. Proteomic analysis of proteins co-immunoprecipitated with one or the other antibody are not performed either. I think that these two type of standard experiments will contribute to clarify the possible differences between the antibodies.

2.- Authors claim that the fraction of H2A.Z that is recognized by ZAbA is, in some way, associated to the nuclear lamina. One of the experiments to demonstrate this is an immunofluorescence with nuclear halos (Figure 2E). However, the authors do not mention what antibody they use to do this important experiment. This experiment should be performed with both antibodies. In addition, nuclear halos preparation is not described in materials and methods.

3.- It is often difficult to keep track of which antibody has been used in each experiment. Figure panels are often labelled with "H2A.Z". For example, what antibody was used in figure 4C? I suggest writing the antibody used directly on the graph (i.e. H2A.Z (ZAbA) or H2A.Z (ZAbB), not just on the figure legend.

4.- Figure 1F. The quality of the western shown in this figure is very poor. In addition, if the authors want to make conclusions of the amount of H2A.Z released in 1M versus 1.2 M in comparison with the amount of H2A/H2B, they should perform the

experiment at least three times and quantify the different bands. The intensity of the bands in the left part of the gel is much lower than in the right part, so, the conclusion driven by the authors is unclear.

5.- Supplementary figures 4A, 4B. In this western the molecular mass of H2A.Z is about 40 kDa, which is not at all a normal molecular mass for H2A.Z. In addition, the silver staining of Supplementary figure 4C give a lot of bands for H2A.Z.

6.- The authors make a proteomic study of the non-soluble fraction upon NaCl extraction (Suppl. Table 1). They argue that in this fraction they detect H2A.Z, that is not detected when proteins are extracted by MNase treatment. However, the same can be observed for H3 and H4. I do not see that this is a valid argument to propose that H2A.z is in a hard to extract fraction.

7.- How differential gene expression profile was done? Neither RNA-seq not microarray experiments are described in Materials and Methods and data are not deposited in databases. How many replicates were performed? What threshold were used? Why p-values are not shown in Table 2? On top of that, in line 264 authors write: "...were also reflected in dramatic differences of gene expression patterns, comparing wild265 type (wt), DKO H2A.Z1 and DKO H2A.ZdeltaC cells". However, I only see, in Supple. Figure 10, a few genes that change expression, especially when comparing H2A.ZdeltaC with H2A.Z1 expression. This is not a "dramatic" change.

8.- H2A.Z1 ChIP-seq signal of supplementary figure 11 is very different from many other H2A.z ChIP-seq in the bibliography. This have to be commented and clarified by the authors.

9.- The authors suggest that H2A.Z C-terminal tail may be involved in establishing intra- or internucleosomal molecular associations, but they do not provide any experiment to prove it. The authors should reconstitute nucleosomes in vitro with recombinant H2A.Z or H2A.ZdeltaC and determine compaction of chromatin fibers either by nuclease sensitivity, density gradient, or some other biophysical method.

10.- Experiments for figure 3 were performed with permeabilized nuclei (dead cells) incubated during hours with the C9 peptide. What it is the physiological relevance of the chromatin changes observed (for example in H3K9me3)? In fact, these changes were not observed when C9 was introduced into live cells.

11.- The authors suggest that C9 peptide bind specifically the nuclear lamina. Have the authors tested by co-immunoprecipitation whether H2A.Z interacts directly with lamins?

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

General response to comments:

I want to thank the authors for their detailed responses to my suggestions and comments. They have added a wealth of new data that strengthen their story and conclusions.

Minor remarks:

1) The authors are correctly arguing that DT40 DKO cells are not viable. However, Kusakabe et al., 2016, who generated these cells, showed that - after tet-induction – DKO cells proliferated for at least 48 hours normally, then cell division dropped and after 96 hours cells started dying (Fig. 2B, D). The authors were even able to obtain enough cells to generate extracts for immunoblotting (Fig. 2A), showing loss of H2A.Z protein. Therefore, some experiments I previously asked for should have been possible to perform (e.g. testing antibody specificity to investigate if the used H2A.Z antibodies might recognize any other proteins than H2A.Z).

Nevertheless, the authors have addressed most of my concerns and answered in great detail all my questions and have rewritten many part of the manuscript and relabeled figures, thereby improving readability.

2) Please take out exaggerating words such as e.g. "spectacular".

3) While looking at the revised manuscript, I noticed that some data seem to appear in both the Figures as well as the Supplemental Figures. Please check to avoid any duplication of experiments and correct accordingly:

- Figure 1G and Supplemental Figure 3 appear identical
- Figure 1A and Supplemental Figure 2F appear identical

4) Further, I noticed that scale bars in all IF microscopy pictures are missing.

Once these minimal concerns have been addressed, I am satisfied and support publication of the manuscript.

Reviewer #3

(Remarks to the Author)

The authors' responses contain some experimental and confusing explanations that do not support the publication of the paper. In particular, the following points need to be reconsidered:

The response to the question regarding Manders Correlation is insufficient. The authors cannot simply replace "molecule" with "pixel" and maintain the claims made in the figure. What is the basis for considering all objects defined as "pixels" to be signals?

The response to the MNase experiment is inadequate. The additional data presented do not exclude the risk that the results may vary depending on the conditions of sonication. Moreover, it is unclear how the clustering of the heatmap was performed, but it suggests that either significant regions were not extracted by clustering or that appropriate clustering was not performed.

For Main Fig1f, it is disappointing that experimental improvements were not made after conducting sufficient trials. In particular, the response to point 5 raises concerns about the stability of the experimental quality.

Regarding the data in Suppl. Fig. 4D-G, the authors have changed the text to "autofluorescence," but this is not appropriate. Their modified text seems to imply that they cannot set the expression level to zero, but this only adds to the confusion.

Reviewer #4

(Remarks to the Author)

The Imre et al. manuscript is much improved. They answer convincingly some of my questions, although not all. In this manuscript there are new approaches and many interesting data. However, I still see that several important results do not agree with the main hypothesis of the authors.

- The authors suggest that there is a pool of H2A.Z that associates with heterochromatin and can be traced using specific antibodies (ZAbA). They state in the Discussion: "we propose that ZAbA preferentially detects stable nucleosomes residing in peripheral heterochromatin." However, when they destabilize this fraction of H2A.Z using the C9 peptide, they do not detect transcriptional changes associated with heterochromatin alteration, as seen in the RNA-seq experiments of Figure 6. They observe downregulation of over 500 genes from euchromatin, but there is no mention of heterochromatin transcripts. To verify their hypothesis about C9 destabilizing heterochromatin, they should investigate whether major satellite and other repetitive sequences such as LINE are upregulated.
- Similarly, when they perform MS analysis of proteins released by ZAbA-targeted CUT&RUN upon C9 treatment (figure 3N), I would expect to see heterochromatin proteins if their hypothesis is correct. Maybe not only heterochromatin proteins because C9 may also affect other H2a.Z fractions as the authors suggest, but at least some.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

I am sorry to hear that the authors do not fully grasp the intent of the comments despite undergoing revisions. In this revised version, I still believe that there is insufficient data to clearly explain the relationship between the modification of heterochromatin involving the C-terminal region of H2AZ and MNase-seq analysis. Particularly, the MNase-seq analysis does not fully utilize the information from mapped reads, making it unclear where the MNase sensitivity of heterochromatin occurred.

I would like to request careful reconsideration of the following points:

There is a lack of direct analysis demonstrating changes in nucleosome positioning in heterochromatin regions using MNase-seq. It would be valuable to compare the read counts in distinct genomic regions such as promoters, enhancers, and transposon elements to identify where MNase sensitivity is higher. Comparisons based on categorization of regions using histone modifications like ChromHMM could also be beneficial. If MNase sensitivity is increased, it would be expected to detect a higher number of reads in heterochromatin regions such as retrotransposons.

The authors responded in the rebuttal letter that they analyzed the expression changes of transposable elements from RNA-seq analysis. This response addresses the previous comment about the lack of direct evidence regarding whether the modification of heterochromatin activates the transcription of transposable elements. This data provides direct evidence that the structural changes in heterochromatin by H2AZ are involved in transcriptional activation. Therefore, incorporating these data would greatly strengthen the validity of the functional role of the C-terminal region of H2AZ as proposed by the authors.

Version 4:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

After reading the authors' responses, I still feel that the concerns regarding the effect of C9 on heterochromatin have not been fully addressed. I would like to reiterate that my main concern is that the discovery of H2AZ C-terminal relaxing heterochromatin, which is one of the key points of this paper, is not convincing with the sequence data alone. In their explanation requested in the revision, the authors state that this is an MNase-sensitive genome sequencing analysis, different from the normal MNaseSeq. This point made it difficult to judge the validity of their genome sequencing. In addition, concerns about reproducibility, including the establishment of replicates in the analysis, were not addressed.

On the other hand, they did perform assessments using imaging methods other than sequencing, which I appreciate. However, the crucial data on heterochromatin H3K9me3 after C9 addition are not presented in the main text but only in the peer review comments. Finally, you seem to avoid essential responses such as showing additional experiments and analyses to readers, justifying this by pointing to constraints such as word limits in the article structure, which seems scientifically unsound and somewhat dishonest.

I would hope that additional analyses with proper reproducibility (such as replicates) would be performed. Furthermore, to alleviate my concerns, quantitative data from imaging of MNase sensitivity in H3K9me3 chromatin should be included in the main text, especially since the authors mention it as an analysis "not accompanied by bioinformatics analysis." If difficult-to-substantiate events emerge during the revision process, it might also be worth considering focusing on only the definitive aspects for logical development.

Version 5:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The authors have been diligent in addressing my concerns and providing data. In particular, C9 MNase sensitivity, which is one of the key aspects of this study, was well presented by quantitative analysis and improved NGS data.

Minor point

For the pie chart in Fig.4B, the color explanation should be included as shown in Supplemental Fig.19 B and C. It would be helpful for the reader to understand the data.

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Answers to Reviewers' comments

Reviewer #1 (Remarks to the Author):

The manuscript “Epigenetic modulation via the C-terminal tail of H2A.Z” by Imre et al., contains a wealth of – mostly – biochemical and immunofluorescence-based data on the potential role of the C-terminal tail of H2A.Z on chromatin structure and function. The findings that H2A.Zs' C-terminal region influences nucleosome stability, possibly by nucleosome interaction, and contributes to the regulation of gene expression is highly interesting. While the used assays, such as e.g. QINESIn and peptide addition to nuclei are innovative, there are some parts of the manuscript that need clarification or improvement. In general, the presentation of data in Figures is too minimal, graphs lack information. Several controls are missing or are only used in “later” experiments. This makes it difficult to interpret the data, as it is possible that H2A.Z lacking the C-terminal tail is not deposited into nucleosomes, explaining the diffuse nuclear distribution. In conclusion, this is a highly interesting and surprising story that needs some more experiments before publication:

We thank Reviewer for finding our work, in general, interesting and we are grateful for the critical comments that helped us improve the manuscript. As to the general, critical issues raised by Reviewer, the presentation of the figures was revised so as to make them clear. The control experiments requested *all* have been performed (see below in detail). Regarding the overall concern whether the tailless H2A.Z form actually becomes part of chromatin, our main argument is that the *expression of Δ CH2A.Z complement the lethality of DKO DT40 cells*. If the ectopically expressed Δ C is not incorporated into nucleosome, the cells cannot survive. In addition, only the H2A.Z molecule, but not canonical H2A, can complement the lethality of DKO DT40 cells. (See ref. <https://pubmed.ncbi.nlm.nih.gov/26833946/>) In addition, in our earlier ChIP-seq experiments (not included in the revised version), the overall coverage was similar in the case of WT and Δ C DT40.

Major concerns:

1. Characterization of antibodies: As ZAbA and ZAbB antibodies showed different nuclear staining patterns, their specificity needs to be analyzed in greater depth. Is the signal lost in DT40 DKO cells (immunofluorescence and immunoblotting)? Peptide competition experiments with histones from HeLa or DT40 cells can be applied to determine specificity of the antibodies?

Application of the DKO DT40 cells as a control comes up in this review repeatedly. As stated above, these cells are non-viable. Please also note that the DKO is induced only in the presence of tet. Therefore, during the procedure of the production of the Δ C knock-in strain, wt H2A.Z was constantly expressed and then, after the

expression of knocked-in ΔC , the wt H2A.Z was shut down by adding tet. So, the cells did not need to survive without H2A.Z in the process of the establishment of the strain. (The procedure of the strain establishment was described in ref. <https://pubmed.ncbi.nlm.nih.gov/26833946/>.)

Peptide competition experiments may not be appropriate in view of our finding that C9-CF was not immobilized by the polyclonal ZAbA in FCS experiments (data not shown). However, we conducted such experiments using complete, recombinant H2A.Z1; see Suppl. Fig. 5.

2. Supplemental Figure 2D, E: This is a very interesting finding. Can the authors use these high salt fractions for mass spec, immunoblots or IPs to verify that this 15% fraction of H2A.Z is indeed associated with the nuclear lamina?

Please see new Suppl. Table 1. In line with Reviewer's suggestion, the halo samples were analyzed by mass spectrometry. We have found H2A.Z along with most other proteins detected by immunofluorescence. Intriguingly, their mRNA expression levels appear to tightly correlate with that of H2A.Z across the Cellminer NCI60 panel (proteins marked with * in the list), suggesting that the co-occurrence of these proteins in the nuclear lamina deserves further attention in the context of an independent future project.

3. Figure 1F: As the protein concentration of H2A-containing dimers appear to be much higher than the one of the H2A.Z-containing ones, it is difficult to conclude that H2A.Z nucleosomes need higher salt concentrations than H2A ones. A quantitation of different replicates would enhance the validity of the interpretation.

Please consider the following arguments below:

1. The H2A/H2B dimers run as a single band, hence appears to be of higher concentration.
2. Would the salt containing eluent be present in limiting quantities, it might be expected that a higher salt concentration would be required to dissociate the species present at higher concentration. In fact, the opposite is seen. If the salt containing eluent is in abundance, experience based on Fig. S11B of our paper <https://pubmed.ncbi.nlm.nih.gov/28986581/> shows that salt sensitivity (quantitatively characterized by the elution curves) is invariant to the amount of nucleosomes present and exposed to salt. Thus the difference seen cannot be the result of differences in the amount of nucleosomes on the columns.

3. The higher salt concentrations to displace recombinant H2A.Z/H2B dimers actually confirms what is observed for *in vivo* assembled chromatin using exactly the same hydroxyapatite approach (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1137757/>).

In the figure, an arrow points at the sample eluted by 1 M salt: H2A is present, H2A.Z is absent.

4. Supplemental Figure 4A-C: Why is recombinant human H2A.Z running this high (40kDa?) and in different sizes (as if it is modified, which should not happen with recombinant proteins)? Is it tagged? Please indicate.

The tag on the Recombinant Human Histone H2A.Z protein ([ab114184](https://pubmed.ncbi.nlm.nih.gov/27854017/)) is an N-terminal GST-tag. We made new western blots using proteins carrying no tags (<https://pubmed.ncbi.nlm.nih.gov/27854017/>); see new Suppl. Fig. 5. These preparations are of the appropriate molecular weight and contain no extra bands that are impurities in the commercial samples. These data replace the earlier ones in our revised manuscript.

Why are there several bands in the silver stained gel? What does reciprocal labelling mean? How was the efficient stripping procedure controlled? Why did the authors use shRNAs, when they have the KO cells?

The samples of the recombinant (commercially available) proteins apparently contained impurities (see also above). They did not show up in the western blot, just in the silver-stained bands (earlier figure). These were deemed useful as internal controls further confirming that the antibodies did not exhibit nonspecific binding. See new Suppl. Fig. 5 with western blots using protein preparations devoid of impurities present in the commercial samples.

The KO cells are non-viable, as detailed above. (Therefore, near-complete silencing was not an option either, in line with the recently revealed fact that H2A.Z must mark nascent chromatin prior to any transcription restart following DNA replication (<https://pubmed.ncbi.nlm.nih.gov/36750094/>)).

After stripping, the membrane was immersed into the chemiluminescent substrate and checked in the AlphaView geldoc system. The second labeling was initiated only when there was no signal at up to 5 mins exposure. However, to resolve any possible concern regarding this approach, we repeated these experiments in the standard way, as shown in the new Suppl. Fig. 5.

5. Supplemental Figure 4F-H: These data are very interesting. However, it is difficult to conclude that "... H2A.Z silencing induced reorganization of the H3K9me3 heterochromatin and the decreased presence of H2A.Z was mainly due to the disappearance of the variant from H3K9me3-rich peripheral heterochromatin." In Suppl. Fig. 4H that authors show the co-localization coefficient between H2A.Z and H3K9me3. As H2A.Z is depleted, it is expected that the co-localization drops as there is less H2A.Z present. In order to make such statement, the authors should use markers for the different chromatin states and their localizations, such as e.g. H3K27me3 and lamin A. In addition, how does H3K9me3 localize in DT40 DKO compared to WT DT40 cells? Is the same change in pattern quantitatively observed?

H2A.Z should be evenly depleted, if the half-life of the protein is the same in the different nuclear localizations, when its intranuclear landscape should not necessarily change. The fact that it did (Suppl. Fig. 5F-H), raises the possibility that the disappearance of H2A.Z from the different regions of chromatin is different, perhaps due to differential degradation. Perhaps the TSS-proximal H2A.Z levels are maintained on the expense of the heterochromatic localization. This interesting phenomenon has not been further investigated.

The newly added H3K9me3 texture analysis results of Suppl. Fig. 11 suggest that H2A.Z silencing has a more profound effect on the organization of peripheral heterochromatin than what could be explained by changes involving only the H2A.Z-nucleosomes. The H3K27me3 texture did not change at the same time.

Manders coefficient (MCC) is not sensitive to intensity differences within pixels. On the other hand, if the number of H2A.Z-pixels decrease, at unchanged level of H3K9me3 (Suppl. Fig. 5E), the fraction of H2A.Z colocalizing with H3K9me3 should not decrease, as it did.

DKO is non-viable; see above.

6. C-terminal truncation leads to genome architectural changes: a) The authors need to show with *in vitro* nucleosome-assembly assays and *in vivo* mononucleosome-purification experiments that the C-terminal truncated H2A.Z protein is indeed incorporated into nucleosomes. It is possible that this truncated variant is not recognized/bound by the required chaperones (p400 or SRCAP) and therefore not deposited into chromatin.

Based on the arguments mentioned above, we are convinced that the Δ C H2A.Z histones are incorporated into chromatin. Cryo-EM studies already cited in the original MS (refs. <https://pubmed.ncbi.nlm.nih.gov/34643712/> and <https://pubmed.ncbi.nlm.nih.gov/33073403/>) also demonstrate that both the full-length and the truncated H2A.Z are readily incorporated into nucleosomes.

Colocalization of the ΔC protein with DNA was also obvious in Suppl. Fig. 9A, B. Furthermore, all soluble proteins are washed out from the agarose-embedded nuclei, so extrachromosomal histones, if present, should not stay in the samples.

B) Also, the authors should show a scatter plot comparing gene expression profiles of DKO and DKO/ ΔZ with each other. Do they look very similar? Then the truncated variant might not be incorporated into chromatin.

Suppl. Fig. 14 shows scatter plots of correlations between the gene expression data of ΔC and Z1 (or wt) cells. DKO is non-viable; see above.

As detailed previously, we are convinced that ΔC H2A.Z is incorporated into chromatin.

c) The ChIP-seq data in Suppl. Fig. 11 are difficult to interpret. What does the antibody recognize in untransfected DKO cells (control)? Is there an enrichment of ΔZ at +1 and -1 nucleosomes? It does not look like it, arguing that the variant is not deposited into chromatin. As positive control, WT DT40 cells should be used to verify the ZAbA is working in ChIP assays.

DKO is non-viable; see above.

As detailed previously, we are convinced that ΔC H2A.Z is incorporated into chromatin.

Regarding the chip experiment suggested:

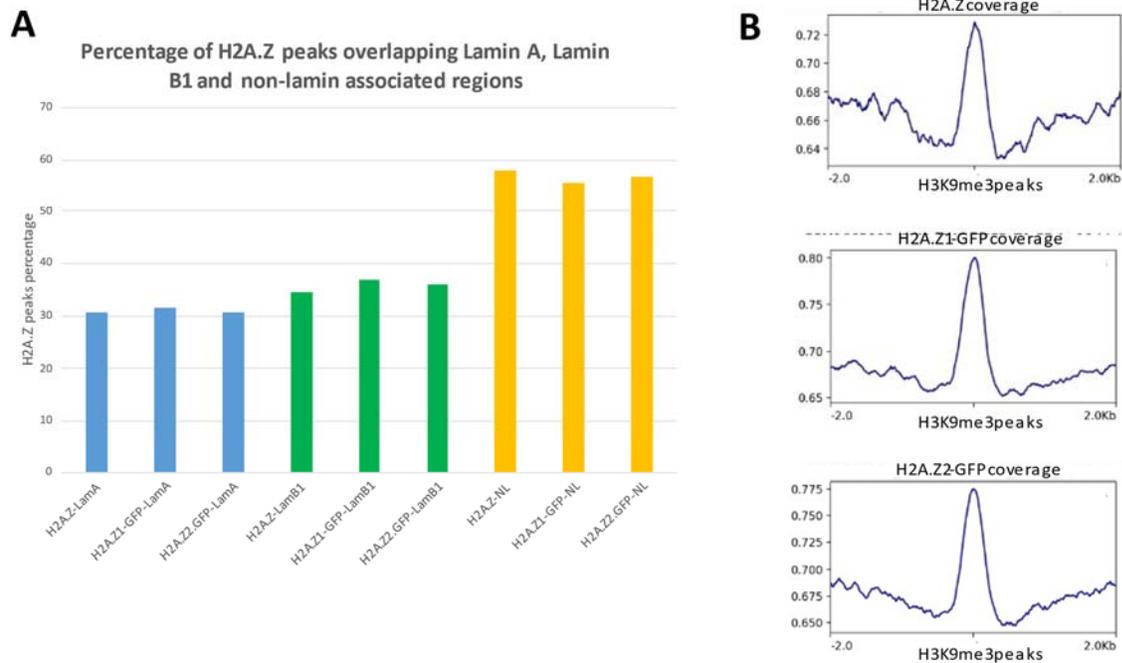
As we write in Discussion that “However, the experimental conditions of ChIP-seq and immunofluorescence studies are very different, as antibody labeling occurs after sonication in the first, while it is applied in the close-to-native state of the agarose-embedded, permeabilized nuclei in the second. Therefore, the results of the two procedures do not necessarily coincide.”

It is also to be emphasized that mapping by ChIP-seq reflects an ensemble feature and reflects both the enrichment of the trait at the locus and the number of nuclei carrying H2A.Z at a particular location, as opposed to immunofluorescence (IF) labeling which is sensitive to the degree of accessibility of the antibody to antigen, and *can* quantitatively reflect the amount of antigen per individual nuclei. Some of these considerations are also relevant for the ChIP-seq vs. IF comparison of the GFP-tagged histones.

The results of the ChIP-seq experiment of earlier Suppl. Fig. 11 are indeed of poor quality, suggesting that neither ZAbA nor ZAbB are chip-grade antibodies (as indicated in the catalogue but less convincingly stated in our correspondence with the company), in line with the fact that no ChIP-seq data have been published/deposited for ZAbA and ZAbB so far. These low-quality data are *not included* in the revised version. However, ChIP-seq data have been deposited for several antibodies used in our studies, allowing a definitive conclusion. These are detailed below to convince Reviewer, but *referred to only summarily in the manuscript*.

We have shown that, just like Abcam 97966 the prototypic ZAbA, Abcam 4174 (Suppl. Fig. 2A) and Abcam 18262 (directed against acetylated H2A.Z according to the catalogue; shown actually not to be specific for acetylated H2A.Z in Suppl. 2L but indeed H2A.Z-specific according to the sensitivity of labeling to silencing of H2A.Z Suppl. Figs. 5, 6) also detect stable H2A.Z, just like ZAbA. The ChIP-seq data compiled below and obtained using these two latter antibodies reveal that H2A.Z is present *in both euchromatic and heterochromatic environment* ([https://www.cell.com/molecular-cell/pdfExtended/S1097-2765\(15\)00345-7](https://www.cell.com/molecular-cell/pdfExtended/S1097-2765(15)00345-7), <https://pubmed.ncbi.nlm.nih.gov/31949157/>). We have also tested in our elution assay a *fourth* H2A.Z antibody (Merck 07-594; see new Suppl. Fig. 2C) also successfully used in ChIP-seq experiments, that revealed localization to both compartments (<https://www.nature.com/articles/s41467-020-14743-w>): this antibody also proved to recognize stable H2A.Z-nucleosomes. There are parallel Abcam 4174 ChIP-seq and anti-EGFP / EGFP-H2A.Z-ChIP-seq data deposited ([https://www.cell.com/molecular-cell/pdfExtended/S1097-2765\(15\)00345-7](https://www.cell.com/molecular-cell/pdfExtended/S1097-2765(15)00345-7)), also showing that the histone variant is present in both compartments. The CUT&RUN mapping data available for H2A.Z (<https://pubmed.ncbi.nlm.nih.gov/30655336/>) also detect the histone in both compartments.

We further analyzed the ChIP-seq data of ref. <https://pubmed.ncbi.nlm.nih.gov/30655336/> to see if H2A.Z resides also in heterochromatin regions (since usually the papers discussing the ChIP-seq results on H2A.Z focus on its presence in euchromatin):



These reveal that the histone variant is readily incorporated into chromatin regions representing different forms of heterochromatin.

Panel A: Bar chart showing the percentage of H2A.Z (detected by ab4174, a ZAbA-like antibody (see Suppl. Fig. 2A), GFP tagged H2A.Z1 and H2A.Z2 (detected by anti-GFP) peaks overlapping Lamin A, Lamin B1 and non-lamin associated regions in SK-MEL-147 cells.

Panel B.: Anchor plots showing the H2A.Z, H2A.Z1-GFP and H2A.Z2-GFP coverage around H3K9me3 peaks in SK-MEL-147 cells.

H2A.Z data (GSE68222) were downloaded from this publication: <https://pubmed.ncbi.nlm.nih.gov/26051178/>.

K27ac shows ChIP-seq coverage of H3K27ac in HeLa cells, downloaded from ENCODE database (ENCFF388WMD).

K27me3 shows ChIP-seq coverage of H3K27me3 in HeLa cells, downloaded from ENCODE database (ENCFF958BAN).

Hela_LMNA shows lamin A associated regions in HeLa cells, downloaded from GEO database (GSM1376181).

Hela_LMNB1 shows lamin B1 associated regions in HeLa cells, downloaded from GEO database (GSM1541019).

SKMel147-EGFP_Z1 and **SKMel147-EGFP_Z1_Peaks** tracks show the ChIP-seq coverage and high confidence peaks of ChIP-seq experiments performed on a EGFP tagged H2A.Z1 expressing SK-MEL-147 cell line using anti-GFP antibody.

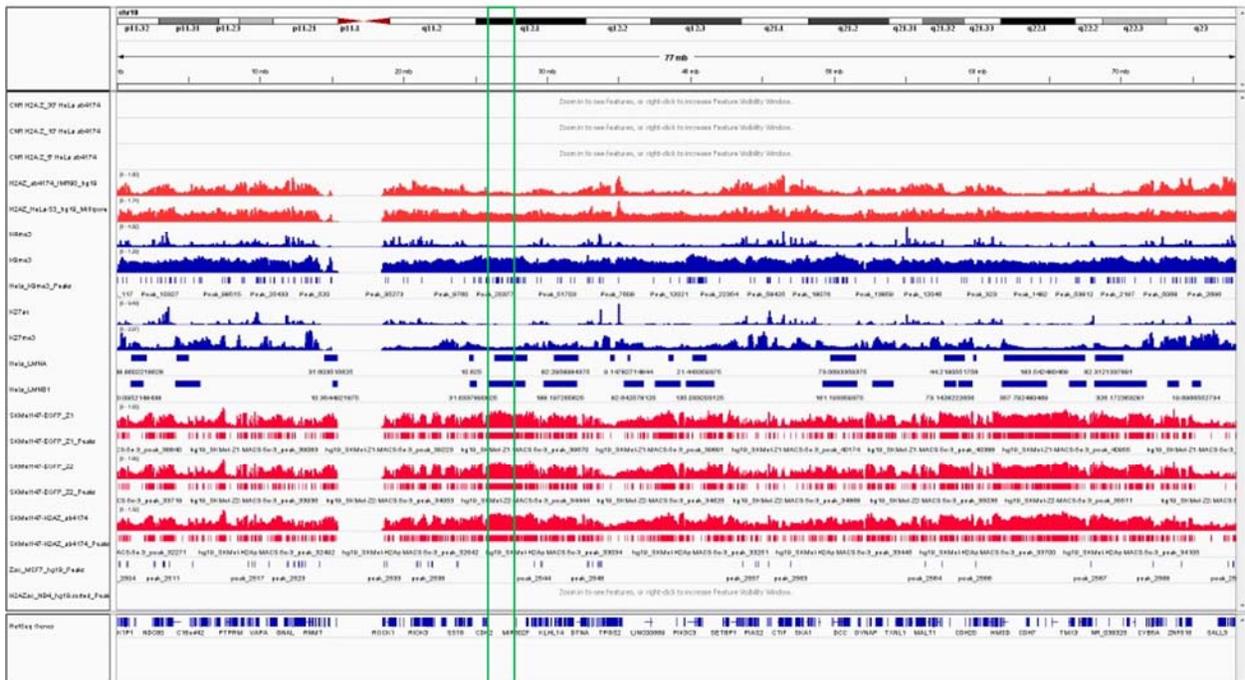
SKMel147-EGFP_Z2 and **SKMel147-EGFP_Z2_Peaks** tracks show the ChIP-seq coverage and high confidence peaks of ChIP-seq experiments performed on a EGFP tagged H2A.Z2 expressing SK-MEL-147 cell line using anti-GFP antibody.

SKMel147-H2AZ-ab4174 and **SKMel147-H2AZ-ab4174_Peaks** tracks show the ChIP-seq coverage and high confidence peaks of ChIP-seq experiments performed on native SK-MEL-147 cell line using anti-H2A.Z antibody (ab4174).

The last 6 tracks above were downloaded from the GEO database; publication: (<https://pubmed.ncbi.nlm.nih.gov/26051178/>).

Zac_MCF7_hg19_Peaks shows a high confidence peakset of a ChIP-seq experiment detecting acetylated H2A.Z using Abcam ab18262 antibody in MCF7 cells, data downloaded from GEO database (GSM3336876).

H2AZac_NB4_hg19.sorted_Peaks shows ChIP-seq coverage of acetylated H2A.Z using the Abcam ab18262 antibody in NB4 cells, downloaded from GEO database (GSM749523).



d) How do DKO cells survive treatment with DNA damaging agents (Suppl. Fig. 12)? Does ΔZ have any effect?

DKO cells are nonviable, as detailed above.

7. Peptide-experiments: the authors should use a scrambled C9 peptide as negative control in all experiments involving this peptide (not just in Figure 6).

We repeated all the experiments in the manuscript using C9 and the scrambled peptide in parallel (see in Figs. 3, 5, 6 & in Suppl. Figs. 6, 16, 18 and 21).

The FCS experiments were repeated so as to compare the effect of nucleosomes or DNA on the diffusion of C9-CF and on that of a CF-labeled scrambled nanomeric peptide. See *new* Suppl. Fig. 18. The latter, *control peptide did not bind* at a detectable level to the nucleosomes containing either H2A or H2A.Z, or to DNA. This finding is significant because it shows that the binding of C9, in contrast, is much above nonspecific binding and its binding sites include DNA. We thank Reviewer for requesting the scrambled control also for this experiment, what has led to this important conclusion.

8. The authors speculate that the C-terminal peptide might bind to possible molecular partners. This would indeed be one good explanation for the observed effects after peptide addition. Here it is crucial to perform peptide-binding assays (protocol by e.g. Wysocka, ScienceDirect, 2006) followed by mass spectrometry, to identify potential binding partners. These should be validated in IPs with H2A.Z WT and ΔC , as well as with depletion studies, looking at the changes in H2A.Z localization. In case the peptide binds nucleosomes, this should be shown by peptide-binding assays with mononucleosomes followed by mass spectrometry or immunoblotting.

New mass spectrometry experiments have been performed, see Suppl. Tables 1, 2, and 4.

As for detection of C9-interactors in general, pulling down of many DNA-binding proteins could occur without direct interaction with C9, in view of our FCS measurements that reveal DNA binding of C9 what may probably be very significant when the interacting tail and DNA are juxtaposed; therefore, the suggested experiment may not be informative. Furthermore, the detergent present in the protocol could interfere with binding of the peptide. Notwithstanding, we performed the suggested measurement.

When the proteins immunoprecipitated by biotin-conjugated C9 or biotin-conjugated scrambled nanomer were analyzed by mass spectrometry in the case of WT and Δ C DT40 cells, the following results were obtained:

Proteins detected in the lysates of WT DT40 cells by biotinylated C9 at >5x abundance compared to biotinylated SCR. MS analyses was performed as described in Materials and Methods. Nucleolar proteins are not listed.

*: undetectable in the SCR sample.

General transcription factor Iii,
HMG-14A,
HMG-17,
HMGN2,
HMGN4
HMGN5
*H2A.Z**

We conclude that the nuclear proteins bound by C9 include H2A.Z.

Nuclear proteins detected by MS in the C9-immunoprecipitates at 10x higher abundance in the lysate of Δ C cells compared to WT are listed below. Only those proteins are listed that were immunoprecipitated at least 5x more with C9 as compared to the scrambled peptide. Nucleolar proteins are also not listed.

Armadillo repeat gene deleted in velocardiofacial syndrome
PIN2 (TERF1) interacting telomerase inhibitor 1
Pleiotropic regulator 1
tRNA splicing endonuclease subunit 54
SREK1 interacting protein 1

We conclude that the spectrum of proteins pulled down by C9 is different in the case of cell lysates prepared from WT and Δ C DT40 cells.

Although the list of proteins binding C9 >5x as compared to SCR were different in the case of WT vs Δ C DT40 cells, with only the first list containing some interesting nuclear proteins (General transcription factor Iii, HMG-14A, HMG-17, HMGN2, HMGN4 and HMGN5, e.g.), we could not come to any meaningful conclusion about the spectrum of proteins bound by C9.

These observations need to be explored further and have not been incorporated into the manuscript.

Minor concerns:

1. According to the histone variant nomenclature (Talbert et al., Epigenetics & Chromatin, 2012) it would be preferable to call the H2A.Z variants H2A.Z.1, H2A.Z.2.1 or H2A.Z.2.2.

The antibodies detect both (see Suppl. Fig. 2A, B), and also the mass spec measurements did not distinguish the isotypes, so we cannot make such a distinction. Distinction has been made in the text only when isotype-specific literature data are referred to. We included a statement to that effect in the revised Results section (highlighted).

We thank Reviewer for correcting the erroneous usage of nomenclature. The text has been corrected.

2. General: the labelling of figures and graphs is extremely minimal. Without reading the legend, it is impossible to understand the results. To make it easier for readers, add info about the experiment (e.g. in y-axis and on top of graphs).

The ZAbA or ZAbB antibody specificities are shown now everywhere.

When this could be achieved without making the figures overcrowded, information to help the reader more readily grasp the messages of the figure were added.

3. Please specify which H2A.Z isoform (H2A.Z.1 or H2A.Z.2.1) was used in the experiments (e.g. Figure 1).

The isoform is specified now in the legend for Fig. 1G.

4. Please add a graphic of H2A.Z isoform sequences and indicate the antibody epitopes. This way the reader gets a better understanding about the different regions recognized by the two antibodies.

Added in Suppl. Fig. 1C. This was possible only for ZAbA in the lack of more detailed information made available by the companies for the other antibodies.

5. In the result section, the authors refer to Suppl. Fig. 2A-C for the characterization of different H2A.Z antibodies. In this Figure, these data sets cannot be found.

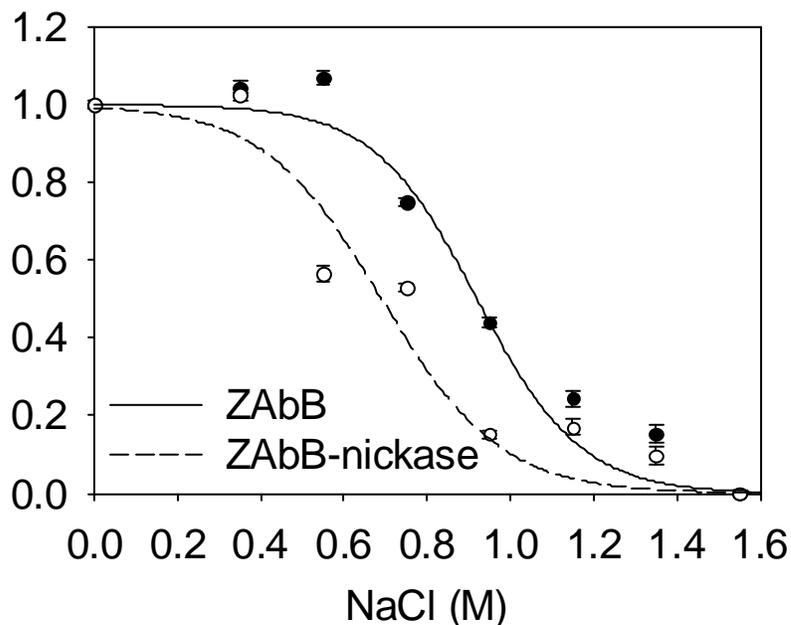
6. Supplemental Figure 2F: How does H3 (which is more stable than H2B that was used as control) behaves after nickase usage? Does it show a similar slope as H2A.Z? And how does the data look when the ZAbB antibody is used in this assay?

The antibodies used are specified now in all figures.

H3 (and H4) are sensitive to nicking in the intercalation elution version of our assay, while the dimers are sensitive to nicking in the salt elution format. (See our

paper: <https://pubmed.ncbi.nlm.nih.gov/28986581/>.)

We performed a nicking experiment using ZAbB in the salt elution format as suggested by Reviewer; the results are shown below:



This has not been incorporated into the manuscript, to avoid its overburdening with dispensable detail.

7. Supplemental Figure 2L: Labelling of the figure is different to the legend. Were DKO cells used, expressing either WZ H2A.Z or the 5KR H2A.Z mutant or were WT DT40 cells used instead as control?

Corrected.

8. Supplemental Figure 3C: Did the authors use GFP-PWWP2A or PWWP2A-GFP? The tag at the C-terminus, next to the PWWP domain, might interfere with its function.

PWWP2A was tagged at the N-terminus (GFP-PWWP2A).

9. Supplemental Figure 3D, E): I am missing a figure showing that the N- or C-terminal localization of the Tag (GFP) does not influence the stability, as stated in the text.

Fluorescent protein tags were on the C-terminus in the case of measurements shown on most of the figures, except for Suppl. Fig. 4B, where GFP was N-terminal. (Underlined now in the legend of the latter figure.)

10. Supplemental Figure 3F: What is PI? Propidium Iodide?

DNA is stained using the Propidium Iodide (PI) dye, as indicated in the revised version. Instead of “PI”, the panels are labeled now as “DNA”.

11. Supplemental Figure 3H: Why is the DNA signal so strong in the nucleoli? Was Propidium iodide staining without RNase digest used and are DNA as well as RNA seen?

No RNase treatment was used, the nucleoli stain intensively because of their ribosomal dsRNA content.

12. Supplemental Table 2: A heatmap (with replicates) would be better.

We think that the current form of presentation may be sufficient, especially in view of our new RNA-seq data shown in Fig. 6.

13. Supplemental Figure 4F: Labelling of panels is missing.

Fields of view labeling now added.

14. Supplemental Figure 4G: Why is there still H2A.Z signal present in the shRNA transfected cells? When the signal was technically amplified, it should be shown with and without enhancement.

It is not possible to reach complete silencing since it would kill the cells within one cell cycle; see <https://pubmed.ncbi.nlm.nih.gov/36750094/>.

Results obtained following the strategy described for H2A.Z in <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2099601/>, using the same cells and constructs and evaluated by LSC gating on the transfected cells, clearly demonstrate the significantly reduced labeling by the anti-H2A.Z antibodies. See new Suppl. Fig. 6G, H.

The authors thank Reviewer again for the encouraging remarks as well as the critical comments that helped improve our manuscript.

Reviewer #2 (Remarks to the Author):

The authors investigate the role of H2A.Z isotypes, highlighting their high stability and exploring its tethering to other nuclear structures such as other histones, nucleosomes and lamin. They also investigate the effect of a peptide representing the last 9 C-terminal aminoacids (C9) by knock-out, live-cell insertion of the peptide, characterizing its role in modulating chromatin accessibility.

It is a very well-written and enjoyable paper featuring many relevant techniques and outlining a comprehensive, well-thought story of H2A.Z structural and functional role. I honestly can't fault it, the authors managed to address every remark I might have had in the next few sentences.

I strongly recommend it for publication

The authors thank Reviewer for a most encouraging evaluation.

Reviewer #3 (Remarks to the Author):

Imre L et al argue that the localization of H2A.Z-nucleosomes to euchromatin or heterochromatin depends on the 9 amino acids of the C-terminal tail. They have established an assay system that distinguishes the majority of euchromatin from heterochromatin using antibodies with different reactivity to QINESIn, which they previously reported. They also show that the 9 amino acids of the C-terminal tail of H2A.Z are important for its localization to heterochromatin. Furthermore, they show that the addition of the 9-amino acid peptide biases the localization of H2A.Z to euchromatin. This finding of differential localization of H2A.Z-nucleosomes to euchromatin and heterochromatin as a phenomenon provides new insights into H2A.Z for chromatin and related fields. On the other hand, its mechanism has yet to be described.

We thank Reviewer for evaluating our work as providing new insights and address the critical remarks regarding the mechanistic aspects below.

Major points:

The impact of localization on heterochromatin in ΔC is presumed to be of minor magnitude. The authors should demonstrate with data to clearly state what the biological impact is. For example, in the salt elution profiles in Sup Fig6C, D, global H3K27me3 and H3K9me3 are unchanged between DKO H2A.Z1 (CTRL) and H2A.Z1 ΔC (ΔC). On the other hand, in Fig. 2D, the localization of H3K9me3 is reduced in H2A.Z ΔC . This indicates that the effect of H2A.Z ΔC in maintaining heterochromatin formation is minor. Furthermore, the fact that there are few genes with variable expression in RNA-seq analysis also provides evidence that H2A.Z ΔC has little effect on maintaining heterochromatin formation.

We discuss in the first subsection of Discussion the various observations suggesting that the tail-dependent changes involve both euchromatin and heterochromatin. Regarding euchromatin, the most direct and biologically certainly relevant evidence is provided by the gene expression changes elicited by the C9 peptide introduced into live melanoma cells (new Fig. 6.). As for heterochromatin, the H3K9me3 landscape was affected in a tail-dependent manner based on colocalization data (see e.g. Fig. 5B, C), texture analyses (Suppl. Fig. 11A-D), and the sensitization also of repetitive DNA to MNase by C9 treatment of nuclei (Suppl. Fig. 17C).

Fig. 2A and Suppl. Fig. 8A vs. Suppl. Fig. 8B, C demonstrate that the difference between nucleosomal stability features measured in DKO H2A.Z1 (CTRL) vs H2A.Z1 ΔC (ΔC) DT40 nuclei are specific for nucleosomes containing H2A.Z and the stability of nucleosomes carrying H3K27me3 and H3K9me3 is indistinguishable in the two cell types. These data are related to *stability only, not localization*; the change of the latter parameter is demonstrated in Fig. 2C, D.

The differences in gene expression appear to involve more genes than what are listed in Suppl. Table 3 (identical with the earlier Suppl. Table 2) showing only the genes exhibiting the largest differences. As added to its *revised legend*, comparing DKO/ Δ C to DKO/Z1, 74 genes had log₂ signal ratio >1.5 (upregulated) and 29 had < -1.5 (downregulated). Comparing DKO/ Δ C to WT, 293 genes had log₂ signal value >1.5 and 209 had < -1.5, out of 24,530 genes.

In view of the possible *secondary changes* of gene expression evolving in the different DT40 cells as compared to wt, we have compared the gene expression profiles of melanoma cells before and shortly after acute treatment with C9, as shown in *new Fig. 6*. These data demonstrate that ~620 genes were affected, mostly downregulated. This is in line with changes in euchromatin.

Since H3.3, which is present in both euchromatin and heterochromatin, is involved in the repression of transposon expression, transposon expression may also be elevated in H2A.Z1 Δ C. The C-terminal tail of H2A.Z may function during perturbation, such as in response to stress.

This is an intriguing question, also in view of ref.

<https://pubmed.ncbi.nlm.nih.gov/31366887/>.

In the wake of Reviewer's question we have found a single transposon related sequence (gypsy retrotransposon integrase 1) among the microarray data (DT40 cells), what exhibited a < 2x difference between the different samples. No transposon related sequence showed up in the RNA-seq experiment.

The authors need a clear explanation for their choice of Manders Correlation in co-localization analysis. In a co-localization analysis where the correspondence is the same (when comparing the localization of the same molecule in wt and mut), as in this case, a scatter plot of the two-color fluorescence signal should be created to visualize the distribution of the data. If there are no outliers or anomalies in the signals, the Pearson coefficient should be the first candidate for analysis. Manders Correlation can discuss whether the signals are spatially located in the same place, but not the correlation of co-localization. Therefore, we may not be able to address whether the change in H2A.Z localization affected the decrease in H3K9me3.

We used MCC to assess what fraction of pixels occupied by one entity also contain the other entity. How bright the pixels are, is not considered in this calculation. We have corrected "molecules" to "pixels" in the revised version.

See <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3074624/>:

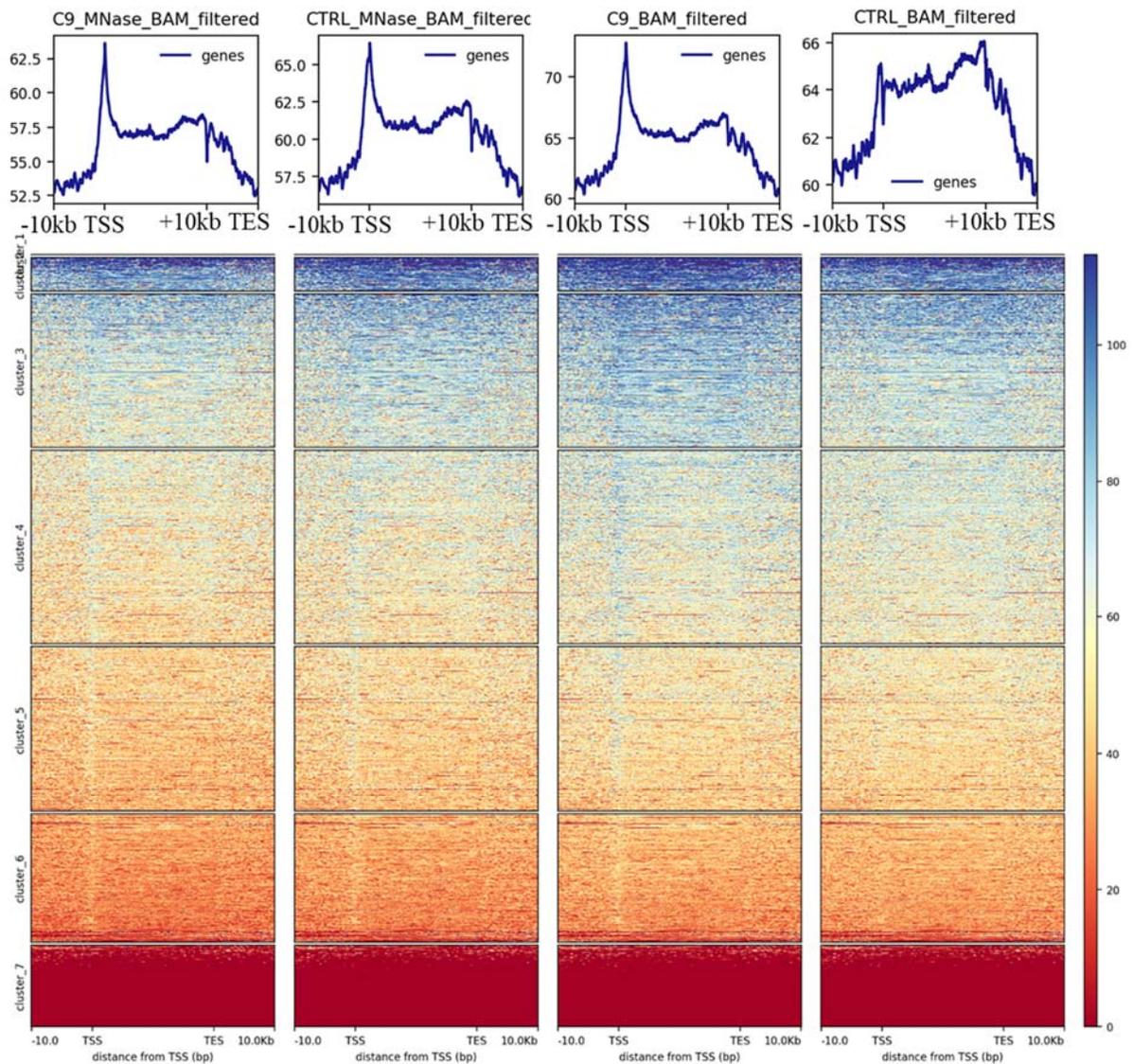
“Whereas PCC provides an effective statistic for measuring overall association of two probes in an image, it has the major shortcoming that it indirectly (and sometimes poorly) measures the quantity that is typically at the heart of most analyses of colocalization in cell biology: the fraction of one protein that colocalizes with a second protein. This quantity can be measured via Manders' Colocalization Coefficients (MCC)”

The -MNase-seq analysis makes no sense at all. The authors discuss the distribution of CTRL - CTRL(MN), and C9 - C9(MN) processed signals, but subtracting individually standardized data may not reflect the original data. In such an analysis, individual heatmap and aggregation plots for CTRL, CTRL(MN), C9, and C9(MN) can be created and compared to analyze differences in localization. Differences in the number of reads and fragment length after MNase treatment may also be altered as differences in susceptibility to MNase. These are also subject to discussion and should be indicated.

The increased overall MNase sensitivity ensuing upon treatment of HeLa nuclei with C9 (Fig. 3F) called for studying it at the genome level among the same conditions of nuclease treatment.

When the distribution of read coverages were compared, the MNase-digested and undigested samples containing identical amounts of DNA were sequenced, yielding approximately identical overall coverage; therefore, *only the shape of the curves*, not their relative position along the Y axis, should be compared. As the metaplot analyses of Fig. 3G shows, the TSS-TESS intervals comprising annotated genes are overrepresented relative to their chromosomal environment likely due to the relative resistance of heterochromatin to sonication <https://pubmed.ncbi.nlm.nih.gov/29272703/>. Upon C9 treatment, coverage is increased at the TSS relative to the gene bodies, indicating decondensation of chromatin; there was no further change of the shape of the metaplot following MNase treatment as judged by the naked eye. When the effect of C9 on the read *coverage-differences* between MNase digested (MN) and undigested (CTRL) nuclei, i.e. on MNase accessibility, were calculated, conspicuous changes were detected at large regions and particular sites; see Suppl. Fig. 17C-K.

Following Reviewer's critique, we have made the heatmap constructed after K-means clustering and the aggregation plots suggested; see below. Only the plot is shown in Fig. 3G, since the heatmap would not add to that information.



We have calculated the effect of the peptide in several different ways (C9/C9_MNase vs. CTRL/CTRL_MNase, C9-C9_MNase vs. CTRL-CTRL_MNase, CTRL_MNase/C9_MNase, etc.; all of these with and without normalization and using log2 values or the data themselves. All exhibited spectacular changes at the TSS and CTCF sites (not shown here).

Let us cite Fig. 1 of <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8100287/> as a typical example when differences can hardly be noticed when the individual heatmaps or aggregation plots are compared, but the calculated changes are revealing (panels a, b vs c,d).

Minor points:

The text is long and difficult to read from Fig. 1 to Fig. 2. The author should think about the structure of the text, for example, making the Supp data the main Fig.

Having considered the possible options of structural changes, we still find that it is more logically built up in its current structure. We also feel that such changes would be unfavorable in view of the other reviews that did not criticize the structure of the MS. In order to make the MS shorter and tighter, the data on the binding of C9-CF to the lamina have been omitted and some parts moved to the supplement. Since several interdependent phenomena have been observed, their demonstration and discussion takes inevitably longer volume than usual.

In Suppl. Fig. 2E, data with different salt concentrations should be shown as representative data.

“Representative images are shown” has been added to the legend (these data of the earlier Suppl. Fig. 2E have become panel F of Fig. 1).

Suppl. Fig. 3I Why is the salt tolerance of H2A.Z stronger than that of K9me3? It would be more convincing if there is a counterstain such as H3K4me3.

In the context of an ongoing other project in our lab, we have compared the stability of nucleosomes marked by different PTMs and could group them in two main categories, with minor differences similar to what was seen between H2A.Z and H3K9me3, within each group. In view of those observations the difference between the two elution curves mentioned was considered of no obvious biological significance.

Suppl. Fig. 4A-C H2A.Z is the correct band around 14kDa, but why does it react at 40kDa? Also, two main bands are visible at different locations in the silver staining. The author should explain these logically.

New western blots have been made using proteins of higher purity than the commercial ones (that were also tagged) (New Suppl. Figs. 5A-C).

In Suppl. Fig. 4D-G, the author claims that "The background signals detected in the still viable cells exhibiting near-complete silencing were minimal for all In Suppl. What is the background in this case?"

We meant autofluorescence. However, we realize that this statement is confusing and have omitted it from the revised manuscript.

The KO cells are non-viable (see ref <https://pubmed.ncbi.nlm.nih.gov/26833946/>). Therefore, near-complete silencing was not an option, also in line with the recently revealed fact that H2A.Z must mark nascent chromatin prior to any transcription re-start following DNA replication (<https://pubmed.ncbi.nlm.nih.gov/36750094/>).

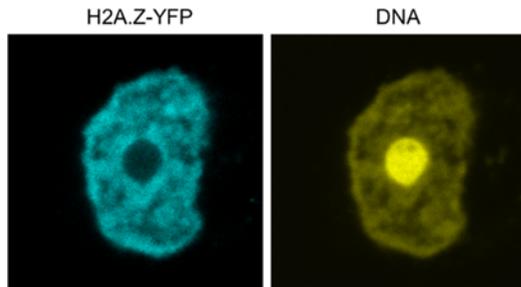
We have performed a *new silencing experiment* using the same cells, constructs and strategy described in <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2099601/>, and gating on the GFP-positive cells. See *new Suppl. Fig. 6G, H*.

The specificity of the ZAbA antibody, used to detect all the tail-related changes and differences, was also supported by the fact that H2A.Z was detected by mass spectrometry in the high salt eluted fraction following treatment with 1 M salt and following the CUT&RUN protocol (see *revised Suppl. Table 2* and *new Suppl. Table 1, 4*). In the revised manuscript we include *new elution data obtained with a 4th H2A.Z-specific antibody* that also recognizes nucleosomes that contain the histone variant in a form that is as salt stable as H3 or H4, not like the canonical H2A or H2B.

In Suppl. Fig. 3F, To clearly show the difference in localization of the C- and N-terminal tags, it is recommended to include the actual immunostaining data of the C-terminal tag for comparison, instead of subtracting it from the reference.

A typical image of a C-terminally tagged H2A.Z1 is shown below. The tagged histone doesn't show the peripheral rim-like labeling seen when ZAbA was used. We have not compared the localization of the N- vs. C-terminally tagged histones. A recent AFM study (<https://pubs.acs.org/doi/full/10.1021/acs.nanolett.2c04346>) has demonstrated that sliding of H2A.Z-containing nucleosomes along the DNA is very sensitive to the N-terminal part of H2A.Z, so we think that the N-terminal tag has an influence on the stability feature measured also in our assay. On the other

hand, the C-terminal tag is expected to directly interfere with interactions involving the tail.



The authors thank Reviewer for the critical comments that helped improve our manuscript.

Reviewer #4 (Remarks to the Author):

The manuscript by Imne et al., describes the nuclear distribution and stability of H2A.Z containing nucleosomes using different antibodies and propose important roles for the C-terminal last 9 amino acids of H2A.Z protein. The manuscript is sometimes difficult to follow, contains inaccuracies and conclusions are often not supported by the data. In addition, the manuscript does not contain solid new information for the field.

The text was made more readily readable, the inaccuracies pointed out by Reviewer have been corrected, the conclusions readdressed in our detailed response below. We cannot agree with Reviewer's general evaluation referring to "the lack of solid new information for the field" as none of the H2A.Z tail-dependent effects have been observed before, they are all of considerable magnitude, they are of biological impact as confirmed by the newly added RNA-seq data and may lead to effective novel means of epigenetic modulation.

Main concerns

1.- The authors describe different stability of H2A.Z in the nucleosomes and different immunofluorescence data using two different antibodies that they call ZAbA and ZAbB. They try to argue that both antibodies recognize different populations of H2A.Z. Throughout the manuscript it is not clear what the molecular basis of these differences are. However, an obvious experiment such as a comparison between the ChIP-seq signals of both antibodies is not performed. Proteomic analysis of proteins co-immunoprecipitated with one or the other antibody are not performed either. I think that these two type of standard experiments will contribute to clarify the possible differences between the antibodies.

Regarding a ChIP-seq validation of antibody performance, we write in Discussion: "ChIP sequencing (ChIP-seq) data available for different cell types reveal that two of the antibodies detecting unusually stable nucleosomes (Abcam 4174 and Millipore 07-594; see Suppl. Fig. 2A and C, respectively, and refs. (52,53)) give peaks both in euchromatin and heterochromatin. The ChIP-seq data obtained using H2A.Z-GFP and anti-GFP antibodies were very similar to parallel ChIP-seq data obtained with Abcam 4174 (51). However, the experimental conditions of ChIP-seq and immunofluorescence studies are very different, as antibody labeling occurs after sonication in the first, while it is applied in the close-to-native state of the agarose-embedded, permeabilized nuclei in the second. Indeed, the distribution of H2A.Z reflected by CUT&RUN mapping, where antibody labeling is performed among conditions comparable to those of our elution assay, exhibits only partial overlap with ChIP-seq datasets (54). In view of the different preparation steps and also the ensemble character of the above genomic approaches, those data are not expected to reflect the picture obtained by immunolabeling using ZAbA or ZAbB."

The last statement above is illustrated by the differences between CUT&RUN and chipseq peaks in ref.

<https://www.biorxiv.org/content/10.1101/2022.03.30.486382v2>.

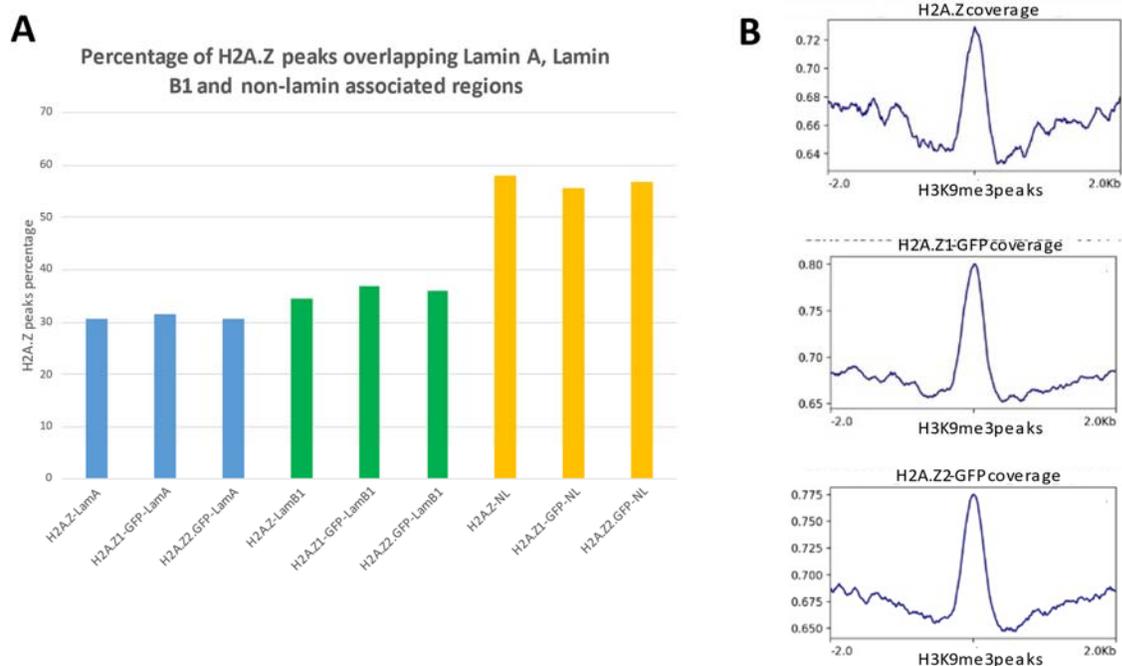
Mapping by ChIP-seq delivers ensemble information and reflects both the enrichment of the trait at the locus and the number of nuclei carrying H2A.Z at a particular location, as opposed to immunofluorescence (IF) labeling which is sensitive to the degree of accessibility of the antibody to antigen, and *can* quantitatively reflect the amount of antigen per individual nuclei. Some of these considerations are also relevant for the ChIP-seq vs. IF comparison of the GFP-tagged histones.

The results of the ChIP-seq experiment of earlier Suppl. Fig. 11 are indeed of poor quality, suggesting that neither ZAbA nor ZAbB are chip-grade antibodies, in line with the fact that no ChIP-seq data have been published/deposited for ZAbA and ZAbB so far. These low-quality data are not included in the revised version. However, ChIP-seq data have been deposited for several antibodies used in our studies, allowing a definitive conclusion. These are detailed below to convince Reviewer, but *referred to only summarily in the manuscript*, as cited above.

We have shown that, just like Abcam 97966 the prototypic ZAbA, Abcam 4174 (Suppl. Fig. 2A) and Abcam 18262 (directed against acetylated H2A.Z according to the catalogue; shown actually not to be specific for acetylated H2A.Z in Suppl. 2L but indeed H2A.Z-specific according to the sensitivity of labeling to silencing of H2A.Z Suppl. Figs. 5, 6) also detect stable H2A.Z, just like ZAbA. The ChIP-seq data compiled below and obtained using these two latter antibodies reveal that H2A.Z is present *in both euchromatic and heterochromatic environment* ([https://www.cell.com/molecular-cell/pdfExtended/S1097-2765\(15\)00345-7](https://www.cell.com/molecular-cell/pdfExtended/S1097-2765(15)00345-7), <https://pubmed.ncbi.nlm.nih.gov/31949157/>). We have also tested in our elution assay a *fourth* H2A.Z antibody (Merck 07-594; see new Suppl. Fig. 2C) also successfully used in ChIP-seq experiments, that revealed localization to both compartments (<https://www.nature.com/articles/s41467-020-14743-w>): this antibody also proved to recognize stable H2A.Z-nucleosomes. There are parallel Abcam 4174 ChIP-seq and anti-EGFP / EGFP-H2A.Z-ChIP-seq data deposited ([https://www.cell.com/molecular-cell/pdfExtended/S1097-2765\(15\)00345-7](https://www.cell.com/molecular-cell/pdfExtended/S1097-2765(15)00345-7)), also showing that the histone variant is present in both compartments. The CUT&RUN mapping data available for H2A.Z (<https://pubmed.ncbi.nlm.nih.gov/30655336/>) also detect the histone in both compartments.

We further analyzed the ChIP-seq data of ref. <https://pubmed.ncbi.nlm.nih.gov/30655336/> to see if H2A.Z resides also in

heterochromatin regions (since usually the papers discussing the ChIP-seq results on H2A.Z focus on its presence in euchromatin):



These reveal that the histone variant is readily incorporated into chromatin regions representing different forms of heterochromatin.

Panel A: Bar chart showing the percentage of H2A.Z (detected by ab4174, a ZAbA-like antibody (see Suppl. Fig. 2A), GFP tagged H2A.Z1 and H2A.Z2 (detected by anti-GFP) peaks overlapping Lamin A, Lamin B1 and non-lamin associated regions in SK-MEL-147 cells.

Panel B.: Anchor plots showing the H2A.Z, H2A.Z1-GFP and H2A.Z2-GFP coverage around H3K9me3 peaks in SK-MEL-147 cells.

H2A.Z data (GSE68222) were downloaded from this publication: <https://pubmed.ncbi.nlm.nih.gov/26051178/>.

panels are often labelled with “H2A.Z”. For example, what antibody was used in figure 4C? I suggest writing the antibody used directly on the graph (i.e. H2A.Z (ZAbA) or H2A.Z (ZAbB), not just on the figure legend.

The identity of the antibodies used to label H2A.Z has been indicated in all the figures of the revised manuscript.

4.- Figure 1F. The quality of the western shown in this figure is very poor. In addition, if the authors want to make conclusions of the amount of H2A.Z released in 1M versus 1.2 M in comparison with the amount of H2A/H2B, they should perform the experiment at least three times and quantify the different bands. The intensity of the bands in the left part of the gel is much lower than in the right part, so, the conclusion driven by the authors is unclear.

Concerning Fig. 1F please consider the arguments below:

1. The H2A/H2B dimers run as a single band, hence appears to be of higher concentration.
2. Would the salt containing eluent be present in limiting quantities, it might be expected that a higher salt concentration would be required to dissociate the species present at higher concentration. In fact, the opposite is seen. If the salt containing eluent is in abundance, experience based on Fig. S11B of our paper <https://pubmed.ncbi.nlm.nih.gov/28986581/> shows that salt sensitivity (quantitatively characterized by the elution curves) is perfectly invariant to the amount of nucleosomes present and exposed to salt. Thus, the difference seen cannot be the result of differences in the amount of nucleosomes on the columns.
3. The higher salt concentrations to displace recombinant H2A.Z/H2B dimers actually confirms what is observed for in vivo assembled chromatin using exactly the same hydroxyapatite approach (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1137757/>).

In the figure, an arrow points at the sample eluted by 1 M salt: H2A is present, H2A.Z is absent.

5.- Supplementary figures 4A, 4B. In this western the molecular mass of H2A.Z is about 40 kDa, which is not at all a normal molecular mass for H2A.Z. In addition, the silver staining of Supplementary figure 4C give a lot of bands for H2A.z.

The tag on the Recombinant Human Histone H2A.Z protein ([ab114184](https://pubmed.ncbi.nlm.nih.gov/27854017/)) is an N-terminal GST-tag. We have made new western blots using the untagged proteins of <https://pubmed.ncbi.nlm.nih.gov/27854017/>. These preparations contain no extra

bands that are impurities in the commercial samples; see *new western blots* of Suppl. Figs. 5, 6.

6.- The authors make a proteomic study of the non-soluble fraction upon NaCl extraction (Suppl. Table 1). They argue that in this fraction they detect H2A.Z, that is not detected when proteins are extracted by MNase treatment. However, the same can be observed for H3 and H4. I do not see that this is a valid argument to propose that H2A.z is in a hard to extract fraction.

We have repeated the mass spectrometric measurement of the CUT&RUN experiment using also a secondary antibody to facilitate MNase cleavages. Now the peptides detected clearly identify H2A.Z in the MNase-digest. (The peptides detected in our earlier mass spectrometric experiments could come from canonical H2A or H2A.Z alike.) We also observed its enrichment upon C9 pretreatment. Please see *new Suppl. Table 4*.

7.- How differential gene expression profile was done? Neither RNA-seq not microarray experiments are described in Materials and Methods and data are not deposited in databases. How many replicates were performed? What threshold were used? Why p-values are not shown in Table 2? On top of that, in line 264 authors write: "...were also reflected in dramatic differences of gene expression patterns, comparing wild265 type (wt), DKO H2A.Z1 and DKO H2A.Z Δ C cells". However, I only see, in Supple. Figure 10, a few genes that change expression, especially when comparing H2A.Z Δ C with H2A.Z1 expression. This is not a "dramatic" change.

Database deposition of the microarray (and the new RNA-seq) data is specified in the revised manuscript. We thank Reviewer for noticing this lapse. The missing details have also been included in the Materials and Methods section of the revised manuscript.

The differences between the wt/H2A.Z1 knock-in versus Δ C DT40 lines involve more genes than what are listed in Table 2, showing only the genes exhibiting the largest differences. As added to the *revised legend* of Suppl. Table 3, Comparing DKO/ Δ C to DKO/Z1, 74 genes had log₂ signal ratio >1.5 (upregulated) and 29 had <-1.5 (downregulated). Comparing DKO/ Δ C to WT, 293 genes had log₂ signal value >1.5 and 209 had <-1.5, out of 24,530 genes.

However, we must agree that the phrase 'dramatic' is exaggerated and the text has been re-worded.

In view of the possible *secondary changes* of gene expression evolving in the different DT40 cells as compared to wt, we have compared the gene expression profiles of HeLa cells before and shortly after acute treatment with C9, as shown in

new Fig. 6. These data demonstrate that ~620 genes were affected, mostly down-regulated.

(This is more, than what was detected in DT40, still would not call it dramatic, indeed.)

8.- H2A.Z1 ChIP-seq signal of supplementary figure 11 is very different from many other H2A.z ChIP-seq in the bibliography. This have to be commented and clarified by the authors.

The original ChIP-seq curves showed TSS-proximal accumulation of H2A.Z signals, as expected. These low-quality data obtained with these apparently not chip-grade antibodies were omitted from the revised version.

Please see our response above to main concern #1.

9.- The authors suggest that H2A.Z C-terminal tail may be involved in establishing intra- or internucleosomal molecular associations, but they do not provide any experiment to prove it. The authors should reconstitute nucleosomes in vitro with recombinant H2A.Z or H2A.Z Δ C and determine compaction of chromatin fibers either by nuclease sensitivity, density gradient, or some other biophysical method.

The cryo-EM studies alluded to in the original manuscript (<https://pubmed.ncbi.nlm.nih.gov/33073403/>, <https://pubmed.ncbi.nlm.nih.gov/34643712/>) demonstrate that (1) both the full-length and the truncated H2A.Z are readily incorporated into nucleosomes, (2) the H2A.Z-containing mononucleosomes have a more open structure while (3) the nucleosome arrays are more compact. Our data and interpretation can be interpreted to fit these recent cryo-EM structures, as described in Discussion.

The FCS data obtained with reconstituted nucleosomes of Fig. 4 clearly show the ability of the tail to bind unmodified, canonical nucleosomes as well as naked DNA. In the revised manuscript we include *new FCS results on H2A.Z-nucleosomes*, comparing the binding of C9-CF with that of the labelled scrambled nanomer; see *new Suppl. Fig. 18*. The latter, *control peptide did not bind* at a detectable level to the nucleosomes containing either H2A, H2A.Z, or to DNA. This finding is important because it shows that the binding of C9 is much above nonspecific binding and its binding sites include DNA. Therefore, we feel safe to conclude that interfiber or intranucleosomal interactions via the H2A.Z C-terminal tail *could involve tail-DNA binding*. We do not state that the FCS data prove that such links actually exist.

The role of a possible tail-nucleosome-core or tail-DNA interactions will need to be further explored quantitatively taking into consideration proximity relationships favoring the tail-DNA and tail-histone interactions, using a wider salt concentration series and comparing different length Widom sequences. This would certainly exceed the limits of this manuscript which is already packed with data.

10.- Experiments for figure 3 were performed with permeabilized nuclei (dead cells) incubated during hours with the C9 peptide. What is the physiological relevance of the chromatin changes observed (for example in H3K9me3)? In fact, these changes were not observed when C9 was introduced into live cells.

All major effects observed on permeabilized nuclei (nucleosome destabilization, changes of chromatin landscape and nuclease sensitivity) were reproduced when C9 was introduced into live HeLa cells. In melanoma cells; see *new Fig. 6*. In melanoma cells, changes of chromatin landscape and gene expression were demonstrated. These changes appeared to be rather specific as dominantly downregulation occurred, the downregulated genes could be associated with non-random pathways and the genes downregulated reside in specific Giemsa-bands.

The extent and spectrum of molecular interactions between C9 and the chromatin may indeed be different when added to nuclei and to live cells. Therefore, the analogous changes in nuclear organization and chromatin structure in live cells is quite remarkable, especially in view of the fact that the changes observed in live cells ensue after just one day following a 2 hrs treatment with peptide-cyclodextrin.

How exactly the different effects of C9 treatment are interrelated requires further studies. The complexity of the phenomena may be demonstrated by the fact that myc downregulation (investigated in the wake of refs.

<https://pubmed.ncbi.nlm.nih.gov/34423893/> and

<https://pubmed.ncbi.nlm.nih.gov/26051178/> of the original manuscript), proved not to be effected at the transcriptional level according to the RNA-seq data. (Therefore, this finding was omitted from the revised version to further streamline the manuscript.)

The physiological relevance has been demonstrated in the RNA-seq experiment shown in *new Fig. 6*.

11.- The authors suggest that C9 peptide bind specifically the nuclear lamina. Have

the authors tested by co-immunoprecipitation whether H2A.Z interacts directly with lamins?

We show that what remains associated with nuclei after 2 M salt from the ZAbA-reactive H2A.Z species, colocalizes with the two concentric layers of the lamina. These preparations were also subjected to mass spectrometric analyses in the wake of Reviewer's remark; the results shown in *new Table 1* are considered as antibody-independent evidence for the presence of some of the proteins detected by immunofluorescence (including H2A.Z), tightly bound to the lamina.

Intriguingly, the mRNA expression levels of these proteins appear to tightly correlate with that of H2A.Z across the Cellminer NCI60 panel (proteins marked with * in the list; <https://discover.nci.nih.gov/rsconnect/cellminercdb/>), suggesting that the co-occurrence of these proteins in the nuclear lamina deserves further attention in the context of an independent future project.

The binding of C9 to the lamina was not confirmed (nor disproved) by our new mass spectrometric measurements), so, and also to make the manuscript tighter, we omitted these data from the revised version.

The authors thank Reviewer for the critical comments that helped improve our manuscript.

Response to Reviewers' comments (*responses in italics*)

Reviewer #1 (Remarks to the Author):

General response to comments:

I want to thank the authors for their detailed responses to my suggestions and comments. They have added a wealth of new data that strengthen their story and conclusions.

Minor remarks:

1) The authors are correctly arguing that DT40 DKO cells are not viable. However, Kusakabe et al., 2016, who generated these cells, showed that - after tet-induction – DKO cells proliferated for at least 48 hours normally, then cell division dropped and after 96 hours cells started dying (Fig. 2B, D). The authors were even able to obtain enough cells to generate extracts for immunoblotting (Fig. 2A), showing loss of H2A.Z protein. Therefore, some experiments I previously asked for should have been possible to perform (e.g. testing antibody specificity to investigate if the used H2A.Z antibodies might recognize any other proteins than H2A.Z). Nevertheless, the authors have addressed most of my concerns and answered in great detail all my questions and have rewritten many part of the manuscript and relabeled figures, thereby improving readability.

We must heed Reviewer's arguments regarding the potential usefulness of the tet-induced DKO cells. Our earlier response was based on a misunderstanding of Reviewer's suggestion. Indeed, choosing an early timepoint after tet-induction of DT40 DKO cells could have worked and would have been an appropriate control, perhaps obviating the need for the silencing experiments. Based on the many genes up- and downregulated at 72 hrs after addition of tet, we felt that too many changes occur at complete shutdown of H2A.Z, so we felt it safer to do the traditional silencing approach using the very same siRNA constructs proved to be useful for a similar purpose before.

2) Please take out exaggerating words such as e.g. “spectacular”.

The exaggerating term is replaced with 'remarkable' in the new version.

3) While looking at the revised manuscript, I noticed that some data seem to appear in both the Figures as well as the Supplemental Figures. Please check to avoid any duplication of experiments and correct accordingly:

- Figure 1G and Supplemental Figure 3 appear identical
- Figure 1A and Supplemental Figure 2F appear identical

Supplemental Figure 3 shows the full gel of Figure 1G, to abide by a formal requirement of the journal. Supplemental Figure 2F shows the same curves as Figure 1A, but without background correction ('normalization to 0'), i.e. the fluorescence intensity measured at the highest salt concentration is not subtracted. This is mentioned in the text as well as described in our original methods paper describing QINESIn. (See source data for the raw data.)

4) Further, I noticed that scale bars in all IF microscopy pictures are missing.

Corrected. We apologize for the lapse and thank Reviewer for noticing.

Once these minimal concerns have been addressed, I am satisfied and support publication of the manuscript.

Remark: We have noticed that one of the melanoma cell lines we worked with was mislabeled in the previous version of the manuscript, so it was corrected. Identification of both melanoma cell lines was based on our RNA-seq data, as mentioned now in the new version of the Reporting Summary.

We thank Reviewer for the overall supportive opinion, the insightful and helpful comments and important suggestions.

Reviewer #3 (Remarks to the Author):

The authors' responses contain some experimental and confusing explanations that do not support the publication of the paper. In particular, the following points need to be reconsidered:

The response to the question regarding Manders Correlation is insufficient. The authors cannot simply replace "molecule" with "pixel" and maintain the claims made in the figure. What is the basis for considering all objects defined as "pixels" to be signals?

We believe that our response to the same question of the first review fully answers the questions raised by Reviewer in this and the previous review, and also well explains why we replaced molecule with pixel:

We used MCC to assess what fraction of pixels occupied by one entity also contains the other entity. How bright the pixels are, is not considered in this calculation. We have corrected "molecules" to "pixels" in the revised version.

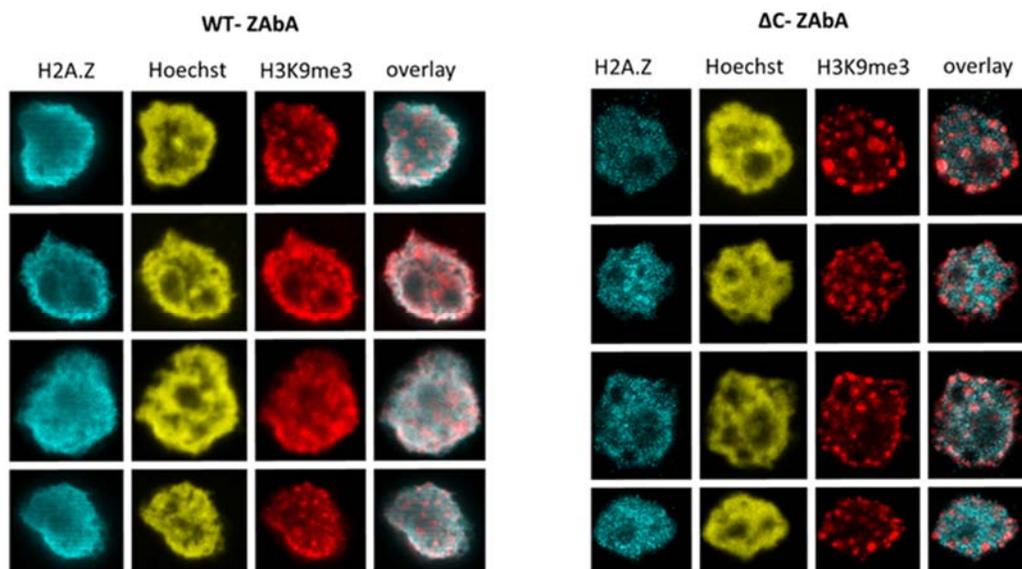
See <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3074624/>:

"Whereas PCC provides an effective statistic for measuring overall association of two probes in an image, it has the major shortcoming that it indirectly (and sometimes poorly) measures the

quantity that is typically at the heart of most analyses of colocalization in cell biology: the fraction of one protein that colocalizes with a second protein. This quantity can be measured via Manders' Colocalization Coefficients (MCC)”

We simply wanted to determine in what fraction of the pixels occupied by one molecule the other molecule is also present, irrespective of their exact local concentrations. This is measured by the MCC, it gave consistent results and proved to be sensitive enough for our purposes, while the SCR peptide always gave negative results using the same approach.

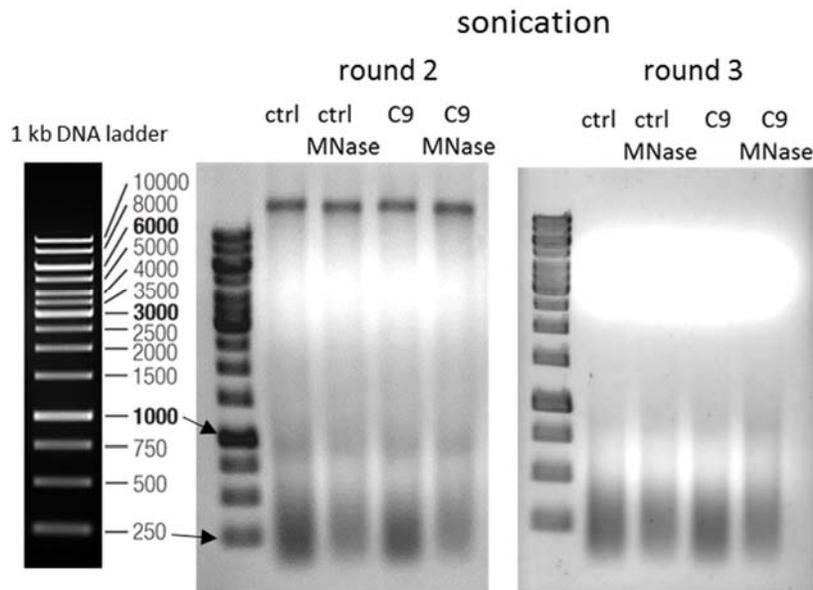
To convince Reviewer, we have included STED images in the newly revised version (see below; copied from new Suppl. Fig. 11F,G) that confirm the conclusions made based on the Manders coefficient calculation conducted according to our reasoning: H2A.Z immunofluorescence becomes scattered, H3K9me3 topography also appears to be altered and the overlap between the H2A.Z-containing and H3K9me3-containing pixels is diminished in the nuclei of cells expressing the truncated version.



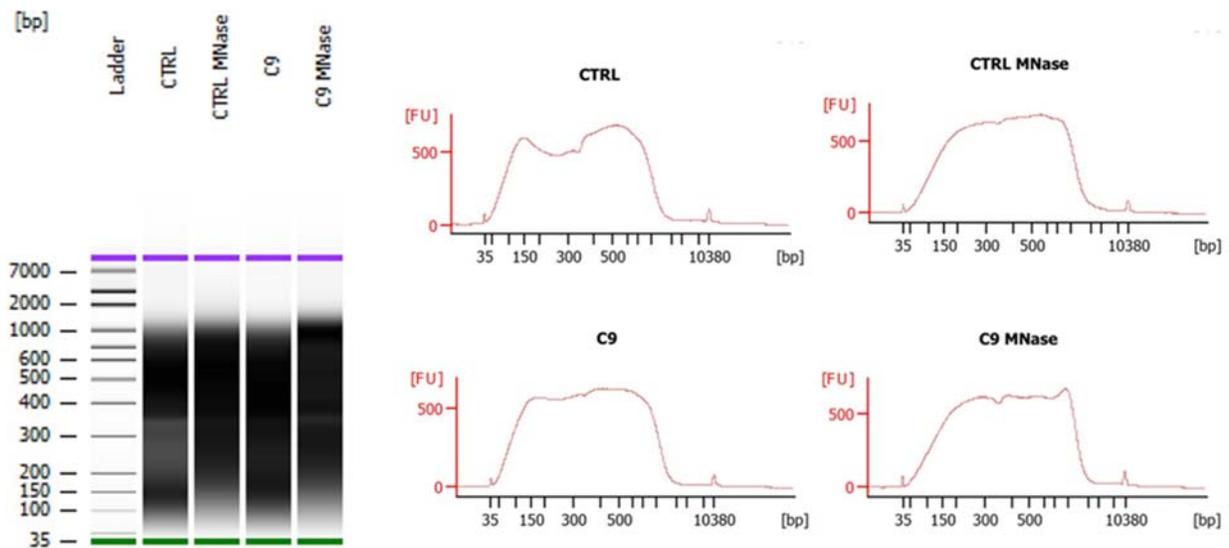
The response to the MNase experiment is inadequate. The additional data presented do not exclude the risk that the results may vary depending on the conditions of sonication. Moreover, it is unclear how the clustering of the heatmap was performed, but it suggests that either significant regions were not extracted by clustering or that appropriate clustering was not performed.

K-means/hierarchical clustering was performed with deepTools package, plotHeatmap tool using "--kmeans" option, where regions with similar score distributions were sorted to specific groups.

Regarding the conditions of sonication, the gel photo below demonstrates that the 3rd round of sonication yielded completely fragmented DNA, which samples were sent for sequencing.



Changes in calculated coverage ratios are the consequence of changes in the average size of the fragments carrying the locus analyzed based on read coverage data. Overall MNase digestibility was altered by C9 treatment, as demonstrated (Fig. 3). Therefore, DNA fragment size distributions are expected to be somewhat different for the different samples. Indeed, upon quality check prior to the generation of sequencing library, the samples exhibit subtle differences (see below). These differences were blurred after library generation (not shown) but became manifest in the read coverage maps (Fig. 3).



In summary, the sonication conditions were optimized and the fragment size distributions behave as expected. As we pointed out in our previous response, very similar local changes in MNase sensitivity could be detected using different ways of normalization.

We also stress that we did not include the heatmaps in the manuscript, did not use them in drawing any conclusion. They were shown only to reflect on Reviewer's critical remark in the previous review.

For Main Fig1f, it is disappointing that experimental improvements were not made after conducting sufficient trials. In particular, the response to point 5 raises concerns about the stability of the experimental quality.

We cannot find any remark of Reviewer 3 on "Main Fig1f" in the first review. Also, we cannot find "point 5" (there was no numbering of the comments in this review). Based on the content of the remarks we assume that the comments of Reviewer 4 are actually alluded to by Reviewer 3. In that case, please consider the argument below, in addition to those mentioned earlier.

Following nucleosome assembly, we performed supercoiling assays so as to ensure that both reconstituted H2A- and H2A.Z-containing chromatin had the same number of nucleosomes. Therefore, we are confident that each reconstitution had the same number of nucleosomes. We included this methodical detail in the newly revised text of the relevant Methods section.

Regarding the data in Suppl. Fig. 4D-G, the authors have changed the text to "autofluorescence," but this is not appropriate. Their modified text seems to imply that they cannot set the expression level to zero, but this only adds to the confusion.

The term “autofluorescence” was used only in our previous response, not in the text.

The cells start to die at near-complete silencing of H2A.Z, what we wanted to avoid in the silencing experiments.

Considering that we got very similar results using any of the 4 different (commercial) antibodies, 2 of which were also documented in papers containing ChIP-seq results, the large body of western blot experiments performed using recombinant proteins (of two different sources in the initially submitted and in the revised version) and our further arguments in the previous response to reviewers’ comments, we don’t think that there is any doubt regarding the specificity of the antibody labeling in our experiments.

To add a further argument regarding ZAbA labeling used in the experiments related to our main observations: H2A.Z staining not only decreases significantly in the periphery of H2A.ZAC DT40 cells but also becomes reorganized to foci, as compared with H2A.Z1 nuclei, when examined in the superresolution images above and in the [new Suppl. Fig. 11F,G](#). These pictures are hardly compatible with any possible scenario of nonspecific staining.

We thank Reviewer for a thorough scrutiny of our work that has led to its significant improvement in the course of the review process.

Finally, let us also add that the question raised in the earlier review on transposon sequences can be better addressed now, as we have further analyzed our RNA-seq data using the bioinformatic approach of Hammel M. et al. (<https://pubmed.ncbi.nlm.nih.gov/29508296/>). The transposable element (TE) annotation file for hg38 was downloaded through their website (<http://hammelllab.labsites.cshl.edu/software>), and was used to analyze the expression of TEs from the bam files using the featurecounts and DESeq2 programs on the Galaxy.eu server (<https://usegalaxy.eu>). Out of the more than 4.7 million TEs listed in the annotation file, only about 7000 TEs had detectable expression (raw counts > 20), and 251 had significantly different expression (adj. p value ≤ 0.05 and $\text{abs}(\log_2\text{FC}) \geq 1.2$). The 96 upregulated TEs included mostly LINES (62), plus 16 DNA transposons, 12 LTRs, and 6 SINEs. The 155 downregulated TEs were skewed towards SINEs (94), in addition to 27 LINES, 17 DNA transposons, 14 LTRs, 2 satellites and 1 retroposon.

These observations appear to be in line with Reviewer’s prediction made in the previous review. The limitations of our analysis were that only polyadenylated TE transcripts could be detected and quantified reliably in our data, and that only a very small percentage of all TEs was detected.

Remark: We have noticed that one of the melanoma cell lines we worked with was mislabeled in the previous version of the manuscript, so it was corrected. Identification of both melanoma cell lines was based on our RNA-seq data, as mentioned now in the new version of the Reporting Summary.

Reviewer #4 (Remarks to the Author):

The Imre et al. manuscript is much improved. They answer convincingly some of my questions, although not all. In this manuscript there are new approaches and many interesting data. However, I still see that several important results do not agree with the main hypothesis of the authors.

- The authors suggest that there is a pool of H2A.Z that associates with heterochromatin and can be traced using specific antibodies (ZAbA). They state in the Discussion: "we propose that ZAbA preferentially detects stable nucleosomes residing in peripheral heterochromatin." However, when they destabilize this fraction of H2A.Z using the C9 peptide, they do not detect transcriptional changes associated with heterochromatin alteration, as seen in the RNA-seq experiments of Figure 6. They observe downregulation of over 500 genes from euchromatin, but there is no mention of heterochromatin transcripts. To verify their hypothesis about C9 destabilizing heterochromatin, they should investigate whether major satellite and other repetitive sequences such as **LINE** are upregulated.
- Similarly, when they perform MS analysis of proteins released by ZAbA-targeted CUT&RUN upon C9 treatment (figure 3N), I would expect to see **heterochromatin proteins** if their hypothesis is correct. Maybe not only heterochromatin proteins because C9 may also affect other H2a.Z fractions as the authors suggest, but at least some.

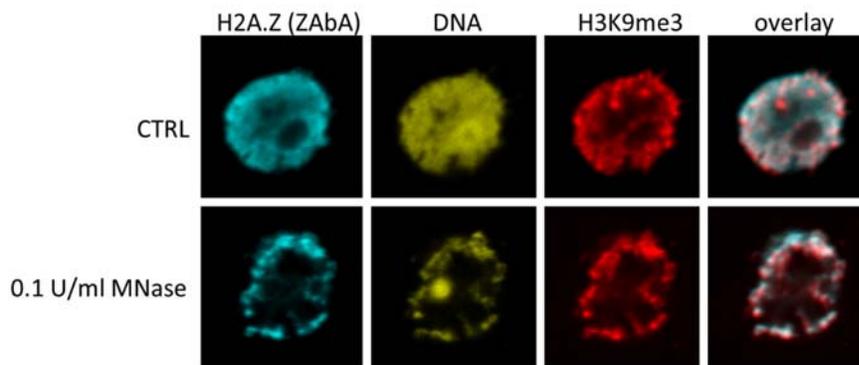
First let us consider the concerns above in general terms. Regarding the relationship between transcriptional changes and other tail-dependent effects or C9-modulated parameters, currently there is no model for the function(s) of H2A.Z in transcriptional regulation what would be able to reconcile the numerous reported observations suggesting opposite roles. (This is alluded to in this review paper ([https://www.cell.com/trends/genetics/fulltext/S0168-9525\(21\)00287-0](https://www.cell.com/trends/genetics/fulltext/S0168-9525(21)00287-0)) as: "It is now clear that H2A.Z does not follow 'one size fits all' and the complex biology of H2A.Z, including PTMs, isoforms, and diverse nucleosome composition, can all play a role in the different transcriptional outcomes.") The published data cited in our manuscript on the presence and possible functioning of H2A.Z in heterochromatin, together with those of ours under review herein, add further layers of complexity.

We share the view that the entire field still lacks a model based on which the effect of the presence or absence of H2A.Z on gene expression could be unambiguously interpreted and predicted.

Specifically, let us address the CUT&RUN-related question first. Two CUT&RUN experiments combined with MS were conducted. In the first experiment we followed the conventional CUT&RUN protocol applying only the H2A.Z specific antibody before pAG/MNase labeling. In this experiment several heterochromatin-resident proteins could be detected (HP1 $\alpha/\beta/\gamma$, LMNA/B1, MATR3, SAFB) but the H2A.Z histones, unambiguously, could not be identified (Suppl. Table 2). In the second experiment (Suppl. Table 4), the H2A.Z specific antibody was further labeled with a secondary antibody to recruit more pAG/MNase to the target. In this case,

H2A.Z proteins were detectable, but heterochromatin-related proteins were not. We attribute this to H2A.Z crosslinking, based on the following experimental observation (see figure below): when nuclei were treated with MNase after H2A.Z crosslinking (using both primary and secondary antibodies), only peripheral heterochromatin remains in the nuclei, euchromatic regions were digested and disappeared from the nuclei. This result is included in the new version as Suppl. Fig. 17P. Apparently, the secondary antibody facilitated DNA cleavages around euchromatic H2A.Z nucleosomes but prevented the escape of the cut-out molecular complexes from within the heterochromatin.

Let us add here that to our best knowledge, the successful combination of CUT&RUN and mass spec analyses has not been described earlier.



Using the sensitized CUT&RUN procedure, heterochromatic H2A.Z, along with its interacting partners, stays chromatin-bound upon MNase digestion in accordance with our mass spec results.

Thus, the H2A.Z and other proteins detected in the sensitized assay (Suppl. Table 4 and the Volcano plot of Fig. 3N) was from the euchromatic regions.

We include a remark on this aspect in the newly revised manuscript.

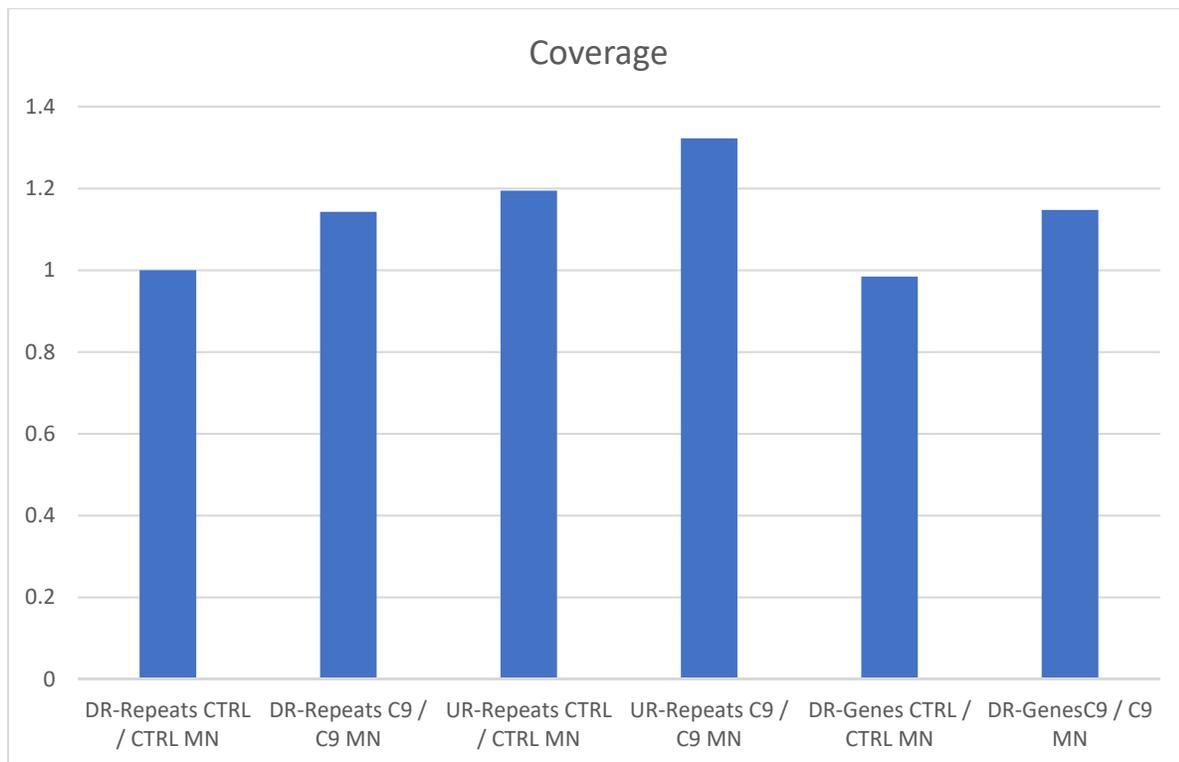
Whether the architectural changes in heterochromatin, also detected in the live cell experiment using C9, become manifest at the level of gene expression, has not been specifically investigated in our submitted work. Although C9 increased MNase digestibility at LINE sequences, this may not necessarily lead to changes in gene expression in view of the fact that all the genes down-regulated by C9 are outside the late-replicating regions encompassing most LINE-related noncoding sequences (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7884787/>; <https://pubmed.ncbi.nlm.nih.gov/30352618/>). Furthermore, the proportion of full-length LINE sequences that are more likely to be expressed is a few %. In addition, the RNA-seq procedure employed by us focuses on poly A-tailed transcripts, which is a minority in the case of LINE-related messages. Therefore, we may not necessarily detect changes in the expression of heterochromatin-derived transcripts even if they occur.

Nonetheless, spurred by Reviewer's comment, we have looked at the changes in the expression of repetitive/transposable elements in general (LINEs, SINEs, LTRs, DNA transposons, satellites, etc.), based on the comparison of C9-treated and scrambled peptide-treated samples, using the

bioinformatic approach of Hammel M. et al. (<https://pubmed.ncbi.nlm.nih.gov/29508296/>). The transposable element (TE) annotation file for hg38 was downloaded through their website (<http://hammelllab.labsites.cshl.edu/software>), and was used to analyze the expression of TEs from the bam files using the featurecounts and DESeq2 programs on the Galaxy.eu server (<https://usegalaxy.eu>). Out of the more than 4.7 million TEs listed in the annotation file, only about 7000 TEs had detectable expression (raw counts > 20), and 251 had significantly different expression (adj. p value ≤ 0.05 and $\text{abs}(\log_2\text{FC}) \geq 1.2$). The 96 upregulated TEs included mostly LINES (62), plus 16 DNA transposons, 12 LTRs, and 6 SINEs. The 155 downregulated TEs were skewed towards SINEs (94), in addition to 27 LINES, 17 DNA transposons, 14 LTRs, 2 satellites and 1 retroposon.

Thus, we have examples of transposable elements with increased transcriptional activity in response to C9 treatment. The limitations of our analysis were that only polyadenylated TE transcripts could be detected and quantified reliably in our data, and that only a very small percentage of all TEs was detected, which is not representative of all heterochromatic genomic regions.

When the C9-induced changes in MNase sensitivity, as measured in *HeLa* nuclei, were calculated at the C9-sensitive coding and repetitive element-derived transcripts, specified in the *melanoma* cell, an increment at these regions was systematically found, as shown in the figure below:



DR: down-regulated; UR: upregulated

This result seems to support the possibility that changes in nuclease sensitivity and in transcriptional activity may be interrelated. A serious limitation of this conclusion is that the source (cells) of the MNase and RNA-seq experiment was different, perhaps a lesser concern in the case of the repetitive elements.

We are aware that elucidating the detailed mechanistic relationships between the C9- or tail-dependent changes of chromatin structure and gene expression over the heterochromatin will require further analyses using assays measuring nascent rather than steady-state RNA levels and its extension beyond the poly A-tailed mRNA population. This is among our plans. We also strive to compare different melanoma lines in terms of their response to C9 in correlation with the metabolic pathways affected.

Remark: We have noticed that one of the melanoma cell lines we worked with was mislabeled in the previous version of the manuscript, so it was corrected. Identification of both melanoma cell lines was based on our RNA-seq data, as mentioned now in the new version of the Reporting Summary.

We thank Reviewer for the insightful comments that have helped us further improve the manuscript.

Response to Reviewer 3

I would like to clarify that the method referred to by Reviewer 3 as MNase-seq was **NOT** MNase-seq.

In the MS, we never call our approach “MNase-seq”, moreover the latter concept and our approach are mentioned in the same sentence (see below) such that the two are clearly distinguished:

“This MNase concentration is in the lower end of the usual concentration range of the enzyme applied in MNase-seq studies performed on fixed⁴⁷ or nonfixed cells⁴⁸, applied, however, in the absence of loosely bound proteins that were released upon preparation of the nuclei in our studies.”

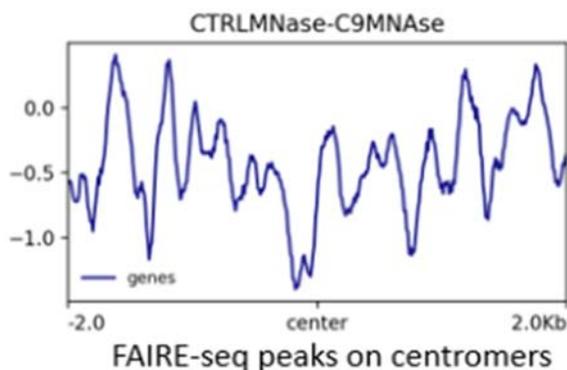
where ref. 48 is:

Chereji RV, Bryson TD, Henikoff S. Quantitative MNase-seq accurately maps nucleosome occupancy levels. *Genome biology* 20, 198 (2019).

Notwithstanding, to avoid similar misunderstandings we have added a note where our procedure comes up first in the text of the MS in the recently submitted version. (“Note: we sequenced the DNA remaining in the agarose-embedded nuclei after digestion (see Materials and Methods), differently from the ‘MNase-seq’ procedures where the DNA of the whole chromatin digest is the input for sequencing, among the several other differences (see e.g ref. 48).”)

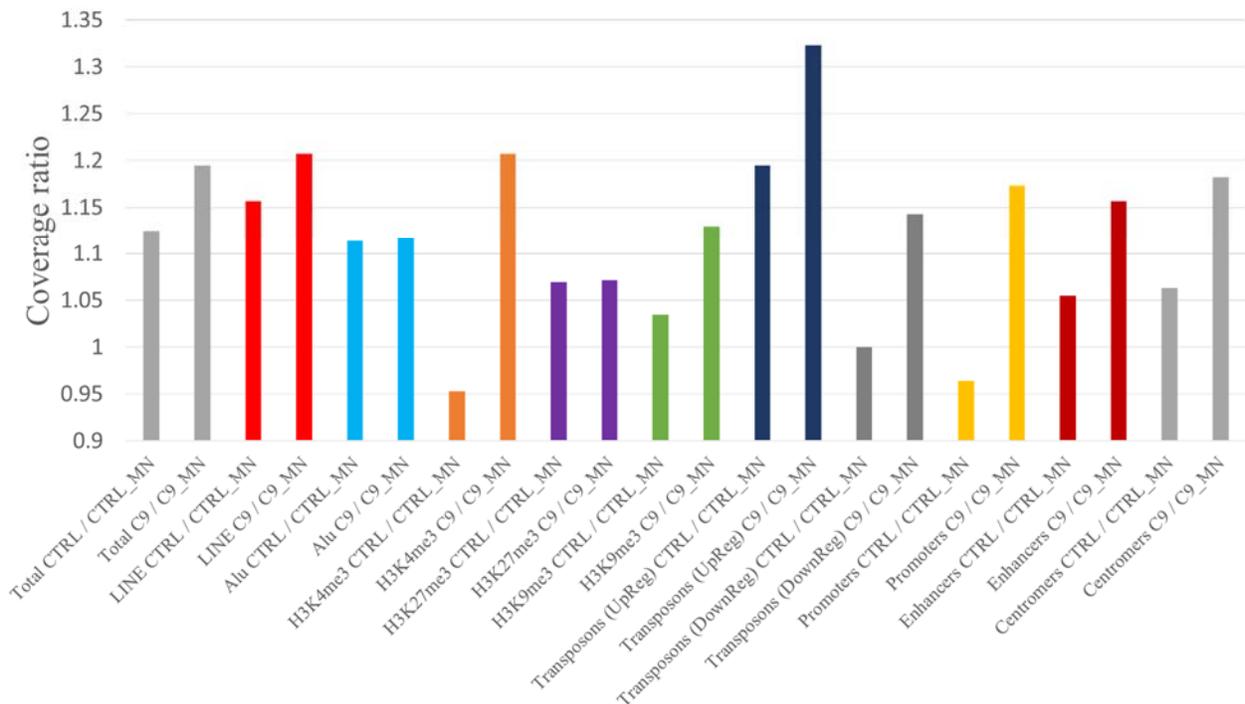
We were studying the DNA remaining in the agarose-embedded nuclei after digestion or exposure to salt in **ALL** our approaches, including the sequence analyses of MNase digestion. This uniform approach allows the direct comparison of the results of the different experiments.

The two kinds of sequencing results (of our method and of MNase-seq) are related, but are not readily convertible (see e.g. <https://www.nature.com/articles/ncomms11485>). Nevertheless, spurred by Reviewer’s comment, we attempted to assess nucleosome positioning based on the comparison of the read coverages obtained in our experiments with the FAIRE-seq datasets available for the same cell line, with the results below for the **centromeric** regions:



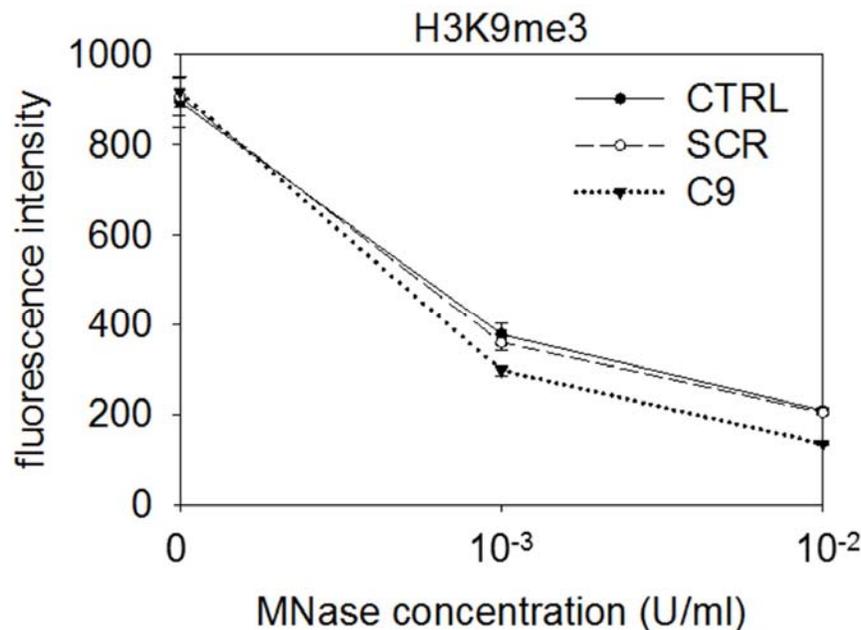
Periodicity resembling that of nucleosomes is obviously maintained, so our peptide did affect the positioning of the nucleosomes in a major heterochromatic region; furthermore, the effect appears to be non-random. (We analyzed the DNA content also in the eluted fraction and observed similar periodicity of the CTRL_MNase – C9_MNase coverage difference; not shown.) The conclusion above is based on the results of standard Illumina sequencing and alignment procedures with their inaccuracies at repetitive sequences. In addition, it may be affected by the differences between our protocol and that of MNase-seq (and also of FAIRE-seq). For these reasons and to avoid overloading of the manuscript which is already packed with data, we prefer not to include the above analyses, only the extended Suppl. Fig. 17C (see below) in the MS. We have plans to perform a *bona fide* MNase-seq study in a separate project.

Reviewer expressed concern over our conclusion that heterochromatin was affected by C9 in the lack of evidence that the nucleosomes were rearranged. Since we did not perform ‘MNase-seq’, we did not assess nucleosome positioning. Our question was if the increase in MNase sensitivity upon C9 treatment included also heterochromatin in addition to euchromatic regions. This question was clearly answered already in the original Suppl. Fig. 17C. This panel was supplemented now with analyses of the other regions mentioned by Reviewer (including those of retrotransposons). We believe that Suppl. Fig. 17C in its current form fully takes care of the issue raised by Reviewer: „unclear where the MNase sensitivity of heterochromatin occurred”. Obviously, **heterochromatic regions are among the sequences sensitized to MNase by C9 (see new Suppl. Fig. 17C copied below). If the second column of the same color is higher than the first, it means sensitization in that chromatin region (heterochromatin: red, green, blue, dark grey, light grey):**



Effect of C9 on MNase sensitivity. Coverage ratios comparing the MNase treated (MN) and non-treated samples for both C9 treated (C9) and untreated (CTRL) samples were calculated for the whole genome (total – indicated with grey), LINE (red) and Alu (light blue) repetitive elements, H3K4me3 (yellow), H3K27me3 (purple) and H3K9me3 (green) peaks, transposons upregulated in our RNA-seq (UpReg – dark blue), downregulated in our RNA-seq (DownReg - dark grey), promoter (yellow) and enhancer (dark red) elements and centromeres (grey). Peaksets used were downloaded from ENCODE database, data accession numbers are indicated in Suppl. Methods. Centromere and Alu/LINE elements were downloaded using the UCSC Main Table Browser and RepeatMasker.

Since the overall concern of Reviewer was related to the strength of our evidence regarding the statement that C9 affected also the heterochromatin, we mention that the global sensitivity of H3K9me3 chromatin to MNase was increased by C9 when compared with either the control or the SCR treated sample, as measured over the entire nuclear volume in a population of HeLa nuclei; see figure below. This result is very similar to what is shown for the decrease of total DNA content in Fig. 3F. This simple experiment, involving no bioinformatic analyses, dissolves any doubt regarding our above statement.



The sensitivity to MNase of H3K9me3 chromatin was also increased in the case of DT40 DKO/ Δ C DT40 cells when compared with DKO/Z1 (data not shown).

These data serve as further arguments to convince Reviewer; they are considered superfluous to be incorporated into the MS.

In a **second remark** Reviewer suggests incorporation of data mentioned in our previous rebuttal letter. These results have been incorporated into the MS (see Supplementary Methods and Supplementary Discussion; highlighted).

We thank Reviewer for a most thorough review that allowed us to improve our MS significantly.

Answers to the critical comments of Reviewer 3.

We thank Reviewer for considering our last revision. We are also thankful for the critical remarks: amending the manuscript along the lines suggested has led to a more complete and more focused study, we believe.

Regarding the remaining concerns we have made several new experiments as well as amendments to the text and figures.

The *quantitative (LSC-based) measurement of MNase sensitivity* of chromatin around H3K4me3 and H3K9me3 nucleosomes (Fig. 3G, H; new data) clearly shows that C9 pretreatment did increase digestion around nucleosomes in both euchromatin and heterochromatin. (The genomic distribution of these PTMs is shown in the pie charts of Fig. 4 and adjoining supplementary figures.)

MNase digestibility of the peripheral, mostly constitutive heterochromatin and of the central nuclear region was studied separately and quantitatively by CLSM (see these new data in Fig. 3K, L). Chromatin in both regions was more extensively digested upon treatment with C9.

Furthermore, we have analysed the sequence environment of the nuclease sensitive nucleosomes by CUT&RUN. As the new data of Fig. 4 and Suppl. Fig. 19A-E show, the distribution of DNA fragments released by pAG-MNase among the different sequence categories was very similar with and without treatment of the nuclei with the C9 peptide, in both euchromatic and heterochromatic environment.

The whole-genome sequencing experiment was repeated. As Suppl. Fig. 21C shows, the distribution of read coverage over the different sequence categories was very similar in the C9-treated and control samples following MNase treatment, in two independent experiments. We would like to underline that each sample was analysed now on its own, not involving subtraction or ratio calculation between samples, what, as Reviewer pointed out, made the results included in the previous versions of the manuscript *difficult to judge*. For the same reason and also because no conclusion is based on them, the data on local changes elicited by C9 (at the TSS, H2A.Z and CTCF sites) have been omitted from the current, revised manuscript. This way we could *focus on only the definitive aspects*, supported by several lines of evidence, as suggested by Reviewer.

In summary, the C9-increased digestibility has been observed everywhere in the genome rather uniformly, through the spectacles of several different approaches, suggesting that C9 has a general nucleosome perturbing effect, in line with its general nucleosome binding ability demonstrated in the FCS experiments which are also alluded to in the revised abstract. This strong effect of the tail peptide parallels the role of the C-terminal tail in determining global chromatin features in the DT40 system.

We call Reviewer's attention also to additional amendments implemented in the revised version. We have added new data in Fig. 3J to further confirm that chromatin rearrangement indeed happened upon C9 treatment of the nuclei, i.e. the changes seen in the immunofluorescence staining patterns cannot be explained by accessibility/antigen masking-related phenomena. We further confirmed the same using Flag-tagged H2A.Z as shown in the new data of Suppl. Fig. 18. We also included a pie chart in Fig. 7 (earlier Fig. 6) showing the Giemsa band-distribution of genes randomly changing in the control cells upon culturing (see these new data in Fig. 7K).

The text was also thoroughly revised and amended to improve its clarity.

We hope that the added data and implemented changes in the manuscript have dissolved Reviewer's remaining concerns.

Gabor Szabo

corresponding author, on behalf of all the coauthors

Answer to Reviewer 3

We thank Reviewer for accepting our last version of the MS. The color code for pie chart of Fig.4B is now included with the figure and Supplementary Fig 19 is restructured accordingly.