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Dissecting the Binding Mechanism of the Linker Histone in Live Cells: An Integrated FRAP Analysis

Timothy J. Stasevich, Florian Mueller, David T. Brown and James G. McNally

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

1st Editorial Decision 16 September 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. You will see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the referees' criticisms in an adequate manner.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper deals with the DNA-binding properties of the linker histone H1. Several models exist in the field on how the so-called S1 and S2 regions and the C-terminal domain of H1 are involved in its binding to DNA, where either binding of the C-terminal tail preceeds specific binding of the globular domain or vice versa, or both domains participate in binding initiation.

Here, the authors use sophisiticated FRAP-analyses (complemented with salt-extractions) which suggest that the C-terminal domain is the first to establish DNA binding. It than obtains a different structure (or gets structure from an unstructured state when not bound) allowing S1 and S2 regions in the globular domain to bind cooperatively.

This is an excellent paper which advances technology (the FRAP-field) as well as biology (the chromatin-field): the authors not only show that FRAP is a very strong tool to unravel molecular mechanisms underlying chromatin dynamics, but also apply it to unravel H1 DNA-binding mechanisms. In addition they make clear that the method is feasible to study other systems.

Suggestion:
The paper presents very interesting data on H1 cooperative binding, but does not answer the question whether structure of the C-terminus invoked by DNA-binding on its own can change DNA conformation and if this is sufficient for proteins with S1 and S2 affinities to bind to the sites indicated by triangles in Figure 7 irrespective of whether they are attached to the C-terminus. It would strengthen the papers impact if FRAP-analyses would be presented where the GFP-tagged C-terminus was studied, preferrably with the globular domain deleted instead of mutated. FRAP could than be performed in the presence or absence of S1 and/or S2 and/or S1S2 in untagged (maybe overexpressed) form. This would elucidate if synergism exists if the binding regions are not attached to one another.

Minor comments:

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The authors state that they used C-terminal GFP-tagging to allow optimal omparison between the different constructs, but since the C-terminus is absent in most, would it not have been more logical to tag the N-terminus? The S1 and S2 are not deleted in the constructs but only made inactive by mutations, so the N-terminus seems to be present in all variants.

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Table 1 (page 23) summarises the results of FRAP-analysis of all combinations of the three constructs, S1, S2 and C, expressed in GFP-tagged form. The reader could get a better view of what is implicated by the data if also the analysis is shown of binding behaviour of the GR, and a little discussion, especially on the analysis in the right columns where bound/free fractions are given, which I could not find in the text.

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In addition, a better explanation/discussion on the bound/free parameter (rightmost columns of Table 1) could be given in the main text. The authors now split the data in slow and fast state components which both seem to have a DNA-bound and and freely mobile fraction, of which the ratios (bound/free) are given. However, it would in my view be more clear if the two bound fractions (long an short residence times) were given next to the freely mobile fraction, since this is just one fraction. These would then add up to 100% which makes the comparison clearer. Moreover, in the present form the table suggests, at least to the less expert reader, that the free fraction of the slow component is another pool of molecules than the free fraction of the fast component, suggesting two states in which the molecule can be prior to DNA-binding, which is probably not the case (and not depicted in the model in Figure 7).

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Strikingly, in Table 1, there seems to be a 2:1 ratio between the bound/free ratios of the slow and fast states of almost all variants studied. I wonder if this requires some in depth considerations/speculations.

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There are publications not mentioned in the manuscript using FRAP-analyses alternative to the one presented here also to obtain fast and slow DNA-binding components, for instance on replication factors (Xouri et al., EMBO j 26:1303-14 2007). Also, FRAP was used previously, in combination with acceptor photobleaching FRET, to show comformational changes upon DNA-binding of the androgen receptor allowing subsequent coregulator binding (van Royen et al., JCB 177:63-72, 2007).
In these papers a similar type of analysis (revealing slow and fast binding states and conformational changes by FRAP) of the same type of molecular mechanisms (DNA-binding of proteins that act on chromatin) is presented. Therefore a clearer picture would be sketched of the state-of-the-art technology available in the field if these would be included in the discussion of the technology.

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In this ms Stasevich et al. use a relatively new mode of FRAP to investigate the binding of H1 to chromatin in vivo. With the linescan approach, the initial bleaching of a disc is followed by repeated scans over a single line through the center of the bleached region. This method allows more rapid sampling of the recovery process, and provides a wealth of quantitative information. Recovery kinetics for three H10 mutations, alone and in combination, are generated, and used to develop a model for H10 binding that in which a high degree of cooperativity is involved. Reassuringly, the binding affinities of the different constructs is strongly correlated with their salt elution profiles. The paper is well written and adds considerably to our understanding of the complexity of H1 binding revealed by the new technique.
However, there are some important aspects of the manuscript that require clarification or reconsideration:
1. Perhaps because of the focus on FRAP-derived parameters, some basic questions regarding the biology of the system that appear to have been ignored. Inspection of the fluorescence images shows a wide variation in H10 distribution between the different constructs, with strong foci seen in several cases. In many constructs, these foci seem to be centered on, but much larger than the Hoechst-staining pericentromeric clusters. What does this imply? Generally, experimental conditions that result in unusually large or prominent deposits are indicative of unhealthy cells, in which the protein in question is aggregated. The authors should comment on the very different fluorescence patterns seen in the constructs (Fig 1, Supp Fig 4).
In this regard, it is important to document the amount of the H10 per nucleus in comparison with the (a) the wildtype GFP-construct and (b) the amount of 'normal' non-GFP H10.

2. In the paper on the theory of the linescan approach (Braeckmans et al, 2007), the importance of confining data sets to fluorescently homogenous regions was stressed. In this ms, the authors state that they took this precaution. However, it is difficult to see how this could be achieved in e.g. S2C where the fluorescence is extremely heterogeneous. In this respect, it is important to know the FRAP kinetics of these heavy accumulations of GFP-H10 mutants.
In this respect, while the image of the wildtype-GFP nucleus in Fig 1 shows relatively homogenous fluorescence, the one provided in supplementary fig 4 appears strikingly more heterogeneous (and more like those shown in previous publications on GFP-H10 by these authors). Again, this raises the issue of the extent to which the 'uniform euchromatic regions' are really uniform as well as the inherent variability in individual cells.

3. The inclusion of a comparison between euchromatin and heterochromatin using the spot method of FRAP is helpful, but of more value would be a direct comparison between the recovery curves for the linescan and spot scan methods.

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Referee #3 (Remarks to the Author):

This is an excellent manuscript that I highly recommend for publication in EMBO J. The paper makes two important contributions. The first, the general methodology introduced in this manuscript for studying and quantifying cooperativity should prove to be a very valuable tool for the study of the complexity of molecular interactions in living cells. The second is the significant advance that this study provides in understanding the cooperativity of binding between globular and C-terminal domains of histone H1.
I have one minor criticism that can be addressed by revision to the text and, in particular, the discussion. While I agree with the general interpretation on the binding of the C-terminus, the binding of the C-terminus may still represent an oversimplification. The authors should note that, like the globular domain, specific DNA binding motifs have been identified in the C-terminus. That is, while the initial interaction of the C-terminus may be non-specific and reflect the high charge density, the reorganization of the C-terminus into a structured binding may directly involve the ST/PKK motifs, of which there are differences between the numbers and spacing of these domains, which have previously been shown to function as DNA binding domains by the Thomas laboratory. The discussion might include, for example, a statement that the described technique could also be used to test cooperativity and exclusivity in the predicted specific DNA binding domains found within the C-termini of the individual histone H1 variants. It is notable, in this respect, that, while the globular domain is absolutely conserved between most human or mouse H1 variants and highly conserved between H1o and the remaining somatic variants, the individual variants do show significant differences in binding. A second point that should probably be noted is that the position of the EGFP tag has the potential to impact upon the binding of the C-terminus. While the measurements of the residency times show similarity with previously published results obtained using an N-terminal tag, in the absence of directly testing the impact of N-terminal versus C-terminal tagging in the same experimental system leaves some uncertainty as to the potential impact of placing the tag so close to regions that are important for chromatin binding. I do not, however, feel that this limitation in any way detracts from the significance of the paper or its suitability for publication and do not feel that it is important to complete this control prior to publication.

1st Revision - authors' response 23 November 2009

Response to referee comments:

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the field on how the so-called S1 and S2 regions and the C-terminal domain of H1 are involved in its binding to DNA, where either binding of the C-terminal tail proceeds specific binding of the globular domain or vice versa, or both domains participate in binding initiation.

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Response to suggestion: We initially thought this experiment was worth trying, but after thinking about it further we realized it would be virtually impossible to interpret given the constraints of the H1 system. The problem is getting sufficient expression of the mutant H1 constructs to compete with endogenous H1 levels, which are very high (almost 1:1 with nucleosomes and so ~10 million per cell due to the contribution of multiple genes). We have tried to over-express mutant constructs but have been unable to detect accumulation to levels greater than ~5% of the endogenous levels in the best cases (DTB, unpublished observations). Thus, if we co-transfect the construct suggested by the reviewer into S1, S2, or S1S2, we would expect the two exogenous constructs to interact less than 5% of the time (since most of the time they would interact with endogenous H1, if anything). The impact on a FRAP recovery curve would therefore be 5% at best, an amount that is hard to distinguish from the noise in a typical FRAP experiment.

Comment 1: The authors state that they used C-terminal GFP-tagging to allow optimal comparison between the different constructs, but since the C-terminus is absent in most, would it not have been more logical to tag the N-terminus? The S1 and S2 are not deleted in the constructs but only made inactive by mutations, so the N-terminus seems to be present in all variants.

Response to comment 1: There is debate about whether to use an N-terminal tagged or C-terminal tagged version of H1 for experiments in live cells. Funayama et. al (Funayama et al., 2006) have suggested N-terminal tagged H1 significantly alters cell growth, morphology, and viability, but Hendzel et al. (Hendzel et al., 2004) have found that an N-terminal tagged H1 binds better according to FRAP. When we compared FRAP of N-tagged and C-tagged H1, the C-tagged version recovered more slowly and thus appeared to bind more stably than the N-tagged version (DTB unpublished observations). Thus we felt the C-terminal tag was the better choice for our experiments.

Comment 2: Table 1 (page 23) summarises the results of FRAP-analysis of all combinations of the three constructs, S1, S2 and C, expressed in GFP-tagged form. The reader could get a better view of what is implicated by the data if also the analysis is shown of binding behaviour of the GR, and a little discussion, especially on the analysis in the right columns where bound/free fractions are given, which I could not find in the text.

Response to comment 2: We have added data for GR into Table I and have expanded our discussion of how bound/free fractions are calculated from raw FRAP data in the middle paragraph of p. 8 of

the main text.

Comment 3: In addition, a better explanation/discussion on the bound/free parameter (rightmost columns of Table 1) could be given in the main text. The authors now split the data in slow and fast state components which both seem to have a DNA-bound and and freely mobile fraction, of which the ratios (bound/free) are given. However, it would in my view be more clear if the two bound fractions (long and short residence times) were given next to the freely mobile fraction, since this is just one fraction. These would then add up to 100% which makes the comparison clearer. Moreover, in the present form the table suggests, at least to the less expert reader, that the free fraction of the slow component is another pool of molecules than the free fraction of the fast component, suggesting two states in which the molecule can be prior to DNA-binding, which is probably not the case (and not depicted in the model in Figure 7).

Response to comment 3: We agree this would make things clearer, so we have added two columns to Table I to show the fast bound fraction, the slow bound fraction, and the free fraction (which together always add to 100%). We have also kept the total bound/free fraction column for easy access when calculating cooperativity.

Comment 4: Strikingly, in Table 1, there seems to be a 2:1 ratio between the bound/free ratios of the slow and fast states of almost all variants studied. I wonder if this requires some in depth considerations/speculations.

Response to comment 4: We have given this some thought, but don't believe this reflects either a deeper meaning or some sort of artifact. In fact we have obtained ratios experimentally that are either significantly less or significantly more than 2:1. The S2 mutant shown in Table I had a fast to slow ratio of 0.7:1, whereas the 3K0 and S1 mutants had ratios much greater than 2:1 (these ratios are not reported in Table I because the data are equally well fit by a one parameter effective diffusion model, and so we reported only that fit). As a more direct test of whether there might be some artifact in our procedure that would bias our estimates to a 2:1 ratio, we performed Monte Carlo simulations of diffusion and binding with fast to slow ratios ranging from 9:1 to 1:9. As we now show in Supplementary Fig. S3, fits to simulated data consistently predicted the correct ratio, suggesting the fitting analysis did not bias our measurements.

Comment 5: It is stated on page 9, that the gamma-factor for cooperativity is calculated by dividing the red region in Figure 5 by the sum of two white regions and that the result should > 1 if cooperativity occurs. However, if cooperativity would lead to doubling of bound/free, gamma would be one and if cooperativity would be a bit less it would be regarded as no cooperativity, since than gamma < 1. Did the authors mean 'sum of red and white regions divided by sum of the two white'?

Response to comment 5: No, we meant that the red portion should be divided by the product of the two white portions. We now explicitly state in the text and also in the caption of Fig. 5 that we are dividing by the product (before we had just said "divided by the two white portions").

Comment 6: In page 10 of the supplemental material there is a mistake: references 7 and 8 are the same but termed '2008a' and '2008b'.

Response to comment 6: Thanks for catching this. We have corrected this mistake.

Comment 7: There are publications not mentioned in the manuscript using FRAP-analyses alternative to the one presented here also to obtain fast and slow DNA-binding components, for instance on replication factors (Xouri et al., EMBO j 26:1303-14 2007). Also, FRAP was used previously, in combination with acceptor photobleaching FRET, to show comformational changes upon DNA-binding of the androgen receptor allowing subsequent coregulator binding (van Royen et al., JCB 177:63-72, 2007). In these papers a similar type of analysis (revealing slow and fast binding states and conformational changes by FRAP) of the same type of molecular mechanisms (DNA-binding of proteins that act on chromatin) is presented. Therefore a clearer picture would be sketched of the state-of-the-art technology available in the field if these would be included in the discussion of the technology.

Response to comment 7: Thanks for pointing this out. We have expanded our discussion of the

current technology and cited the publications discussed above. This includes (1) an expanded discussion of FRAP technology in the last paragraph of the Introduction (final paragraph, p3); (2) a comment in the third paragraph of the Discussion (bottom of p.12/ top of p.13) about how acceptor photobleaching FRET can also be used to quantify conformational changes induced by DNA binding; and (3) appropriate citations related to effective diffusion and fast binding at the end of the fourth paragraph of the Results section (bottom paragraph p.5/top paragraph, p.6).

Referee #2 (Remarks to the Author):

In this ms Stasevich et al. use a relatively new mode of FRAP to investigate the binding of H1 to chromatin in vivo. With the linescan approach, the initial bleaching of a disc is followed by repeated scans over a single line through the center of the bleached region. This method allows more rapid sampling of the recovery process, and provides a wealth of quantitative information. Recovery kinetics for three H10 mutations, alone and in combination, are generated, and used to develop a model for H10 binding that in which a high degree of cooperativity is involved. Reassuringly, the binding affinities of the different constructs is strongly correlated with their salt elution profiles. The paper is well written and adds considerably to our understanding of the complexity of H1 binding revealed by the new technique. However, there are some important aspects of the manuscript that require clarification or reconsideration:

Response: We thank the referee for careful reading of the manuscript and for expressing interest in our work.

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Response to comment 1: The large foci surrounded by pericentromeric clusters that the reviewer refers to are nucleoli, not protein aggregates. Many of the mutant constructs seem to be enhanced in nucleoli, which may reflect a more non-specific interaction (perhaps with rRNA's?) that is uncovered upon loss of the normally higher affinity for chromatin. We now discuss this heterogeneity in more detail in the second paragraph of the Results section (top paragraph, p.5)

Comment 1b: In this regard, it is important to document the amount of the H10 per nucleus in comparison with the (a) the wildtype GFP-construct and (b) the amount of 'normal' non-GFP H10.

Response to comment 1b: We have shown in an earlier paper (Misteli et al., 2000) that the wild-type H1-GFP is less than 5% of the endogenous H1 (probably much less) and the mutants, by comparison of intensity and the amount of fluorescent material extracted from isolated nuclei, are less than that. This was mentioned in the Methods section of the manuscript, under "Constructs and cell lines" (bottom of p.16).

Comment 2: In the paper on the theory of the linescan approach (Braeckmans et al, 2007), the importance of confining data sets to fluorescently homogenous regions was stressed. In this ms, the authors state that they took this precaution. However, it is difficult to see how this could be achieved in e.g. S2C where the fluorescence is extremely heterogeneous. In this respect, it is important to know the FRAP kinetics of these heavy accumulations of GFP-H10 mutants.

In this respect, while the image of the wildtype-GFP nucleus in Fig 1 shows relatively homogenous fluorescence, the one provided in supplementary fig 4 appears strikingly more heterogeneous (and more like those shown in previous publications on GFP-H10 by these authors). Again, this raises the issue of the extent to which the 'uniform euchromatic regions' are really uniform as well as the inherent variability in individual cells.

Response to comment 2: In fact, the images in Supplementary Fig. S6 are not so representative because they were chosen to highlight heterochromatin. To provide the reader a better idea of the kinds of regions we selected for the FRAP measurements, we now include a Supplementary figure that shows this explicitly. Supplementary Fig. S1 includes five images for each mutant cell line showing where we performed the photobleach. As can be seen, all cells had reasonably homogeneous regions within which FRAP measurements were performed.

Even though we did our best to avoid heterogeneity, we share the reviewerís concern that contributions from heterogeneous regions outside of the FRAP measurement zone could still potentially alter our diffusion and binding estimates. The model we used to fit the data assumes fluorescence is evenly distributed and thus does not incorporate outlying heterogeneity. We were reassured, however, by the self-consistency of our results. In particular, the standard deviation of our fits is small even though each fit corresponds to data from a single cell having a highly variable number of nucleoli, heterochromatic foci, etc. Furthermore, our estimates for the H1 wild-type agree with those from an earlier study (Carrero et al., 2004) and our predicted bound fractions–the only parameters used to dissect the H1 binding mechanism–are in line with our salt-extraction experiments. Together, these self-consistencies suggest that the heterogeneity does not significantly affect our conclusions. In general, however, we agree that heterogeneity can lead to erroneous results, so we now discuss this limitation in the top paragraph on p. 13 of the Discussion.

Although we have characterized the kinetics of each construct in heterochromatic foci (Supplementary Figs. S6 and S7), we have chosen not to characterize the kinetics of each construct in nucleoli. Analysis of nucleoli is typically not done in FRAP studies of nuclear proteins, even though the nucleoli frequently show a markedly different staining from the rest of the nucleus.

Comment 3: The inclusion of a comparison between euchromatin and heterochromatin using the spot method of FRAP is helpful, but of more value would be a direct comparison between the recovery curves for the linescan and spot scan methods.

Response to comment 3: We cannot directly compare our recovery curves for the line vs. spot procedures because the bleach spot sizes and number of bleach iterations used were different. As a result, the curves would not be expected to overlay. However, we can fit data from line and spot FRAP (when the number of bleach iterations is the same) and compare the predicted diffusion constants and binding rates. In fact we had done that in the original version of the manuscript for both GFP and for GFP-GR and found agreement between the two methods. In considering the reviewer's comments, we decided to expand this comparison to two more FRAP experiments with recovery times in between GFP (very fast) and GFP-GR (slow). For this purpose, we chose two of the H1 mutant constructs S1S2 (one of the faster constructs) and the C construct (one of the slower constructs). Here we have also found agreement between the line and spot procedures. This argues that the line FRAP procedure is appropriate for the full range of recovery times seen in the H1 mutants. We have added these new data to Supplementary Fig. S2.

Comment 4: In their calculations, the authors appear not to have taken into account the differing masses of the various H10 constructs. These differences could affect the diffusion coefficients used to determine the fast binding parameters.

Response to comment 4: We did account for the different masses of the H1 constructs, but the point was buried in the Supplemental Material. We now mention it in the second paragraph on p. 8 of the main text.

Comment 5: In the text, the high mobility seen with loss of either the C-terminus or globular domains is interpreted as indicating cooperativity. However, it is not clear why C-terminal (rather than globular domain) binding is featured as the initial event in Fig 7.

Response to comment 5: We reasoned that if H1 binding proceeds via a preferred sequence, then removal of the region that initiates binding should eliminate more binding than removal of the other regions. Among the three binding regions examined, removal of the C-terminal tail eliminated far more binding than removal of S1 alone or S2 alone (bound/free ~ 1 in S1S2 vs. bound/free ~ 14 in S2C and ~20 in S1C). This suggests the C-terminal tail initiates the majority of binding events. In

concordance with this, the C-terminal tail binds far better on its own than either S1 or S2 bind on their own (bound/free of ~7 in C vs. 0.7 in S1 or 0.4 in S2), suggesting the tail alone does not rely on another region to bind. Finally, we note that the cooperativity factor gamma between S1 and S2 is approximately zero, but between S1 and C is ~2.6 and between S2 and C is ~2.4. In other words, S1 and S2 cannot bind simultaneously without the C-terminal tail, so the globular domain cannot initiate binding on its own. Together, these independent observations strongly suggest binding of H1 is for the most part initiated by the C terminal tail.

To make this point clearer, we have expanded the discussion in the last paragraph on p. 11 of the main text.

Comment 6: Blue/green is a bad choice to represent GFP and Hoechst fluorescence respectively since the blue is barely visible by itself, and completely dwarfed by green in the overlays. Green-red of green-orange would make it much easier to interpret the images.

Response to comment 6: We have changed the coloring in Supplementary Figs. S6 and S7 to green/red to better illustrate the overlap.

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This is an excellent manuscript that I highly recommend for publication in EMBO J. The paper makes two important contributions. The first, the general methodology introduced in this manuscript for studying and quantifying cooperativity should prove to be a very valuable tool for the study of the complexity of molecular interactions in living cells. The second is the significant advance that this study provides in understanding the cooperativity of binding between globular and C-terminal domains of histone H1.

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Response to comment 1: These are good suggestions. We agree that binding domains within the C-terminal tail could themselves also be analyzed in the same manner to further elucidate their cooperation or lack thereof, especially considering the potential role of these regions in distinguishing the H1 variants. We now mention this in the section entitled "Complex binding of the H10 C-terminal tail" in the Discussion (bottom of p.13/top of p.14), and cite relevant work from the Thomas and Hendzel laboratories.

We also now note in the Discussion section (top of p. 13) several limitations of our approach, including the possible complications introduced by a C-terminal tag of the GFP on H1.

REFERENCES

1. Carrero,G., Crawford,E., Hendzel,M.J., and de Vries,G. (2004). Characterizing fluorescence recovery curves for nuclear proteins undergoing binding events. Bull Math Biol, 66, 1515-1545.

2. Funayama,R., Saito,M., Tanobe,H., and Ishikawa,F. (2006). Loss of linker histone H1 in cellular senescence. Journal of Cell Biology, 175, 869-880.

3. Hendzel,M.J., Lever,M.A., Crawford,E., and Th'Ng,J.P.H. (2004). The C-terminal domain is the primary determinant of histone H1 binding to chromatin in vivo. Journal of Biological Chemistry, 279, 20028-20034.

4. Misteli,T., Gunjan,A., Hock,R., Bustin,M., and Brown,D.T. (2000). Dynamic binding of histone H1 to chromatin in living cells. Nature, 408, 877-881.

2nd Editorial Decision 16 December 2009

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again. In general, the referees are now positive about publication of your paper. Still, referee 2 feels that there is one issue that still needs to be addressed (see below) before we can ultimately accept your manuscript. Furthermore, there is one editorial issue. Please format the references in EMBO J. format (in alphabetical order, no numbering).

I would therefore like to ask you to deal with the issues raised. Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

In this revision, the authors have provided adequate responses to the majority of the reviewers' comments. However, there is one important point that is not satisfactorily addressed, namely the location of several H1 constructs in a few large foci. In their response and new paragraph on p5, the authors suggest that these often large foci represent nucleoli and that since they don't use these regions for FRAP, there is nothing to be concerned about. However, situations like this raise the issue of whether the abnormal localization reflects a compromised nuclear metabolism and that data are being collected on unhealthy cells. The explanation put forward for the distribution in large foci suggests that the nucleoli themselves are affected (number, size). An image set like that in Supp Fig 6 that includes a nucleolar marker, or phase/DIC image where nucleoli are readily identifiable should be provided, as well as a discussion of the possible side effects of a clearly abnormal intranuclear distribution of H1 and nucleoli.

2nd Revision - authors' response 29 January 2010

Response to referee comments:

Referee #2 Suggestion

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Response: We obtained DIC images of the H1 cell lines to determine the nature of the foci seen in the H1 mutant constructs. Overlay of the GFP images with the DIC images showed that the larger H1 foci correspond to nucleoli, the borders of which could be seen in the DIC images (see Supplementary Fig. S1A). Similarly, overlay with the Hoechst images revealed that the remaining smaller accumulations correspond to heterochromatic foci (this is expected since the wild type H1 is also enhanced at heterochromatic foci, as seen in both Supplementary Figs. S1A and S6). For example, the S2C construct co-localizes with many smaller heterochromatic foci (appearing in both the GFP and Hoechst images) as well as with four larger nucleoli that have identifiable borders in the DIC image. The remaining mutant constructs follow a similar pattern, although the degree of co-localization to both nucleoli and heterochromatin varies. We therefore conclude that aberrant H1 localization is indeed within nucleoli.

To see if the number or size of nucleoli was altered in the mutant cell lines, we counted the number of nucleoli present in DIC images of the different cell lines and found no obvious differences. Just as the wild-type, all mutant cell lines contained a wide range of cells having from 1-6 nucleoli with an average of ~3 nucleoli per cell. For example, in Supplementary Fig. S1A the 3KO cell has 5 nucleoli, but in Supplementary Fig. S1B some 3K0 cells have just 2 or 3. The same holds for the other constructs. In addition, the variability in the size of the nucleoli within the wild-type cells was similar to the variability seen in mutant cells. This makes it virtually impossible to distinguish the cell lines based on DIC images or Hoechst images alone, suggesting the abnormal co-localization to nucleoli does not significantly impact their morphology within the cell.

To account for these observations, we now refer to Supplementary Fig. S1A in the Results section of the manuscript, pointing out the abnormal nucleolar localization of the H1 mutant constructs and the normal heterochromatic localization. We also include a caveat in the Discussion on the top of p. 13 noting that this abnormal nucleolar localization could alter cell metabolism and potentially alter the FRAPs in the mutant cell lines.

