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A model for mitotic inheritance of histone lysine methylation

Mo Xu, Weixiang Wang, She Chen and Bing Zhu

Corresponding author: Bing Zhu, National Institute of Biological Sciences, Beijing

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Correspondence:	01 July 2011
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Transaction Report:

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

1st Editorial Decision 27 June 2011

Thank you for the submission of your research manuscript to EMBO reports. It has been sent to three referees, and so far we have received reports from two of them, which I copy below. As both referees feel that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. As soon as we receive the third report it will be forwarded to you.

Referee #1 has one major concern, which is the interpretation of the data presented in figures 3 and 4. Regarding figure 3, the referee considers that the conclusions derived do not fit the results shown and this should be clarified or corrected if necessary. A similar concern applies to figure 4 as referee considers that the conclusion reached does not take into account results presented in figure 3. Referee #3 is concerned with the effect of a prolonged cell-cycle arrest, as it could affect cell viability and significantly alter the results observed. This concern is shared with referee #1. Referee #3 also thinks that prevailing views of histone metabolism should be incorporated into the model shown in figure 5 and the general significance of the "buffer model" in transcriptional control should be discussed.

Given the reviewers constructive comments and the potential interest of your study, I would like to

give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports 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.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

RE: Comments on Xu et.al 2011 peer review for EMBO reports

In this study the authors utilized SILAC technology to investigate mitotic dynamics of individual histone H3 lysine methylation marks. The first notable observation presented is that newly incorporated histones do not acquire equal levels of lysine methylation compared to parental histones, even after one complete cell cycle. This is an interesting new finding that is obtained from a well-controlled experiment and merits to be reported (Figure 1). The transient decrease of the relative abundance of the lysine methyl marks during S-phase (Figure 2) is expected, given the bulk synthesis of histones during the S-phase. The next two observations relate to the maintenance and turnover of methylation in arrested cells. The conclusions drawn from these experiments appear to be overinterpretations, and the model presented (Figure 5) overlooks well-established spreading capabilities of repressive histone lysine methylation marks. In this reviewers' opinion the authors should re-consider the interpretation of the results presented in Figures 3 and 4. Moreover, the model - if one required - should include alternative explanations of their observations.

Major points:
(1) In their interpretation of the observations presented in Figure 3 (prolonged arrest at G1/S boundary), authors state that the abundance of the most of the methylation marks examined did not change, and four methylation marks changed slightly after 24h arrest. However, the relative abundance of the H3K27me0K36me0 is decreased about 40% in 24h (Figure 3D). Therefore, the above statement appears to be incorrect interpretation of the data. Furthermore, the relative abundance of the H3K9me0 is progressively decreasing up to 30% as the cells continue to arrest (Figure 3B). This is accompanied by the decrease in relative abundance of H3K9me1 and increase in that of H3K9me2, suggesting that there is an active conversion of me1 to me2 during the prolonged arrest. However, the authors interpret these data as a 'steady state' that is maintained by preventing histone methylation and promoting active histone de-methylation. The data presented in figure 3 suggests that there is an active methylation (rather than de-methylation) that is operating during the prolonged replication arrest, assuming that H3.1 levels are internally controlled in the SILAC experiment and there is no preferential loss/degradation of H3K9me0 histones. The authors should clarify/correct interpretation of these data.

(2) In the experiment presented in Figure 4, authors claim to measure the turnover of the methyl marks during the prolonged replication arrest by heavy metabolic labeling the precursor of the methyl moiety. However, this measurement doest not reflect an absolute dynamics or turnover of the preexisting H3K9me2 marks. This also includes the active conversion of H3K9me1 to H3K9me2, which is evident when Figures 3B and 4C are considered together. Therefore, it might be misleading to talk about the measurement of the absolute turnover rate of the H3K9me2.

Other points:
(3) A prolonged arrest of 72h with HU after double thymidine treatment is a significant amount of stress exerted to the cell. Authors did not present a FACS profile of these cells used for experiments

presented in Figures 3 and 4. These profiles (where apoptotic cells are not gated out) should be presented to understand the relative amount of apoptotic cells present in the culture and physiological nature of these experiments. Moreover, the differences observed at 48h and 72h time points in figure 3 should also be clearly discussed in the text.

(4) Figure 1A and 2A: authors should mark the time points for each treatment/release, in order to increase the readability of the figure and supplementary FACS data.

(5) Figure 3A: it is not labeled whether the two histone sources used in the SILAC experiment are mixed in 1:1 ratio, therefore internally normalized?

(6) The dynamics of histone methylation at heterochromatin regions, including changes of H3K9me during S-phase and its subsequent recovery, has been documented using alternative methods (e.g. see Chen et Nature 451:734). The authors should include this in their discussion of the work presented.

Overall, this is a good paper.

Referee #3:

In this manuscript, the authors, use stable isotope labeling by amino acids (SILAC) based quantitative mass spectrometry to address the stability and regeneration of histone methylation modifications linked to epigenetic regulation. The question the authors address is an important one for the epigenetics field and would have wide interest to the readership of EMBO Reports. Histone modifications, in particular histone methylation, have been linked to the phenomenon of epigenetic inheritance yet a mechanism demonstrating how these marks are propagated faithfully through mitotic cell generations has not been developed. The authors use a cell-cycle arrest protocol (double thymidine block) to arrest HeLa cells in G1/S and then to metabolically label the cells to monitor the kinetics of histone methylation marks through a single cycle. They show that higher histone methylation states are not completely regenerated during transit through a single cell cycle. To determine if the inability to regenerate fully the higher methylation states was actively prevented by histone demethylase activity or if the effect was due to inhibition of histone methyltransferase activity, the authors use an extended cell cycle arrest protocol. This protocol involves an initial double thymidine block with two subsequent hydroxyurea treatments and a metabolic labeling step to pulse with labeled amino acid. The authors' data suggest that histone H3 lysine 9 (H3K9) dimethylation is maintained in equilibrium with other H3K9 methylation states that might be due to active demethylation. However, their data suggests that another histone H3 methylation mark, lysine 79 (H3K79), is likely regulated by controlling histone methyltransferase activity.

Overall, I found the studies presented in this manuscript both important and intriguing. However, I do have significant technical concerns regarding the prolonged cell-cycle arrest experiments outlined in Figures 3 and 4. The single greatest concern is the possibility that the combination of double hydroxyurea (HU) treatment, which in itself creates DNA replication stress, combined with the double thymidine block treatment for the extended timepoints (up to 72 hours), may be significantly affecting the viability of the cell population. The authors provide no data regarding the viability of the cells at the 48 and 72 hour time points. Do these cell cycle arrest conditions push the cells into senescent and/or apoptotic pathways or is the entire population of cells viable? Could the differential effects on H3K9me2 and H3K79me establishment and maintenance be skewed by sub-populations of cells undergoing senescence or apoptosis since the authors are examining bulk histone populations and not discriminating between possible sub-populations of cells? I believe these are key issues to address before the authors can be confident of their interpretations of the data from Figures 3 and 4.

The other concern is in regard to the authors' "buffer model" presented in Figure 5. Although their model contains important points, it should be presented in conjunction with other aspects of histone metabolism that demarcate transcriptionally active genomic regions versus transcriptionally silent regions. The authors' data only addresses bulk histone populations so it is possible that at defined regions of regulatory DNA for transcriptionally active genes, other mechanisms may be involved in

the maintenance of transcriptional memory. Specifically, the idea that transcription-dependent incorporation of post-translationally modified histone H3.3 on and/or near the transcriptional start sites of active genes could act in concert with biases in the underlying DNA sequences to lower the density of replication specific histone H3.1 containing nucleosomes. The "buffer model" may be more relevant in the maintenance of tissue restricted genes that have been initially silenced through active means and then maintained silent via "buffering". A more thorough incorporation of their model into the prevailing views of this process would enhance the well-roundedness of the manuscript.

Additional Correspondence 01 July 2011

Thank you again for the submission of your research manuscript to EMBO reports. As I mentioned in my previous e-mail, your manuscript had been sent to three referees but only two reports had been received. We have just received the third report, which I paste below. Referee #2 is also positive in line with the other two referees, but s/he has raised some concerns that should be addressed in your revised manuscript.

Referee #2 is mainly concerned with two issues. The first one is shared with the other two referees and relates to the effects of the long cell cycle arrest in cell viability. The second major concern refers to the proof-of-concept of your model, and the referee suggests performing ChIP analysis on a selected locus to follow the methylation changes throughout a full cell cycle. I believe that this kind of experiment might fall outside the scope of EMBO reports given the length restrictions and the experiments already suggested by this and the other referees.

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REFEREE REPORT:

Referee #2:

Chromatin modifications, comprising histone marks and DNA methylation, convey epigenetic information determining transcriptional activity of underlying genes and thereby the differential potential and cell fate. S-phase represents a critical window for maintenance of epigenetic information as recycled parental and newly deposited histones are combined on replicated DNA strands. It is assumed that post-translational modifications (PTMs) on parental histones act as a blueprint for new histones leading to full restoration of chromatin organization and structure. Thus the question of how new histones acquire the PTM profile of parental histones is central to epigenetics.

The manuscript of Xu et al. approach this challenging question taking advantage of the state-of-the-art mass-spectrometry technology combined with SILAC. By analysing histone H3 methylation (lysines K9, K27, K36, K79) the authors show that the kinetics of new histones reaching the methylation level of parental histones is rather slow, consistent with a previous work studying H3K9 and K27 methylation (Scharf et al., 2009).
In the current study of Xu et al., new histones appear not to reach the methylation level of parental histones even after one complete cell cycle (Figure 1). Despite this rather unexpected result, the global methylation levels on the other hand appear to be maintained (Figure 2). On this basis the authors conclude that after replication new methyl groups are added to both new and old histones - and this ensures the

epigenetic state of the target locus, rather than full restoration of mehtylation patterns on new histones. This is an important finding that should be published, but the authors need to address the points listed below in order to support their strong conclusions.

Major comments

1.
The authors apply long (up to 72 hrs) HU treatment to arrest cells and address methylation dynamics. The histone H3 methylation levels remained largely unchanged, suggesting an active mechanism securing steady-state levels in these cells (Figure 3). In the same set-up the authors also analyze turnover of histone methylation. Whereas turnover of H3K9me2 appeared very dynamic, this was not the case for K9me3 and K79me1/2. These are interesting observations that support the proposed model. However, long HU treatment (> 6hrs) induce sever DNA damage including DSBs and a major concern is thus that these cells experiencing massive damage (possibly dying) do not recapitulate the situation in long term arrested (i.e. quiescent or differentiated) cells. The experiments in figure 3 and 4 should therefore be repeated in G1 arrested cells (e.g., contact inhibition or serum starvation of primary cells).

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The peptides analyzed in figure 1 can carry additional modifications (H3K9ac, H3K14ac, H3S10P; H3S28P). The authors should provide data or information on how this will influence their results.

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The authors propose a far-reaching 'buffer model' suggesting histone methylation levels could be maintained at 'regional' level rather than at 'local' level of individual nucleosomes may be true on genome-wide level. As a proof of principle they should select a locus broadly enriched in H3K27me3 or H3K9me3 and follow how the level changes through a full cell cycle by ChIP (from G1/S to G1/S). Their model predicts that the me3 level is reduced to half throughout the locus after replication, but that the total level will be restored in G1 of the following cell cycle.

Minor comments

1. The authors should expand on the discussion of figure 1 & 2 and state clearly how they reach the conclusions illustrated in their model.

2. The authors should mention that also Sharf et al., 2009 found by MS approach that H3K27me3 restoration is slow.

3. It would be interesting to follow the 'new' histones throughout the next cell cycle and see when the me3 levels are restored. Did the authors try allowing the cells to entre the next S phase without Lysine 8?

Point-by-point response to reviewers' comments

Editor's comments:

Referee #1 has one major concern, which is the interpretation of the data presented in figures 3 and 4. Regarding figure 3, the referee considers that the conclusions derived do not fit the results shown and this should be clarified or corrected if necessary. A similar concern applies to figure 4 as referee considers that the conclusion reached does not take into account results presented in figure 3. Referee #3 is concerned with the effect of a prolonged cell-cycle arrest, as it could affect cell viability and significantly alter the results observed. This concern is shared with referee #1 Referee #3 also thinks that prevailing views of histone metabolism should be incorporated into the model shown in figure 5 and the general significance of the "buffer model" in transcriptional control should be discussed. Referee #2 is mainly concerned with two issues. The first one is shared with the other two referees and relates to the effects of the long cell cycle arrest in cell viability. The second major concern refers to the proof-of-concept of your model, and the referee suggests performing ChIP analysis on a selected locus to follow the methylation changes throughout a full cell cycle. I believe that this kind of experiment might fall outside the scope of EMBO reports given the length restrictions and the experiments already suggested by this and the other referees.

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(1) In their interpretation of the observations presented in Figure 3 (prolonged arrest at G1/S boundary), authors state that the abundance of the most of the methylation marks examined did not change, and four methylation marks changed slightly after 24h arrest. However, the

relative abundance of the H3K27me0K36me0 is decreased about 40% in 24h (Figure 3D). Therefore, the above statement appears to be incorrect interpretation of the data.

(We apologize for our misleading sentence in the previous version. We wanted to say that the changes were subtle between 24 h and 72 h, and we used "after" 24 h in the previous version. We recognized that it was a very misleading sentence. So we have rephrased our statement as "The levels of most methylation states on H3.1 histones continued to change during the first 24 hours of additional HU arrest. Generally, the lower methylation states tended to decrease whereas the higher methylation states tended to increase, suggesting a further conversion from the lower methylation states to the higher methylation states (Figs. 3B-E). However, during 24-72 h extended G1/S phase arrest, histone methylation levels tended to reach a steady state.")

Furthermore, the relative abundance of the H3K9me0 is progressively decreasing up to 30% as the cells continue to arrest (Figure 3B). This is accompanied by the decrease in relative abundance of H3K9me1 and increase in that of H3K9me2, suggesting that there is an active conversion of me1 to me2 during the prolonged arrest. However, the authors interpret these data as a 'steady state' that is maintained by preventing histone methylation and promoting active histone de-methylation. The data presented in figure 3 suggests that there is an active methylation (rather than de-methylation) that is operating during the prolonged replication arrest, assuming that H3.1 levels are internally controlled in the SILAC experiment and there is no preferential loss/degradation of H3K9me0 histones. The authors should clarify/correct interpretation of these data.

(We thank the reviewer's careful consideration on this issue. To provide a better explanation, we measured the relative abundance of different modification states on peptide H3:K9-R17 in HeLaS3 cells arrested for 24 hrs. As shown in our new Fig. S8, H3K9me0/1/2/3 accounted for about 13%, 7.5%, 32.6% and 47.1%. This ratio explains why a 30% decrease of H3K9me0 and 10% decrease of H3K9me1 resulted to only about 10% increase of H3K9me2 and almost no change of H3K9me3 during 24-72 hours in Fig. 3B. On the other hand, although there was an increase of H3K9me2 during 24-72 hrs, the alternation per unit time was significantly lower compared to dividing cells. This suggests the level of H3K9me2 was restricted in long-term arrested cells. We have rephrased our statement to "These data suggest that in cells experiencing an extended G1/S-phase arrest, upon "maturation" of the methylation states, the levels of higher methylation states tend to be maintained at relatively stable levels, which suggest the existence of active mechanisms that restrict the activity of histone methyltransferases and/or that promote active histone demethylation.")

(2) In the experiment presented in Figure 4, authors claim to measure the turnover of the methyl marks during the prolonged replication arrest by heavy metabolic labeling the precursor of the methyl moiety. However, this measurement doest not reflect an absolute dynamics or turnover of the preexisting H3K9me2 marks. This also includes the active conversion of H3K9me1 to H3K9me2, which is evident when Figures 3B and 4C are considered together. Therefore, it might be misleading to talk about the measurement of the absolute turnover rate of the H3K9me2.

During 24-72h the overall abundance of H3K9me2 (Fig. 3B) increased for about 10%. Even if all these 10% H3K9me2 carry two new methyl groups, the corresponding ratio shown in

Fig. 4C should change from 37%:29%:34% at 24h to 33.6%:26.4%:40% at 72h. However, in our experiment, the ratio at 72h was 15%:24%:61%, which suggests that during 24-72h, significant amounts of heavy methyl groups on H3K9me2 peptides appeared in the form of turnover. These sentences were added into the text.

Other points:

(3) A prolonged arrest of 72h with HU after double thymidine treatment is a significant amount of stress exerted to the cell. Authors did not present a FACS profile of these cells used for experiments presented in Figures 3 and 4. These profiles (where apoptotic cells are not gated out) should be presented to understand the relative amount of apoptotic cells present in the culture and physiological nature of these experiments. Moreover, the differences observed at 48h and 72h time points in figure 3 should also be clearly discussed in the text.

This is a point shared by the other two reviewers. According to reviewer 1's suggestion, we performed FACS analysis for these cells. As shown in the new Fig. S6, at the 0 and 24 h time points (which were treated by HU for 12 and 36 h respectively), the sub-G1 peak (apoptotic cells) accounted for 1.6%-1.7% of total cells, which were similar to untreated cells. For cells at the 48 and 72 h time points, the sub-G1 peak accounted for 4.6%-4.7% of total cells. Given that there were only less than 5% of apoptotic cells in the population, our conclusions would not be altered.

We also would like to point out that the H3 histones studied in this report were purified H3.1 histones (Fig. S1). And we did not observe any incorporation of new H3.1 histones under the HU treatments (Fig. 4B), which is an indication that repair-coupled histone deposition was too low to have any contribution to our study.

(4) Figure 1A and 2A: authors should mark the time points for each treatment/release, in order to increase the readability of the figure and supplementary FACS data.

We thank the reviewer's suggestion, and we have added the above information into the figures.

(5) Figure 3A: it is not labeled whether the two histone sources used in the SILAC experiment are mixed in 1:1 ratio, therefore internally normalized?

We thank the reviewer's careful reading. We added "mixed in approximately 1:1 ratio" in Fig 2A and Fig 3A. In our experiments, we mixed the histones in approximately 1:1 ratio. The actual mixing ratios were determined by the backbone peptides and the relative abundance of each modified peptide was normalized to the ratio of backbone peptides.

(6) The dynamics of histone methylation at heterochromatin regions, including changes of H3K9me during S-phase and its subsequent recovery, has been documented using alternative methods (e.g. see Chen et Nature 451:734). The authors should include this in their discussion of the work presented.

We have incorporated the above suggestion into our manuscript and cited the study mentioned above.

Overall, this is a good paper.

Referee #2:

Chromatin modifications, comprising histone marks and DNA methylation, convey epigenetic information determining transcriptional activity of underlying genes and thereby the differential potential and cell fate. S-phase represents a critical window or maintenance of epigenetic information as recycled parental and newly deposited histones are combined on replicated DNA strands. It is assumed that post-translational modifications (PTMs) on parental histones act as a blueprint for new histones leading to full restoration of chromatin organization and structure. Thus the question of how new histones acquire the PTM profile of parental histones is central to epigenetics.

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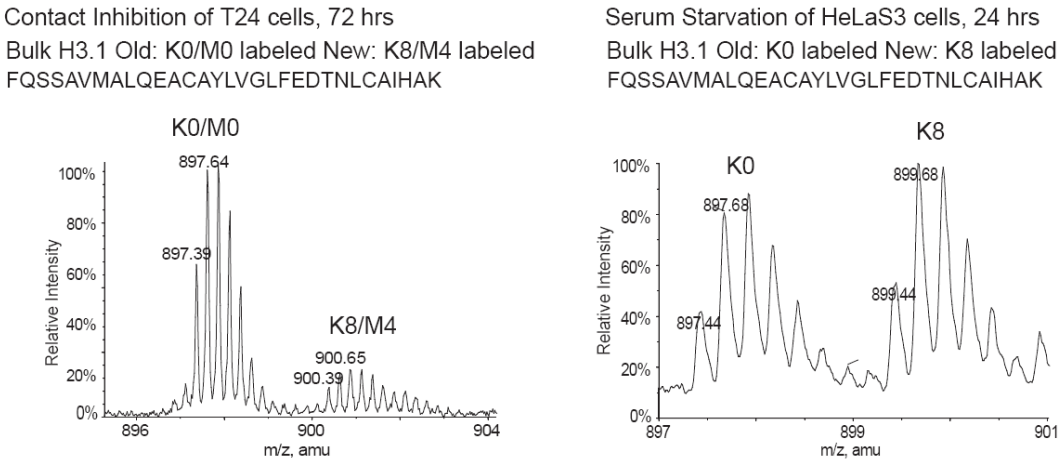
Major comments

1. The authors apply long (up to 72 hrs) HU treatment to arrest cells and address methylation dynamics. The histone H3 methylation levels remained largely unchanged, suggesting an active mechanism securing steady-state levels in these cells (Figure 3). In the same set-up the authors also analyze turnover of histone methylation. Whereas turnover of H3K9me2 appeared very dynamic, this was not the case for K9me3 and K79me1/2. These are interesting observations that support the proposed model. However, long HU treatment (6hrs) induce sever DNA damage including DSBs and a major concern is thus that these cells experiencing Massive damage (possibly dying) do not recapitulate the situation in long term arrested (i.e. quiescent or differentiated) cells. The experiments in figure 3 and 4 should therefore be repeated in G1 arrested cells (e.g., contact inhibition or serum starvation of primary cells).

We attempted to perform the suggested experiments with contact inhibition and serum starvation. The contact inhibition experiments were performed with T24 bladder carcinoma

cells, which were known to enter into G0 phase after saturation (PNAS 1997, 94: 12075-12080). However, using K8/M4 labeling, we found that a small proportion newly synthesized H3.1 can still be incorporated into chromatin during contact inhibition, which indicated the cell cycle was not fully blocked. (Figure pasted below, left panel). We also tried serum starvation, which also failed to fully arrest the HeLaS3 cells (Figure pasted below, right panel). Although both contact inhibition and serum starvation can arrest the cells to certain extent, we failed to fully arrest the cells with these approaches. Therefore, we were unable to perform the experiments further, because the incorporation of newly synthesized H3.1 in that portion of unarrested cells would jeopardize our experiments.



Cell viability and DNA damage during HU treatment is a point shared by the other two reviewers. According to reviewer 1's suggestion, we performed FACS analysis for these cells. As shown in the new Fig. S6, at the 0 and 24 h time points (which were treated by HU for 12 and 36 h respectively), the sub-G1 peak (apoptotic cells) accounted for 1.6%-1.7% of total cells, which were similar to untreated cells. For cells at the 48 and 72 h time points, the sub-G1 peak accounted for 4.6%-4.7% of total cells. Given that there were only less than 5% of apoptotic cells in the population, our conclusions would not be altered.

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2. The peptides analyzed in figure 1 can carry additional modifications (H3K9ac, H3K14ac, H3S10P; H3S28P). The authors should provide data or information on how this will influence their results.

We appreciate the reviewer's constructive suggestion. We included the K8/K0 curve and overall abundance curve of peptides carrying H3K9ac and H3K14ac into our Figs S3A, S5A, S7A and S8. As shown in these figures, in the case of H3:K9-R17 peptides containing both methylation and acetylation, the shape of their curves were highly similar to those of peptides

that only contain the corresponding methylation states, which is an indication of the fast turnover of histone acetylation. We also attempted to analyze the re-establishment of H3S10p and H3S28p. However, the abundance of these modifications was too low to be detected and quantified.

3. The authors propose a far-reaching 'buffer model' suggesting histone methylation levels could be maintained at 'regional' level rather than at 'local' level of individual nucleosomes may be true on genome-wide level. As a proof of principle they should select a locus broadly enriched in H3K27me3 or H3K9me3 and follow how the level changes through a full cell cycle by ChIP (from G1/S to G1/S). Their model predicts that the me3 level is reduced to half throughout the locus after replication, but that the total level will be restored in G1 of the following cell cycle.

We are currently performing similar experiments, at the genome-wide level. But we agree with the editor that these experiments are beyond the scope of our current story. Finally, as a proof of principle, we would like to refer to a previous publication pointed out by reviewer 1 that H3K9me2 experiences a similar transient decrease at the fission yeast pericentric heterochromatin (Chen et al Nature 2008, 451:734-737). We have cited this paper in our discussion.

Minor comments

1. The authors should expand on the discussion of figure 1 & 2 and state clearly how they reach the conclusions illustrated in their model.

We thank the reviewer's constructive comment and add more sentences into the discussion. But we cannot elaborate further since the manuscript is very close to the word count limit.

2. The authors should mention that also Sharf et al., 2009 found by MS approach that H3K27me3 restoration is slow.

We cited the above paper. In our introduction, we also mentioned their attempt in determining the restoration of H3K27me3. MALDI-TOF experiments were used to study the H3K27 and K36 methylation in that report, which could not distinguish the isobaric ions, including H3K27me3, H3K36me3, H3K27me2/K36me1 and H3K27me1/K36me2. Therefore, it was not clear what exactly was slow.

3. It would be interesting to follow the 'new' histones throughout the next cell cycle and see when the me3 levels are restored. Did the authors try allowing the cells to enter the next S phase without Lysine 8?

Ideally, to perform the suggested experiment, we need a third label in the next S phase, because we need the initial K0 labeled histones as an internal reference for quantifying our K8 labeled histones. However, if we introduce a three-label combination; it would greatly increase the complexity for our quantification and only allow us to do some limited analysis. For example, in our Figure 4, where we introduced a third label, we became unable to analyze the K27/K36 methylation states due to overlapping peaks from the isobaric ions and ions with close m/z values. Moreover, we have already shown that the old histones continued to get

new modifications, and we do not think this conclusion would be altered.

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Overall, I found the studies presented in this manuscript both important and intriguing. However, I do have significant technical concerns regarding the prolonged cell-cycle arrest experiments outlined in Figures 3 and 4. The single greatest concern is the possibility that the combination of double hydroxyurea (HU) treatment, which in itself creates DNA replication stress, combined with the double thymidine block treatment for the extended time points (up to 72 hours), may be significantly affecting the viability of the cell population. The authors provide no data regarding the viability of the cells at the 48 and 72 hour time points. Do these cell cycle arrest conditions push the cells into senescent and/or apoptotic pathways or is the entire population of cells viable? Could the differential effects on H3K9me2 and H3K79me establishment and maintenance be skewed by sub-populations of cells undergoing senescence or apoptosis since the authors are examining bulk histone populations and not discriminating between possible sub-populations of cells? I believe these are key issues to address before the authors can be confident of their interpretations of the data from Figures 3 and 4.

This is a point shared by the other two reviewers. According to reviewer 1's suggestion, we performed FACS analysis for these cells. As shown in the new Fig. S6, at the 0 and 24 h time points (which were treated by HU for 12 and 36 h respectively), the sub-G1 peak (apoptotic cells) accounted for 1.6%-1.7% of total cells, which were similar to untreated cells. For cells at the 48 and 72 h time points, the sub-G1 peak accounted for 4.6%-4.7% of total cells. Given

that there were only less than 5% of apoptotic cells in the population, our conclusions would not be altered.

We also would like to point out that the H3 histones studied in this report were purified H3.1 histones (Fig. S1). And we did not observe any incorporation of new H3.1 histones under the HU treatments (Fig. 4B), which is an indication that repair-coupled histone deposition was too low to have any contribution to our study.

The other concern is in regard to the authors' "buffer model" presented in Figure 5. Although their model contains important points, it should be presented in conjunction with other aspects of histone metabolism that demarcate transcriptionally active genomic regions versus transcriptionally silent regions. The authors' data only addresses bulk histone populations so it is possible that at defined regions of regulatory DNA for transcriptionally active genes, other mechanisms may be involved in the maintenance of transcriptional memory. Specifically, the idea that transcription-dependent incorporation of post-translationally modified histone H3.3 on and/or near the transcriptional start sites of active genes could act in concert with biases in the underlying DNA sequences to lower the density of replication specific histone H3.1 containing nucleosomes. The "buffer model" may be more relevant in the maintenance of tissue restricted genes that have been initially silenced through active means and then maintained silent via "buffering". A more thorough incorporation of their model into the prevailing views of this process would enhance the well-roundedness of the manuscript.

We agree with the reviewers' point that our "buffer" model is "more relevant in the maintenance of tissue restricted genes that have been initially silenced through active means and then maintained silent via 'buffering' " and we specifically pointed out that our model is for explaining the epigenetic silencing. To our understanding, the maintenance of "active marks" may not be an "epigenetic" event, which might be a consequence of active transcription, although Trithorax mediated Polycomb derepression might be an exception. We have added more discussions related to these issues into the text.

2nd Editorial Decision 28 September 2011

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Yours sincerely,

Editor
EMBO Reports