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Breaking the HAC Barrier: Histone H3K9 acetyl/methyl balance regulates CENP-A assembly

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1st Editorial Decision 25 November 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports from three expert reviewers, which are copied below. I am pleased to inform you that all referees appreciate the potential interest of your findings and would in principle support publication in the journal, pending adequate revision. In this respect, they all raise a number of specific concerns and open question. Some of these points (in particular referee 3's first major point, to examine a wider range of histone modifications) in my opinion probably exceed the scope of the current study, and some others (e.g. referee 1's point 3) may not necessarily require additional experimentation for being adequately addressed. Nevertheless, there are also a number of well-taken requests and straightforward experimental suggestions likely to benefit the overall conclusions and insights of the study, such as points 1 and 4 of referee 1, or referee 2's question for relating the presently studied epigenetic marks with those reported in your earlier work.

In light of these reports, I would like to invite you to prepare a revised version of the manuscript along the lines raised in the referees' comments. In this respect, please be reminded that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all the specific points raised at this stage in the process. When preparing your letter of response to the referees' comments, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Ohzeki et al. uses integrated and episomal alpha satellite arrays which include Tet operators to examine the role of histone modification on the establishment and assembly of centromeric chromatin. H3K4 methylation has been shown previously to inhibit the formation of CENP-A containing chromatin. The authors correlate the inefficient HAC formation in HeLa cells with an increased level of Suv39h and increased H3K9me4. They go on to demonstrate that increasing methylation at an alphoid containing array limits CENP-A deposition, while increasing acetylation increases CENP-A recruitment and HAC stability. Overall this is an interesting and well conducted study that significantly extends our knowledge of how modification of histone H3 may affect centromere assembly and inheritance.
However, two major concerns persist which are addressed in the specific comments below. The first is whether HAT activation which the authors demonstrate to facilitate de novo CENP-A deposition underlies the endogenous mechanism of CENP-A deposition during the G1 phase of the cell cycle. The second is to what degree the modification changes can account for limited HAC formation in a variety of cell types.

1. HAT activity is up-regulated at the array by fusing HATs to TetR. The authors need to demonstrate whether CENP-A deposition driven at the array by HAT targeting is occurring with the same timing as endogenous CENP-A. When TetR HAT fusions constitutively targeted to the tetO alphoid array is HJURP recruited only in G1 or is CENP-A deposition uncoupled from the cell cycle. Certainly if the spike of H3 acetylation demonstrated in Figure 7 is what is mimicked by HAT targeting we would expect CENP-A deposition to occur throughout the cell cycle when HATs are targeted. The two possible outcomes may delineate roles of HAT activity in centromere deposition.

2. The authors show that increasing HAT activation, decreasing methyltransferase activity or targeting CENP-A deposition factors leads to increased CENP-A deposition. However this does not determine whether these approaches are equally efficient at creating a stable epigenetically maintained centromere. How does targeting Mis18 or HJURP compare in the efficiency of HAC formation to targeting HATs?

3. The authors also show in Figure 3, D-F as well as Supp. 8c that the HAT activity is not required to maintain CENP-A at the alphoid array. As the authors state, this suggests that the HAT activity may just be required for initial CENP-A establishment. This raises the question of how long HAT activity needs to be present initially to establish stable HAC formation. It evens seems that there is an increase in CENP-A levels from weeks 4-6 (Figure 3c vs. Supp. 8c). Why do the authors think there is an increase in CENP-A levels once the HAT domains are removed while H3K9ac levels

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remain stable?

4. In Figure 4 the authors combine looking at HAT-induced CENP-A assembly with depleting known CENP-A assembly players, Mis18 and HJURP. They conclude that while depleting Mis18 does decrease CENP-A levels as previously shown, that they can rescue this depletion in CENP-A levels by targeting the HAT domains. The authors also show an increase in H3Kac following mitotic release. This argues that the Mis18 complex may have a role in recruiting a HAT to endogenous centromeres. Does Mis18 siRNA eliminate localization of p300 or PCAF to centromeres and/or eliminate the spike in H3 acetylation observed in early G1? Does siRNA against p300 or PCAF alter the acetylation of endogenous centromeric alphoid DNA and recruitment of HJURP?

5. The correlation between Suv29h, and H3K9Me3 levels at alphoid DNA and the potential to form HAC in HeLa versus HT1080 cells is very intriguing. The degree to which these changes account for HAC efficiency in a range of cell types is an important aspect of this study that is not full addressed The authors state " Surprisingly, HeLa cells, TIG7 human fetal primary and hTERT-BJ1 immortalized fibroblasts, could efficiently assemble CENP-A chromatin de novo, but declined rapidly (Fig. 1b,c and Supplementary Fig. S2 and S3)." However, I did not see the data on CENP-A de novo assembly in the TIG7 and hTERT-BJ1 cells. Furthermore the change in HAC efficiency in response to HAT targeting in these cell types is also required to fully substantiate the assertion that modification H3K9 is general mechanism of controlling CENP-A deposition. Also, does HAT targeting further increase the HAC efficiency in HT1080 cells, or does the incomplete efficiency (granted that 30% is pretty good) in HT1080 cells represent a different barrier to HAC formation?

Minor points:

1. Page 6, first line, the sentence is not finished.

2. The sentence at the end of the last paragraph on page 10 is also difficult to understand.

3. Ectopic is spelled incorrectly on the graph in Figure 6c

4. The sentence at the end of the 3rd paragraph on page 8 is confusing.

5. Figure 2f, It is unclear why the 4 day bar is green.

6. Figure 7C, Why do CENP-A signals not change between mitotically arrested cells and those released in G1?

Referee #2 (Remarks to the Author):

Centromeric chromatin has a unique nucleosomal organization that includes interspersion of nucleosomes that contain either CENP-A or H3. How centromere assembly is initiated and how CENP-A is recruited to sites of kinetochore assembly by the chaperone HJURP are questions that many in the field are keen to address. This group has used a clever protein tethering strategy on non-essential synthetic human artificial chromosomes to address the importance of acetylated chromatin in centromere assembly and function. The authors show that centromeric chromatin contains acetylated H3K9 at a crucial time in the cell cycle between late anaphase and early G1, corresponding with timing of Mis18, HJURP and new CENP-A recruitment to centromeres. At other times in the cell cycle, presumably after centromere assembly has been initiated, H3K9 acetylation decreases and H3K9 methylation increases. They also show that HAT-fusion proteins can bypass the need for Mis18, but not HJURP, in centromere/CENP-A assembly. These results imply that HJURP depends on histone acetylation to recruit or incorporate new CENP-A to centromeric sites. The authors show that once acetylated chromatin is established by tethering HAT-fusions to alpha

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satellite DNA, continued tethering is not necessary and the chromatin state is propagated over many cell divisions. The authors argue that moving between H3K9 acetylated and methylated states is important for maintaining normal centromeric chromatin organization and assembly.

Another interesting aspect of the study is the finding that HT1080 cells, THE cell line historically used to create human artificial chromosomes (HACs), express less SUV39H1 (the H3K9 methylase) than HeLa cells and subsequently HT1080 centromeres are more euchromatic. HACs have always been more readily generated in HT1080 cells than in HeLa cells. The authors show that SUV39H1 depletion in HeLa cells leads to HAC formation at rates similar to HT1080 cells, suggesting that relaxing the amount of heterochromatin in HeLa promotes de novo centromere assembly. The converse experiment - over-expressing SUV39H1 in HT1080s - resulted in the inability to make HACs. They also show that other cell lines, such as TIG7 and hTERT-BJ1 also have higher amounts of H3K9me than HT1080. This is an important result that will relieve many of the cell line constraints that have existed for over a decade in the HAC field.

The authors show tethering HAT-fusion proteins to HACs and ectopic alpha satellite arrays results in "hyper-assembly" of CENP-A on the entire HAC sequence and non-centromeric regions of the HAC. I could not find the data supporting this for the non-centromeric sequences. Was this tested by ChIP? How do they know for sure that CENP-A covers the entire HAC sequence molecularly? And why does it only do so in 33% or 66% of the cells? What accounts for such cell-to-cell variation in the spreading/hyper-incorporation of CENP-A even in the same line?

The highest levels of H3K9ac were detected after metaphase and in late telophase and G1, at the time when CENP-A assembly, mediated by HJURP, occurs. Is mitotic exit required for the pulse of H3K9ac? How do the authors put these new results in context with their previous H3K4me2/H3K36me2 study published in EMBO J? Specifically, how inter-dependent are H3K4me2, H3K36me, H3K9ac and transcription for CENP-A assembly? Are the H3K4me2 and H3K36me marks present at the same time as H3K9ac or do they occur at different times in the assembly/maintenance cycle?

Referee #3 (Remarks to the Author):

This paper aims to gain insight into the regulation of CENP-A assembly into centromeric chromatin, and focuses specifically on the role of H3K9 methylation and acetylation in that process. The authors show that tethering histone acetyltransferases or a methyltransferase to artificial chromosome constructs, either stimulates or inhibits de novo centromere formation, respectively. They also show that tethering acetyltransferases to artificial chromosomes bypasses the requirement for Mis18alpha for de novo CENP-A assembly, but that CENP-A assembly still requires HJURP. The authors show that H3K9 acetylation levels at endogenous centromeres peak at a time correlated with CENP-A assembly. This paper provides a significant advance in human artificial chromosome formation, and makes an important link between acetylation to de novo centromere formation. The authors propose an H3K9 acetyl/methyl switch that regulates centromere formation. In general, the experiments in this manuscript are well done and the results are convincing. However, the conclusions that H3K9 acetylation is the specific modification governing new centromere formation and that this represents an acetyl/methyl switch represent one of several possible interpretations for the data presented. This paper provides several important advances in the centromere formation field and I recommend this paper for publication once the authors have addressed the points listed below.

Major Points:

It is unclear why the authors choose to look specifically at H3K9 methylation and acetylation, and not at a wider range of modifications that could also be present at centromeres. It is possible that general acetylation or acetylation of an alternative specific residue, not specific acetylation on H3K9, could affect de novo CENP-A assembly. This would suggest a mechanism very different from their acetyl/methyl switch hypothesis. The authors should examine whether other

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modifications of H3 and H4 might also correlate with centromere formation particularly in light of the fact that pCAF and P300 are known to modify several different lysine residues in histones.

The authors argue that centromere formation is controlled by an "acetyl/methyl switch". A 'switch' implies that a single histone residue is either acetylated or methylated, and that if you increase one, you will decrease the other. The authors' data in Figures 2c and 7c and e shows that there is not a strong inverse correlation between H3K9 methylation and acetylation, which would be expected if the system were truly a "switch". One likely alternative is that the relative balance of these modifications throughout the chromatin could drive the propensity for centromere formation in different directions. The authors should either experimentally demonstrate switch like behavior akin to the phosphor/methyl switch controlling HP1 binding to chromatin or should change the language to more accurately describe this observation.

The authors note that their results raise the question of 'whether CENP-A assembly induced by acetylation of H3K9 is biologically relevant.' To address this, the authors perform ChIP experiments. However, it is not clear that the observed spike in acetylation is statistically significant. It is possible that another assay aside from ChIP might be able to better resolve this phenomenon. The authors acknowledge that identification of the naturally occurring HAT is paramount, and could also help to reinforce the biological significance. The authors do not need to identify the endogenous HAT for publication of this manuscript but improving the data for the naturally occurring H3K9 acetyl spike would significantly strengthen the manuscript.

Minor Points:

Although not necessary for publication of this manuscript, tethering a catalytically inactive acetyltransferase or methyltransferase would ensure that the activity is relevant for the phenotype rather than just the protein binding to chromatin.

In the text describing Figure 4, the authors state that they want to "test whether the same chromatin modifiers... also affect CENP-A maintainence at an established HAC kinetochore". They demonstrate this point nicely in Figure 3f but this is not what they are testing in this figure.

Why are very few HA-CENP-A labeled endogenous centromeres seen in Figures 4c and 5c.

The presentation of data in Figure 4g and 5e is confusing. One appropriate conclusion from this data is that the Mis18 depletion leads to an increase in CENP-A hyper assembly in cells that only have CENP-A at the HAC, which is not accounted for in the authors' results. It is not clear what the authors are attempting to demonstrate, it seems like this figure could be excluded.

The last sentence of the 4th paragraph of the results does not seem to be a complete sentence.

The values cited in the text for Figure 5e do not match the values actually displayed on the graph.

What is the significance of the differences seen in Figure 3c?

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Response to the referees' comments

Response to the referee #1

Referee #1 (Remarks to the Author):

The manuscript by Ohzeki et al. uses integrated and episomal alpha satellite arrays which include Tet operators to examine the role of histone modification on the establishment and assembly of centromeric chromatin. H3K4 methylation has been shown previously to inhibit the formation of CENP-A containing chromatin. The authors correlate the inefficient HAC formation in HeLa cells with an increased level of Suv39h and increased H3K9me4. They go on to demonstrate that increasing methylation at an alphoid containing array limits CENP-A deposition, while increasing acetylation increases CENP-A recruitment and HAC stability. Overall this is an interesting and well conducted study that significantly extends our knowledge of how modification of histone H3 may affect centromere assembly and inheritance.
However, two major concerns persist which are addressed in the specific comments below. The first is whether HAT activation which the authors demonstrate to facilitate de novo CENP-A deposition underlies the endogenous mechanism of CENP-A deposition during the G1 phase of the cell cycle. The second is to what degree the modification changes can account for limited HAC formation in a variety of cell types.

1. HAT activity is up-regulated at the array by fusing HATs to TetR. The authors need to demonstrate whether CENP-A deposition driven at the array by HAT targeting is occurring with the same timing as endogenous CENP-A. When TetR HAT fusions constitutively targeted to the tetO alphoid array is HJURP recruited only in G1 or is CENP-A deposition uncoupled from the cell cycle. Certainly if the spike of H3 acetylation demonstrated in Figure 7 is what is mimicked by HAT targeting we would expect CENP-A deposition to occur throughout the cell cycle when HATs are targeted. The two possible outcomes may delineate roles of HAT activity in centromere deposition.
Thank you for this important question. To determine the precise cell cycle timing of HAT-induced CENP-A assembly, we carried out new experiments using SNAP-CENP-A. We tethered tetR-EYFP-fused HATs for two hours by controlling doxycycline and labeled newly synthesized SNAP-CENP-A during this period (new Figure S13). These results show that newly synthesized SNAP-CENP-A can be assembled on the ectopic tetO alphoid DNA integration site following as little as two-hours of HAT tethering. Under these conditions, we estimated the cell cycle position by using Cyclin B staining (Silva et al., 2011) and found that CENP-A assembly induced by HAT tethering occurred only in Cyclin B negative (G1) cells. In addition, the "Orange" type assembly (Figure 4G, 5E) also occurred in G1 phase. (This might have happened in mid or late G1 when endogenous centromeres had already finished CENP-A replenishment.) Only HJURP tethering showed ectopic CENP-A assembly in Cyclin B positive (S/G2) cells. Presumably, other G1-specific factors might be required in the steps leading from HAT tethering to CENP-A deposition.

Reference
Silva MC, Bodor DL, Stellfox ME, Martins NM, Hochegger H, Foltz DR, Jansen LE. Cdk activity couples epigenetic centromere inheritance to cell cycle progression. Dev Cell. 2012 Jan 17;22(1):52-63.

2. The authors show that increasing HAT activation, decreasing methyltransferase activity or targeting CENP-A deposition factors leads to increased CENP-A deposition. However this does not determine whether these approaches are equally efficient at creating a stable epigenetically maintained centromere. How does targeting Mis18 or HJURP compare in the efficiency of HAC formation to targeting HATs?

We added new HAC formation data with hMis18 alpha and HJURP tethering in a new Figure S17. Tethering of either supported HAC formation in HeLa cells to an extent similar to that seen with HAT tethering (Figure 3E and Figure S17). In controls, tethering of tetR alone did not induce HAC formation. Thus, these new data combined with our original data indicate that tethering HAT activity, hMis18 alpha or HJURP are all similarly efficient at breaking the barrier for efficient CENP-A assembly, subsequent kinetochore formation and stable HAC formation in HeLa cells.

3. The authors also show in Figure 3, D-F as well as Supp. 8c that the HAT activity is not required to maintain CENP-A at the alphoid array. As the authors state, this suggests that the HAT activity may just be required for initial CENP-A establishment. This raises the question of how long HAT activity needs to be present initially to establish stable HAC formation.

We demonstrated in several ways that HAT activity on alphoid DNA induces de novo CENP-A assembly (Figure 3-5) and that once established CENP-A assembly is stably maintained (Figure 3F). In addition, we also showed a transient increase in H3K9ac on endogenous centromeric alphoid DNA and HAC alphoid DNA during mitotic exit (Figure 7C) and showed that this spike is diminished on the tetO HAC following Suv39H1 tethering (Figure 7E). Suv39h1 tethering also decreased CENP-A assembly (Figure 3C and Figure S7) and destabilized established HAC centromeres (Figure S9). Together, these data suggest that tethered HAT activity is only needed until HAC centromere establishment. After that, HAC centromeres are presumably maintained by endogenous HAT activity as part of the normal centromere assembly mechanism.

It evens seems that there is an increase in CENP-A levels from weeks 4-6 (Figure 3c vs. Supp. 8c). Why do the authors think there is an increase in CENP-A levels once the HAT domains are removed while H3K9ac levels remain stable?

Figure 3C results were obtained with bulk transformants, whereas Figure S8 results were obtained with singly isolated cell lines. Thus, although premature CENP-A assemblies that did not result in centromere establishment were inactivated during culturing of HeLa cells with stronger Suv39h1 activity, once established, centromeres stably maintained CENP-A assembly, presumably by a mechanism involving intrinsic HAT activity (as revealed by our detection of a transient increase of H3K9ac on endogenous centromeric alphoid DNA and HAC alphoid DNA - Figure 7C). As a result, CENP-A levels were decreased in bulk transformants but not in established HAC clones.

4. In Figure 4 the authors combine looking at HAT-induced CENP-A assembly with depleting known CENP-A assembly players, Mis18α; and HJURP. They conclude that while depleting Mis18α; does decrease CENP-A levels as previously shown, that they can rescue this depletion in CENP-A levels by targeting the HAT domains. The authors also show an increase in H3Kac following mitotic release. This argues that the Mis18 complex may have a role in recruiting a HAT to endogenous centromeres. Does Mis18 siRNA eliminate localization of p300 or PCAF to centromeres and/or eliminate the spike in H3 acetylation observed in early G1? Does siRNA against p300 or PCAF alter the acetylation of endogenous centromeric alphoid DNA and recruitment of HJURP?

This is a good question that raises the important issue of "What is the endogenous HAT responsible for the spike of H3K9ac activity during mitotic exit (and therefore for Mis18 alpha recruitment and CENP-A assembly)". Importantly, we do not believe that either p300 or PCAF is the endogenous HAT in G1 phase. Indeed in our experiments, both HAT domains were forced to target to alphoid^{tetO} DNA by expressing them as TetR fusion proteins. We examined the potential role of both proteins in endogenous centromere

assembly. As described below, our data do not provide strong evidence to support a direct relationship between Mis18 alpha and p300(or PCAF) at this time. To date, we have tethered six HATs to alphoid DNA and all showed similar positive effects on CENP-A assembly.

The question therefore becomes “What is the endogenous HAT recruited by Mis18 complex to centromeres and promoting CENP-A assembly”. To date at least 17 lysine acetyl transferases (KATs) have been identified in human (Allis et al, 2007). Redundancy of HAT proteins may increase the difficulty of interpretation of individual siRNA experiments, however the tethering experiments argue strongly for functional correlations between HAT activity and CENP-A. Thus, our data suggest the existence of another endogenous HAT protein that is recruited to centromeres by the Mis18 complex in early G1 phase. The identity of this HAT is an important subject for future research.

We did the following experiments to ask if either p300 or PCAF could be the endogenous HAT:

(i) We checked p300 and PCAF localization at metaphase centromeres. However, both the endogenous p300 and PCAF could localize centromere even following hMis18 alpha depletion (data not shown). p300 and PCAF may be recruited to metaphase centromere by other centromere proteins but not by hMis18 alpha.

(ii) Assembly of newly synthesized CENP-A (as in Figure 4F, 5D) was not affected by siRNA depletion of p300 and/or PCAF. This could be explained by a redundancy of HAT proteins because CBP and GCN5 are very similar to p300 and PCAF. A more likely possibility is that another HAT protein localizes to G1 centromeres and induces H3K9 acetylation during mitotic exit.

At this time, we do not show these data in the manuscript, however, some of the relevant results can be added if it were felt to be useful to extend further the Supplementary information.

Reference

Allis CD, Berger SL, Cote J, Dent S, Jenuwien T, Kouzarides T, Pillus L, Reinberg D, Shi Y, Shiekhattar R, Shilatifard A, Workman J, Zhang Y. New nomenclature for chromatin-modifying enzymes. Cell. 2007 Nov 16;131(4):633-6.

5. The correlation between Suv39h, and H3K9Me3 levels at alphoid DNA and the potential to form HAC in HeLa versus HT1080 cells is very intriguing. The degree to which these changes account for HAC efficiency in a range of cell types is an important aspect of this study that is not full addressed. The authors state " Surprisingly, HeLa cells, TIG7 human fetal primary and hTERT-BJ1 immortalized fibroblasts, could efficiently assemble CENP-A chromatin de novo, but declined rapidly (Fig. 1b,c and Supplementary Fig. S2 and S3)." However, I did not see the data on CENP-A de novo assembly in the TIG7 and hTERT-BJ1 cells.

We showed de novo CENP-A assembly activity in TIG7 and hTERT-BJ1 cells in the original Figure S3C, but did not include the data showing the decrease of CENP-A assembly. We have now included those data for TIG7, hTERT-BJ1 and U2OS (human osteosarcoma) cells in a new Figure S3C. As observed in HeLa, all those cells showed initial CENP-A assembly, with levels subsequently declining during culturing.

Furthermore the change in HAC efficiency in response to HAT targeting in these cell types is also required to fully substantiate the assertion that modification H3K9 is general mechanism of controlling CENP-A deposition. Also,

does HAT targeting further increase the HAC efficiency in HT1080 cells, or does the incomplete efficiency (granted that 30% is pretty good) in HT1080 cells represent a different barrier to HAC formation?

This is a good question. We added a new Figure S17 containing new data from HAC formation assays using tetR-EYFP-alone or tetR-EYFP-PCAF tethering in HT1080, HeLa and U2OS cells. U2OS centromeres have high levels of H3K9me3, similar to HeLa cells (new Figure S3B, C). As expected, we observed no HAC formation when the synthetic alphoid DNA array was introduced together with tetR-EYFP-alone in HeLa and U2OS cells. In contrast, HAT tethering supported HAC formation in U2OS as well as HeLa cells. Interestingly, HAT tethering in HT1080 cells did not significantly enhance HAC formation. Other factors, affecting the chromatin assembly balance - possibly including heterochromatin formation counteracting HAT activity - may also influence stable HAC formation as we discussed in the Discussion.

Minor points:

1. Page 6, first line, the sentence is not finished.

We added the missing words "have low levels of centromeric H3K9me3."

2. The sentence at the end of the last paragraph on page 10 is also difficult to understand.

We added "by tetR-EYFP-Suv39h1 tethering" to the end of the sentence.

3. Ectopic is spelled incorrectly on the graph in Figure 6c

We corrected this spelling.

4. The sentence at the end of the 3rd paragraph on page 8 is confusing.

We modified that paragraph. Please see the main text.

5. Figure 2f, It is unclear why the 4 day bar is green.

We agree with this. We changed the color to gray.

6. Figure 7C, Why do CENP-A signals not change between mitotically arrested cells and those released in G1?

According to current CENP-A replenishment models, CENP-A is deposited in (early) G1 phase, and CENP-A molecules are divided between the replicated DNA strands during S phase. Cytologically, this distribution is almost equal between sister chromatids in metaphase, however, a unit size and structure of CENP-A distribution is still unclear (e.g. mononucleosomes, oligonucleosomes or blocks of longer chromatin domains). We assume that if the CENP-A distribution unit is similar in size to ChIP input DNA (in our case ~500bp average = ~ three nucleosomes), the apparent ChIP enrichment would be less affected because each input DNA molecule still might have at least a single CENP-A molecule even after replication. In addition, it is possible that CENP-A is localized at centromeres in early G1 phase but not yet stably incorporated into mature nucleosomes (Perpelescu et al, 2009). Such CENP-A might be less efficiently detected by ChIP assays.

Response to the referee #2

Referee #2 (Remarks to the Author):

Centromeric chromatin has a unique nucleosomal organization that includes interspersion of nucleosomes that contain either CENP-A or H3. How centromere assembly is initiated and how CENP-A is recruited to sites of kinetochore assembly by the chaperone HJURP are questions that many in the field are keen to address. This group has used a clever protein tethering strategy on non-essential synthetic human artificial chromosomes to address the importance of acetylated chromatin in centromere assembly and function. The authors show that centromeric chromatin contains acetylated H3K9 at a crucial time in the cell cycle between late anaphase and early G1, corresponding with timing of Mis18, HJURP and new CENP-A recruitment to centromeres. At other times in the cell cycle, presumably after centromere assembly has been initiated, H3K9 acetylation decreases and H3K9 methylation increases. They also show that HAT-fusion proteins can bypass the need for Mis18, but not HJURP, in centromere/CENP-A assembly. These results imply that HJURP depends on histone acetylation to recruit or incorporate new CENP-A to centromeric sites. The authors show that once acetylated chromatin is established by tethering HAT-fusions to alpha satellite DNA, continued tethering is not necessary and the chromatin state is propagated over many cell divisions. The authors argue that moving between H3K9 acetylated and methylated states is important for maintaining normal centromeric chromatin organization and assembly.

Another interesting aspect of the study is the finding that HT1080 cells, THE cell line historically used to create human artificial chromosomes (HACs), express less SUV39H1 (the H3K9 methylase) than HeLa cells and subsequently HT1080 centromeres are more euchromatic. HACs have always been more readily generated in HT1080 cells than in HeLa cells. The authors show that SUV39H1 depletion in HeLa cells leads to HAC formation at rates similar to HT1080 cells, suggesting that relaxing the amount of heterochromatin in HeLa promotes de novo centromere assembly. The converse experiment - over-expressing SUV39H1 in HT1080s - resulted in the inability to make HACs.

We thank referee #2 for his/her positive comments. However, at some points, we may need to describe our results more precisely. Suv39h1 depletion in HeLa cells induced ectopic CENP-A assembly at alphoid DNA integration sites (Figure 2F), but HAC formation was not tested in those cells. This is because we had previously tested HAC formation in Suv39h1dn mouse embryonic fibroblast cells. In those cells we could detect a three-fold increase of CENP-A assembly at ectopic sites (Okada et al, 2007), but could not observe HAC formation (data not shown). Those results suggested that not only the reduction of negative regulation but also an increase in some form of positive regulation (such as acetylation as reported here) is needed for HAC centromere formation. Suv39h1 over-expression restored centromeric H3K9me3 levels in HT1080 cell (Figure 2B), and tethering of Suv39h1 on transfected alphoid^{tetO} in HT1080 cell diminished both HAC formation activity (Figure 3E) and CENP-A assembly (original Figure S7, present S8). We did not check HAC formation in Suv39h1 over-expressing HT1080 cells.

They also show that other cell lines, such as TIG7 and hTERT-BJ1 also have higher amounts of H3K9me than HT1080. This is an important result that will relieve many of the cell line constraints that have existed for over a decade in the HAC field.

The authors show tethering HAT-fusion proteins to HACs and ectopic alpha satellite arrays results in "hyper-assembly" of CENP-A on the entire HAC sequence and non-centromeric regions of the HAC. I could not find the data supporting this for the non-centromeric sequences. Was this tested by ChIP? How do they know for sure that CENP-A covers the entire HAC sequence molecularly?

Referee #2s' comment is correct. Our data did not demonstrate CENP-A assembly on flanking non-alphoid^{tetO} sequences. To clarify, we replaced "entire HAC" with "entire alphoid^{tetO} signal". CENP-A expansion to flanking DNA sequence is not discussed further in this manuscript.

And why does it only do so in 33% or 66% of the cells? What accounts for such cell-to-cell variation in the spreading/hyper-incorporation of CENP-A even in the same line?

That variation may reflect the cell cycle regulation of CENP-A assembly. To explore this, we added new data from a short-term HAT tethering experiment in which Cyclin B staining was used to distinguish G1 from S/G2 cells (new Figure S13) (Fujita et al, 2007; Silva et al, 2011). We found that HAT tethering induced CENP-A assembly only in Cyclin B negative (G1) cells. HAT-induced assembly may require other CENP-A deposition factors that are expressed or activated in G1 phase. In contrast, HJURP could induce CENP-A assembly even in Cyclin B positive cells (S/G2 phase). HJURP may be a direct CENP-A deposition factor, explaining why it exhibited 100% CENP-A assembly in the experiment of Figure 5.

Reference

Silva MC, Bodor DL, Stellfox ME, Martins NM, Hochegger H, Foltz DR, Jansen LE. Cdk activity couples epigenetic centromere inheritance to cell cycle progression. Dev Cell. 2012 Jan 17;22(1):52-63.

The highest levels of H3K9ac were detected after metaphase and in late telophase and G1, at the time when CENP-A assembly, mediated by HJURP, occurs. Is mitotic exit required for the pulse of H3K9ac?

In our result, H3K9ac was at quite low levels on the centromere DNA during a 6 hours metaphase arrest. A reproducible transient increase in H3K9ac levels was detected after mitotic exit (Figure 7A-C). Therefore our data indicates that mitotic exit is required for the pulse of H3K9 acetylation.

Silva et al. recently reported that Mis18BP1 centromere localization is prevented by mitotic cyclin dependant phosphorylation (Silva et al, 2011). Phosphorylation-resistant Mis18BP1 mutants localized to centromeres in G2 phase but could not induce CENP-A deposition. It is possible that mitotic exit might result in activation of other factors required for CENP-A assembly that regulate HAT activity and/or localization.

How do the authors put these new results in context with their previous H3K4me2/H3K36me2 study published in EMBO J? Specifically, how inter-dependent are H3K4me2, H3K36me, H3K9ac and transcription for CENP-A assembly? Are the H3K4me2 and H3K36me marks present at the same time as H3K9ac or do they occur at different times in the assembly/maintenance cycle?

In the previous study referred to by the referee, we looked at levels of histone modifications in asynchronous populations of cells and also at cells in metaphase. Under those conditions, we would not see significant levels of H3K9ac on the centromeric DNA (since it is found only during/after mitotic exit). Thus, we believe that H3K4me2 and H3K36me3 can exist in the absence of H3K9ac. Whether H3K9ac

can exist in the absence of H3K4me2 and H3K36me3 is an interesting question that could be addressed in another study.
In the present study, we have found that H3K4me2 is also higher on alphoid DNAs in HT1080 cells compared with that in HeLa cells (Figure S1 and our unpublished result). Crosstalk between H3K4me2 and H3K9ac at centromeres through transcription might be possible. Such cooperative crosstalk for centromere assembly could also be addressed in a future study.

Response to the referee #3

Referee #3 (Remarks to the Author):

Breaking the HAC barrier: Histone H3K9 acts as an acetyl/methyl switch to regulate CENP-A assembly

J. Ohzeki et. al.

This paper aims to gain insight into the regulation of CENP-A assembly into centromeric chromatin, and focuses specifically on the role of H3K9 methylation and acetylation in that process. The authors show that tethering histone acetyltransferases or a methyltransferase to artificial chromosome constructs, either stimulates or inhibits de novo centromere formation, respectively. They also show that tethering acetyltransferases to artificial chromosomes bypasses the requirement for Mis18alpha for de novo CENP-A assembly, but that CENP-A assembly still requires HJURP. The authors show that H3K9 acetylation levels at endogenous centromeres peak at a time correlated with CENP-A assembly. This paper provides a significant advance in human artificial chromosome formation, and makes an important link between acetylation to de novo centromere formation. The authors propose an H3K9 acetyl/methyl switch that regulates centromere formation. In general, the experiments in this manuscript are well done and the results are convincing. However, the conclusions that H3K9 acetylation is the specific modification governing new centromere formation and that this represents an acetyl/methyl switch represent one of several possible interpretations for the data presented. This paper provides several important advances in the centromere formation field and I recommend this paper for publication once the authors have addressed the points listed below.

Major Points:

It is unclear why the authors choose to look specifically at H3K9 methylation and acetylation, and not at a wider range of modifications that could also be present at centromeres. It is possible that general acetylation or acetylation of an alternative specific residue, not specific acetylation on H3K9, could affect de novo CENP-A assembly. This would suggest a mechanism very different from their acetyl/methyl switch hypothesis. The authors should examine whether other modifications of H3 and H4 might also correlate with centromere formation particularly in light of the fact that pCAF and P300 are known to modify several different lysine residues in histones.
Centromere chromatin and proteins are likely to have many post-translational modifications. Among those modifications, we found H3K9me3 was substantially lower in HAC-competent HT1080 cells but higher in HeLa and other cells that are not competent in HAC formation (Figure 1D, E). We also found that the H3K9-specific methyltransferase Suv39h1 decreases CENP-A assembly and can destabilize HACs (Figure 3C, previous Figure S7, S9 → present S8, S10). In addition, HAT tethering on alphoid DNA induces the H3K9ac modification and is accompanied by de novo CENP-A assembly (Figure 3C, 4,

5). Furthermore, we also showed that of the transient increase in H3K9ac on endogenous centromeric alphoid DNA and HAC alphoid DNA during mitotic exit (Figure 7C) is diminished by Suv39H1 tethering (Figure 7E). These are all reasons why we think that H3K9 is a critical residue for CENP-A assembly.

However, we also agree with referee #3 that acetylation of other residues on H3 in addition to K9 could also have important roles in CENP-A assembly. Furthermore, acetylation of other non-histone proteins could also turn out to be important for the process. Thus, we have addressed in the Discussion, "The HAT normally responsible for *de novo* CENP-A assembly and its key substrates in addition to H3K9 remain to be identified" (p13). Although we are interested in further characterizing centromere chromatin modifications other than H3K9ac/me3, to extend this analysis to the vast array of other possible histone modifications is beyond the scope of this manuscript.
(About the balance H3K9ac/me3, please see also our comments in the paragraph below.)

The authors argue that centromere formation is controlled by an "acetyl/methyl switch". A 'switch' implies that a single histone residue is either acetylated or methylated, and that if you increase one, you will decrease the other. The authors' data in Figures 2c and 7c and e shows that there is not a strong inverse correlation between H3K9 methylation and acetylation, which would be expected if the system were truly a "switch". One likely alternative is that the relative balance of these modifications throughout the chromatin could drive the propensity for centromere formation in different directions. The authors should either experimentally demonstrate switch like behavior akin to the phosphor/methyl switch controlling HP1 binding to chromatin or should change the language to more accurately describe this observation.

H3K9ac and H3K9me3 occur on same residue, therefore, the two modifications are mutually exclusive. However, our ChIP detection did not show a clear inverse correlation of these modifications. H3K9ac/me3 switching might also be affected by other cellular activities including histone deactetylases and/or histone demethylases. Furthermore H3K9 can adopt me1, me2 and unmodified forms. We agree that H3K9ac/me3 switching is not as simple as phospho/dephospho switching. At this time, therefore, according to the suggestion of referee #3, we changed "acetyl/methyl switch" to read "acetyl/methyl balance" in the text and the title.

The authors note that their results raise the question of 'whether CENP-A assembly induced by acetylation of H3K9 is biologically relevant.' To address this, the authors perform ChIP experiments. However, it is not clear that the observed spike in acetylation is statistically significant. It is possible that another assay aside from ChIP might be able to better resolve this phenomenon. The authors acknowledge that identification of the naturally occurring HAT is paramount, and could also help to reinforce the biological significance. The authors do not need to identify the endogenous HAT for publication of this manuscript but improving the data for the naturally occurring H3K9 acetyl spike would significantly strengthen the manuscript.

We stained metaphase cells and cells released from mitotic arrest as shown in Figure 7 with H3K9ac antibodies. Generally, metaphase centromeres and chromosomal arms have very little H3K9ac staining. In contrast, cells released from mitotic arrest had relatively positive H3K9ac staining. However, specific detection of H3K9ac signal at centromeres was difficult to resolve in the presence of higher H3K9ac signals elsewhere on the chromosomes. At this time, ChIP is only way to detect H3K9ac on alphoid DNA specifically.

Minor Points:

Although not necessary for publication of this manuscript, tethering a catalytically inactive acetyltransferase or methyltransferase would ensure that the activity is relevant for the phenotype rather than just the protein binding to chromatin.

In addition to Suv39h1, there is a second H3K9 trimethylase, SETDB1. Previous studies showed that indirect recruitment of SETDB1 using the tTS or KAP1 induced H3K9me3 formation and HAC destabilization (Nakano et al, 2008; Cardinale et al, 2009). Those results support the idea that H3K9 methylation is important for centromere inactivation. Concerning PCAF, tethering of a point mutation in the core of the PCAF HAT domain (E570Q; Clements et al, 1999) reduced newly synthesized CENP-A assembly relative to that seen with PCAFHD (8% vs 40%). We could add those data if necessary.

References

Clements A, Rojas JR, Trievel RC, Wang L, Berger SL, Marmorstein R. Crystal structure of the histone acetyltransferase domain of the human PCAF transcriptional regulator bound to coenzyme A. EMBO J. 1999 Jul 1;18(13):3521-32.

In the text describing Figure 4, the authors state that they want to "test whether the same chromatin modifiers... also affect CENP-A maintainence at an established HAC kinetochore". They demonstrate this point nicely in Figure 3f but this is not what they are testing in this figure.

The referee is correct. We tested assembly of newly synthesized CENP-A. Therefore, we changed "CENP-A maintenance" to read "newly synthesized CENP-A assembly".

Why are very few HA-CENP-A labeled endogenous centromeres seen in Figures 4c and 5c.

The pictures in the original figure were of single focal planes, therefore, few endogenous centromeres were observed. We subsequently took a set of multiple Z-stack pictures and made max intensity projections. We have now replaced the single focal pictures with max intensity projections. Some cells are showing that endogenous centromeres are not labeled with HA-CENP-A as examples for orange bars in Figure 4G and 5E (please see also our response to referee#1 comment1).

The presentation of data in Figure 4g and 5e is confusing. One appropriate conclusion from this data is that the Mis18 depletion leads to an increase in CENP-A hyper assembly in cells that only have CENP-A at the HAC, which is not accounted for in the authors' results. It is not clear what the authors are attempting to demonstrate, it seems like this figure could be excluded.

One of important point of Figure 4G is that CENP-A assembly induced by the HAT tethering is still effective even after hMis18 depletion. Under those conditions, endogenous centromeres showed no CENP-A assembly. Figure 5E shows that de novo CENP-A deposition induced by hMis18 tethering occurred with the same timing as CENP-A assembly on endogenous centromeres. We think the increase or decrease of the "Orange" population represents these points well. Therefore, we would prefer not to remove these figures.

The last sentence of the 4th paragraph of the results does not seem to be a complete sentence.

We added the missing phrase "have low levels of centromeric H3K9me3."

The values cited in the text for Figure 5e do not match the values actually displayed on the graph.

We have corrected these values.

What is the significance of the differences seen in Figure 3c?

We added a new Figure S6 to present the t-test values.

2nd Editorial Decision 08 March 2012

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by referee 1 (see comments below), and I am happy to inform you that the referee is satisfied with your responses and finds the revised manuscript now in principle suitable for publication in our journal. Before acceptance, I would kindly ask you to follow the referee's suggestion to include the additional supporting dataset that you mentioned and offered in your response letter. Therefore, I am returning the study to you for one final minor revision, following which we should be able to swiftly proceed with formal acceptance and production of the paper!

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The revised manuscript from Ohzeki et al. details the influence of methylation and acetylation on HAC formation and could have an important impact on the experimental use of HACs. The article nicely details the influence of acetylation on the CENP-A deposition pathway and provides some of the most significant data for the role of histone modification in centromere specification since Mis18 was identified.
Overall these experiments are well conducted and many of the points raised in my initial review have been adequately addressed. My only remaining reservation is the degree to which the authors have addressed the endogenous HAT activity. As part of their rebuttal, the authors offer to include relevant data and I would encourage them to do so, perhaps even within the primary data set. The localization of p300 and PCAF to centromeres is used as support for the endogenous role of acetylation at the centromere, so I think it is important to include the data that relates these two proteins to CENP-A assembly. In particular the data showing that p300 and PCAF knockdown does not alter CENP-A loading and Mis18 knockdown does not alter p300 or PCAF recruitment. I will readily concede that there are several KATs and redundancy could be an issue, but these two experiments together appear to suggest that P300 and PCAF recruitment play a role different from the CENP-A deposition pathway at centromeres.

2nd Revision - authors' response 11 March 2012

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Response to the referees' comments

Response to the referee #1

Referee #1 (Remarks to the Author):

The revised manuscript from Ohzeki et al. details the influence of methylation and acetylation on HAC formation and could have an important impact on the experimental use of HACs. The article nicely details the influence of acetylation on the CENP-A deposition pathway and provides some of the most significant data for the role of histone modification in centromere specification since Mis18 was identified. Overall these experiments are well conducted and many of the points raised in my initial review have been adequately addressed. My only remaining reservation is the degree to which the authors have addressed the endogenous HAT activity. As part of their rebuttal, the authors offer to include relevant data and I would encourage them to do so, perhaps even within the primary data set. The localization of p300 and PCAF to centromeres is used as support for the endogenous role of acetylation at the centromere, so I think it is important to include the data that relates these two proteins to CENP-A assembly. In particular the data showing that p300 and PCAF knockdown does not alter CENP-A loading and Mis18 knockdown does not alter p300 or PCAF recruitment. I will readily concede that there are several KATs and redundancy could be an issue, but these two experiments together appear to suggest that P300 and PCAF recruitment play a role different from the CENP-A deposition pathway at centromeres.

We added these two data requested by referee #1 in the new Supplementary Figure S19 and referred to them in the Discussion (Page 13) "The HAT normally responsible for *de novo* CENP-A assembly and its key substrates in addition to H3K9 remain to be identified (Supplementary Figure S19).".