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

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Introduction

The kinetochore is responsible for accurate chromosome segregation. However, the mechanism by which kinetochores assemble and are maintained remains unclear. Here we report that de novo CENP-A assembly and kinetochore formation on human centromeric alphoid DNA arrays is regulated by a histone H3K9 acetyl/methyl balance. Tethering of histone acetyltransferases (HATs) to alphoid DNA arrays breaks a cell type-specific barrier for de novo stable CENP-A assembly and induces assembly of other kinetochore proteins at the ectopic alphoid site. Similar results are obtained following tethering of CENP-A deposition factors hMis18 or HJURP. HAT tethering bypasses the need for hMis18, but HJURP is still required for de novo kinetochore assembly. In contrast, H3K9 methylation following tethering of H3K9 tri-methylase (Suw391h1) to the array prevents de novo CENP-A assembly and kinetochore formation. CENP-A arrays assembled de novo by this mechanism can form artificial chromosomal elements (HACs) that are propagated indefinitely in human cells.

The fundamental question addressed by this study is how different chromatin fates are generated on alphoid DNA in human cells and what kind of chromatin directs functional centromere/kinetochore assembly. We found that competency for stable CENP-A assembly and de novo kinetochore assembly are correlated with the acetylation status of H3K9 on alphoid DNA in several different cell types. We therefore decided to manipulate H3K9 modifications during de novo kinetochore assembly using a synthetic alphoid DNA array carrying multiple tet operator (tetO) sequences that allow the tethering of chromatin modifiers into the array as tet repressor (tetR) fusions (Nakano et al, 2008; Cardinale et al, 2009; Bergmann et al, 2011).

Chromatin modifications are thought to regulate functional kinetochore assembly and maintenance by an epigenetic mechanism. Recent studies of normal centromeres also suggest a possible involvement of canonical histone H3-containing nucleosomes in kinetochore function. In humans, CENP-A nucleosomes are localized to only a portion of the megabase-sized alphoid DNA arrays, where they are organized as multiple clusters interspersed with histone H3 nucleosomes (Blower et al, 2002; Sullivan and Karpen, 2004; Ribeiro et al, 2010). Canonical H3 nucleosomes co-purify with CENP-A in oligonucleosomes (Ando et al, 2002), and some classes of CENPs (e.g. CENP-T, -W) are suggested to bind only to H3 nucleosomes (Hori et al, 2008). Thus, epigenetic CENP-A-mediated kinetochore assembly could also be affected by the surrounding H3 histone state. Thus, functional kinetochore formation and maintenance may be influenced by additional factors that determine the modification status of centromeric chromatin.

The fundamental question addressed by this study is how different chromatin fates are generated on alphoid DNA in human cells and what kind of chromatin directs functional centromere/kinetochore assembly. We found that competency for stable CENP-A assembly and de novo kinetochore assembly are correlated with the acetylation status of H3K9 on alphoid DNA in several different cell types. We therefore decided to manipulate H3K9 modifications during de novo kinetochore assembly using a synthetic alphoid DNA array carrying multiple tet operator (tetO) sequences that allow the tethering of chromatin modifiers into the array as tet repressor (tetR) fusions (Nakano et al, 2008; Cardinale et al, 2009; Bergmann et al, 2011).
Tethering of tetR-EYFP-p300 or tetR-EYFP-PCAF, two histone acetyltransferase (HAT) domains that promote acetylation of H3K9, results in assembly of newly synthesized CENP-A on exogenous alphoid DNA arrays. Remarkably, HAT induction of de novo CENP-A chromatin assembly requires HJURP but bypasses the need for hMis18x, and spontaneously nucleates assembly of an outer kinetochore on the artificial DNA arrays. Indeed, in a technological breakthrough, these HAT-induced de novo CENP-A arrays can even lead to the formation of stable HACs that are maintained indefinitely in human cell lines that have previously proven refractory to HAC formation. Together, our data reveal that CENP-A assembly appears to be controlled by a histone H3K9ac/me3 balance that acts upstream of HJURP.

## Results

### Cell-type-dependent chromatin assembly on transfected human alphoid DNA

De novo kinetochore assembly is efficient in HT1080 cells. However, neither stable de novo kinetochore formation nor CENP-A assembly on exogenous alphoid DNA occurs in many other commonly used human cell lines, including HeLa (Figure 1A and Supplementary Figure S1).

Surprisingly, HeLa cells, TIG7 human fetal primary, hTERT-BJ1 immortalized fibroblasts and U2OS osteosarcoma cells, all efficiently assemble CENP-A chromatin de novo, but CENP-A levels declined rapidly during subsequent cell culture (Figure 1B, C, Supplementary Figure S2 and S3C). The decrease in CENP-A levels on transfected alphoid DNA in HeLa cells was accompanied by a progressive increase in the heterochromatin-associated modification, H3K9me3 (Figure 1C).

Detailed ChIP analysis of the chromatin modification status at several endogenous centromeres revealed that alphoid DNA appears more euchromatic in HT1080 cells than in HeLa (Figure 1D). Using CENP-A and CENP-B as controls, H3K9ac, a euchromatic modification, was readily detected on HT1080 alphoid DNA, but was much lower at HeLa centromeres (Figure 1D). In addition, HT1080 cells had substantially lower levels of H3K9me3 on alphoid DNA than on other repetitive DNA sequences, including satellite 2, D4Z4 and DYZ1. In contrast H3K9me3 levels on alphoid DNA were significantly higher in HeLa, TIG7, hTERT-BJ1 and U2OS cells (Figure 1D and Supplementary Figure S3). The ChIP data were confirmed by a stronger H3K9me3 staining intensity at mitotic centromeres in HeLa cells (Figure 1E).

### Suv39h1 negatively regulates de novo CENP-A assembly on alphoid DNA at ectopic site

The histone methyltransferase Suv39h1 may be one critical factor responsible for this difference between HT1080 and HeLa alphoid DNA chromatin. HT1080 cells express only 50% of the relative level of Suv39h1 mRNA found in HeLa cells (Figure 2A). Suv39h1 over-expression increased both levels of the enzyme itself and H3K9me3 on centromeric alphoid DNAs in HT1080 cells (Figure 2B). These results fit with the observations that mouse cells doubly null for Suv39h1 and Suv39h2 (Suv39hdn) have low levels of centromeric H3K9me3 (Peters et al., 2001).

Suv39h1 depletion by RNAi revealed a remarkable inverse correlation between CENP-A and H3K9me3 levels on an alphoid DNA array integrated ectopically on a chromosomal arm in HeLa cells (HLW-Int-09; Figure 2C-F).

These results suggest that Suv39h1 suppresses ectopic CENP-A incorporation, presumably by maintaining H3K9me3 levels on alphoid DNA. However, Suv39h1 depletion alone and the accompanying transient increase in CENP-A were not sufficient for functional kinetochore formation on ectopic alphoid DNA arrays (Okada et al., 2007). Additional regulatory factors must be required for functional kinetochore formation de novo on alphoid DNA.

### HAT recruitment breaks the barrier for de novo kinetochore assembly

Several observations suggest that histone acetyltransferases may be required for functional CENP-A assembly and subsequent kinetochore formation de novo (Nakano et al., 2003; Okamoto et al., 2007). Furthermore, the acetyltransferases p300 and PCAF [p300/CREB associated factors (Yang et al., 1996)] both localize at functional, but not at inactive, centromeres (Supplementary Figure S4) (Craig et al., 2003; Choi et al., 2009).

To test the hypothesis that histone acetylation might antagonize H3K9me3 and promote functional CENP-A assembly, we expressed tetR-EYFP fused to the histone acetyltransferase (HAT) domains of p300 or PCAF in HeLa cells (Figure 3A). Into the tetR-EYFP expressing cells, we then introduced a 50 kb synthetic DNA array based on the α21-I alphoid dimer sequence with a tetO site where the CENP-B box would be on one monomer (pWT02R; Figure 3A and Supplementary Figure S5) (Ebersole et al., 2003; Kim et al., 2009). In this system, tetR fusion proteins bound to tetO sites within the synthetic alphoid DNA arrays can directly modify the chromatin environment at a single centromere or locus in human cells.

Tethering of either HAT domain fusion (tetR-EYFP-p300HD or tetR-EYFP-PCAFHD) to the synthetic alphoid DNA enhanced H3K9ac modification and CENP-A assembly, as demonstrated by time-course ChIP assays (Figure 3B, C and Supplementary Figure S6). In contrast, tethering of the tetR-EYFP-Suv39h1 fusion increased H3K9me3 levels and also decreased CENP-A assembly. This raised the question whether HAT domain recruitment could stimulate de novo kinetochore formation.

Remarkably, stable HACs bearing the synthetic α21-I alphoidrep fusion repeat were detected in HeLa cell lines expressing tetR-EYFP-p300HD or tetR-EYFP-PCAFHD (in 8 or 14% of cell lines, respectively; Figure 3B, D and E and Supplementary Figure S7). Importantly, HAC formation was never detected when the synthetic α21-I alphoidrep fusion repeat was introduced into cells expressing tetR-EYFP or tetR-EYFP-Suv39h1 (Figure 3E). Similarly, alphoidrep-based HAC formation was never observed in HT1080 cells expressing tetR-EYFP-Suv39h1 (Figure 3E and Supplementary Figure S8).

Although exogenous HAT activity is required for initial de novo kinetochore formation in HeLa cells, once established, the de novo kinetochores no longer require this exogenous activity to maintain their structure and function. We initially observed that HACs were stably maintained in cell clones that no longer expressed the tetR-EYFP-HAT fusion construct, presumably due to the silencing of retrovirus integration sites. We therefore, directly tested whether de novo...
kinetochores remained functional following forced dissociation of the HAT domain fusions by culturing cells for more than 60 days in the presence of doxycycline (Figure 3F). Microscopic and ChIP analyses showed that the HACs remained mitotically stable and capable of recruiting inner and outer kinetochore proteins CENP-A, -C, -T, hKNL1, Hec1, hDs1 and hMis12 (Supplementary Figure S9A, B) in the absence of bound exogenous HAT fusion proteins (Figure 3F; Loss of tetR fusion binding to the HAC was confirmed by ChIP analysis (panel C). Primer set for synthetic 11mer repeats was used for quantitative PCR. Error bars, s.d. (n = 2).

(D) Chromatin modifications on human repetitive DNAs. ChIP assay was carried out with normal IgG and indicated antibodies. Primer sets used for quantitative PCR are specific to 5S ribosomal DNA (5S Ribo), satellite 2 (Sat2), D4Z4 repetitive DNA (D4Z4), DYZ1 repetitive DNA (DYZ1), Alu elements (Alu), 17 alphoid (17a), 21-I alphoid (21a, 21b), 21-II alphoid (21c), X alphoid (Xa, Xb) and Y alphoid DNA (Ya, Yb, Yc) sequences. More information for these primers is shown in Supplementary Figure S3A and Supplementary Table S2. Columns indicate non-alphoid elements (Alu), 17 alphoid (17a), 21-I alphoid (21a, 21b), 21-II alphoid (21c), X alphoid (Xa, Xb) and Y alphoid DNA (Ya, Yb, Yc) sequences.

Thus, HAT domain recruitment to the synthetic α21-I alphoid tetO array renders HeLa cells competent for de novo kinetochore formation.

Centromere chromatin modifications regulate newly synthesized CENP-A assembly
Kinetochore maintenance requires the targeting of newly synthesized CENP-A to centromeres during mitotic exit/early G1 (Jansen et al., 2007). To test whether the same chromatin modifiers that potentiate de novo kinetochore assembly also affect newly synthesized CENP-A assembly at an established HAC kinetochore, we transiently cotransfected constructs expressing HA-tagged CENP-A (HA-CENP-A) plus various tetR-EYFP-fusion proteins into tetO-HAC containing HeLa cells (HeLa-HAC-R5; Figure 4A and B). We then asked if HA-CENP-A (a mark for newly assembled CENP-A) assembled on the HAC and endogenous centromeres at 24 h (i.e. one complete cell cycle in HeLa cells) after transfection.

Tethering of tetR-EYFP alone did not affect the assembly of newly synthesized HA-CENP-A onto either the HAC or endogenous centromeres (Figure 4C and Supplementary Figure S10A). In contrast, tethered tetR-EYFP-Suv39h1 specifically reduced HA-CENP-A assembly on the HAC centromere (Figure 4C and Supplementary Figure S10B). This was coupled with destabilization of the HAC, detected as lagging chromosomes and micronuclei (Supplementary Figure S10C-G).

Figure 1 Cell type specific chromatin modifications on transfected and endogenous alphoid DNA. (A) Summary of the HAC formation assay. The pWTR11.32 plasmid, which contains 60 kb of α21-I 11mer repeat (shown in panel B), was transfected to HT1080 or HeLa cell. Single transformants were isolated and analyzed for chromosomal events by FISH and microscopy. Examples of HAC and integration are shown as merged images. Signals in pictures indicate DNA (gray), BAC plasmid DNA (red) and CENP-A (green). (B and C) Time-course ChIP analysis. The pWTR11.32 or pMTR11.32 plasmid (panel B) was transfected to HT1080 or HeLa cell. Transfectants were cultured under presence of selective drug (G418), and harvested at 2, 3 and 4 weeks after transfection. ChIP assay was carried out with normal IgG and indicated antibodies (panel C). Primer set for synthetic 11mer repeats was used for quantitative PCR. Enrichment against normal IgG, 0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100% HAC CENP-A+ Integration CENP-A+ No CENP-A 70% 100% Integration CENP-A+ No CENP-A.

| Panel | Description |
|-------|-------------|
| A     | HAC formation / integration |
| B     | Wild type repeat | Mutant repeat |
| C     | HT1080 | HeLa |
| D     | HT1080 | HeLa |
| E     | DNA | H3K9me3 | CENP-A | Merged |

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Figure 2 Suv39h1, histone H3K9 tri-methylase, negatively regulates ectopic CENP-A assembly. (A) Suv39h1 expression level. Total RNA was purified from each cell line, reversely transcribed and quantified by real-time PCR. Suv39h1 mRNA amounts were normalized by HPRT RNA. Vertical axis indicates relative Suv39h1 mRNA level (%).

Unexpectedly, tethering of p300HD or PCAFHD induced HA-CENP-A hyper-assembly not only at the HAC centromere, but covering the entire alphoid DNA array on the HAC in a significant proportion of cells (34 and 40% in Figure 4C and Supplementary Figure S10B).

We next tested whether the known canonical CENP-A deposition factors hMis18a and HJURP are involved in this HAT-induced CENP-A assembly. We first depleted hMis18a or HJURP by siRNA knockdown and then tethered tetR-fused HAT proteins to the synthetic alphoid array (Figure 4D, E). hMis18a depletion reduced HA-CENP-A assembly at both endogenous centromeres and the HAC centromere (Figure 4F). However, stable HA-CENP-A assembly continued on alphoid DNA with tethered HAT fusions following hMis18a depletion, which blocks CENP-A assembly on endogenous centromeres (Figure 4G, orange bars). The cell population, which had newly assembled HA-CENP-A on alphoid DNA but no HA-CENP-A signals on endogenous centromeres, was relatively increased after hMis18a depletion (Figure 4G, orange bars. \( P < 0.05 \)). These results indicate that tethering of HAT fusions can partially rescue HA-CENP-A assembly in the absence of hMis18a.

Importantly, HJURP depletion dramatically reduced HA-CENP-A assembly both on endogenous host centromeres and on the HAC. Furthermore, neither was rescued by tethering of HAT-fusion proteins to the HAC alphoid DNA array (Figure 4F, G). Thus, HJURP is required for HAT-mediated CENP-A assembly.

Given that HAT tethering can potentiate de novo kinetochore formation on a HAC and induce HA-CENP-A hyper-assembly covering non-centromeric regions of the HAC (Figures 3 and 4), we next tested whether HAT-tethering can induce de novo CENP-A assembly on a chromosomal arm. We did this using a stable cell line (HeLa-Int-03), which carries an ectopic alphoid DNA array (Supplementary Figure S11).

Tethering of tetR-EYFP-p300HD or tetR-EYFP-PCAFHD induced HA-CENP-A hyper-assembly on the ectopic array in 27 and 47% of cells, respectively (Figure 5A-E). A similar effect was observed after tethering the CENP-A assembly factors, tetR-EYFP-hMis18a or tetR-EYFP-HJURP (HA-CENP-A hyper-assembly in 32 and 100% of cells, respectively—Figure 5C-E). CENP-A assembly at the ectopic site induced by tetR-EYFP-HJURP hMis18a tethering was diminished by HJURP depletion (Figure 5F, G), consistent with Barnhart et al. (2011). In controls, tethering of tetR-EYFP alone or tetR-EYFP-Suv39h1
did not induce HA-CENP-A assembly at the ectopic site (Figure 5C–E). Moreover, no specific enhancement of the assembly of newly expressed HA-tagged histone H3.1 nor H3.3 was observed on the ectopic alphoidtetO array by the HAT tetherings in addition to the usual assembly patterns of those histone H3 (Figure 5H,I and Supplementary Figure S12).

**Figure 3** Recruiting of histone acetyl-transferases induced de novo kinetochore formation in HeLa cell. (A) The expression constructs and BAC plasmid used in this Figure. TetR-EYFP gene was fused with Suv39h1, p300 HAT domain (p300HD) or PCAF HAT domain (PCAFHD). HeLa cell lines expressing these tetR-EYFP fusions were generated by retrovirus infection, and these cells were transfected with z21-I alphoidtetO DNA containing plasmid (pWTO2R; see Supplementary Figure S5). (B) Schematic timetable for ChIP and HAC assay. (C) Time-course ChIP analysis. Cells transfected by plasmid pWTO2R were harvested at 2, 3 and 4 weeks after transfection. Normal IgG and a set of specific antibodies were used for ChIP. A set of primers for z21-I alphoidtetO 2mer (tetO-2mer) was used for quantitative PCR. Columns indicate the results obtained with cells expressing tetR-EYFP (green), tetR-EYFP-Suv39h1 (blue), tetR-EYFP-p300HD (pink) or tetR-EYFP-PCAFHD (red) fusions, respectively. Error bars, s.d. (n = 3). (D) Examples of a HAC (p300-HAC-13) formed in HeLa cell. Metaphase cells were spread and stained with DAPI (blue), anti-CENP-A antibody (green) and BAC DNA probe (red). BAC DNA probe visualizes a vector region of the introduced pWTO2R construct. Scale bar, 3 μm. (E) Summary of HAC formation. Bars indicate a frequency of HAC formation in the cells expressing protein fusions. Error bars, s.d. (n = 2). Chi-square test of the predominant pattern for HAC formation frequency indicated significant differences. Asterisks * or ** indicate P values, (P < 0.05) or (P < 0.005), respectively. (F) HAC stability without HAT tethering. HAC containing cells were cultured for 60 days under presence of doxycycline (no tetR binding condition; left panel) and absence of selective drug (permissive condition for HAC loss). The number of HAC retention rate in 30–50 spread metaphase cells was scored by FISH using input BAC DNA specific probes (right panel). HAC loss rate was calculated with HAC retention rates at day 0 (N0) or at day 60 (N60) using the following formula: N60 = N0 × (1 – R)60 (Ikeno et al., 1998). All HAC cell lines showed high stability (HAC loss rate < 0.001).
Next, we determined when during the cell cycle HAT tethering induces CENP-A assembly. We tethered tetR-EYFP fusion proteins for 2 h by controlling the presence and absence of doxycycline, and detected new CENP-A assembly both in Cyclin B positive and negative cells (Supplementary Figure S13E). Taken together, HAT activity is sufficient to trigger the specific assembly of newly synthesized CENP-A on alphoid DNA in G1 phase.
HAT tethering induces de novo functional kinetochore assembly at the ectopic site

We next investigated whether ectopic CENP-A assembly driven by chromatin acetylation or tethered hMis18α or HJURP can induce assembly of the outer kinetochore in HeLa cells (Figure 6A). CENP-A assembled on ectopic alphoid tetO arrays was maintained in metaphase cells, where the ectopic HA-CENP-A was
always detected as an extended region weakly stained with DAPI (Figure 6B, C). HA–CENP-A-coated arrays were observed in 21 or 77% of metaphase cells expressing tetR-EYFP-hMis18α or tetR-EYFP-HJURP, respectively (compared to 32 and 100% in interphase cells). HAT-induced CENP-A assembly was apparently less stable on mitotic chromosomes (7% of mitotic cells, compared with 27–47% of interphase cells—Figures 5E and C).

Remarkably, the essential inner or outer kinetochore markers CENP-T, -I and -E (Hori et al., 2008; Santaguida and Musacchio, 2009) assembled on the ectopic array following CENP-A assembly (red arrowheads, Figure 6B, D and Supplementary Figure S14). These proteins accumulated at greater levels than at the centromeres of host chromosomes (Figure 6B, green arrowheads). Such an induced hyper-assembly of kinetochore proteins at the ectopic sites appears to result in bundling of an excess amount of microtubules and results in aberrant spindle formation. As a result these cells appear to be arrested in mitosis (Supplementary Figure S15C). In contrast, kinetochore assembly was not observed on CENP-A assembled nonspecifically on whole chromosomal arm regions (Supplementary Figure S16), consistent with Van Hooser et al. (2001) and Gascoigne et al. (2011).

A HAC assay with α21-I alphoidtetO BAC accompanied by transient transfection of these tetR-EYFP fusion-expressing plasmids confirmed that induced kinetochores like in those shown in Figure 6 can acquire stable maintenance and full function as stable artificial chromosomes (Supplementary Figure S17A). In HeLa cells, HAC formation was efficiently supported by co-transfection with tetR-EYFP-PCAFHD, tetR-EYFP-hMis18α and tetR-EYFP-HJURP but not with tetR-EYFP alone (Supplementary Figure S17C). Furthermore, tetR-EYFP-PCAFHD also supported HAC formation in U2OS cells where again no HAC formation was seen with tetR-EYFP alone.
Centromere H3K9 acetylation normally occurs in a short time window following metaphase

Although forced HAT tethering can induce CENP-A and kinetochore protein assembly on alphoid\textsuperscript{tetO} DNA, the level of H3K9 acetylation on endogenous alphoid DNA is normally very low—almost undetectable in unsynchronized HeLa cells (Figure 1D). This raises the question of whether CENP-A assembly induced by acetylation of H3K9 is biologically relevant. If centromere acetylation does normally occur, it may be during only a brief cell cycle window—possibly coinciding with the localization of hMis18\textalpha and HJURP to centromeres. HJURP centromere localization is maximal at two hours after release from a metaphase arrest, and rapidly decreases thereafter (Dunleavy et al, 2009)

Indeed, ChIP analysis revealed that H3K9 acetylation levels increased temporarily on endogenous and HAC centromere alphoid DNAs at one hour after release from a metaphase arrest, but fell again by three hours after the release (Figure 7A–C). The temporary increase in H3K9ac can be blocked by tethering tetR-EYFP-Suv39h1 and CENP-A levels also fell following tethering of tetR-EYFP-Suv39h1 (Figure 7E and Supplementary Figure S18; compare dox+dox+ to dox– dox–).

These results confirm the presence of intrinsic acetylation activity on centromere chromatin, and show that this activity is apparently restricted to a short time window from anaphase through early G1. Taken together these results indicate that CENP-A assembly is regulated by the H3K9 acetyl/methyl balance. If H3K9 is acetylated, the chromatin can bind chaperones such as HJURP and assemble CENP-A chromatin. If it is trimethylated, CENP-A assembly is inhibited.

Figure 7 Centromere acetylation occurs within a short time window following metaphase. (A) Schematic diagram for cell sample preparation. Cells were arrested in metaphase for six hours, and then harvested and released to G1 phase. Mitotically arresting (pale blue), one hour post release (orange), three hours post release (green) and random culture cells (gray) were harvested. (B) Examples of phase contrast microscope images for cells at each time points. Scale bar, 20\,\mu m. (C) Centromere acetylation in HeLa cell. ChIP assay was carried out with normal IgG and a set of specific antibodies using samples indicated in panel A. A set of primers was used for quantitative PCR (top). Error bars, s.d. (n = 3). P-values obtained with t-test are indicated. (D) Schematic diagrams for Suv39h1 tethering. A HeLa-HAC-05 cell line expressing tetR-EYFP-Suv39h1 was established in the presence of doxycycline (dox). Three days before sample preparation, tetR-EYFP-Suv39h1 tethering to HAC was induced by dox washout. Then a set of four cell samples shown in panel A was harvested and used for ChIP. (E) Suv39h1 tethering represses the increase of centromeric H3K9ac level. HeLa-HAC-R5 cells expressing Suv39h1 were cultured with presence or absence of doxycycline for three days, and then harvested similar to that shown in panel A. ChIP assay was carried out with a set of specific antibodies. A set of primer was used for quantitative PCR (top). Error bars, s.d. (n = 3). P-values obtained with t-test are indicated.

(Supplementary Figure S3, S17B and C). Interestingly, HAC formation was not dramatically increased in HT1080 cells if the \textalpha 21-I alphoid\textsuperscript{tetO} BAC was introduced together with a constitutively expressing tetR-EYFP-PCAFHD.

These results demonstrate that tethering of HAT activity or Mis18\textalpha can induce HJURP-dependent de novo CENP-A chromatin assembly and subsequent assembly of a functional kinetochore on the alphoid DNA array.

Centromere H3K9 acetylation normally occurs in a short time window following metaphase

Although forced HAT tethering can induce CENP-A and kinetochore protein assembly on alphoid\textsuperscript{tetO} DNA, the level of H3K9 acetylation on endogenous alphoid DNA is normally very low—almost undetectable in unsynchronized HeLa cells (Figure 1D). This raises the question of whether CENP-A assembly induced by acetylation of H3K9 is biologically relevant. If centromere acetylation does normally occur, it may be during only a brief cell cycle window—possibly coinciding with the localization of hMis18\textalpha and HJURP to centromeres. HJURP centromere localization is maximal at two hours after release from a metaphase arrest, and rapidly decreases thereafter (Dunleavy et al, 2009).

Indeed, ChIP analysis revealed that H3K9 acetylation levels increased temporarily on endogenous and HAC centromere alphoid DNAs at one hour after release from a metaphase arrest, but fell again by three hours after the release (Figure 7A–C). The temporary increase in H3K9ac can be blocked by tethering tetR-EYFP-Suv39h1 and CENP-A levels also fell following tethering of tetR-EYFP-Suv39h1 (Figure 7E and Supplementary Figure S18; compare doxycycline +/− doxycycline). These results confirm the presence of intrinsic acetylation activity on centromere chromatin, and show that this activity is apparently restricted to a short time window from anaphase through early G1. Taken together these results indicate that CENP-A assembly is regulated by the H3K9 acetyl/methyl balance. If H3K9 is acetylated, the chromatin can bind chaperones such as HJURP and assemble CENP-A chromatin. If it is trimethylated, CENP-A assembly is inhibited.

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Discussion

Centromeric chromatin acetylation induces de novo heritable kinetochore assembly

Tethering of histone acetyltransferases (HATs) induces de novo assembly of CENP-A and functional kinetochore on ectopic alphoid<sup>α</sup>o DNA, and can culminate in de novo formation of stable human artificial chromosomes (HACs). HAT-induced de novo CENP-A assembly appears to mimic the natural process. It requires the activity of specific CENP-A deposition factor HJURP. 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). Nonetheless, this observation that tethered HAT activity in canonical H3 chromatin can induce de novo CENP-A and outer kinetochore assembly by adjusting the modification status of H3K9 represents a major step towards understanding the epigenetic regulation of kinetochore assembly.

Recent exciting studies demonstrated that tethering of CENP-C and CENP-T (Gascoigne <em>et al</em>, 2011) or HJURP (Barnhart <em>et al</em>, 2011) to an ectopic LacO array induced the assembly of a functional outer kinetochore. However, whether those kinetochore-like structures were stably inherited was not tested. Here, we show that kinetochores formed de novo by targeted induction of CENP-A assembly direct accurate segregation of the resulting HACs for many generations without any requirement for continued tethering of the exogenous HAT. Thus, our data suggest that proper assembly of CENP-A chromatin is sufficient for long-term epigenetic maintenance of centromere activity.

H3K9 ac/me3 are positive and negative regulators of CENP-A assembly, respectively

The notion that CENP-A assembly may normally be linked to chromatin acetylation (Nakano <em>et al</em>, 2003; Fujita <em>et al</em>, 2007; Okamoto <em>et al</em>, 2007) is strongly supported by our detection of a pulse of histone H3 acetylated on lys 9 (H3K9ac) during a brief window following release from a mitotic arrest. This timing corresponds remarkably well with the observed localization of hMis18a and HJURP at kinetochores (Fujita <em>et al</em>, 2007; Foltz <em>et al</em>, 2009; Dunleavy <em>et al</em>, 2009) and is the cell cycle window in which CENP-A assembly normally occurs (Jansen <em>et al</em>, 2007; Silva <em>et al</em>, 2012).

Although Suv39h1 over-expression increased levels of H3K9me3 on centromeric alphoid DNA, the functions of endogenous centromeres on host chromosomes were not impaired. However, tethering of Suv39h1 to the alphoid<sup>α</sup>o kinetochore blocked the pulse of centromeric H3K9 acetylation normally seen during mitotic exit and interfered with the assembly of newly synthesized CENP-A on the established HAC centromere. Thus, although kinetochores do contain limited H3K9me3-containing chromatin regions (Ribeiro <em>et al</em>, 2010), the CENP-A chromatin core in the active kinetochore must be protected from Suv39h1-induced H3K9 tri-methylation during mitotic exit.

Regulation of the balance between H3K9ac (promoting CENP-A assembly) and H3K9me3 (inhibiting it) may be critical not only for de novo kinetochore assembly in our artificial system, but also for genome stability. The extremely large secondary kinetochores formed by induction of CENP-A assembly at ectopic sites apparently caused abnormal bundling of spindle microtubules and resulted in a mitotic arrest. This suggests that kinetochore geometry must be regulated appropriately on endogenous alphoid DNA—possibly to avoid formation of merotelic attachments. Adjusting the balance between H3K9 acetylation and methylation might provide a modulation mechanism to minimize inappropriate CENP-A assembly and the formation of ectopic centromeres on native chromosomes.

The role of centromeric heterochromatin may vary in different organisms. In fission yeast, heterochromatin is important not only for sister chromatid cohesion, but also for de novo CENP-A assembly (Hayashi <em>et al</em>, 2004; Grewal and Jia, 2007; Ishii <em>et al</em>, 2008; Kagansky <em>et al</em>, 2009). Understanding this contrast between fission yeast and human CENP-A assembly clearly requires additional study.

Breaking the HAC barrier

Since the first HAC formation assay, it has been unclear why de novo kinetochore formation could occur in HT1080 cells but not in other popular cell lines, such as HeLa. Indeed, in some quarters, this was taken to suggest that HAC formation in HT1080 might in some way be an aberrant process. Here, we suggest a very simple H3K9 acetyl/methyl balance model to explain this host cell specificity for HAC formation. Assembly of a core of CENP-A sufficient to establish an epigenetically stable active centromere appears to require H3K9ac, and if the balance is tipped in favor of H3K9me3, then the CENP-A that assembles initially is gradually lost and stable kinetochores do not form.

We propose that tethering of HAT activity to the input alphoid DNA array breaks the kinetic barrier provided by the very brief window of acetylation that occurs during mitotic exit. This appears to allow sufficient time for CENP-A to assemble into ‘core’ regions of sufficient size to be stably maintained (Alonso <em>et al</em>, 2007; Okamoto <em>et al</em>, 2007). Thus the synthetic tetO-alphoid/tetR-fusion tethering system now allows us to induce de novo kinetochore assembly on both newly introduced synthetic alphoid DNA arrays as well as pre-existing arrays integrated into chromosome arms. This ability to induce the formation of stable minichromosomes in HeLa, U2OS and other popular cell lines offers a powerful approach to analyzing epigenetic centromere/kinetochore formation and maintenance.

Materials and methods

Cell culture and transfection

HT1080 (tetraploid) and HeLa cells were grown in Glutamax I (Invitrogen) supplemented with 10% FBS at 37°C in 5% CO2 atmosphere. For transfections, Lipofectamine 2000 (Invitrogen), Lipofectamine (Invitrogen) or FuGENE HD (Roche) was used for siRNA, BAC plasmid DNAs (pWTTR11.32, pMTR11.32, pw/M11.64 and pWT02R) or usual plasmid vectors, respectively. Retrovirus infection method used for tetR-fusions expression was previously described (Nakano <em>et al</em>, 2008). For depletion experiments, siRNAs for Suv39h1 were obtained from Dharmaco as a pool (D-009604-01, D-009604-02, D-009604-04 and D-009604-06), and siRNA sequence for hMis18a or HJURP depletion was referred Fujita <em>et al</em> (2007) or Dunleavy <em>et al</em> (2009), respectively. siRNA sequence for p300 or PCAF was CAGAGCAGUCCUGAUAUGTT and GGUUGUUAUCUGU UUCCGUAATT, respectively. Control siRNAs were obtained from Dharmaco (siGFP) and Ambion (siNegative). Cell lines used in this study are shown in Supplementary Table S4.

ChIP

Cells were trypsinized and harvested in a centrifuge tube. Cells were washed with PBS and then fixed with 0.61% (for tetR-EYFP or EFYP
fusions) or 0.3% (for histones) formaldehyde at 25°C for 10 min. ChIP procedure was previously described (Ohzeki et al., 2002). Antibodies used for ChIP are shown in Supplementary Table S1. Immuno-precipitated DNAs were de-fixed at 65°C for more than 4 h and purified by phenol/chloroform extraction following proteinase K treatment. Purified DNA was quantified by the competitive PCR (Supplementary Figure S2) or real-time PCR (BIORAD). For real-time PCR detection, SYBR Green I containing reagent was used.

Preparation of mitotic cells and chromosome spreads

For mitotic arrest, cells were treated with 350 nM of the highly reversible microtubule destabilizing drug, TN-16 (WAKO; Kitagawa et al., 1995; Perpelescu et al., 2009), for 2 to 6 h in the growth medium. Mitotic cells were harvested by pipetting, washed with PBS, incubated in a hypotonic buffer (20 mM Tris pH7.4, 1 mM EGTA and 40 mM KCl) and then spread on cover glass (Matsunami) by Cytospin3 (Shandon). Following immuno-staining and/or FISH protocols (Ikeda et al., 1998; Ohzeki et al., 2002). For ChIP with mitotic or post mitotic cells (Figure 7), mitotically arrested cells were harvested with the method described above, firstly. Then a portion of the mitotic cells was fixed for ChIP, and the remaining were washed with PBS two times and plated in petri dish. After 1 h incubation, unattached mitotic cells were washed out with PBS by pipetting. Attached cells were harvested for ChIP analysis at each time point. ChIP procedure is described in above section.

Supplementary data

Supplementary Information are available at The EMBO Journal Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Conflict of interest

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