Supplementary Information

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

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Supplementary Material and Methods

Construction of synthetic alphoid DNA arrays and expression vectors

The α21-I-EcoRI-2mer sequence was amplified with two primer sets; 2mer-F1 and 2mer-R2 for the first half, and 2mer-F2 and 2mer-R1 for the last half of alpha 21-I EcoRI 2mer. These two PCR products were independently cloned into a pUC vector and confirmed by DNA sequencing. Then, two fragments were combined and SpeI and NheI sites were added at the ends by a general cloning method. This synthetic dimer was named as α21-I alphoid^{tetO} 2mer (Supplementary Figure S5). This α21-I alphoid^{tetO} 2mer was cut out from the vector with NheI and SpeI, purified from agarose gel, self ligated and used for rolling circle amplification (RCA). RCA reaction was carried out with phi29 polymerase (NEB) and short primer set for alphoid DNA sequences (Ebersole et al, 2005; Kim et al, 2009). RCA products were co-transfected to yeast cells along with a cloning vector, termed pHook, and assembled arrays were recovered by transformation-associated recombination (TAR) cloning. pHook is BAC108L-based plasmid created in this study, which contains Kanamycin/neomycin selection marker cassette, yeast *ARS* and *CEN* sequences and specific hook sequences for α21-I alphoid^{tetO} 2mer. Details of pHook vector sequence are available upon request. YACs carrying alphoid DNA arrays were purified and transfected to *E. coli* cells, and then single colonies were picked up for insert length screening by PFGE gel electrophoresis. Using this procedure, we obtained ~50-kb arrays of α21-I alphoid^{tetO}-2mer repeats in the pHook vector. The final construct was termed pWTO2R, and used for tetR-fusions tethering.

The tetR-EFYP gene was amplified from the pFB-tetR-EYFP-neo plasmid (Nakano et al, 2008) with primers tetR-F and EYFP-R. PCR product was cut with Eco52I and EcoRI and cloned into NotI and EcoRI site of pQCXIP (Clontech). The constructed plasmid was named as pQC-TRYF-IP. The genes used for tetR-EYFP fusion proteins were obtained by RT-PCR. PCR product of Suv39h1, p300 HAT domain (amino acid 1283-1673) or PCAF HAT domain (amino acid 451-832) was cut with XhoI and NotI (for Suv39h1 and p300) or SalI and NotI (for PCAF), and cloned into XhoI and NotI site of pQC-TRYF-IP. Primer DNA sequences used in this study are shown in Supplementary

Table S3. These plasmids were transfected to amphopack 293 cells (Clontech) with pVSV-G (Clontech) plasmid, and produced retrovirus were harvested and infected to HT1080 or HeLa cells according to a previously described method (Nakano et al, 2008). For plasmid-based tetR-EYFP-fusion proteins expression, the genes were cloned into the pJETY3 vector. pJETY3 was constructed from pJTI Fast DEST vector (Invitrogen). Briefly, EF1 promoter, tetR-EYFP gene and IRES-Hyg gene sequences were inserted between PstI (1801) and EcoRI (4375) sites of pJTI Fast DEST vector by multistep DNA subcloning. Details of pJETY3 vector sequence are available upon request. PCR products of Suv39h1, p300HD, PCAFHD, hMis18α and HJURP genes were cloned into pJETY3.

Cell staining (in Supplementary Figure S9)

HeLa-HAC-R5 cells were transiently transfected with a plasmid expressing tetR-EYFP. Transfection with 2 μg plasmid DNA was carried out using Nucleofector Kit R (Amaxa) at programme setting I-013, essentially according to the manufacturer's instructions. Cells were subsequently seeded onto no. 1.5 glass coverslips in pre-warmed DMEM + GlutaMAX-I (Invitrogen) supplemented with 10% FBS and Penicillin / Streptomycin. For analysis of unperturbed mitoses two days after transfection, cells were fixed in 4% PFA / PBS for 10 minutes at room temperature prior to permeabilization in 0.2% PBS / Triton X-100 for 5 minutes and subsequent immuno-fluorescence staining as described below. For analysis of HAC kinetochore structure, transfected cells were incubated in the presence of 0.1 μg/ml Karyo MAX Colcemid (Invitrogen) for 5 hours at 37°C followed by brief hypotonic swelling in 75 mM KCl prior to fixation as above.

Immuno-fluorescence staining was essentially carried out as described previously (Cardinale et al, 2009). In brief, PFA fixed cells were pre-blocked in 3% BSA in PBS / 0.2% Tween (PBS-T) at 37°C following PFA fixation and Triton permeabilization. Incubation with primary antibodies diluted in 1% BSA in PBS-T was performed in a humid chamber at 37°C for one hour, followed by washing in PBS-T and incubation with fluorescently labeled (Texas Red or Cy5) secondary antibody (Jackson Research), diluted in 1% BSA / PBS-T, for 30 minutes at 37°C. Coverslips were subsequently mounted in VectaShield with DAPI (Vector Labs).

Z-stacks with a spacing of 0.2 μm were acquired on a DeltaVision microscope setup based on an Olympus IX-71 inverted microscope stand coupled to a Photometrics Cool Snap HQ camera. Stage, filters, shutter and camera were controlled by SoftWorx (Applied Precision), and an Olympus UPlanSApo 100x oil immersion objective (numerical aperture of 1.4) was used during image acquisition. All images were acquired at 1x1 binning and deconvolved with SoftWorx. Maximum intensity projections of the relevant HAC-containing sections were generated for display purposes.

Supplementary Figures and legends

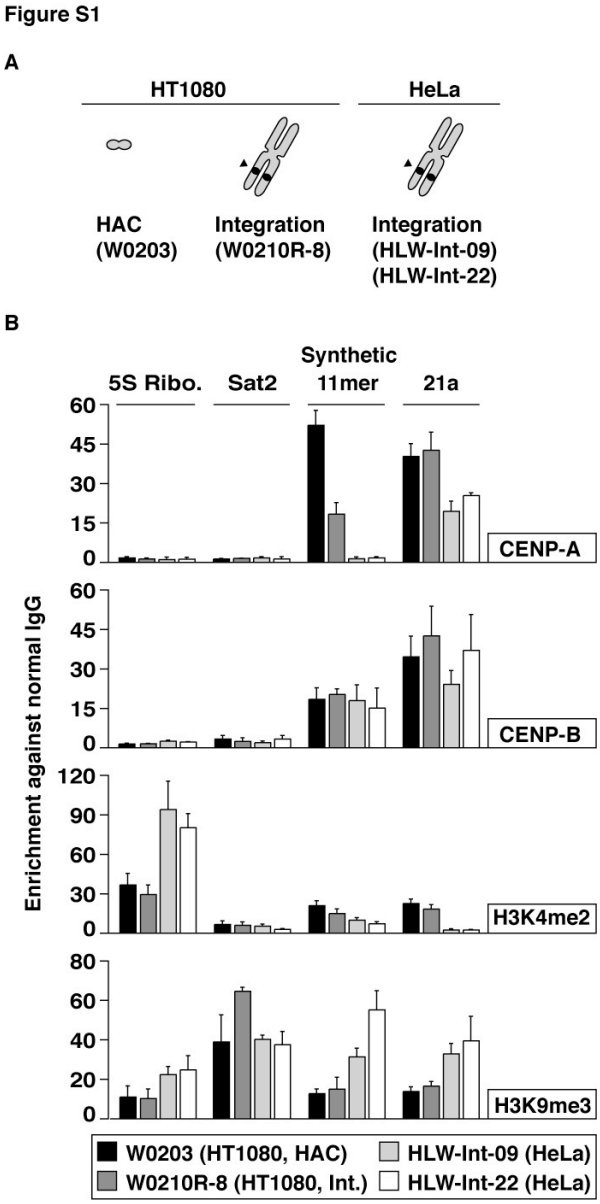

Figure S1 Cell type specific chromatin modifications and *de novo* CENP-A assembly activity on alphoid DNA. (**A**) Cell lines obtained from HAC formation assay with HT1080 and HeLa cells. pWTR11.32 plasmid (see Figure 1B) was transfected into HT1080 or HeLa cells. HT1080 transformants, W0203 (HAC) and W0210R-8 (Integration) were obtained in previous work (Ohzeki et al, 2002). HeLa transformants, HLW-Int-09 (Integration) and HLW-Int-22 (Integration), were obtained in this work. (**B**)

No CENP-A was assembled on the ectopic alphoid DNA integration sites in HeLa cells. ChIP analysis was carried out with normal IgG and indicated antibodies. PCR primers used for quantitative PCR are shown at the top. Error bars, standard deviation (n=3).

Figure S2

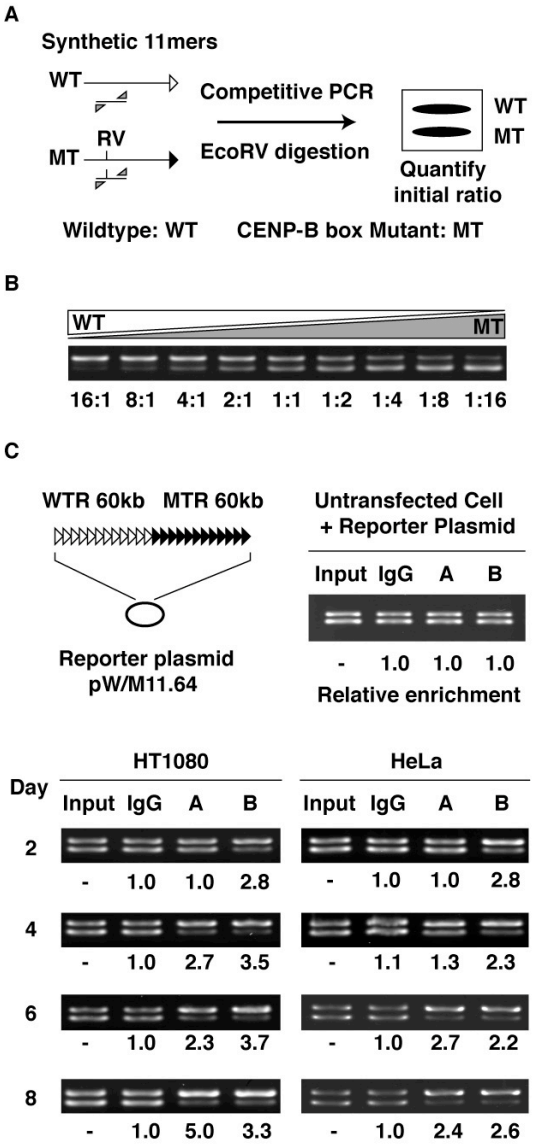

Figure S2 HeLa cell has de novo CENP-A assembly activity on transfected alphoid DNA. (**A**) A schematic diagram of competitive PCR detection using the synthetic alphoid DNAs. The α21-I 11mer wild type high order repeating (HOR) unit and the CENP-B box (CENP-B binding site) mutant 11mer HOR unit can be amplified with the same primer set keeping initial ratio of these DNA copies. PCR product from CENP-B box mutant 11mer contains two nucleotide substitutions, which produced a recognition site of restriction enzyme, EcoRV. PCR products derived from each alphoid DNA can be distinguished by EcoRV digestion and agarose gel electrophoresis. (**B**) Example of

competitive PCR. The α21-I 11mer (WT) and CENP-B box mutant (MT) DNA were mixed at several ratios, and amplified by competitive PCR. PCR products were digested with EcoRV and applied on agarose gel electrophoresis. (**C**) Transient transfection and ChIP assay with a reporter plasmid. The pW/M11.64 contains both 60 kb of α21-I 11mer and 60 kb of CENP-B box mutant 11mer repeat (Ohzeki et al, 2002). The pW/M11.64 was transfected to HT1080 or HeLa cell, and harvested 2, 4, 6 and 8 days after transfection for following ChIP assay. A competitive PCR detection was carried out with immuno-precipitated and input DNA. Capital letters indicate; ChIP sample with normal IgG (IgG), anti-CENP-A (A) and anti-CENP-B (B). Upper and lower band indicates PCR product derived from wild type and mutant 11mer repeat DNA, respectively.

Figure S3

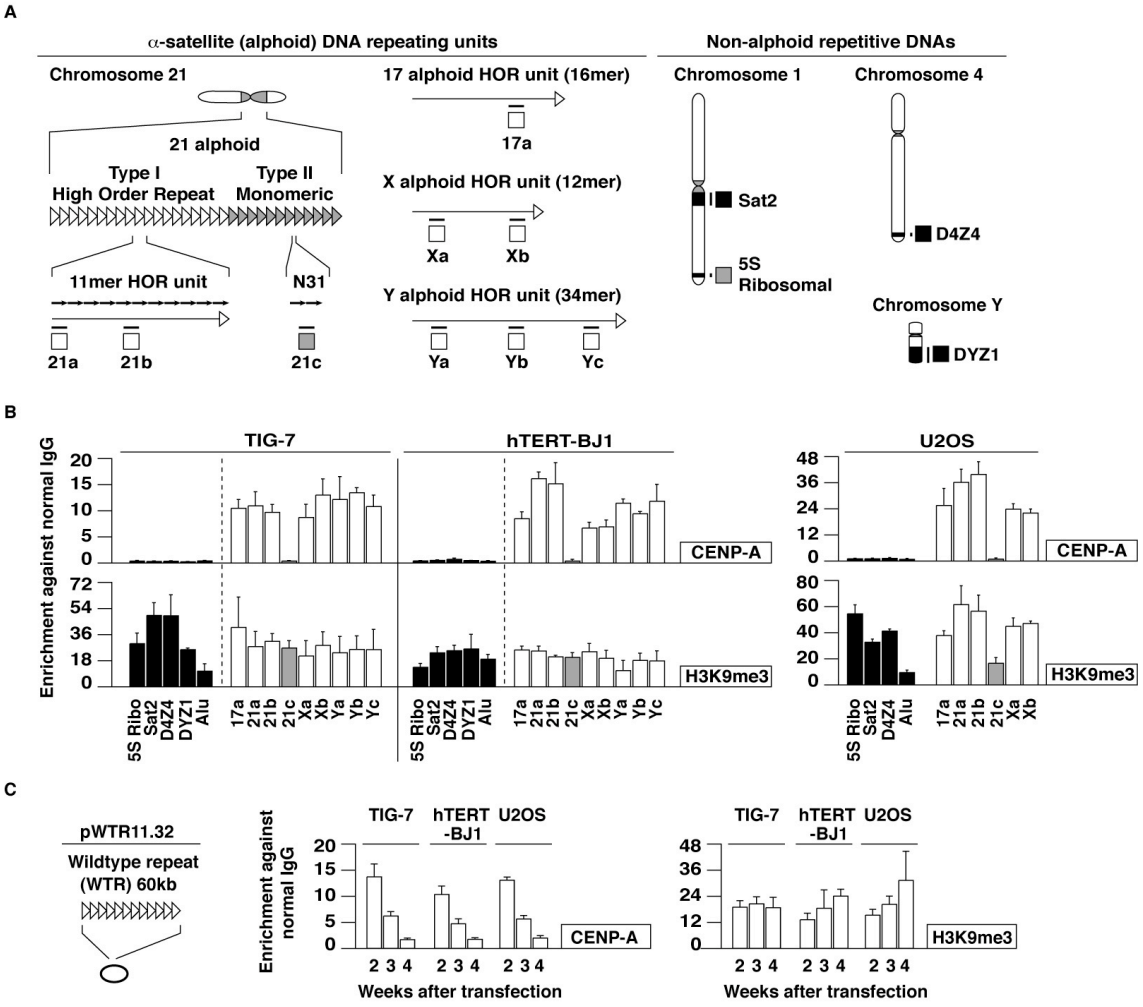

Figure S3 H3K9me3 and CENP-A assembly in human primary fibroblasts. (**A**) Schematic diagram of primers for human alphoid DNAs or other repetitive DNA sequences used in this study. Although human alphoid DNA is basically repetitive sequence of 171bp monomers, each chromosome's alphoid DNA has sequence variations. Type I alphoid is homogeneous repeat of chromosome specific HOR unit. Type II alphoid is diverged monomeric repeat (Willard and Waye, 1987: Ikeno et al, 1994). These indicated HOR DNA sequences are known as D17Z1 (17 alphoid), D21Z1 (21 alphoid), DXZ1 (X alphoid) and DYZ3 (Y alphoid). All the HOR except DYZ3 contain CENP-B box. Sat2, D4Z4 and DYZ1 were analyzed as controls for heterochromatic repeat. 5S ribosomal DNA sequence was used as a control for transcribed repetitive DNAs. One

additional set of primers was designed for *Alu* elements that are dispersed traces of retrotransposon. The primer DNA sequences are shown in Supplementary Table S2. (**B**) ChIP profiling of centromere chromatin with TIG-7, hTERT-BJ1 and U2OS cells. ChIP analysis was carried out with normal IgG and indicated antibodies. PCR primers used for quantitative PCR are shown at bottom. Vertical axis indicates enrichment against normal IgG control. Columns indicate non-alphoid repetitive DNA controls (black), type I alphoid DNA (white) and type II (gray), respectively. Error bars, standard deviation (n≥3). (**C**) Time-course ChIP analysis. The pWTR11.32 plasmid (left panel) was transfected to TIG-7, hTERT-BJ1 or U2OS 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. Primer set for synthetic 11mer repeats was used for quantitative PCR. Error bars, standard deviation (n=3).

Figure S4

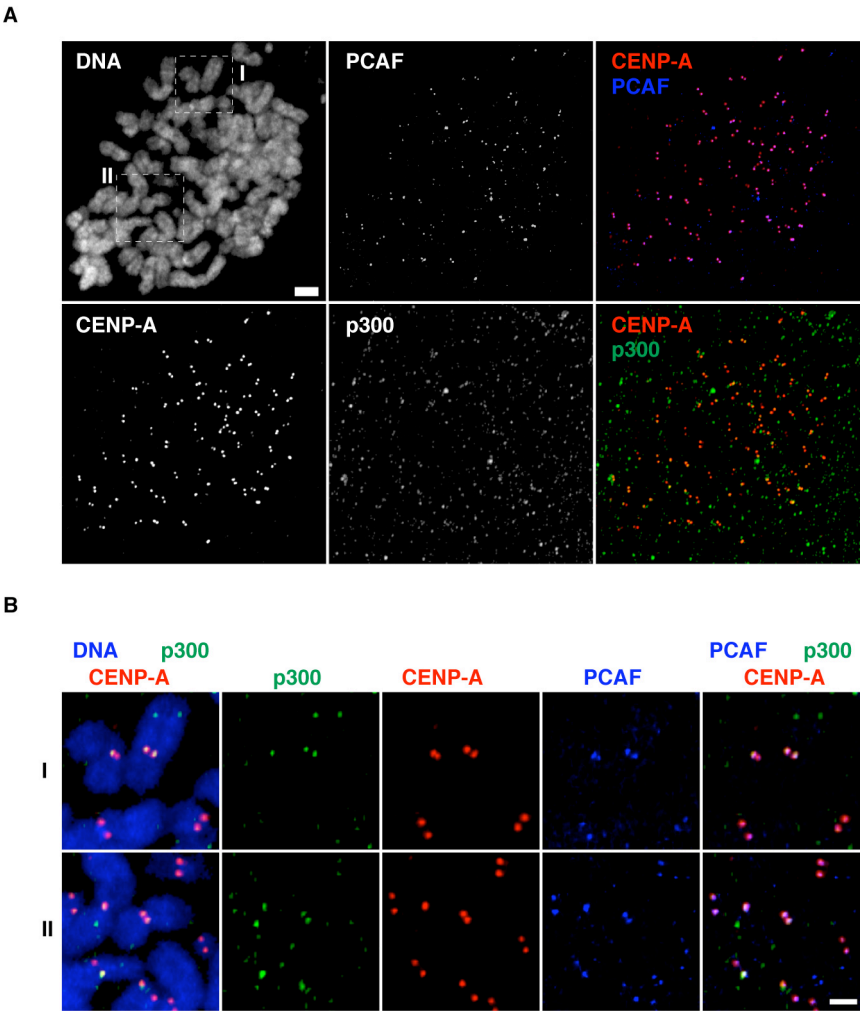

Figure S4 p300 and PCAF localize at kinetochore. (**A**) Mitotic HeLa cells were spread on cover glass, and stained with anti-CENP-A (red), anti-p300 (green) and anti-PCAF (blue) antibodies. PCAF signals were detected on all kinetochores. p300 signals were also detected on many kinetochores. Similar result was obtained with HT1080 cells. Scale bar, 10 μm. (**B**) Magnified images of the panel A. Selected two boxed areas shown in the panel a (top left, I and II) were magnified. (Left most panel) DNA, p300 and CENP-A signals are shown in blue, green or red, respectively. (Other panels) p300, CENP-A and PCAF signals are shown in green, red and blue, respectively. Scale bars, 3 μm.

Figure S5

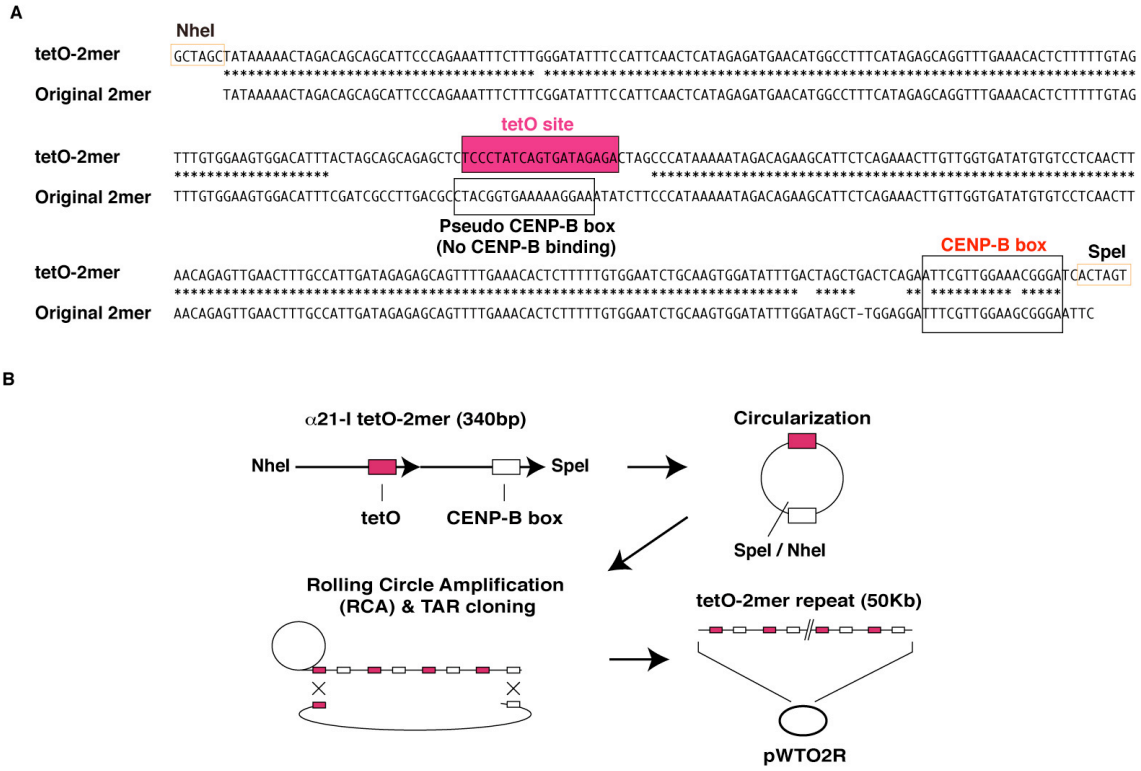

Figure S5 Construction of α21-I alphoid^{tetO} repeats. (**A**) DNA sequence of original α21-I-EcoRI-2mer and tetO are shown. The tetO site (red box) was embedded at the position of the pseudo CENP-B box sequence. Recognition sites for restriction enzyme NheI or SpeI were added at the ends of α21-I alphoid^{tetO} 2mer sequence (orange boxes). (**B**) Schematic diagram for α21-I alphoid^{tetO} 2mer repeat construction. The α21-I alphoid^{tetO} 2mer repeating unit was cut out from a cloning vector with NheI and SpeI. Then this fragment was circularized and used as a template for rolling circle amplification (RCA) reaction. RCA products were assembled into tandem repeat arrays by transformation-associated recombination (TAR) cloning method (Ebersole et al, 2005; Kim et al, 2009). For this study, we used a TAR BAC isolate containing a 50 kb repeat of α21-I alphoid^{tetO} 2mer sequence (pWTO2R).

Figure S6

Antibodies	tetR-EYFP-fusions	Weeks after transfection	Calculated p-Values		
			vs Suv39	vs p300	vs PCAF
CENP-A	tetR-EYFP-Alone	2 week	0.008	0.082	0.138
		3 week	0.040	0.181	0.032
		4 week	0.010	0.092	0.002
	tetR-EYFP-Suv39h1	2 week	-	0.006	0.005
		3 week	-	0.009	0.015
		4 week	-	0.019	0.000
	tetR-EYFP-p300HD	2 week	-	-	0.837
		3 week	-	-	0.948
		4 week	-	-	0.166
H3K9ac	tetR-EYFP-Alone	2 week	0.438	0.022	0.005
		3 week	0.760	0.079	0.012
		4 week	0.473	0.088	0.073
	tetR-EYFP-Suv39h1	2 week	-	0.034	0.026
		3 week	-	0.177	0.013
		4 week	-	0.082	0.002
	tetR-EYFP-p300HD	2 week	-	-	0.064
		3 week	-	-	0.050
		4 week	-	-	0.514
H3K9me3	tetR-EYFP-Alone	2 week	0.264	0.038	0.098
		3 week	0.108	0.368	0.303
		4 week	0.164	0.123	0.006
	tetR-EYFP-Suv39h1	2 week	-	0.029	0.157
		3 week	-	0.020	0.021
		4 week	-	0.107	0.023
	tetR-EYFP-p300HD	2 week	-	-	0.086
		3 week	-	-	0.561
		4 week	-	-	0.023

$p<0.05$	$0.05<p<0.2$

Figure S6 P-values of t-test for Figure 3C.

Figure S7

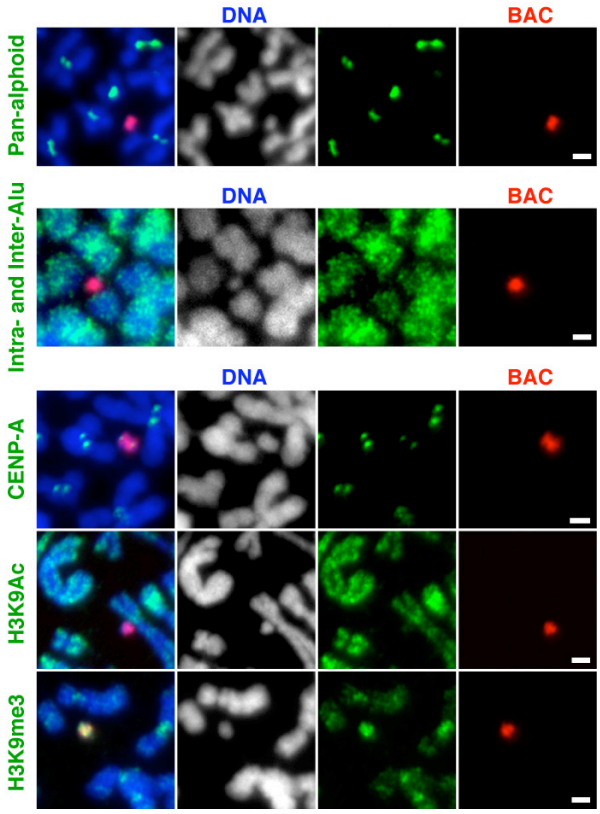

Figure S7 De novo tetO-HAC formed in HeLa cells. HAC containing HeLa cells, p300-HAC-13, were arrested at mitotic phase and harvested. Then the cells were spread and stained with BAC DNA probe specific to introduced pWTO2R plasmid (red) and DAPI (blue). (top) HAC was stained with Pan-alphoid DNA probe with excess amount of unlabeled α21-I alphoid^{tetO} 2mer DNA. Although pan-alphoid DNA probe (green) can hybridize all alphoid DNA families, unlabeled DNA competed out pan-alphoid DNA signal on tetO-HAC (detailed method was described in Ikeno et al, 1998), indicating tetO-HAC was formed only with α21-I alphoid^{tetO} 2mer DNA. (middle) Intra- and inter-*Alu* PCR probe (green) stains almost all chromosomal arm regions, but did not on tetO-HAC, indicating no detectable recruitment of host DNA fragment into *de novo* tetO-HAC. (bottom) Examples of metaphase tetO-HAC staining. Green signals obtained with indicated antibodies. Scale bars, 3 μm.

Figure S8

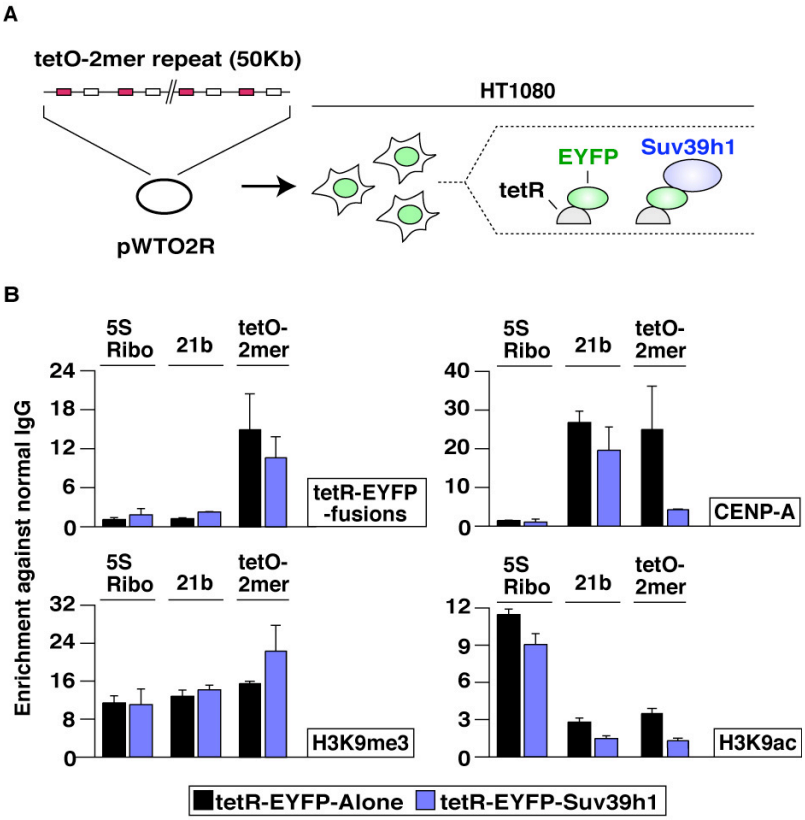

Figure S8 Suv39h1 tethering prevents de novo CENP-A assembly in HT1080 cell. (**A**) Schematic diagram for panel G. pWTO2R plasmid was transfected to HT1080 cells expressing tetR-EYFP or tetR-EYFP-Suv39h1 fusion protein. These tetR-EYFP and tetR-EYFP-Suv39h1 expressing cells were created by infection of retroviral expression vector. (**B**) Suv39h1 tethering inhibited *de novo* CENP-A assembly in HT1080 cells. pWTO2R transfected HT1080 cells were cultured under presence of selective drug (G418), and harvested for ChIP at two weeks after transfection. ChIP was carried out with normal IgG and indicated antibodies. Indicated primers were used for quantitative PCR (top). Error bars, standard deviation (n=3).

Figure S9

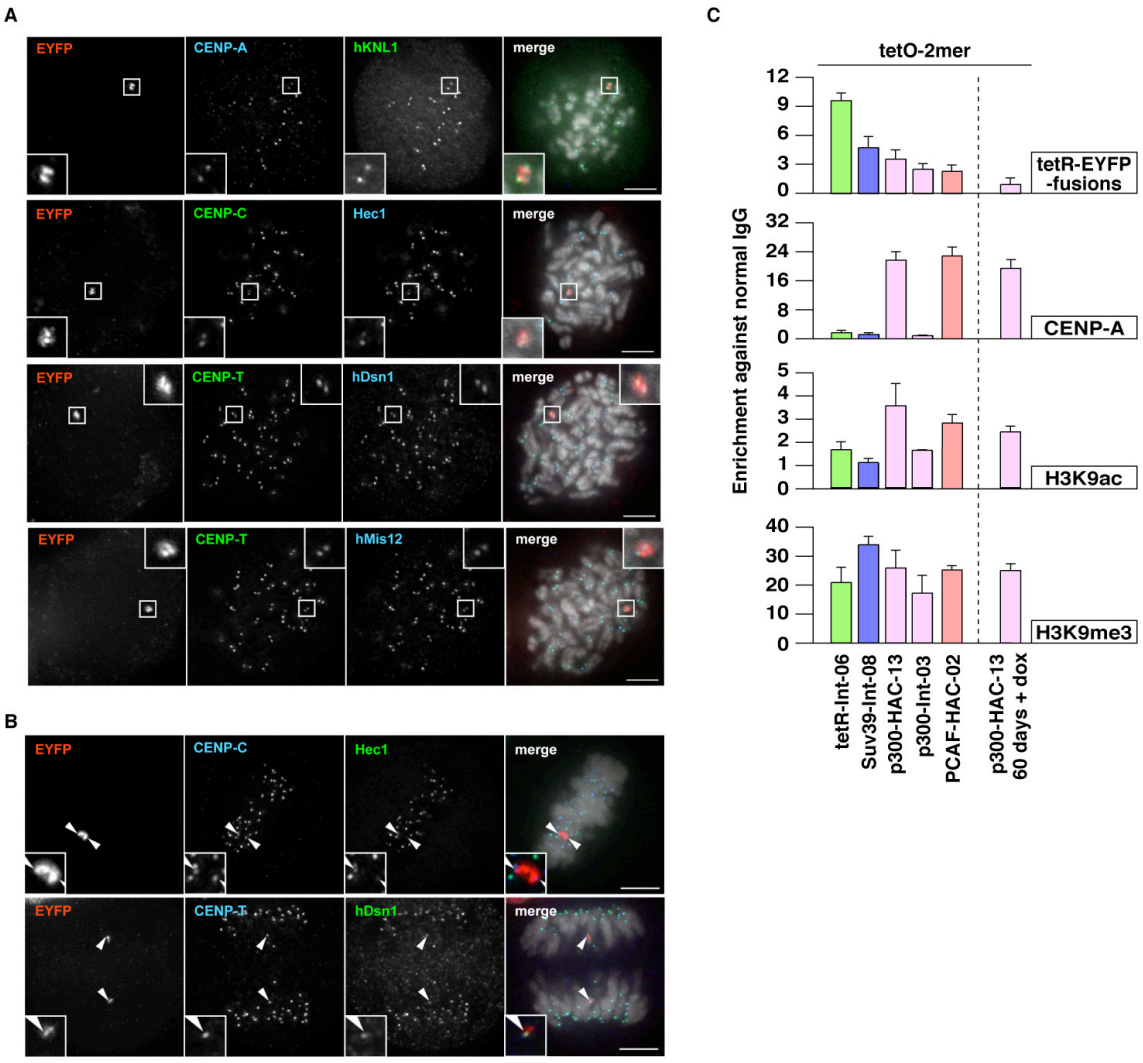

Figure S9 HAC kinetochore is stably maintained without further HAT tethering. (**A**) p300-HAC-13 cell was cultured under presence of doxycycline (no tetR binding condition) for more than 60 generations and single colonies were isolated. HeLa-HAC-R5 cell was one of these isolated cell lines, which lost detectable tetR-EYFP-fusion expression during culturing. This HeLa-HAC-R5 cell was transfected with a plasmid construct expressing tetR-EYFP. Cells were blocked in colcemid and co-stained for the indicated centromere and kinetochore components after brief hypotonic treatment. CENP-A, CENP-C and CENP-T are the inner kinetochore components (first from top, second from top and bottom two panels, respectively). Furthermore, outer kinetochore components were assembled as determined by staining for the KMN components

Hec1/Ndc80, hDsn1, hMis12 and hKNL1 (top to bottom panels). (**B**) In unperturbed mitotic cells, the HAC sister kinetochores (arrowheads) achieved alignment on the metaphase plate, with HAC kinetochores being under tension (top panel), and HAC sister chromatids segregated properly during subsequent anaphases (bottom panel) in all cells analyzed. Scale bar, 5 μm. (**C**) ChIP analysis of α21-I alphoid^{tetO} (pWTO2R) transformants. Indicated cell lines were obtained from HeLa cells expressing tetR-EYFP (tetR-Int-06), tetR-EYFP-Suv39h1 (Suv39-Int-08), tetR-EYFP-p300 HAT domain (p300-HAC-13 and p300-Int-03) and tetR-PCAF HAT domain (PCAF-HAC-02), respectively. p300-HAC-13 and PCAF-HAC-02 are HAC cell lines. tetR-Int-06, Suv39-Int-08 and p300-Int-03 carry ectopic α21-I alphoid^{tetO} DNA integration sites. ChIP was carried out with normal IgG and indicated antibodies. Primer set for synthetic α21-I alphoid^{tetO} 2mer (tetO-2mer) was used for quantitative PCR. Error bars, standard deviation (n=3).

Figure S10

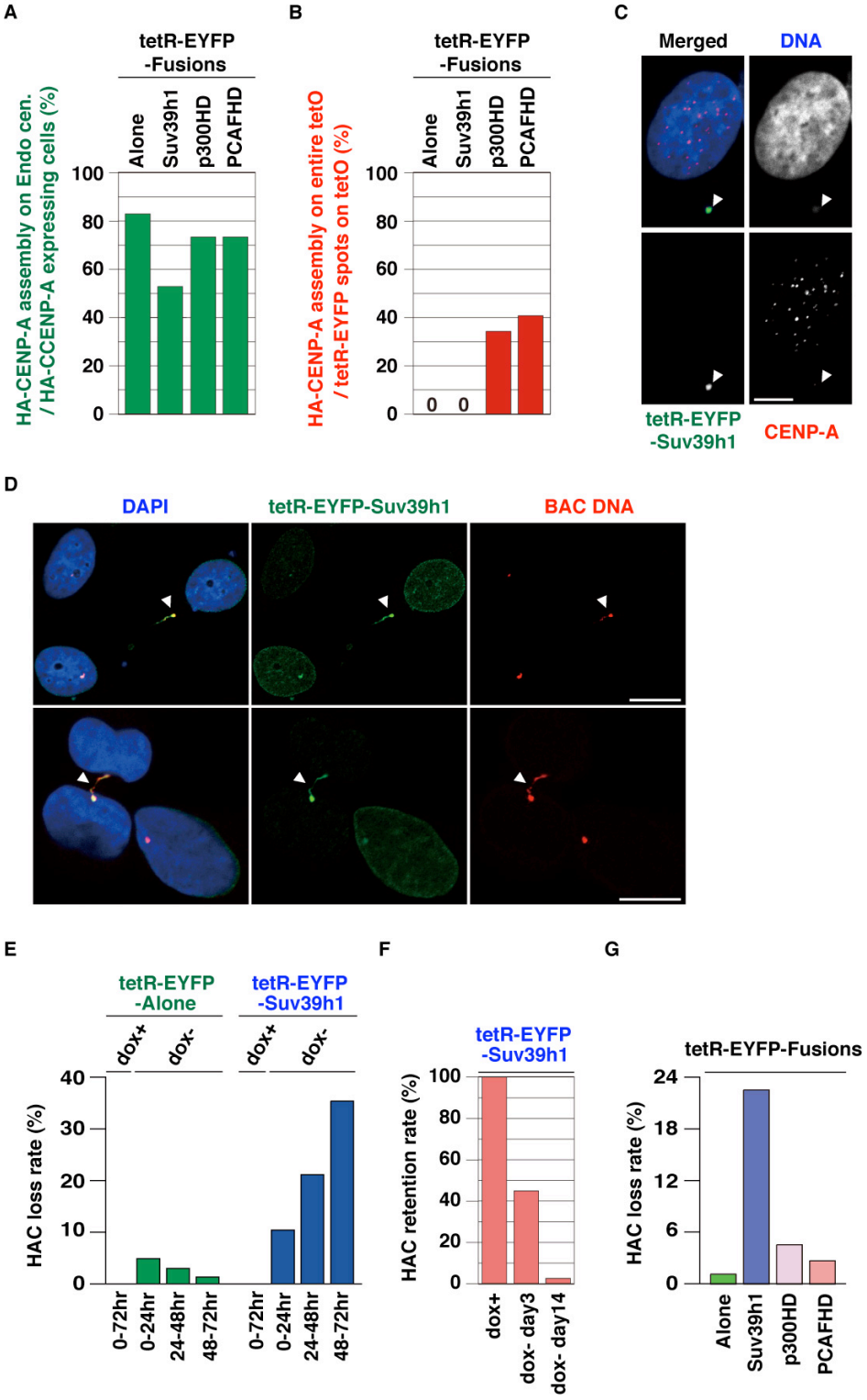

Figure S10 HAT tethering induces new-CENP-A assembly and Suv39h1 tethering

destabilizes tetO-HAC. (**A**) Frequency of HA-CENP-A assembly on endogenous centromere was counted in each sample shown in Figure 4C (n≥200). (**B**) Frequency of HAT tethering-induced expanded HA-CENP-A assembly was counted in each sample shown in Figure 4C (n≥200). (**C**) Destabilized tetO-HAC had lost CENP-A assembly. HeLa HAC-R5 cells expressing tetR-EYFP-Suv39h1 were stained with DAPI, anti-GFP (recognize EYFP, green) and anti-CENP-A (red). Arrowheads indicate a destabilized tetO-HAC outside of nuclei. Scale bar, 5 μm. (**D**) Examples of destabilized tetO-HAC detected by FISH. HeLa HAC-R5 cells expressing tetR-EYFP-Suv39h1 were stained with DAPI, anti-GFP (recognize EYFP, green) and BAC DNA specific probe (red). Arrowheads indicate destabilized tetO-HAC. (**E**) Suv39h1 tethering cumulatively destabilizes tetO-HAC. tetR-EYFP or tetR-EYFP-Suv39h1 was expressed in HeLa-HAC-R5 cells. Tethering of these proteins were induced by doxycycline removal. Cells were fixed at each time point and stained as shown in panel D. Destabilized tetO-HAC was counted (n≥200) and HAC loss rate was calculated. (**F**) HAC retention rates of Suv39h1 tethered cells were also counted with the same method shown on panels D and E. (**G**) HAC loss rates for 14 days with tetR-EYFP fusions shown in Figure 4A. HAC loss rate was calculated with HAC retention rates at day 0 (N_0) or at day 14 (N_{14}) using the following formula: $N_{14}=N_0 \times (1-R)^{14}$ (Ikeno et al, 1998).

Figure S11

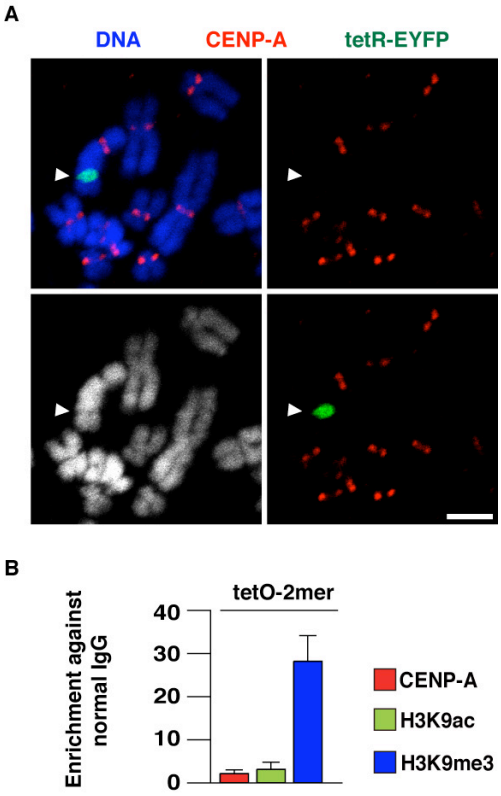

Figure S11 Integrated α21-I alphoid^{tetO} DNA array has no CENP-A assembly. (**A**) There was no CENP-A assembly at ectopic α21-I alphoid^{tetO} integration site. Mitotic HeLa-Int-03 cells transiently expressing tetR-EYFP were spread and stained with DAPI (blue), anti-GFP (recognize EYFP, green) and anti-CENP-A (red). Green signal indicates tetR-EYFP that binds to ectopic integration site of α21-I alphoid^{tetO} DNA array. (**B**) ChIP analysis of HeLa-Int-03 cells. ChIP was carried out with normal IgG and indicated antibodies. A set of primers for synthetic α21-I alphoid^{tetO} repeat (tetO-2mer) was used for quantitative PCR. Error bars, standard deviation (n=3).

Figure S12

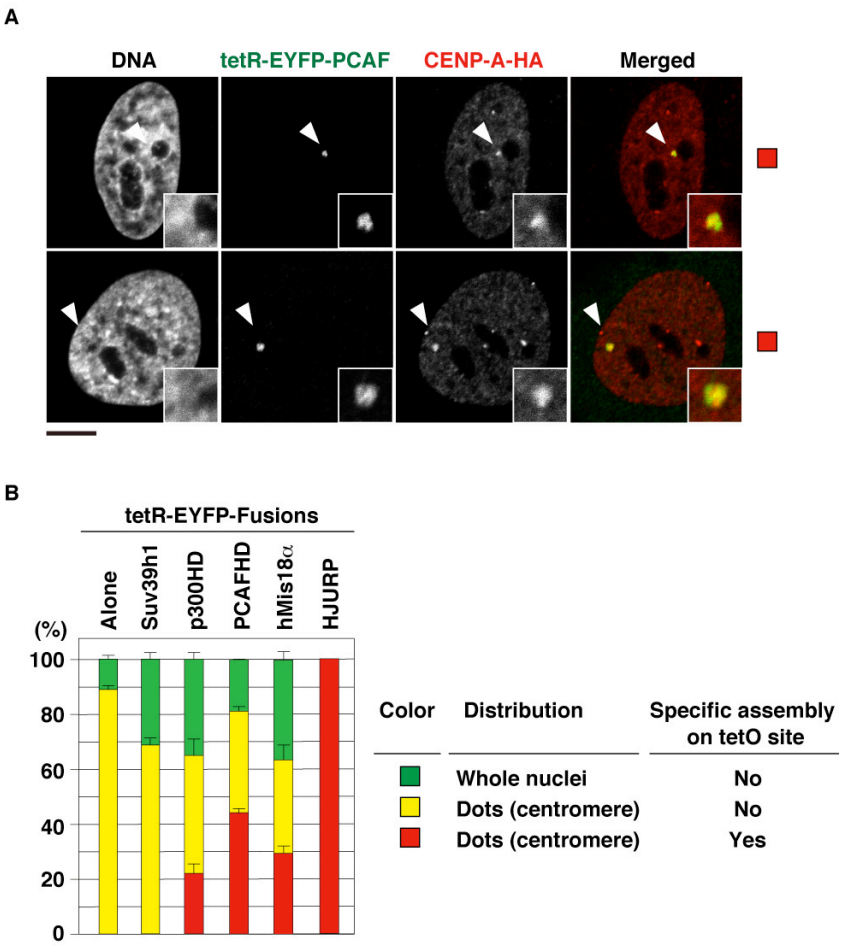

Figure S12 CENP-A-HA assembly on the ectopic alphoid^{tetO} integration site. (**A**) CENP-A-HA was expressed with the same procedure to Figure 5B. Cells were stained with DAPI, anti-GFP (green) and anti-HA (red). Scale bar, 5 μm. (**B**) Distribution of CENP-A-HA with tetR-EYFP-fusions tethering. CENP-A-HA localization patterns were divided as whole nuclei (green column), dots signals on only endogenous centromere (yellow column) and dots signals on centromere and tetO site. Cells containing tetR-EYFP spots were counted in each sample (n≥100). Error bar, standard deviation (n=3). There was no large difference between HA-CENP-A and CENP-A-HA.

Figure S13

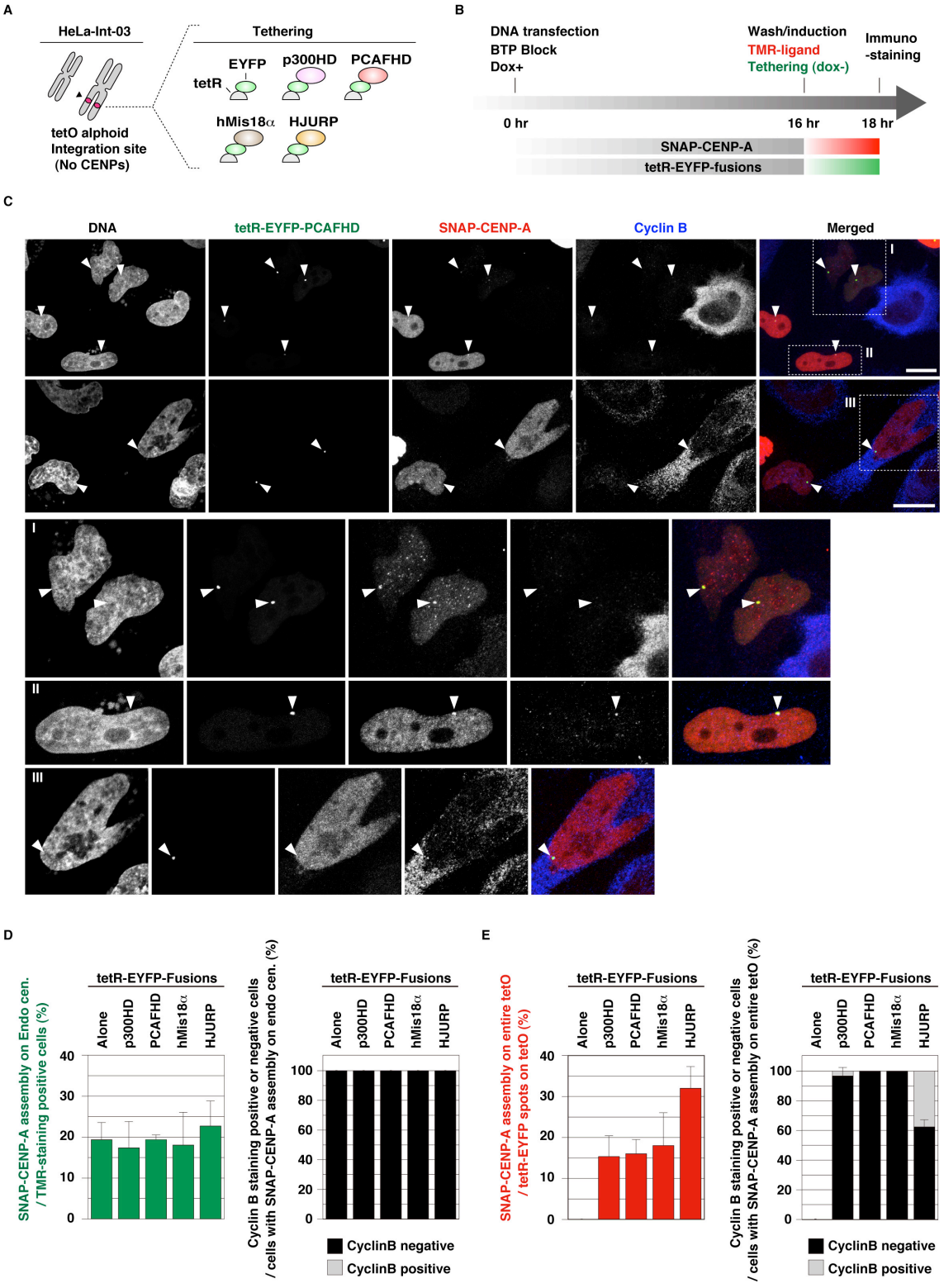

Figure S13 CENP-A assembly induced by HAT tethering occurred in the Cyclin B

negative (G1) cells. (**A**) Schematic diagram for used cell and tetR-EYFP-fusions. (**B**) Schematic timetable for a short-term tethering assay. HeLa-Int-03 cells were co-transfected with SNAP-CENP-A and a set of tetR-EYFP-fusion expression vectors. During first 16 hours, SNAP-cell-Block (BTP; NEB) was added to block SNAP tag (final 1μM). Doxycycline (20ng/ml) was also added to prevent tetR-EFYP-Fusions binding to tetO sites. At 16 hours after transfection, cells were washed with PBS three times and culture medium (BTP and doxycycline free) was added. Thirty minutes later, cells were washed again with PBS 3 times and culture medium containing 1 μM SNAP-cell-TMR-Star (TMR-ligand; NEB) was added. Cells were washed with PBS 3 times, fixed with formaldehyde and then used for immuno-staining. (**C**) Representative images of newly synthesized SNAP-CENP-A assembly on ectopically integrated alphoid^{tetO} DNA. Cells were stained with DAPI, anti-GFP (green) and anti-Cyclin B (Blue). TMR-labeled SNAP-CENP-A is shown as Red. Arrowheads indicate alphoid^{tetO} DNA integration sites. Scale bar, 10 μm. (**D**) (left panel) Frequency of SNAP-CENP-A assembly on endogenous centromere per total TMR-staining positive cells was counted in each sample (n≥100). Error bar, standard deviation (n=3). (Right panel) Frequencies of Cyclin B negative cells per cells with SNAP-CENP-A positive signals at centromeres were counted (n=10~24). Error bar, standard deviation (n=3). (**E**) (left panel) Frequency of SNAP-CENP-A assembly on ectopic alphoid^{tetO} DNA integration sites per TMR-staining positive cells was counted in each sample (n≥100). Error bar, standard deviation (n=3). (Right panel) Frequencies of Cyclin B positive or negative cells per cells with SNAP-CENP-A positive signals at centromeres (n=10~28). Error bar, standard deviation (n=3).

Figure S14

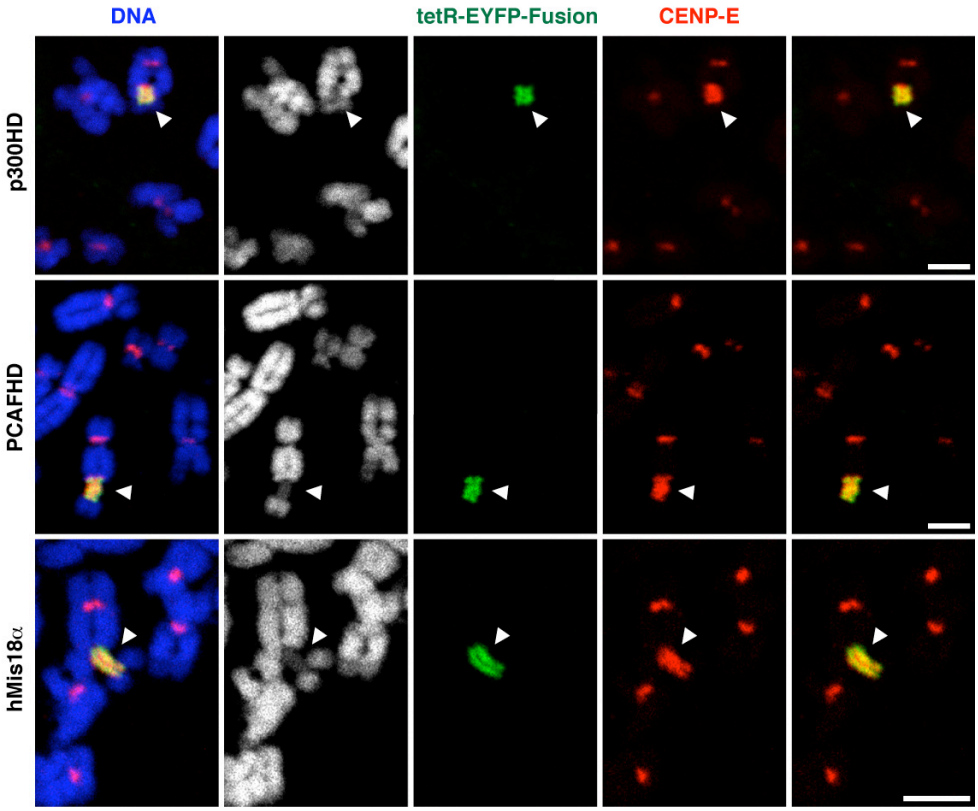

Figure S14 Kinetochore protein assembly on CENP-A deposited α21-I alphoid^{tetO} site. Examples of CENP-E assembly at ectopic α21-I alphoid^{tetO} DNA integration site. HeLa-Int-03 cells were co-transfected with HA-CENP-A and each tetR-EYFP fusion protein expressing vector as shown in Figure 6A. After 48 hours of incubation, mitotically arrested cells were spread and stained with DAPI (blue), anti-GFP and anti-CENP-E (red). Images were obtained by tethering of tetR-EYFP-p300HD (top), tetR-EYFP-PCAFHD (middle) or tetR-EYFP-hMis18α (bottom), respectively. Scale bars, 5 μm.

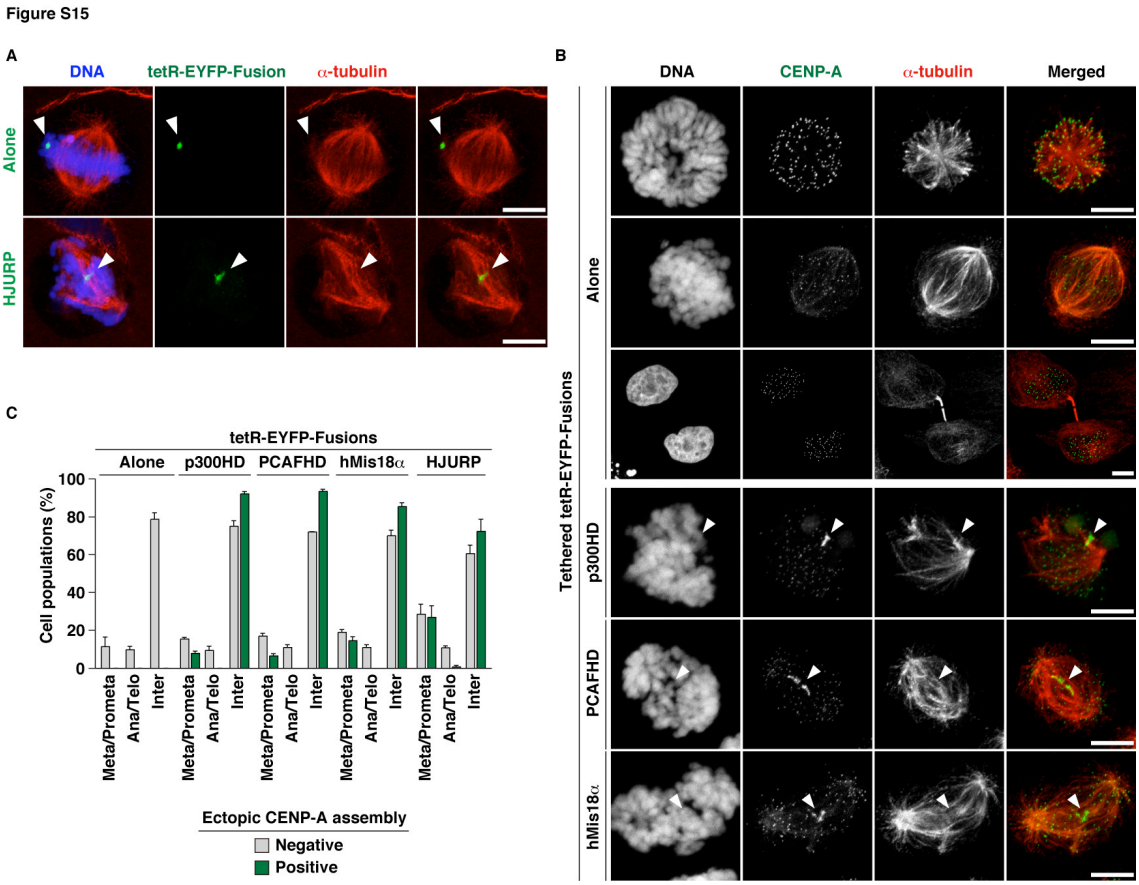

Figure S15 Induced ectopic kinetochores bundle microtubules. (**A**) HeLa-Int-03 cells were stained with DAPI (blue), anti-GFP (green) and anti-α-tubulin (red) antibodies. Tethering of tetR-EYFP-alone did not disturb a symmetric spindle formation (top). In contrast, tethering of tetR-EYFP-HJURP induced bundled microtubules to tetO site and abnormal spindle shape (bottom). Scale bars, 5 μm. (**B**) HeLa-Int-03 cells were stained with DAPI, anti-CENP-A (green) and anti-α-tubulin (red) antibodies. Tethering of tetR-EYFP-fusions except tetR-EYFP-Alone induced huge CENP-A assembly signal and abnormal spindle shape. Scale bars, 5 μm. All tetR-EYFP-fusions were dissociated tetO site by addition of doxycycline one hour before staining. (**c**) Cell cycle distribution of tetR-EYFP-fusions tethered cells. HeLa-Int-03 cells were stained as in panel B. Cells in meta-/prometa-, ana-/telo- and interphase were counted (n≥100). Error bar, standard deviation (n=3).

Figure S16

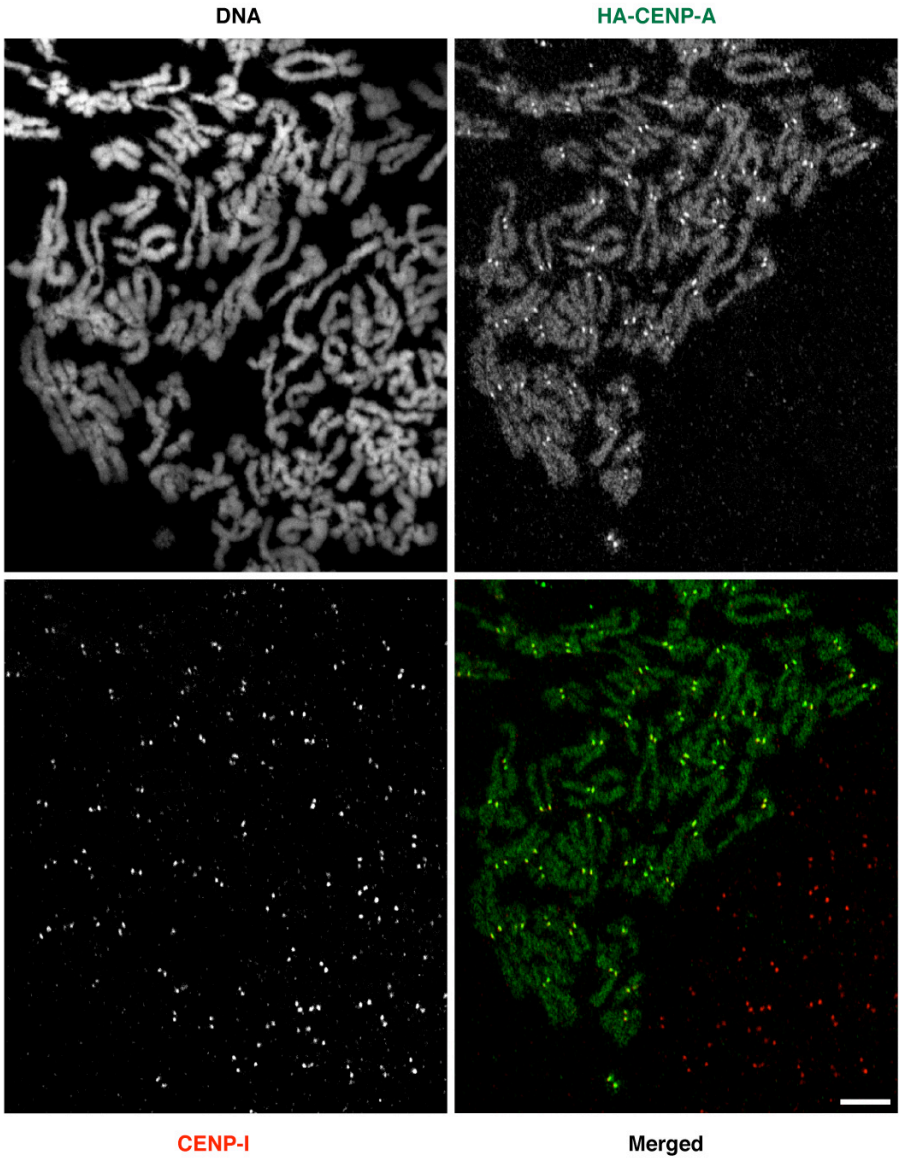

Figure S16 No CENP-I assembled on mislocalized CENP-A. HeLa-Int-03 cells were transfected with HA-CENP-A expressing vector. Mitotically arrested cells were spread and stained with DAPI (blue), anti-HA (green) and anti-CENP-I (red). Approximately 20% of spread showed HA-CENP-A mislocalized signal on the chromosomal arm region (top left in the panels). However, a kinetochore protein CENP-I was localized only at centromere but not on chromosomal arm regions. Scale bar, 10 μm.

Figure S17

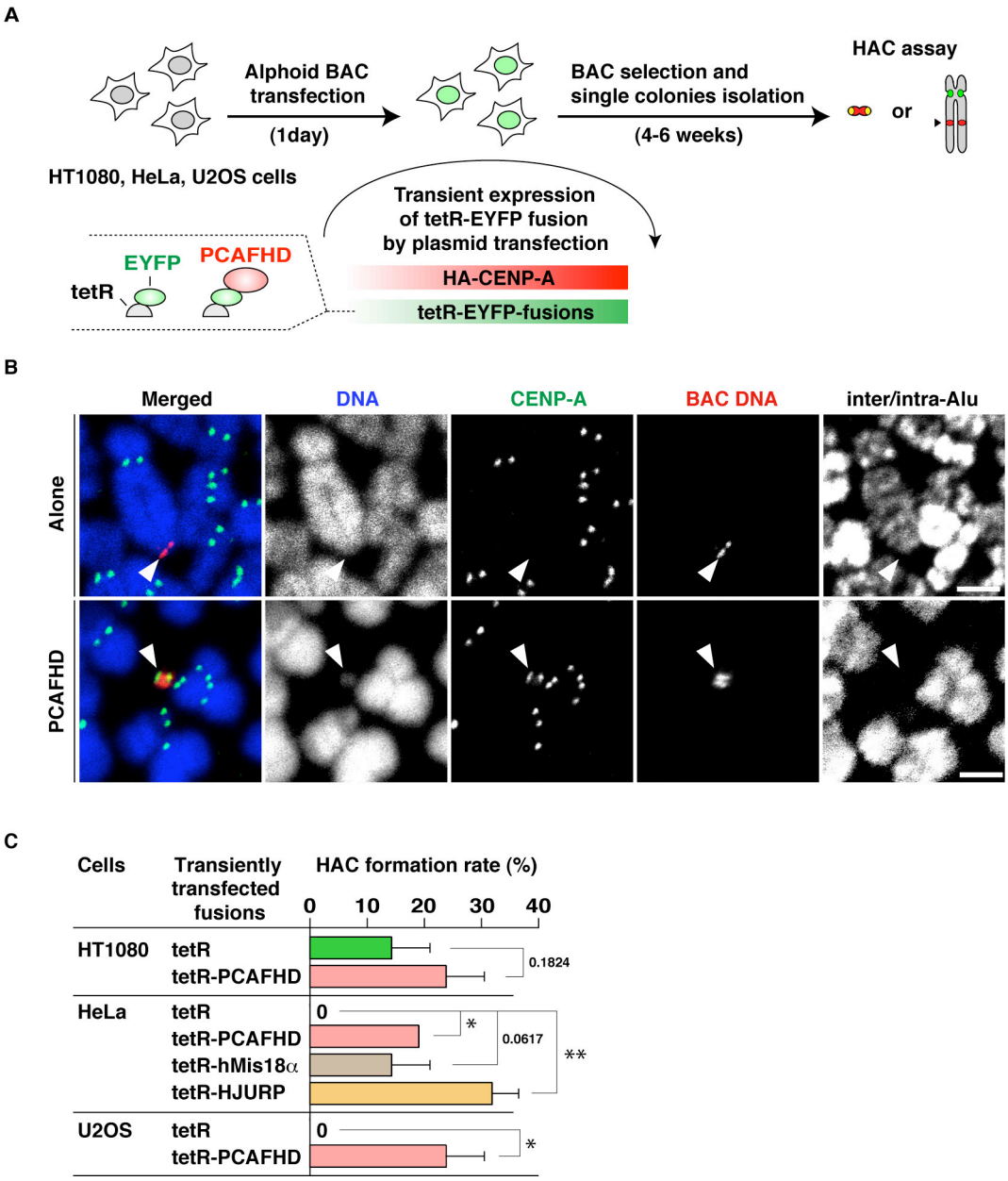

Figure S17 HAT-tethering induced HAC formation in HT1080, HeLa and U2OS cells. (**A**) Scheme for HAC formation. Alphoid^{tetO} DNA containing BAC plasmid (pWTO2R) were transfected to HT1080, HeLa and U2OS cells, respectively. One day after alphoid BAC transfection, tetR-EYFP-Alone or tetR-EYFP-PCAFHD was transiently expressed as shown in Figure 4 and 5. Cells transformed by pWTO2R were selected with G418. Single colonies were isolated and tested whether HAC formation by FISH. (**B**) Examples

of pWTO2R alphoid^{tetO} BAC transformants in U2OS cells (Upper; integration, Lower; HAC formation). Metaphase cells were spread and stained with DAPI (blue), anti-CENP-A antibody (green), BAC DNA probe (red) and intra-/ inter- Alu DNA probe (gray; see also Supplementary Figure S7). Scale bar, 3 μm. Expression of tetR-EYFP-fusion protein was no longer observed in these isolated cell lines. (**C**) Summary of HAC formation. Bars indicate a frequency of HAC formation in the pWTRO2R alphoid^{tetO} BAC transformants. Error bars, standard deviation ($n \geq 2$). P values of chi-square test of the predominant pattern for HAC formation frequency were indicated. Asterisks * or ** indicate P values, ($P<0.05$) or ($P<0.005$), respectively.

Figure S18

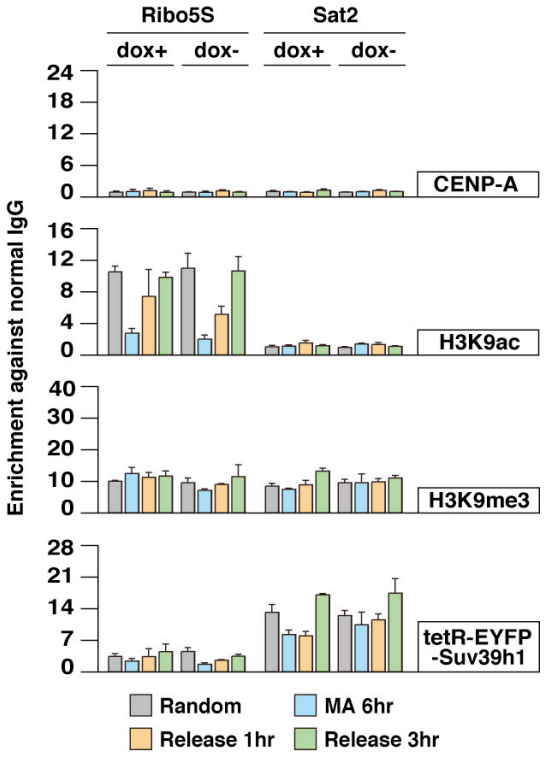

Figure S18 ChIP controls for Figure 7E. tetR-EYFP-Suv39h1 expressing HeLa-HAC-R5 cells were cultured in the presence or absence of doxycycline for three days as shown in Figure 7E, and then the harvested as the same sample sets as described in Figure 7A. ChIP assay was carried out with normal IgG and indicated antibodies. Indicated primer sets were used for quantitative PCR (top). Error bars, standard deviation (n=3).

Figure S19

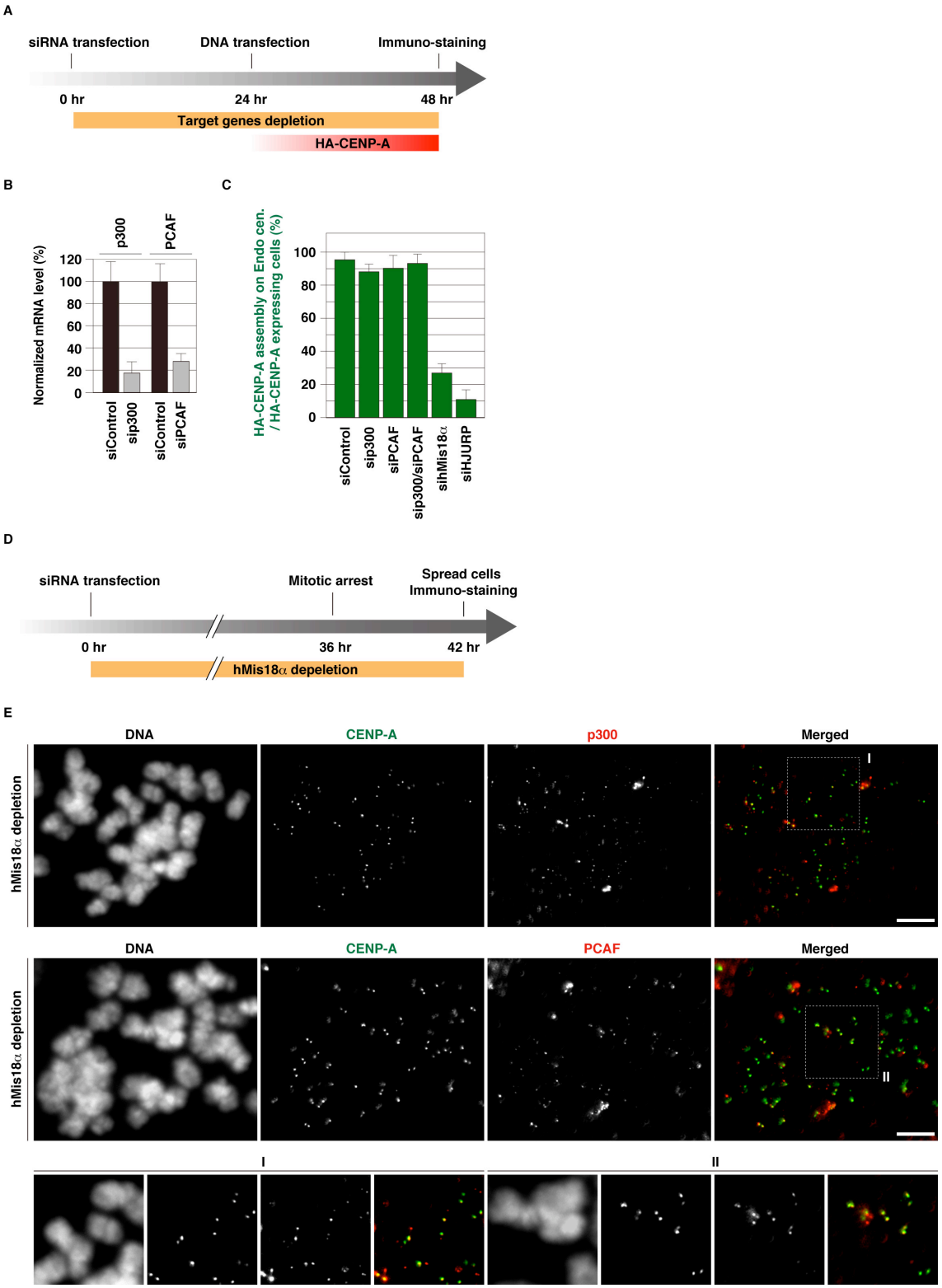
A
siRNA transfection
DNA transfection
Immuno-staining
0 hr
24 hr
48 hr
Target genes depletion
HA-CENP-A
B
p300
PCAF
Normalized mRNA level (%)
120
100
80
60
40
20
0
siControl
sip300
siControl
siPCAF
C
HA-CENP-A assembly on Endo cen. / HA-CENP-A expressing cells (%)
100
80
60
40
20
0
siControl
sip300
siPCAF
sip300/siPCAF
sihMis18α
siHJURP
D
siRNA transfection
Mitotic arrest
Spread cells
Immuno-staining
0 hr
36 hr
42 hr
hMis18α depeletion
E
DNA
CENP-A
p300
Merged
hMis18α depletion
DNA
CENP-A
PCAF
Merged
hMis18α depletion
I
II
I
II

Figure S19 The HAT normally responsible for de novo CENP-A assembly remains to be identified. (**A**) Scheme for new CENP-A assembly assay. (**B**) p300 or PCAF depletion using siRNA. siRNAs for p300 (sip300) and for PCAF (siPCAF) as well as for a negative control (siControl: siNegative, ambion) were used for transfection to HeLa cells. Total RNA was purified two days after transfection and quantified by real-time PCR. p300 or PCAF mRNA levels were normalized by HPRT transcripts. Horizontal axis indicates relative p300 or PCAF mRNA level against a negative control (siControl). Error bar, standard deviation (n=3). (**C**) Frequency of newly synthesized CENP-A assembly. HeLa cells were transfected with siRNAs shown in this panel, respectively. HA-CENP-A assembly frequency on endogenous centromere was counted for each sample as shown in Figure 4 ($n \geq 100$). Error bar, standard deviation (n=3). (**D**) Schematic diagram of metaphase cell preparation. (**E**) p300 and PCAF localized metaphase centromere under hMis18α depletion. (top and middle) Mitotic HeLa cells were spread on cover glass, and stained with anti-CENP-A (green) and anti-p300 (red), or anti-CENP-A (green) and anti-PCAF (red) antibodies. Scale bar, 10 μm. (bottom) Magnified images of the top and middle panels. Selected two boxed areas shown in the top panel (I) and the middle panel (II) were magnified, respectively.

Supplementary Table S1. Antibodies used in this study

Antibodies	Catalog number	Antibody produced in	Usage	Reference
anti-CENP-A (A1)	None	Mouse	Immuno-staining, ChIP	Ohzeki et al, 2002
anti-CENP-A (An1)	None	Mouse	Immuno-staining	Kind gift of Kinya Yoda
anti-CENP-A (r6F2)	None	Rat	Immuno-staining	Kind gift of Kinya Yoda
anti-CENP-B (5E6C1)	None	Mouse	ChIP	Ohzeki et al, 2002
anti-CENP-C (R554)	None	Rabbit	Immuno-staining	Kind gift of Kinya Yoda
anti-CENP-E	None	Rabbit	Immuno-staining	Kind gift of Tim Yen
anti-CENP-I	None	Rat	Immuno-staining	Kind gift of Kinya Yoda
anti-CENP-T	None	Rat	Immuno-staining	Kind gift of Kinya Yoda
anti-hDsn1	None	Mouse	Immuno-staining	Kind gift of Iain Cheeseman
anti-hKNL1	None	Rabbit	Immuno-staining	Kind gift of Iain Cheeseman
anti-hMis12	None	Rabbit	Immuno-staining	Kind gift of Mitsuhiro Yanagida
anti-Hec1 (clone 9G3)	Abcam, ab3613	Mouse	Immuno-staining	
anti-H3K4me2	Upstate, 07-030	Rabbit	ChIP	
anti-H3K4me3	Upstate, 07-473	Rabbit	ChIP	
anti-H3K9ac	Upstate 07-352	Rabbit	Immuno-staining, ChIP	
anti-H3K9ac	MABI, 0305	Mouse	Immuno-staining, ChIP	Kimura et al, 2008
anti-H3K9ac	MABI, 0310	Mouse	Immuno-staining, ChIP	Kimura et al, 2008
anti-H3K9me3	Upstate 07-523	Rabbit	Immuno-staining, ChIP	
anti-H3K9me3	MABI, 0308	Mouse	Immuno-staining, ChIP	Kimura et al, 2008
anti-p300 (clone RW128)	Millipore, 05-257	Mouse	Immuno-staining	
anti-PCAF	Abcam, ab12188	Rabbit	Immuno-staining	
anti-GFP	Roche	Mouse	ChIP for tetR-EYFP	Okada et al, 2007
Anti-GFP	MBL	Rabbit	Immuno-staining	
anti-HA-7	Sigma, H-3663	Mouse	Immuno-staining	
Anti-Cyclin B	Sigma, C-8831	Rabbit	Immuno-staining	

Supplementary Table S2. Primer sequences used for real-time PCR

Name	primer 1 (5' to 3')	primer 2 (5' to 3')
5S Ribosomal DNA	ACGCTGGGTTCCCTGCCGTT	TGGCTGGCGTCTGTGGCACCCGCT
Satellite 2	TCGCATAGAATCGAATGGAA	GCATTCGAGTCCGTGGA
D4Z4	CAGGCCTCCTGGCTGCACCT	TGAGCCCCGGCCGGAATTTCA
DYZ1	TTGGATTGCATTACATACGTG	TGGAGTCAAATGGAATGGTCT
Alu	GGCCGGGCGGTGGCTC	GAGACCGAGTCTCGCTCTGTC
17 alphoid	CAACTCCCAGAGTTTCACATTGC	GGAAACTGCTCTTTGAAAAGGAACC
21-I alphoid a	CTAGACAGAAGCCCTCTCAG	GGGAAGACATTCCCTTTTTCACC
21-I alphoid b	GTAGTTTGTGGAAGTGGAC	CTGAGAATGCTGCTGTCTACC
21-II alphoid	CATCTCACAGAGTAAAGGCT	CAGAATTTTTCTGTCTAGTTTTTGT
X alphoid a	AGATTTGGACCGCTTTGAGGC	CCGTTCAGTTATGGGAAGTTGA
X alphoid b	CCACAGAAAAACTAAACTGAAGC	GGCTTTCAGGCCTTTTCCACCAC
Y alphoid a	CACAGAATGTGTCTGTGTAG	GCATTCTGAGAAACCATTTTGTGC
Y alphoid b	GGAAGTTTCAAGTCTGTGAGGTG	CAACTCGCAGAGTTGACCCTATC
Y alphoid c	CACAGTAGAATGCAAAGGGCTCC	CACAGAAGCATGCTGAGAAACCTC
Synthetic 11mer	TTGTAGTGTCTGGAAGTGGAC	AATTCACTAGCGAATTCCC
tetO-2mer	GTGGAATCTGCAAGTGGATATTTGAC	CTGATAGGGAGAGCTCTGCTGCTAG
HPRT mRNA	GGACCCCACGAAGTGTTGG	CTGGCGATGTCAATAGGACTCCAG
Suv39h1 mRNA	TGCCCTCGGTATCTCTAAGAGG	GAGGATACGCACACACTTGAG
hMis18α mRNA	CATCCTGCTTCGCTGTGTTTCCTG	TTGAGTGAGCACCCCGCGCAGCAC
HJURP mRNA	GCCATCATCACCCCTGGGGTGCAG	GCAACACATGTAGATGAAGGAGCCTC
p300 mRNA	CAGAGCGTATTGTCCATGACTACAAGG	GGCCAGAAATCACCCTCGAAATAAGGC
PCAF mRNA	TACTCCGCCTGCAAGGCCGAGGAGTC	TGCTGCAGGTCGGCTCTGGGGGGAGT

For competitive PCR detection (Supplementary Figure S1), primer set for Synthetic 11mer was used.

Supplementary Table S3. Primers sequences used for DNA construction

Name	primer sequence (5' to 3')
2mer-F1	ATGGGATCCGCTAGCTATAAAAACTAGACAGCAGCATTCCC
2mer-F2	GAGGCTAGCCCATAAAAATAGACAGAAGCATTCTCAG
2mer-R1	CATACTAGTGATCCCGTTTCCAACGAATTCTGAGTCAGCTAGTCAAATATCCACTTGCAG
2mer-R2	TGGACTAGTCTCTATCACTGATAGGGAGAGCTCTGCTGCTAGTAAATGTCCACTTCCACAAACTAC
tetR-F	ATGCGGCCGACCGGTCGCGATGTCTAGATTAGATAAAAGTAAAGTG
EYFP-R	GCGGAATTCCGGATCCGGATCCGCGGCCGCTCACTCGAGTGTTAACGATATCTTGTACAGCTCGTCCATGCCG
Suv39h1-F	ACACTCGAGATGGCGGAAAATTTAAAAGGCTGCAGCG
Suv39h1-R	TCCGCGGCCGCTAGAAGAGGTATTTGCGGCAGGACTCAGTCCC
p300 HATd-F	ACACTCGAGAGGAAAGAAAATAAGTTTTCTGCTAAAAGGTTGCC
p300 HATd-R	TCCGCGGCCGCTAGCATTCATTGCAGGTGTAGACAAAGCGGTCC
PCAF HATd-F	ACAGTCGACATGGAATTAATCAACGAGGTTATGTCTACCATCACGG
PCAF HATd-R	TCCGCGGCCGCTCACTTGTCAATTAATCCAGCTTCC
hMis18α-F	ATGACTAGTGTCGACATGGCAGGCGTTCGGTCACTGAGGTG
hMis18α -R	CATGGATCCAGAATGGAGGACACAGACTAGAGTTC
HJURP-F	ATGCTCGAGGCTAGCATGCTGGGTACGCTGCGCGCCATGGA
HJURP-R	CATGCTAGCCACACTTTTAGTTTCCAATTTTTCTAGCATGAAATCAC

Supplementary Table S4. Cell lines used in this study

Name	Host cell	Input alphoid DNA	Expressing protein	Event	Reference
W0203	HT1080	pWTR11.32	None	HAC	Ohzeki et al, 2002
W0210R-8	HT1080	pWTR11.32	None	Integration	Ohzeki et al, 2002
HLW-Int-09	HeLa	pWTR11.32	None	Integration	This study
HLW-Int-22	HeLa	pWTR11.32	None	Integration	This study
tetR-Int-06	HeLa	pWTO2R	tetR-EYFP	Integration	This study
Suv39-Int-08	HeLa	pWTO2R	tetR-EYFP-Suv39h1	Integration	This study
p300-HAC-13	HeLa	pWTO2R	tetR-EYFP-p300HD	HAC	This study
p300-HAC-16	HeLa	pWTO2R	tetR-EYFP-p300HD	HAC	This study
p300-Int-03	HeLa	pWTO2R	tetR-EYFP-p300HD	Integration	This study
PCAF-HAC-02	HeLa	pWTO2R	tetR-EYFP-PCAF	HAC	This study
HeLa-HAC-R5	HeLa	pWTO2R	Silenced (undetectable) (tetR-EYFP-p300HD)	HAC	This study
HeLa-Int-03	HeLa	pWTO2R	None	Integration	This study
HeLa-HAC-R5 TRYF-Alone	HeLa	pWTO2R	tetR-EYFP	HAC	This study
HeLa-HAC-R5 TRYF-Suv39h1	HeLa	pWTO2R	tetR-EYFP-Suv39h1	HAC	This study

Supplementary references

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Kimura H, Hayashi-Takanaka Y, Goto Y, Takizawa N Nozaki N (2008) The organization of histone H3 modifications as revealed by a panel of specific monoclonal antibodies. *Cell Struct Funct* **33:** 61-73