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CENP-B Controls Centromere Formation Depending on the Chromatin Context

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SUMMARY

The centromere is a chromatin region that serves as the spindle attachment point and directs accurate inheritance of eukaryotic chromosomes during cell divisions. However, the mechanism by which the centromere assembles and stabilizes at a specific genomic region is not clear. The de novo formation of a human/mammalian artificial chromosome (HAC/MAC) with a functional centromere assembly requires the presence of alpha-satellite DNA containing binding motifs for the centromeric CENP-B protein. We demonstrate here that de novo centromere assembly on HAC/MAC is dependent on CENP-B. In contrast, centromere formation is suppressed in cells expressing CENP-B when alpha-satellite DNA was integrated into a chromosomal site. Remarkably, on those integration sites CENP-B enhances histone H3-K9 trimethylation and DNA methylation, thereby stimulating heterochromatin formation. Thus, we propose that CENP-B plays a dual role in centromere formation, ensuring de novo formation on DNA lacking a functional centromere but preventing the formation of excess centromeres on chromosomes.

INTRODUCTION

The centromere is an essential chromosomal domain that is required for accurate inheritance of eukaryotic chromosomes during cell division. A number of protein components of the centromere/kinetochore have been identified, including CENP-A, -B, -C, -E, -F, -H, hMis6 (CENP-I), hMis12, and others (Foltz et al., 2006; Izuta et al., 2006; Okada et al., 2006), many of which are conserved between yeast and humans (Chan et al., 2005). In particular, CENP-A, a centromere-specific histone H3 variant which is highly conserved among eukaryotes, is essential and required for assembly of the most of other centromere/kinetochore components (Howman et al., 2000; Sullivan, 2001; Goshima et al., 2003). Although CENP-A is required to maintain centromere chromatin as an epigenetic marker, the precise mechanism by which it promotes and stabilizes at the metazoan centromere is not yet known.

CENP-B, highly conserved in humans and mice, binds to the 17-bp CENP-B box through its amino-terminal region and dimerizes through its carboxy-terminal region (Earnshaw et al., 1987; Masumoto et al., 1989; Muro et al., 1992; Pluta et al., 1992; Yoda et al., 1992). The CENP-B box is conserved in centromeric human type I α-satellite (alphoid) DNA and mouse minor satellite DNA, even though these satellite sequences otherwise lack sequence homology. Centromere/kinetochore structures form at these two satellite loci (Kipling et al., 1995; Ando et al., 2002; Spence et al., 2002; Guenatri et al., 2004), and biochemical studies demonstrated that the CENP-A associated kinetochore complex contains CENP-B (Suzuki et al., 2004; Foltz et al., 2006). This result suggests that CENP-B forms a structural link between centromeric DNA sequences and the kinetochore. Indeed, this possibility is supported by previous studies showing that type-I alphoid DNA forms de novo human artificial chromosomes (HACs) with high efficiency in human HT1080 cells (Harrington et al., 1997; Ikeno et al., 1998; Ebersole et al., 2000; Mejía et al., 2001; Grimes et al., 2002), and that this reaction including the functional assembly of CENP-A and kinetochore requires alphoid DNA and CENP-B boxes (Ohzeki et al., 2002).

However, roles of CENP-B for centromere assembly have been controversial. Studies in fission yeast show that homologs of CENP-B play a role in forming pericentromeric heterochromatin adjacent to kinetochores (Nakagawa et al., 2002). In human cells, one of the two centromeres on dicentric fusion chromosomes is frequently inactivated even though both centromeres bind CENP-B (Earnshaw et al., 1989; Sullivan and Schwartz, 1995; Warburton et al., 1997). Moreover, in CENP-B knockout

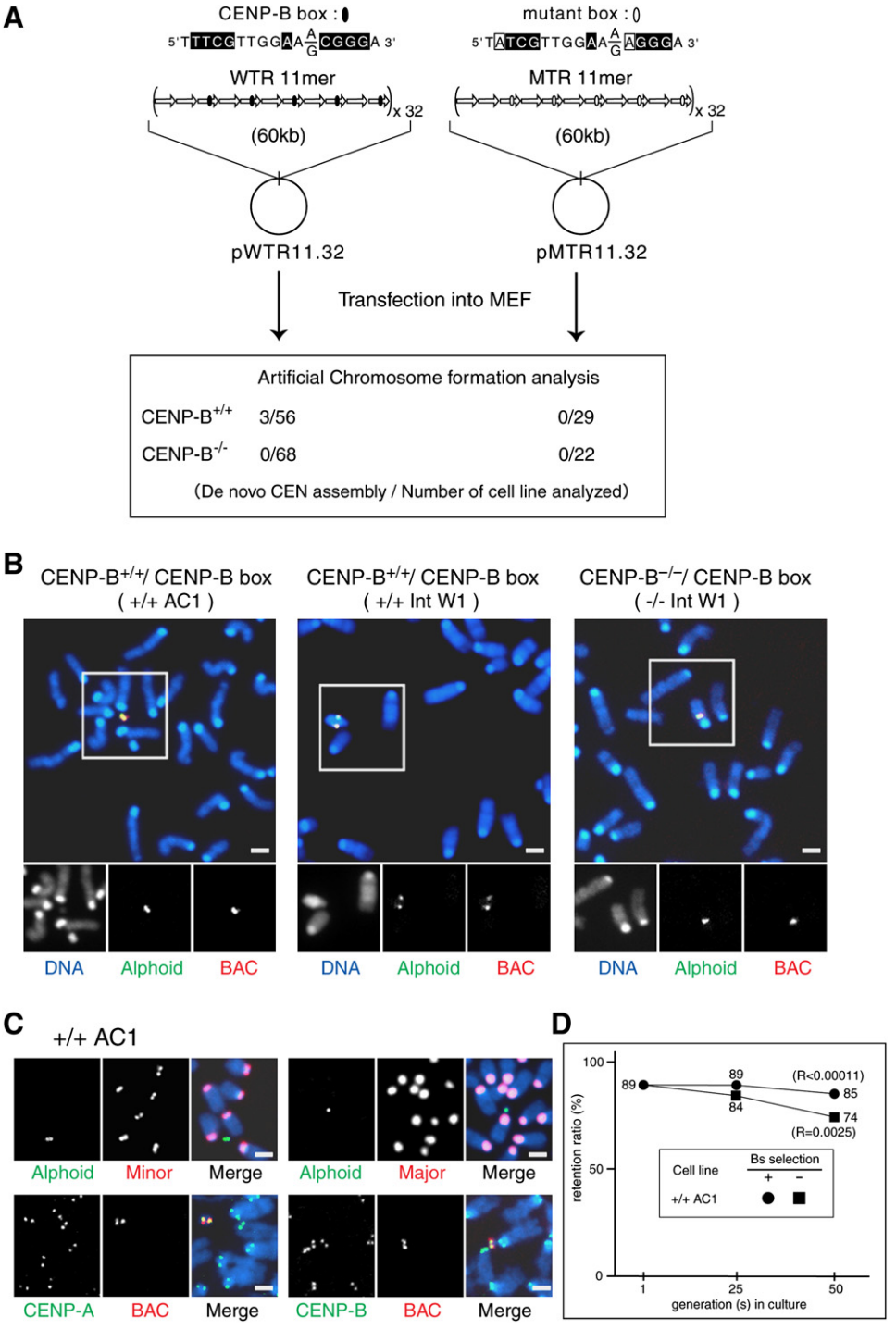

Figure 1. De Novo Artificial Chromosome Formation in Mouse Cells

(A) BAC vector containing synthetic α21-I alphoid sequences (60 kb) with wild-type or mutant CENP-B boxes was transfected into mouse embryonic fibroblasts (MEFs) or MEFs lacking CENP-B. De novo artificial chromosome formation and centromeric chromatin assembly occurred on input CENP-B boxes containing alphoid DNA when transformed in wild-type MEFs (see Table S1 for a more detailed explanation).

(B) FISH analysis of MEFs transfected with BACs carrying human alphoid DNA. Cell lines were as follows: CENP-B$^{+/+}$, extrachromosomal BAC (+/+ AC1); CENP-B$^{+/+}$, integrated BAC (+/+ Int W1); CENP-B$^{-/-}$, integrated BAC (−/− Int W1). FISH probes were for α21-I alphoid DNA (green) or BAC DNA (red).

(C) FISH analysis of +/+ AC1 MEFs with α21-I alphoid probes (green) and mouse minor satellite probes (red) or major satellite probes (red), or BAC probes (red) in combination with anti-CENPs immunofluorescence (green). DAPI (blue). The scale bars represent 2 μm.

mouse cells, functional kinetochores are maintained without CENP-B (Hudson et al., 1998; Kapoor et al., 1998; Perez-Castro et al., 1998). CENP-B is not detected on the Y chromosome centromere or in ''neocentromeres,'' rare functional centromeres in chromosomal arm regions. The neocentromere has no significant centromeric DNA sequence or CENP-B, but includes most other centromeric proteins including CENP-A (Choo, 2001).

The goal of this study was to investigate the intriguing role of CENP-B in de novo centromere assembly. To this end, BAC constructs carrying human alphoid DNA with wild-type or mutant CENP-B boxes were transfected into wild-type mouse embryo fibroblasts (MEFs) or CENP-B-deficient MEFs with or without exogenous CENP-B. The stability and initial/stable chromatin structure of the input alphoid DNA was then assessed using immuno-FISH and chromatin immunoprecipitation (ChIP).

RESULTS

Artificial Chromosome Formation in Mouse Cells

The following experiments examine de novo assembly of functional centromeric chromatin in wild-type and CENP-B-deficient MEFs (see Figure S1A available online). The chromatin assembly substrate for these experiments was a BAC construct carrying a 60 kb fragment of synthetic human alphoid DNA containing 32 tandem copies of the 11-mer unit with wild-type (pWTR11.32Bsr) or mutant CENP-B boxes (pMTR11.32Bsr) (Figure 1A). BAC DNA constructs were transfected into MEFs, selected for resistance to Blasticidin S (Bs) and transformants were analyzed by FISH using probes for α21-I alphoid DNA and BAC vector and by immunofluorescence using antibodies to CENP-B and CENP-A. The results are shown in Figures 1A–1C and Table S1. Alphoid DNA BAC with wild-type CENP-B boxes formed stable centromeric minichromosomes at the rate of 5.2% (3/56 cell lines in two independent experiments) as the predominant fates. While this efficiency is lower than that observed with the same construct in human HT1080 cells (~30% of cell lines) (Ohzeki et al., 2002), artificial chromosomes in MEFs have similar centromeric chromatin assembly. Endogenous murine CENP-A, CENP-B, and CENP-C proteins assembled on the human alphoid DNA, and mouse centromeric minor satellite and pericentromeric major satellite DNA did not colocalize with the nascent centromeres, as indicated by immuno-FISH analysis (Figures 1C and S3A). This result indicates that de novo centromere assembly occurs in wild-type MEFs on BAC constructs carrying human alphoid DNA. However, de novo centromere and artificial chromosome assembly does not occur in CENP-B-deficient MEFs (Figures 1A and 1B, Table S1, $p < 0.05$) or in wild-type MEFs carrying alphoid DNA BAC with mutant CENP-B boxes (Figure 1A and Table S1).

The mitotic stability of the mouse artificial chromosomes was examined in MEFs cultured under nonselective conditions (Figure 1D). This was done by performing FISH analysis of wild-type MEFs carrying a nonintegrated alphoid BAC with wild-type CENP-B boxes (+/+ AC1 cells) in the presence and absence of Bs over approximately 50 population doublings. The results showed high stability of the mouse artificial chromosome with a loss rate per cell division (R) of 0.0025; this result confirms the conclusion that functional centromeres form de novo on the transfected BACs as stable artificial chromosomes.

Stable Assembly of Mouse CENP-A and CENP-B on Human Alphoid DNA

Chromatin immunoprecipitation (ChIP) assay was used to determine whether CENP-A nucleosomes assembled on extrachromosomal (i.e., maintaining as an artificial chromosome) or chromosomally-integrated human alphoid DNA. The analysis was performed as shown in Figures 2A and 2B using competitive PCR. The multiplicity of the alphoid BAC DNA was measured before starting the analysis, as shown in Table 1. +/+ AC1 cells, carrying the extrachromosomal alphoid DNA with wild-type CENP-B boxes, and +/+ Int M1 cells, carrying chromosomally-integrated alphoid DNA with mutant CENP-B boxes (Figure S3B), were mixed in equal amounts, fixed, sonicated, and immunoprecipitated using antibody to CENP-B, CENP-A or normal IgG. The relative recovery of alphoid DNA with wild-type or mutant CENP-B-boxes was then measured by competitive PCR. The results show that the alphoid DNA with a wild-type CENP-B box was enriched 24-fold or 25-fold after immunoprecipitation with anti-CENP-A or anti-CENP-B antibodies, respectively (Figure $2B_b$). This indicates that assembly of mouse CENP-A chromatin occurs on human alphoid DNA with wild-type CENP-B boxes but not on mutant CENP-B boxes, as expected. Control experiments showed no change in the ratio of mutant and wild-type alphoid DNA when the samples were immunoprecipitated with normal IgG (Figure $2B_b$) or in CENP-B deficient MEFs (−/− Int W1 cell) (Figures 1B and $2B_d$). However, the wild-type alphoid DNA fragment was enriched 2- to 4-fold in CENP-$B^{+/+}$ cells with chromosomally-integrated alphoid DNA (+/+ Int W1 cell) (Figure 1B and Figure $2B_c$). Thus, CENP-A and CENP-B assembled with very low efficiency on wild-type alphoid DNA integrated in a mouse chromosome, and with much higher efficiency on extrachromosomal alphoid DNA. The high level of CENP-A assembly on extrachromosomal alphoid BAC with wild-type CENP-B boxes (+/+ AC1 cell) and the low or no CENP-A assembly on chromosomally-integrated alphoid DNA

(D) Mouse artificial chromosome stability. +/+ AC1 cells were cultured in selective (circle) or nonselective (square) medium. The proportion of > 70 metaphase cells containing artificial chromosomes was scored by FISH using α21-I alphoid probes and BAC probes and is shown in the histogram as retention ratio (%). Artificial chromosome loss rate (R) was calculated using the following formula: $N_n = N_1 \times (1\text{-}R)^n$. N_1 and N_n are the rates for artificial chromosome containing cells in these cell lines at the points of 1 and n generation(s), respectively.

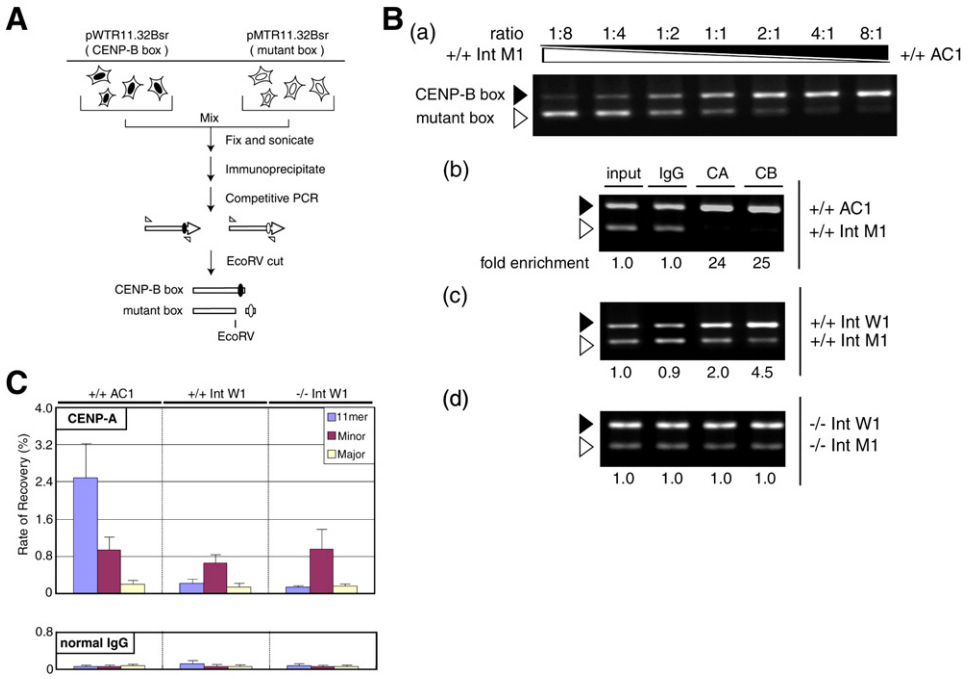

Figure 2. Competitive ChIP Analysis of Centromeric Chromatin in Human Alphoid DNA

(A) Schematic diagram of competitive ChIP analysis. pWTR11.32Bsr-transformed cells (+/+ AC1, +/+ Int W1 or −/− Int W1) were mixed with pMTR11.32Bsr-transformed cells (+/+Int M1 or −/− Int M1).

(B) (a) Competitive PCR products from genomic DNA templates. +/+ AC1 and +/+ Int M1 cells were mixed at different ratios (from 1:8 to 8:1). Black arrowhead indicates the 142-bp PCR fragment from pWTR11.32Bsr (CENP-B box). White arrowhead indicates the 111-bp PCR fragment from pMTR11.32Bsr (mutant box). (b–d) Competitive PCR products from ChIP analysis with normal IgG (IgG), anti-CENP-A antibody (CA), and anti-CENP-B antibody (CB). Relative enrichment of the CENP-B box alphoid versus mutant box alphoid is shown below the panels.

(C) ChIP and real-time PCR analyses were carried out for the introduced alphoid DNA, mouse minor satellite, or major satellite using anti-CENP-A antibody and normal IgG (as a control). Recovery rate of immunoprecipitated DNA against the input DNA is shown in the histogram. Error bars represent the SEM (n = 3).

(+/+ Int W1 cell or −/− Int W1 cell) were confirmed by ChIP and real-time PCR analysis (Figure 2C) referenced by positive (centromeric minor satellite DNA) and negative (pericentromeric major satellite DNA) mouse endogenous controls. These data establish that de novo assembly of CENP-A chromatin occurs on extrachromosomal human alphoid DNA in MEFs. However, this reaction requires functional CENP-B and functional (wild-type) CENP-B boxes in human alphoid DNA and is enhanced by an extrachromosomal location of the heterologous centromeric DNA. Integration of the centromere competent alphoid DNA into an ectopic chromosomal site caused fairly strong suppression in the assembly of CENP-A chromatin.

Transient Assembly of CENP-A and CENP-B in Wild-Type and CENP-B-Deficient MEFs

The assembly of CENP-A chromatin was examined at early time points after DNA transfection using a sensitive modified ChIP assay called transient ChIP. In this assay, centromere assembly was examined on a chimeric BAC construct containing 60 kb human alphoid DNA with wild-type CENP-B boxes adjacent to 60 kb alphoid DNA with mutant CENP-B boxes (Figure 3A). Here, the mutant alphoid DNA served as an internal negative control. The competence of this chimeric alphoid BAC to form de novo centromere assembly was initially tested in human HT1080 cells showing high-efficiency artificial chromosome formation as a control. After transfection, cells were cultured under nonselective conditions and competitive ChIP analysis was carried out after 1–4 days. The results showed that CENP-B assembled on alphoid DNA with wild-type CENP-B boxes within 1 day after transfection, and that wild-type alphoid DNA was enriched 8- to 10-fold in CENP-B immunoprecipitates. CENP-A assembly was also detectable on the third and fourth days after transfection, with 2- to 3-fold enrichment of wild-type alphoid DNA in CENP-A immunoprecipitates (Figure 3B). Other experiments showed that the chimeric alphoid BAC (mutant and wild-type CENP-B boxes) and the wild-type alphoid BAC formed stable human artificial chromosomes with similar efficiency in human HT1080 cells (data not shown).

Transient ChIP with the chimeric alphoid BAC was then carried out in CENP-B$^{+/+}$ and CENP-B$^{-/-}$ MEFs. The results showed that CENP-A and CENP-B assembled on alphoid DNA with wild-type CENP-B boxes within 1 day after transfection into CENP-B$^{+/+}$ MEFs. Wild-type alphoid

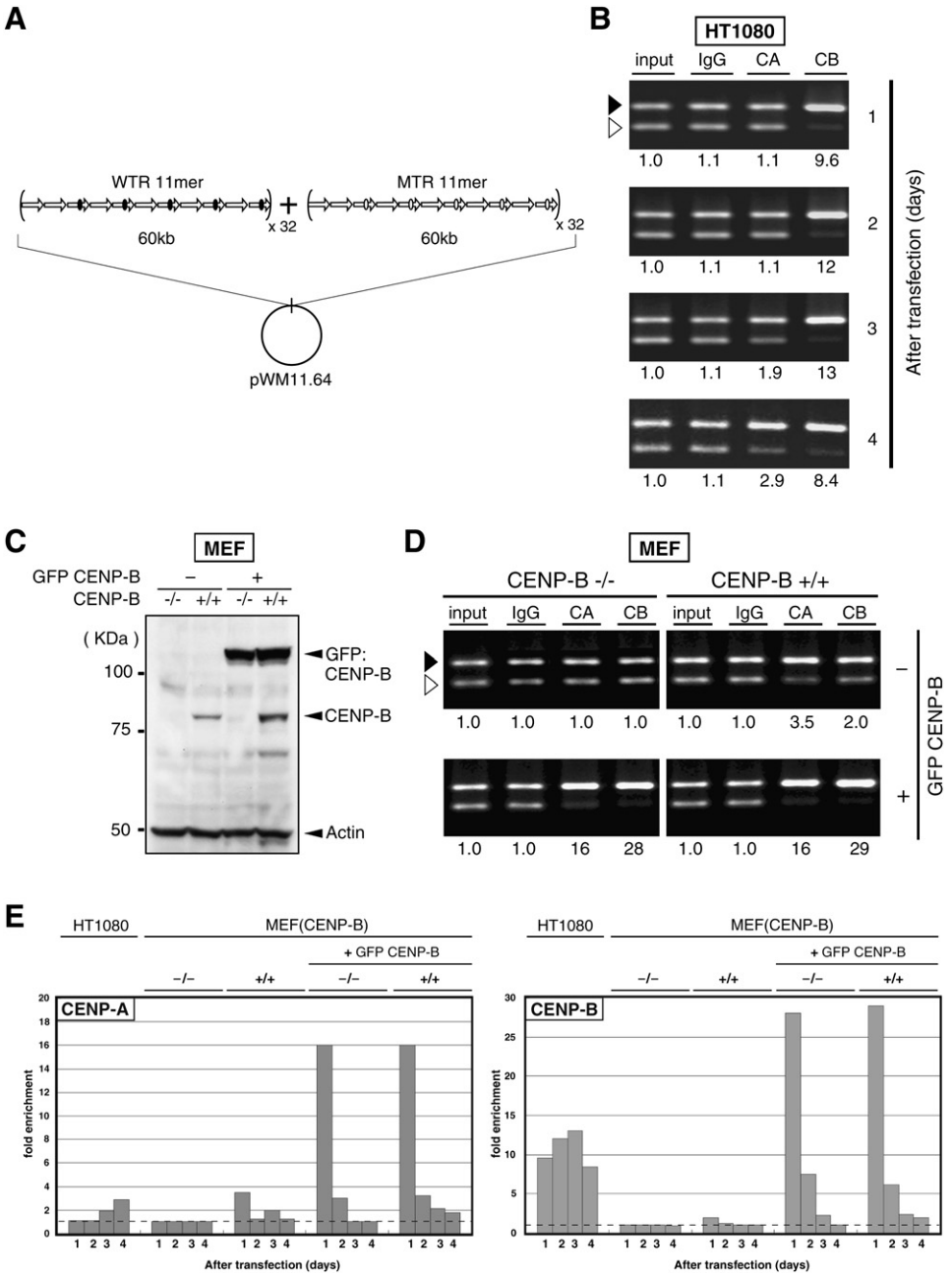

Figure 3. Transient ChIP Assay of Centromeric Chromatin in Human Alphoid DNA in HT1080 Cells or MEFs

(A) BAC-based pWM11.64 vector with chimeric alphoid arrays containing CENP-B boxes and mutant boxes. (B) Competitive ChIP analysis, as in Figure 2 was performed with pWM11.64-transfected HT1080 cells at indicated day(s) after transfection. pWM11.64-introduced MEF cells (CENP-B$^{+/+}$ or CENP-B$^{-/-}$) cotransfected with or without a GFP-CENP-B expressing vector were analyzed by immunoblotting using antibodies against CENP-B (BC1, Suzuki et al., 2004) and Actin (A4700, Sigma) (C) or by competitive ChIP 1 day after transfection (D). (E) Transient ChIP analysis was performed at indicated day(s) after transfection (Figures 3B, 3D, and S4). The dashed line represents the threshold signal of 1-fold, above which significant enrichment for CENP-A and CENP-B states was scored.

DNA was enriched 3.5-fold and 2.0-fold in CENP-A and CENP-B immunoprecipitates, respectively. In contrast, wild-type alphoid DNA was not enriched and CENP-B/A assembly was not detected in CENP-B$^{-/-}$ cells up to 4 days after transfection (Figures 3D and 3E). The lower enrichment of CENP-B assembly on alphoid DNA in CENP-B$^{+/+}$ MEFs may be due to one-eleventh lower level of CENP-B expression in CENP-B$^{+/+}$ MEFs compared to that in HT1080 cells (data not shown). To try to complement the defect in centromere assembly in CENP-B-deficient

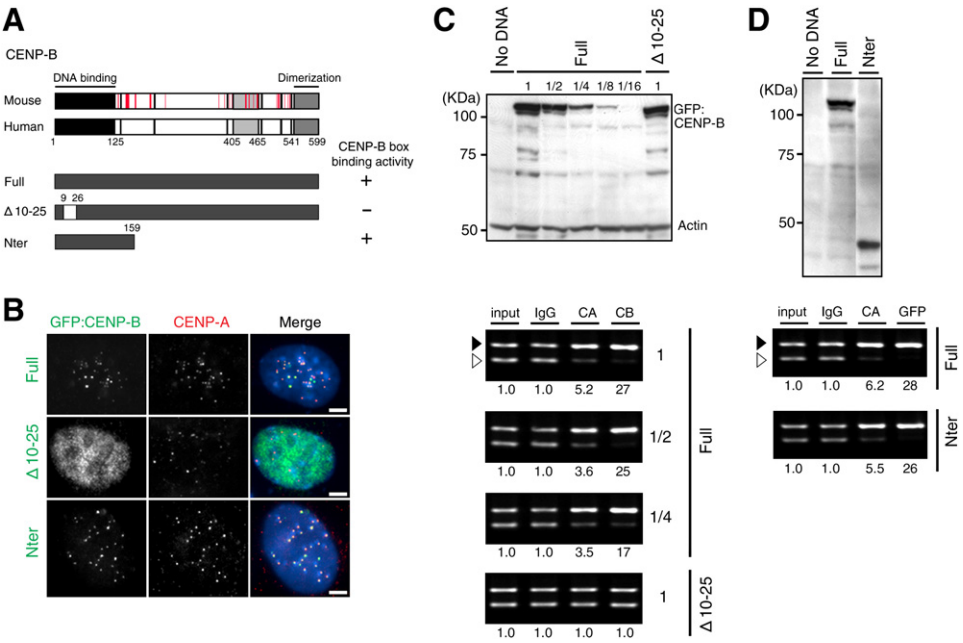

Figure 4. Binding of CENP-B, N-Terminal Fragment, to Alphoid DNA Is Required for Assembly of CENP-A Chromatin

(A) Schematics of CENP-B protein domains. Red lines indicate amino acid differences between human and mouse CENP-B. CENP-B box-binding activity of the full-length CENP-B (Full), the N-terminal fragment (Nter), and the 16 aa truncation (Δ10–25).

(B) CENP-B$^{-/-}$ cells transiently expressing GFP-tagged CENP-B polypeptide (Full, Δ10–25 and Nter) were immunostained with antibodies against GFP (green) and CENP-A (red), and DAPI (blue). The scale bars represent 5 μm.

(C and D) CENP-B$^{-/-}$ cells cotransfected with pWM11.64 and each CENP-B-expressing vectors (Full, Δ10–25, or Nter) were analyzed by immunoblotting using antibodies against CENP-B and Actin ([C] upper panel), or anti-GFP antibody ([D] upper panel), or by transient ChIP analysis ([C] lower panel; [D] lower panel) using anti-GFP antibody instead of anti-CENP-B antibody, as in Figure 3.

cells, an expression plasmid encoding GFP (green fluorescent protein)-tagged CENP-B was cotransfected into CENP-B$^{-/-}$ cells with the chimeric alphoid BAC. Immunodetection confirmed that GFP-tagged CENP-B was expressed and localized to the centromere in CENP-B$^{-/-}$ and CENP-B$^{+/+}$ cells (Figures 3C and 4B). Overexpression of GFP-tagged CENP-B increased the efficiency of CENP-A and CENP-B assembly on wild-type alphoid DNA dramatically. Wild-type alphoid DNA was enriched 16- to 30-fold in CENP-A and CENP-B immunoprecipitates in CENP-B$^{-/-}$ and CENP-B$^{+/+}$ MEFs, when the cells coexpressed GFP-CENP-B (Figure 3D).

Binding of CENP-B to the CENP-B Box Promotes Formation of CENP-A Chromatin

Previous in vitro studies demonstrated that a deletion mutant of amino acids 10-25 of CENP-B failed to bind to the CENP-B box (Figure 4A; Yoda et al., 1992). Here, two DNA binding competent and one DNA binding-defective variants of CENP-B were coexpressed as GFP fusion proteins in CENP-B-defective MEFs, and their ability to support de novo centromere assembly on human alphoid DNA was tested. The three CENP-B variants were full-length CENP-B-GFP, N-ter CENP-B-GFP (including the N-terminal 139 amino acids of CENP-B fused to GFP) and Δ10–25 CENP-B-GFP. Fluorescence microscopy showed that CENP-B-GFP and N-ter CENP-B-GFP colocalized with CENP-A at centromeres in CENP-B$^{-/-}$ MEFs, while CENP-B (Δ10–25) localized to the whole nucleus (Figure 4B). In addition, expression of full-length or N-ter CENP-B supported assembly of CENP-A and CENP-B on human alphoid DNA in CENP-B$^{-/-}$ MEFs, while expression of CENP-B (Δ10–25) did not (Figures 4C and 4D). These results indicated that binding of CENP-B to the CENP-B box is necessary for de novo assembly of centromeric chromatin on human alphoid DNA in MEFs. It should be noted that assembly of CENP-A on human alphoid DNA is dependent on the presence of a functional N-terminal DNA binding domain of CENP-B. This result suggests that the DNA binding domain may play a direct role in recruiting CENP-A to the centromeric satellite DNA and/or in de novo assembly of CENP-A nucleosomes.

Methylation of CpGs in CENP-B Box Loses the Binding of CENP-B and Associates with the Reduction of CENP-A Level

In +/+ Int W1 MEFs, human alphoid DNA with wild-type CENP-B boxes is stably integrated in mouse chromosomal DNA. Figure 2 shows that assembly of CENP-A and CENP-B on alphoid DNA was suppressed in these cells. Moreover, CENP-A, and CENP-B level on input alphoid DNA decreased toward 4 days after transfection in mouse cells, whereas CENP-B level was maintained and CENP-A

level increased after transfection in human HT1080 cells (Figure 3E). Here, we investigated the possibility that epigenetic modifications of chromosomally integrated alphoid DNA, including the CENP-B boxes, might play a role in suppressing de novo centromere assembly at these sites. We confirmed that mouse and human CENP-B have identical specificities for CENP-B box binding and dimer formation (Figure S5), which is consistent with the level of amino acid sequence homology in the N-terminal DNA binding domain and the C-terminal dimerization domain of human and mouse CENP-B (Figure 4A). The 9 bp core of the CENP-B box, which includes two CpG sites, is also 100% conserved in human and mouse DNA (Masumoto et al., 1989; Figure S2). In vitro studies showed that methylation of the CpG sites in the CENP-B box inhibits CENP-B binding (Tanaka et al., 2005a). Furthermore, partial methylation, even hemimethylation of one CpG of the CENP-B box, also inhibits CENP-B binding (Figure S5). Therefore, it seemed possible that methylation of alphoid DNA in MEFs could influence its ability to form de novo centromeric chromatin.

Here, CpG methylation in chromosomal and nonchromosomal alphoid DNA was examined with methylation-sensitive restriction enzyme Aci I, which cuts within the CENP-B box (Figure 5A). The results show that chromosomally integrated alphoid DNA in +/+ Int W1 cells was hypermethylated, but extrachromosomal alphoid DNA in +/+ AC1 cells was not (Figure 5B). Interestingly, chromosomally-integrated alphoid DNA was hypomethylated in CENP-B$^{-/-}$ MEFs (i.e., −/− Int W1 cells; Figure 5B). The result was confirmed by immunoprecipitation of genomic DNA with anti-5meC (Figure S6 and Table 1). These results suggest that CpG methylation may negatively regulate CENP-B binding to the CENP-B box and assembly of CENP-A chromatin. However, neither CpG hypermethylation nor CENP-A assembly occurs on alphoid DNA in CENP-B$^{-/-}$ cells.

The amount of histone H3K9 trimethylation (H3K9me3) in alphoid DNA was also investigated using ChIP and real-time PCR. These experiments revealed a high level of H3K9me3 modification in chromosomally-integrated alphoid DNA in +/+ Int W1 cells, or in extrachromosomal alphoid DNA in +/+ AC1 cells, and a low level of H3K9me3 modification in integrated alphoid DNA in −/− Int W1 cells (Figure 5C). We also examined H3K9me3 modification in other independent cell lines and found that hyper H3K9me3 modification occurred in chromosomally integrated alphoid DNA in CENP-B$^{+/+}$ MEFs. In contrast, this reaction did not occur in CENP-B$^{-/-}$ MEFs (Figure S7B and Table 1). Thus, the presence of endogenous CENP-B is required to induce active assembly of CENP-A chromatin or a suppressed and inactive state at ectopic sites. In contrast, in the absence of CENP-B, human centromere competent satellite DNA assumes to be neither in an active state (assembly of CENP-A and CENP-B) nor in an inactive/suppressed chromatin state (hyper CpG methylation and H3K9me3 hypermodification) efficiently.

CENP-B Mediates Histone H3 Lysine 9 Trimetylation at the Ectopic Sites prior to DNA Methylation in CENP-B Box

The CpG hypomethylation and lack of H3K9me3 modification at chromosomal alphoid DNA in CENP-B$^{-/-}$ MEFs led us to compare the effects of N-ter CENP-B-GFP and full-length CENP-B-GFP on DNA and chromatin modifications in these cells. CENP-A assembly occurred at a low level in cells expressing both polypeptides (Figures 5D and S7C and Table 1); H3K9me3 modification occurred only in cells expressing full-length CENP-B-GFP (Figures 5E and S7B and Table 1), and CpG methylation in CENP-B box was not stimulated by expression of either polypeptide at the same time (Figure 5F). However, CpG methylation of whole alphoid DNA unit (including CENP-B box) analyzed by immunoprecipitation with anti-5meC antibody was increased by expression of full-length CENP-B-GFP (Figure S6 and Table 1). These results indicate that wild-type CENP-B may play a role in assembly or spreading of heterochromatic H3K9me3 chromatin in chromosomally-integrated alphoid DNA, and that the DNA binding domain of CENP-B is not sufficient for this function. We further investigated the effect of CENP-B on chromosomally-integrated alphoid DNA at early time points after transfection with CENP-B expression vector under nonselective conditions. Similarly, only full-length CENP-B promotes H3K9me3 hypermodification within 2-4 days after retroviral infection (Figures 6A and S8A, lower panels). However, no enrichment of CENP-A was detected within 4 days (Figures 6A and S8A, upper panels). A low but reproducible enrichment of CENP-A became detectable toward 12 days after retroviral infection (Figures S8B and 5D). These observations suggest that CENP-A assembly pathway via the N-terminal domain of CENP-B was impaired initially in chromosomally-integrated alphoid DNA. Importantly, full-length CENP-B actively promotes H3K9me3 hypermodification. We found that tagged Suv39h1, H3K9-specific methyltransferase, in MEFs was coimmunoprecipitated with CENP-B in solubilized chromatin fraction (Figure 6B). The result indicates that these proteins exist closely. Indeed, loss of Suv39h1/h2 affects H3K9me3 modification in chromosomally-integrated alphoid DNA. Similar to the reduction of H3K9me3 level on minor and major satellite DNA in Suv39h1/h2 double-null (Suv39h dn) MEFs, the level of H3K9me3 modification in chromosomally-integrated alphoid DNA was also decreased (Figure 6C, left panel). Furthermore, the decrease in H3K9me3 modification in the double mutant correlated with CpG hypomethylation in integrated copies of alphoid DNA (Figure 6C, right panel). These results indicate that Suv39h1/h2 are involved in H3K9me3 modification in chromosomally-integrated alphoid DNA cooperating with CENP-B and a major part of DNA CpG methylation events is dependent on H3K9me3 modification. Thus we propose that CpG hypermethylation in CENP-B boxes occurs after modification of H3K9me3, and that binding of CENP-B to CENP-B boxes may interfere

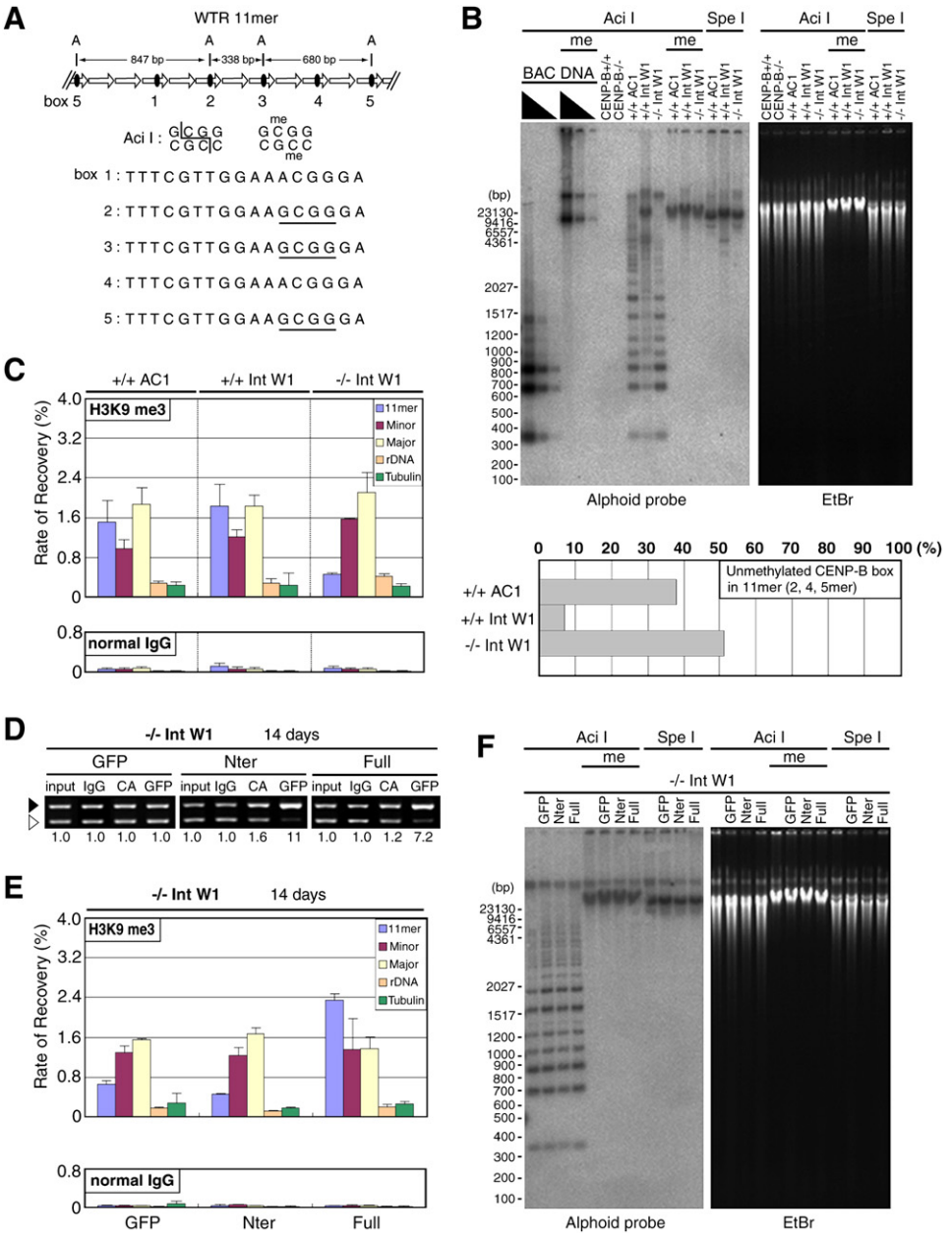

Figure 5. Heterochromatic Modification State in Chromosomally Integrated Alphoid DNA

(A) Positions of methylation-sensitive AciI site within the CENP-B box on the alphoid 11-mer unit.

(B) Upper panel: southern analysis of CpG methylation in integrated human alphoid DNA. Genomic DNA prepared from MEF cell lines (+/+ AC1, +/+ Int W1, and −/− Int W1), was digested with AciI after in vitro treatment with (me) or without CpG methylase (M. SssI), and hybridized with ^{32}P-labeled alphoid probe (left panel). Naked alphoid BAC DNA was used as a control. The input alphoid BAC DNA has a unique SpeI site. Lower panel: the intensities of nonmethylated CENP-B boxes (equivalent to 2-mer 338bp, 4-mer 680bp, and 5-mer 847bp) relative to that of full-methylated DNA with SssI methylase (equivalent to 23 kb; lanes, me) was determined using Image Gauge (Fuji FILM Science Lab 97).

(C) ChIP and real-time PCR analysis of human alphoid DNA, mouse minor satellite, or major satellite with anti-H3K9me3 antibody and normal IgG (as a control). Error bars represent the SEM (n = 3). Cell lines derived from −/− Int W1 expressing GFP (−/− Int W1 GFP), GFP-tagged Nter CENP-B (−/− Int W1 Nter), or GFP-tagged Full CENP-B (−/− Int W1 Full) were obtained under puromycin selection for 2 weeks after retroviral infection and were analyzed in (D)–(F).

(D) Competitive ChIP analysis of centromeric chromatin on integrated human alphoid DNA as shown in Figure 2, using indicated antibodies. −/− Int M1 cells were used as reference cells.

(E) ChIP and real-time PCR analysis for the integrated human alphoid DNA, mouse minor satellite, or major satellite using anti-H3K9me3 antibody and control normal IgG. Error bars represent the SEM (n = 2).

(F) Methylation state of integrated human alphoid DNA.

Table 1. CENPs and H3-K9me3 Assemblies on Introduced Alphoid BAC DNAs

Chromosomal Event/ Cell Line	CENP-B Allele in MEF	Input Alphoid DNA, CENP-B Box (W) or Mutant Box (M)	CENPs Assemblies[a] (Fold Enrichment or Rate of Recovery [%])		H3K9me3 Assembly[a] (Rate of Recovery)	Methylation of Alphoid DNA[b] (Rate of Recovery)	Multiplicity of Input Alphoid DNA (Copies)
			CENP-A	CENP-B			
MAC							
+/+ AC1	+/+	W	++++ (24)	++++ (22)	++ (1.5%)	++[c]	17
Integration							
+/+ Int W1	+/+	W	+ (2.0)	+ (4.5)	+++ (1.8%)	++++ (7.4%)	52
+/+ Int W2	+/+	W	+ (1.2)	+ (1.4)	++++ (3.8%)	nt	41
+/+ Int W3	+/+	W	+ (1.2)	± (1.1)	++++ (2.7%)	nt	11
+/+ Int M1	+/+	M	–	–	nt	nt	35
–/– Int W1	–/–	W	– (1.0)	– (1.0)	+ (0.45%)	+ (0.9%)	22
	+ Nter		+ (1.6)	+++ (11)	+ (0.46%)		
	+ Full		+ (1.2)	++ (7.2)	++++ (2.3%)	++ (2.7%)	
–/– Int W2	–/–	W	– (1.0)	– (1.0)	+ (0.84%)	nt	5
	+ Nter		+ (1.3)	+++ (12)	+ (0.59%)		
	+ Full		+ (1.2)	+++ (10)	++++ (2.3%)		
–/– Int W3	–/–	W	– (1.0)	– (1.0)	+ (0.64%)	nt	20
	+ Nter		+ (2.0)	++ (8.8)	+ (0.88%)		
	+ Full		+ (2.0)	++ (9.2)	++ (1.3%)		
–/– Int M1	–/–	M	–	–	nt	nt	20
Transformed cell mixture							
WT / WTR	+/+	W	± (0.024%)	nt	+++ (1.6%)	++++ (7.9%)	nt
Suv39 dn / WTR	+/+	W	++ (0.065%)	nt	+ (0.58%)	+ (1.8%)	nt
Suv39 dn / MTR	+/+	M	– (0.021%)	nt	± (0.25%)	± (0.8%)	nt

One point two fold enrichment measured by competitive PCR was statistically significant ($p < 0.01$) by a Student's t test, relative to values with control normal IgG.

[a] Relative level of CENP-A, CENP-B and H3K9me3 assembly was determined by ChIP analyses and shown; –, no assembly, ±; very low; +, low; ++, medium; +++, high; ++++, very high.

[b] Relative level of methylated alphoid DNA was determined by methylated DNA immunoprecipitation (MeDIP) and shown; ±, very low; +, low; ++, medium; ++++, very high.

[c] Relative level of methylated CENP-B box compared with +/+ Int W1 and –/– Int W1 cells was determined by the result of Figure 5B (lower panel).

with CpG methylation in CENP-B boxes. Finally, once satellite DNA assumes an inactive state (i.e., at sites of chromosomal integration), this state can be maintained even in the absence of CENP-B by epigenetic mechanisms involving H3K9me3 modification and CpG methylation.

DISCUSSION

De Novo Centromere Assembly in Human Alphoid DNA Requires CENP-B

Our understanding of the mechanism(s) of the assembly of human centromeres comes largely from studies of artificial chromosome formation in human HT1080 cells. These studies helped define the DNA sequences requirements for forming de novo centromeres in human cells. Thus, the requirement for human alphoid DNA and CENP-B boxes is clear, but the mechanism for assembling centromeric proteins, including CENP-A, on alphoid DNA is less well understood. The role of CENP-B is particularly intriguing; despite the conservation of CENP-B and its target binding sequence in human and mouse, several studies suggest that a functional centromere/kinetochore can be maintained in the absence of CENP-B.

The present study demonstrates that human alphoid DNA arrays with wild-type CENP-B boxes are sufficient to direct assembly of CENP-A chromatin, de novo centromere formation, and stable artificial chromosome propagation in mouse CENP-B$^{+/+}$ MEF cells. CENP-B is essential for these events, since de novo assembly of a

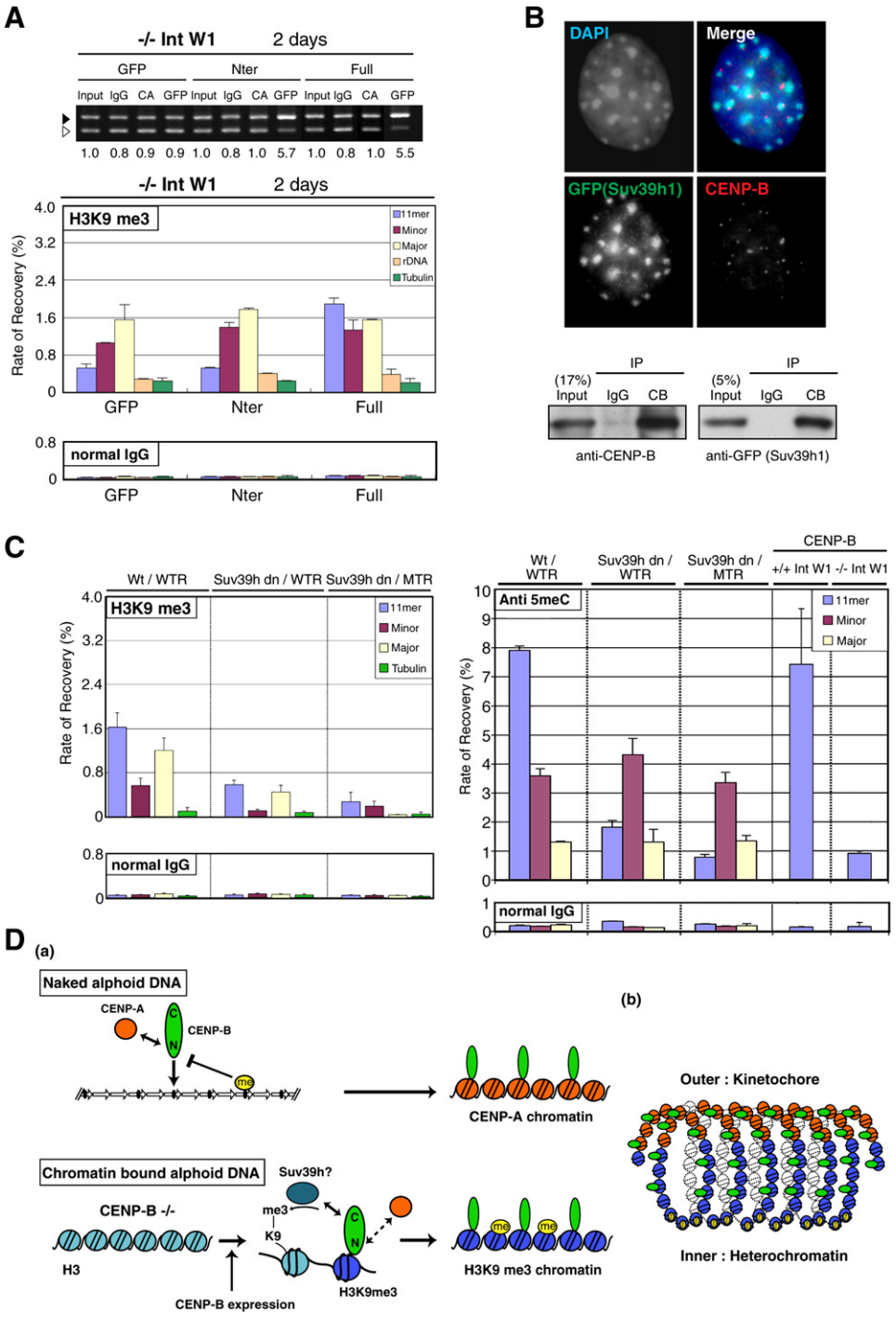

Figure 6. Binding of Full-Length of CENP-B Promotes H3K9me3 Hypermodification in Chromosomally Integrated Alphoid DNA

(A) Each GFP-tagged protein (GFP, Nter CENP-B, full-size CENP-B) was expressed using retroviral infection in −/− Int W1 cells. Two days after infection, cells were harvested for ChIP analysis. Error bars represent the SEM (n = 2).

(B) Upper panel: GFP-tagged Suv39h1 (Suv39h1-GFP) and CENP-B were expressed using retroviral infection in −/− Int W1 cells. At 2 days after the infection, the cells were immunostained with antibodies against GFP (green), CENP-B (red), and DAPI (blue). Lower panel: Solubilized chromatin fractions (a majority is mononucleosome) prepared from −/− Int W1 cells expressing Suv39h1-GFP and CENP-B were immunoprecipitated with anti-CENP-B (CB) antibody (BN1) or normal IgG (IgG) according to the previously described method (Suzuki et al., 2004). Immunoprecipitated samples were analyzed by western blotting with anti-CENP-B or anti-GFP antibody. Lanes labeled Input contain the equivalent of 17% (left panel) or 5% (right panel) of the input protein.

centromere or CENP-A containing chromatin did not occur in CENP-B$^{-/-}$ MEFs and CENP-A chromatin assembly was only rescued by expression of exogenous CENP-B. This indicates that specific assembly of CENP-A chromatin is mediated by and dependent on CENP-B. We conclude that the mechanism of de novo centromere assembly as well as the components involved in this process are highly conserved in human and mouse cells.

Effect of CENP-B Binding to Alphoid DNA on De Novo CENP-A Assembly

The CENP-B box is the only homologous region in human alphoid DNA and mouse minor satellite DNA. Data presented here suggest that human alphoid DNA and mouse minor satellite DNA are interchangeable in promoting de novo centromere chromatin assembly and centromere function in MEFs. In contrast, nonalphoid synthetic repetitive DNA from pBR322 (AT content < 40%) with CENP-B boxes lacks such function in human HT1080 cells (Ohzeki et al., 2002). Centromere sequences tend to be AT-rich (i.e., human alphoid DNA, ~60% AT and mouse minor satellite DNA, ~65% AT: Vafa et al., 1999; Sullivan et al., 2001; Edwards and Murray, 2005). In vitro studies show that binding of CENP-B to the CENP-B box induces a DNA bend of 59 degrees, which in turn induces translational positioning of CENP-A nucleosomes on alphoid DNA (Tanaka et al., 2005b). Present study shows that binding of the CENP-B N-terminal DNA binding domain to the CENP-B box is sufficient to promote assembly of CENP-A chromatin on human alphoid DNA. Moreover, our recent studies showed that both the length of alphoid DNA arrays and the density of CENP-B boxes had a strong effect on nucleation, spreading and/or maintenance of CENP-A chromatin core, and formation of functional kinetochores (Okamoto et al., 2007). Taken together, these results suggest that binding of CENP-B to multiple adjacent CENP-B boxes arrayed in alphoid satellite DNA could promote assembly of stable functional chromatin core with CENP-A nucleosomes, at least in part through the structure of AT-rich repetitive DNA. Alternatively, since a direct interaction between CENP-B and CENP-A could not be confirmed by yeast two-hybrid analyses (Suzuki et al., 2004), additional as yet unidentified factor(s) may promote de novo assembly of CENP-A chromatin and functional centromeres in human alphoid DNA.

CENP-B Binding to CENP-B Boxes Modulates H3K9me3 Hypermodification in Chromosomally Integrated Alphoid DNA

Additional factors may also play a role in assembly or stability of CENP-A chromatin. For example, the low CpG frequency reflecting the AT-richness, lack of CpG methylation and absence of proteins that bind methylated CpG may contribute to the properties and in vivo behavior of centromeric DNA sequences such as human alphoid DNA and mouse minor satellite DNA (Klose and Bird, 2006). In many eukaryotes, the centromere is embedded in heterochromatin which is enriched in HP1 and H3K9me3. Interestingly, even partial CpG methylation in the CENP-B box prevents CENP-B from binding to its target sequence (Figure S5). This is consistent with the observation that human alphoid DNA is hypermethylated when integrated in the mouse chromosome (Figure 5B), and that formation of CENP-A chromatin and CENP-B binding are suppressed at the chromosomally-integrated sites (Figures 2B$_c$ and 2C). Previous reports also suggest that the presence of H3K9me3 is antagonistic to formation of CENP-A chromatin in alphoid DNA (Nakashima et al., 2005; Okamoto et al., 2007). In this study, hyper H3K9me3 modification occurred in alphoid DNA at chromosomally-integrated sites in CENP-B$^{+/+}$ MEFs. In contrast, this reaction did not occur in CENP-B$^{-/-}$ MEFs (Figures 5C and S7B). Remarkably, H3K9me3 hypermodification in CENP-B$^{-/-}$ MEFs in chromosomally integrated human alphoid DNA was restored by expression of full-length but not by the N-terminal region of CENP-B (Figures 5E and S7B). At the same time, CpG methylation did not increase in CENP-B boxes when full-length CENP-B was expressed in CENP-B$^{-/-}$ MEFs (Figure 5F). However, CpG methylation in whole alphoid DNA unit indeed increased (Figure S6). This suggests that CENP-B binding promotes both H3K9me3 hypermodification and CpG methylation in chromosomally-integrated alphoid DNA, without stimulating CpG methylation at its binding site, initially. Several studies in fungi, plants and mammals suggest that H3K9me3 hypermodification is dependent

(C) H3K9me3 level on the transfected alphoid DNA was reduced in the Suv39h1/h2 double-null cells. Alphoid BAC DNAs (pWTR11.32Bsr and pMTR11.32Bsr) were transfected into Wild-type (WT) MEFs and Suv39h1/h2 double-null (Suv39h dn) MEFs, respectively. Transformed cell mixtures were obtained under Bs selection for 6 weeks. pWTR11.32Bsr-transformed Wild-type cells (WT / WTR), pWTR11.32Bsr-transformed Suv39h dn cells (Suv39h dn / WTR), or pMTR11.32Bsr-transformed Suv39h dn cells (Suv39h dn / MTR) were harvested for ChIP analysis using anti-H3-K9me3 antibody (left panel) or for DNA-immunoprecipitation analysis using anti 5-meC antibody (right panel) referenced by highly methylated (CENP-B+/+ Int W1 cells) and low methylated (CENP-B−/− Int W1 cells) alphoid DNA. The immunoprecipitated DNA was quantitated by real-time PCR. Error bars represent the SEM (n = 3).

(D) (a) A model of de novo CENP-A chromatin assembly on transfected human alphoid DNA that is dependent on CENP-B binding. CENP-B binding to CENP-B box (black circles) mediates de novo assembly of CENP-A chromatin on human alphoid DNA (white repetitive arrows) via the N-terminal DNA binding domain of CENP-B. Alternatively, on chromatin bound alphoid DNA at chromosomal integration sites, CENP-B mediates H3K9me3 hypermodification via its central and C-terminal domains dominantly, possibly by recruiting chromatin remodeling and /or modifying complexes (Suv39h is a possible candidate), which concomitantly stimulate CpG methylation in alphoid DNA competing CENP-B binding to CENP-B boxes. (b) A hypothetical model of the natural centromere structure in contiguous AT-rich repetitive DNA. CENP-B may enhance at least three distinctive chromatin states; CENP-A and CENP-B-rich kinetochore, CENP-B-bound H3K9me3 chromatin, and CpG hypermethylated and CENP-B-deficient H3K9me3 heterochromatin. (See Discussion for more details.)

on DNA methylation (Tamaru and Selker, 2001; Jackson et al., 2002; Lehnertz et al., 2003). In mammals, DNA methyltransferases can interact with Suv39h H3K9 methyltransferases (Lehnertz et al., 2003). However, in Suv39h1/h2 double-null (Suv39h dn) MEFs, decreased H3K9me3 correlated with decreases in CpG methylation in pericentromeric major satellite DNA but not in centromeric minor satellite DNA (Lehnertz et al., 2003). We observed the decreased levels of H3K9me3 and CpG methylation in chromosomally integrated human alphoid DNA in Suv39h dn MEFs (Figure 6C). Thus, several mechanisms may regulate formation of heterochromatin in mammalian cells, and one such mechanism is the CENP-B-dependent pathway described above, which operates on human and mouse centromeric satellite DNA in MEFs.

Dual Roles of CENP-B in CENP-A Chromatin Assembly and Trimethylation of H3K9 in Centromeric Satellite DNA

The above results with alphoid DNA at chromosomally-integrated sites suggest that centromere inactivation and heterochromatin assembly correlates with disassembly of active centromere components. This suggestion is consistent with the fact that one centromere in dicentric mouse or human fusion-chromosomes is usually inactive, despite the presence of centromeric satellite DNA and CENP-B. Interestingly, while introduced naked (chromatin-free) alphoid DNA binds the N-terminal region of CENP-B and supports CENP-A chromatin assembly, binding of the same region of CENP-B in chromosomally-integrated (chromatin-bound) alphoid DNA fails to support neither an initial assembly of CENP-A chromatin nor H3K9me3 hypermodification (Figures 6A, 6D$_a$, and S8A). Besides, full-length CENP-B actively enhances H3K9me3 modification in chromosomally-integrated alphoid DNA without stimulating an initial assembly of CENP-A chromatin (Figures 6A, 6D$_a$, and S8A). Although we do not know yet whether CENP-B cooperates directly or indirectly with Suv39h1/h2, tagged Suv39h1 was coimmunoprecipitated with CENP-B in solubilized chromatin fraction, indicating that these proteins exist closely (Figure 6B). Thus, CENP-B does not simply induce CENP-A assembly in alphoid DNA. We propose that CENP-B may play dual antagonistic roles in modulating chromatin structure in centromeric satellite DNA, alternatively modulating assembly of CENP-A chromatin or H3K9me3 hypermodification (Figure 6D$_a$). We also suggest that the former process is mediated by the N-terminal DNA binding domain and the latter process is mediated by other CENP-B domains.

Recent studies indicate that the distinctive clusters of CENP-A chromatin and H3K9me3 chromatin are both present in mouse and human centromeric satellite DNAs (Guenatri et al., 2004; Martens et al., 2005; Nakashima et al., 2005; Lam et al., 2006). We favor the view that CENP-B nucleates CENP-A chromatin or enhances H3K9me3 modification by recruiting chromatin remodeling and/or modifying complexes at each site of centromere region, thereby allowing a CENP-A chromatin cluster or a H3K9me3 cluster to spread across the entire centromere region (Figure 6D$_b$). However, for assembly of the functional centromere, CENP-A chromatin and H3K9me3 chromatin clusters should coexist and be maintained in centromeric satellite DNA by an epigenetic mechanism. Indeed, CENP-A chromatin and H3K9me3 chromatin appear to be in balance in mouse artificial chromosomes carrying human alphoid DNA (Figures 2B$_b$, 2C, 5C and Table 1). In contrast, when human alphoid DNA was at chromosomally-integrated sites, formation of H3K9me3 chromatin was dominant, and de novo centromere assembly was suppressed. In mouse cells, the efficiency of CENP-B binding to alphoid DNA was gradually decreased during 4 days after transfection, whereas the CENP-B binding maintained stable in human HT1080 cells highly competent for artificial chromosome formation (Figure 3E). Although reduction of the H3K9me3 level in Suv39h dn cells did not induce a complete centromere activation, the level of CENP-A assembly was 2.7-fold increased at chromosomally-integrated sites (Figure S9 and Table 1). Thus, the balance between these antagonistic substrates, CENP-A and heterochromatin including H3K9me3, appears to be critical in determining the functional centromere structure on alphoid DNA. We propose that CENP-B acts as AT-rich satellite DNA specific modulator (still associating with a low CpG motives), which dynamically promotes formation of the CENP-A chromatin cluster or spreading of the suppressive heterochromatin on the same satellite DNA. This dynamic chromatin assembly balance between the antagonistic chromatin substrates may enhance not only de novo centromere/artificial chromosome formation but also inactivation of one of centromeres in dicentric fusion chromosomes.

The results presented here agree well with the idea that once established, CENP-A or H3K9me3 chromatin and CpG hypermethylation can be maintained by an epigenetic mechanism in the absence of CENP-B (Figures 2C and 5C). However, the mechanisms that modulate heterochromatin assembly and spreading in mammalian satellite DNA are still very poorly understood. Further studies are needed, especially to better understand the role of CENP-A and CENP-B in these processes. Undoubtedly, this knowledge will be indispensable for development of next-generation human artificial chromosomes and mammalian vectors for gene therapy.

EXPERIMENTAL PROCEDURES

Cell Lines

Mouse embryo fibroblasts (MEFs) from litter mates homozygous for wild-type CENP-B (E18) or homozygous for a CENP-B deletion (CENP-B$^{-/-}$, E8) were immortalized with SV40 large T antigen (Kapoor et al., 1998). CENP-B$^{+/+}$, CENP-B$^{-/-}$, and HT1080 human fibrosarcoma cells were cultured in DME supplemented with 10% (vol/vol) FCS, penicillin, streptomycin, and L-glutamine at 37°C in a 5% CO2 incubator. Cell culture of Wild-type (WT) MEFs and Suv39h1/h2

double-null (Suv39h dn) MEFs was carried out as described (Martens et al., 2005).

Transfection

Purified BAC DNA using a QIAGEN column was transfected into 50% confluent CENP-B$^{+/+}$ or CENP-B$^{-/-}$ cells in 6 well plates with lipofectamine (Invtrogen). Stable transformants were selected with 6 μg/ml blasticidin S (Bs) (Kaken Seiyasu).

ChIP and Competitive PCR/Real-Time PCR

ChIP was performed as previously described (Nakashima et al., 2005). Cells (3–5 × 10^5) were fixed in 0.25% formaldehyde at 22°C for 5 min. After sonication, soluble chromatin (as input) was recovered by centrifugation and immunoprecipitated using 2-10 μg antibody. Antibodies used were anti-mouse CENP-A (8C5), anti-CENP-B (5E6C1), anti-GFP (Roche), and anti-trimethylated H3 lys9 (Upstate). Purified DNA of the immunoprecipitates and of input DNA was quantified by competitive PCR (Ohzeki et al., 2002) or real-time PCR (Nakashima et al., 2005) using the primer specific for synthetic alphoid, BAC vector, minor satellite, major satellite, rDNA, or β-tubulin (see Table S2).

Retroviral Infection of −/− Int W1 MEFs Cells

Recombinant retrovirus was produced by transfecting retroviral constructs (pMX-puro GFP, pMX-puro GFP Nter, or pMX-puro GFP Full) into the virus packaging cell line Plat-E (Morita et al., 2000) using FuGene6 Reagent (Roche). The supernatant was incubated with MEF cells in presence of 5 μg/ml polybrene and plated on 6 well plates. Stable transformants were selected with 5 μg/ml puromycin.

Standard procedures, including descriptions of Construction of BACs and plasmids, Cytological assays, DNA multiplicity by real-time PCR, Methylated DNA immunoprecipitation (MeDIP), Transient DNA transfection, Names of Stable transformants are described in Supplementary Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, two tables, and nine figures and can be found with this article online at http://www.cell.com/cgi/content/full/131/7/1287/DC1/.

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