DNA CpG hypomethylation induces heterochromatin reorganization involving the histone variant macroH2A

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Summary

In mammalian heterochromatin, cytosine bases of CpG dinucleotides are symmetrically modified by methylation. Patterns of CpG methylation are maintained by the action of Dnmt1, the mammalian maintenance cytosine methyltransferase enzyme. We genetically manipulated the levels of CpG methylation and found that extensive chromatin alterations occur in pericentric heterochromatin. Homozygous mutations in Dnmt1 cause severe hypomethylation of pericentric heterochromatin and concomitant chromatin reorganization involving the histone variant macroH2A. Demethylation-induced alterations in macroH2A localization occur in both interphase and mitotic embryonic stem (ES) cells. Heterochromatin protein 1 (HP1) marks interphase pericentric heterochromatin (chromocenters). MacroH2A immunostaining in Dnmt1⁻/⁻ cells becomes coincident with chromocenters detected by HP1 content. MacroH2A, but not HP1, is enriched in nuclease-resistant chromatin fractions extracted from Dnmt1⁻/⁻ cells. Normal localization of macroH2A was restored upon reintroduction of a Dnmt1 transgene into Dnmt1⁻/⁻ cells. MacroH2A localization was also affected in T-antigen-transformed fibroblasts subjected to the conditional mutation of Dnmt1. Together, these results suggest that pericentric heterochromatin can be maintained in the absence of CpG methylation, but in a significantly altered configuration.

Key words: chromatin, methylation, Dnmt1, MacroH2A, centromere, chromocenter

Introduction

The genomes of mammals are organized as chromatin, which is a complex and dynamic assemblage of DNA and proteins. Transcriptionally silent DNA can be roughly divided into two functional compartments – constitutive and facultative heterochromatin. Constitutive heterochromatin contains DNA in a state of obligate transcriptional silence and includes elements such as centromeres, pericentric DNA, telomeres and silenced mobile elements (LINE-1 elements, SINE elements, disabled retroviruses, etc.). In contrast, facultative heterochromatin is conditionally silenced. Facultative heterochromatin includes genes subject to tissue-specific silencing, inactive X chromosomes of female eutherian mammals, imprinted genes and perinucleolar DNA. Though both classes of heterochromatin are organized into nucleosomes, a growing body of evidence reveals that constitutive and facultative heterochromatin differ substantially in their content of variant histones and post-translational histone modifications.

Constitutive heterochromatin is assembled upon mammalian centromeres and surrounding pericentric sequences. The histone H3 variant CENP-A replaces histone H3 in nucleosomes assembled at centromeres (Palmer et al., 1987). Pericentric nucleosomes also contain a high content of histone H3 that is post-translationally modified by methylation at lysine residue 9 (me-H3-K9), a modification that is associated with transcriptional inactivity (Lachner et al., 2001; Maison et al., 2002; Rea et al., 2000). The me-H3-K9 modification serves as a cis-acting binding site for heterochromatin protein 1 (HP1) (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001), an interaction that serves to sequester interphase centromeres in discrete foci (chromocenters). In addition, regions in and around mammalian centromeres are subject to heavy DNA methylation at CpG dinucleotides. In short, constitutive heterochromatin contains a distinct molecular signature consisting of heavy methylation of CpG dinucleotides coupled with chromatin enriched in CENP-A, me-H3-K9 and concomitant association with HP1.

Facultative heterochromatin such as that of the inactive X chromosome (Xi) also contains a distinct molecular signature. Though numerous reports show that CpG islands of silenced X-linked genes are highly methylated, the overall levels of methylation on Xi appear to be reduced relative to the active X chromosome (Xa) and autosomes (Viegas-Pequignot et al., 1988). In addition, the chromatin of Xi is enriched in histone
H3 methylated at lysine 27 (me-H3K27) (Plath et al., 2003; Silva et al., 2003) and macroH2A, a variant of canonical histone H2A, which contains a large C-terminal extension (Costanzi and Pehrson, 1998; Pehrson and Fried, 1992). On a molecular level, MacroH2A interferes with NF-kappaB transcription factor binding and SWI/SNF chromatin remodeling (Angelov et al., 2003). Furthermore, the C-terminal nonhistone domain (NHD) of macroH2A may be involved in ADP-ribosylation of chromatin with potential implications for transcriptional silencing (Ladurner, 2003). The crystal structure of the AF1521 protein from Archaeoglobus fulgidus was recently determined (Allen et al., 2003). This protein is similar to the NHD of macroH2A and probably binds nucleic acids, suggesting a possible role for macroH2A in chromatin condensation.

Methylation of CpG dinucleotides in mice is maintained by the action of Dnmt1, a maintenance DNA methyltransferase that converts hemimethylated CpG dinucleotides to symmetrically methylated dinucleotides (Pradhan et al., 1999). Loss of Dnmt1 results in embryonic lethality, but undifferentiated embryonic stem (ES) cells lacking Dnmt1 function are fully viable (Li et al., 1992). Since ES cells are viable in the absence of CpG demethylation, we used these cells to investigate the molecular composition of demethylated heterochromatin. We show that loss of DNA methylation causes a significant reorganization of constitutive heterochromatin involving the histone variant macroH2A. This finding raises the intriguing possibility that mammalian heterochromatin adopts a default state, reminiscent of facultative heterochromatin, in response to DNA demethylation.

Materials and Methods
Cell culture
Male ES cell lines with genotypes Dnmt1<sup>+/+</sup>, Dnmt1<sup>SS</sup> and Dnmt1<sup>CC</sup> were used. The N allele of Dnmt1 consists of a targeted deletion in the N-terminal coding region of Dnmt1 that causes dramatic reduction of CpG methylation (Li et al., 1992). The S allele of Dnmt1 disrupts the domain that targets the Dnmt1 enzyme to replication forks, resulting in a reduction of CpG methylation due to mislocalization of the enzyme (Lei et al., 1996; Leonhardt et al., 1992; Li et al., 1993). The C allele of Dnmt1 disrupts the methyltransferase domain of the enzyme (Lei et al., 1996). Male ES cell line B9 was derived by introduction of a bacterial artificial chromosome bearing the wild-type Dnmt1 gene into the Dnmt1<sup>CC</sup> cells, which causes extensive remethylation (Biniszewska et al., 2002).

ES cells were grown under standard conditions (on irradiated fibroblast feeder layers) in ES cell medium (ESCM) consisting of DMEM-based medium (Gibco) supplemented with non-essential amino acids, 2 µl/l β-mercaptoethanol and 500 units/ml leukemia inhibitory factor (LIF) on gelatinized plastic tissue culture vessels. Feeders were removed by trypsinizing ES cell cultures and panning out feeder cells by incubation for 30 minutes of growth on plates not treated with gelatine. ES cells were then plated for an additional passage without feeder cells in ESCM supplemented with 1000 units/ml LIF. Colchicine (Sigma) was added to cells 2 hours prior to harvest at a concentration of 1 µg/ml, then cells were trypsinized, washed once, and resuspended in 5 ml of 0.075 M KCl at 37°C for 12 minutes. Cells were then pelleted carefully at 800 rpm in a tabletop centrifuge, resuspended in residual supernatant and dropped onto slides to liberate chromosome spreads, followed by immediate fixation in 4% paraformaldehyde in PBS at 4°C for 5 minutes. Slides were washed in PBS and immediately subjected to immunofluorescence.

Chromatin fractionation
Nucleosomes were prepared essentially as described previously (Thorne et al., 1998). Cells were harvested and washed in ice-cold PBS-butyrate (135 mM NaCl, 250 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM sodium butyrate), then resuspended in cell lysis buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM sodium
butyrate, 4 mM MgCl₂, 0.1% Triton X-100) with protease inhibitors (1 tablet of protease inhibitor cocktail; Roche, cat. no. 1697498), and lysed with 20 strokes of a Dounce homogenizer (type B pestle). Nuclei were pelleted by centrifugation for 10 minutes at 2000 g at 4°C, then resuspended in 3 ml of wash buffer C (250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM sodium butyrate, 4 mM MgCl₂ and protease inhibitors) and layered over a 5 ml sucrose cushion (30% sucrose w/v in wash buffer C). Nuclei were then centrifuged at 2400 g for 5 minutes at 4°C in a swinging bucket rotor and the nuclear pellet was resuspended in 250 µl of wash buffer C. CaCl₂ was added to a final concentration of 1 mM and incubated at 37°C for 8 minutes in the presence of 8 µl of micrococcal nuclease (MNase, 5 units/l). Digestion was stopped by adding EDTA to a concentration of 1 mM and incubated at 37°C for 1 hour. This hypotonic treatment forced nucleosomes into the supernatant (extraction 1). The nuclei were then extracted a second time (3 hours of diffusion), yielding a chromatin fraction containing micrococcal nuclease-resistant polynucleosomes (extraction 2). Chromatin samples were analyzed for DNA content after phenol/chloroform extraction, and protein content was analyzed by SDS-PAGE and western blotting using standard methods.

Results
Demethylation of repetitive DNA by Dnmt1 deletion
We assessed the levels of CpG methylation in three ES cell lines: wild-type J1 cells, an ES cell line with a methyltransferase portion of Dnmt1 (Lei et al., 1996), and ES cell line B9, which was derived by introduction of a bacterial artificial chromosome bearing the wild-type Dnmt1 gene into the Dnmt1<sup>C/C</sup> cells (Biniszkiewicz et al., 2002). Southern blots were performed using the methyl-sensitive restriction enzyme HpaII, and MspI, an enzyme with the same recognition sequence as HpaII, but whose activity is unaffected by CpG methylation (Fig. 1). We found that Dnmt1<sup>C/C</sup> cells contained highly demethylated centromeric repeats and that centromeric methylation was partially restored in rescued cell line B9 (Fig. 1A). Similar analyses showed that Line-1 elements were highly demethylated in Dnmt1<sup>C/C</sup> cells and partially remethylated in B9 (Fig. 1B). Finally we probed blots with Co/T1 DNA (purified repetitive mouse genomic DNA) (Fig. 1C). This showed that the total repetitive DNA of Dnmt1<sup>C/C</sup> cells was highly demethylated, and that methylation was partially restored in B9. It is generally

| Fig. 1. Genomic methylation assays for repetitive sequences. Southern blots of total DNA extracted from wild-type (J1), rescued B9 cells (Dnmt1<sup>C/C</sup> cells containing a Dnmt1<sup>+</sup> BAC) and Dnmt1<sup>C/C</sup> ES cells, cut with HpaII (methyl sensitive) and MspI (methyl insensitive) isoschizomeric restriction enzymes. (A) Southern analysis of equally loaded DNA using a probe that recognizes minor centromere repeats. (B) Identical Southern blot probed with pKS13b, which recognizes open reading frames of Line-1 repetitive transposable elements. (C) Identical Southern blot probed with mouse Co/T1 DNA, which recognizes all highly repetitive sequences in the mouse genome.

| Fig. 2. Production of polyclonal antibodies against macroH2A. (A) MacroH2A consists of an N-terminal region (red) that is 64% identical to canonical H2As followed by a large C-terminal nonhistone domain (NHD) of unknown function (green). The dark green box labeled 1.2 indicates the position of one of two potential leucine zipper domains generated by alternative splicing. A PacI site was introduced into the macroH2A.2 cDNA at the junction of the histone and nonhistone domains (black triangle), allowing fusion of the nonhistone domain to GST. The GST-NHD fusion contains a thrombin cleavage site (open triangle) at the GST-NHD junction, allowing proteolytic cleavage to yield free NHD protein. (B) Recombinant NHD expression in bacterial cells. Lanes 1 and 2: Coomassie Blue-stained total bacterial protein from uninduced (lane 1) and IPTG-induced cells (lane 2) containing a GST-NHD expression plasmid. Lane 3: GST-NHD fusion protein purified from bacterial cells on a glutathione column eluted with reduced glutathione elution. Lanes 4 and 5: Purified NHD protein was eluted from a glutathione resin charged with GST-NHD by cleaving with thrombin. This allowed free NHD fusion protein to be eluted in two washes (samples of these supernatants are shown). (C) NHD protein from the same sample loaded in lane 4 was used to inoculate a rabbit that produced antibodies that recognize recombinant GST-NHD protein (lane 1) and endogenous macroH2A in total ES cell protein (lane 2) by immunoblotting.

| Table 1. Centromere repeats

| Table 2. Line-1 Elements

| Table 3. Total Co/T1 DNA
accepted that CoT1 DNA is composed of the sum total of highly repetitive DNA present in the mouse genome. Since the pattern of bands revealed by the CoT1 DNA probe was highly similar to the combined patterns of the centromeric and Line-1 probes, we conclude that centromeric repeats and Line-1 elements constitute the bulk of murine repetitive DNA that is subject to CpG methylation. In summary, we conclude that Dnmt1C/C cells contain extensively demethylated repetitive DNA and that partial remethylation can be achieved by reintroduction of a Dnmt1 BAC.

Demethylation-induced chromatin remodeling in pericentric DNA
The variant histone macroH2A has previously been shown to associate with pericentric DNA in preimplantation mouse embryos (Costanzi et al., 2000) and spermatocytes (Hoyer-Fender et al., 2004). The results presented in Fig. 1 confirmed that pericentric DNA is highly demethylated upon loss of Dnmt1 function in ES cells. We therefore sought to test whether loss of pericentric DNA methylation influenced the distribution of macroH2A in Dnmt1-mutant ES cells. MacroH2A resembles canonical histone H2A except for the presence of a C-terminal non-histone domain (NHD) roughly twice the size of the histone core domain (Pehrson and Fried, 1992). We purified recombinant NHD protein and used it as an immunogen to create a polyclonal antibody that detected macroH2A but not endogenous H2A in total protein extracted from ES cells (Fig. 2).

We performed immunofluorescence on a panel of male ES cells bearing mutations in Dnmt1 to investigate potential alterations of pericentric chromatin resulting from reductions in levels of CpG methylation (Fig. 3A). These cells included a wild-type line (J1), ES cells with Dnmt1NN, Dnmt1SS, and Dnmt1CC genotypes (which all cause extensive demethylation of the genome), and remethylated ES cell line B9. Interphase nuclei of ES cells of all genotypes contained condensed regions that stained intensely with DAPI (Fig. 3A). These condensed
chromocenter-like foci correspond to the sites of interphase centromeres, and can be identified using CREST antisera, human autoimmune serum from scleroderma patients that specifically recognizes interphase kinetochore proteins (Brenner et al., 1981). Though chromocenters were intact and apparently unaffected by DNA methylation levels, we noted alterations in the appearance of chromocenter histones. We observed a dramatic increase of macroH2A immunostaining in hypomethylated chromocenters (Fig. 3A). In undifferentiated wild-type J1 ES cells, macroH2A exhibited diffuse nuclear staining; a localization pattern observed with several other fully methylated ES cell lines (data not shown). However, in Dnmt1\textsuperscript{NN}, Dnmt1\textsuperscript{SS}, Dnmt1\textsuperscript{CC} cells, we noticed intense concentrations of macroH2A that were coincident with chromocenters. We double-labeled Dnmt1\textsuperscript{CC} cells to visualize macroH2A and CREST antigens and found that concentrations of macroH2A are often adjacent to CREST signals (Fig. 3B). The pericentric concentrations of macroH2A were reversed in rescued cell line B9. We conclude that chromatin remodeling involving macroH2A occurs within interphase pericentric chromatin.

Pericentric DNA is known to associate with heterochromatin protein 1 (HP1) via direct interactions that occur between HP1 and histone H3 methylated at lys 9 (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001). We found that HP1 co-localizes with chromocenters in ES cells regardless of their Dnmt1 genotype (Fig. 3C). However, macroH2A becomes concentrated in demethylated chromocenters marked by HP1 immunostaining.

Because chromocenters contain interphase centromeres and associated pericentric chromatin, we investigated the distribution of macroH2A on metaphase chromosomes (Fig. 4). We found that metaphase centromeres of CpG-demethylated chromosomes exhibited increased signals for macroH2A (Fig. 4B) as compared to normally methylated chromosomes prepared from wild-type cells (Fig. 4A). In addition, we performed combined macroH2A/CREST immunofluorescence on J1 and C/C cells and identified mitotic cells. We found that the macroH2A immunostaining pattern was immediately adjacent to CREST kinetochore staining only in demethylated Dnmt1\textsuperscript{CC} cells (Fig. 4C).

**MacroH2A content in demethylated nuclease-resistant chromatin**

The above results suggested that heterochromatin acquires a substantial content of macroH2A when CpG methylation levels are significantly reduced. We therefore conducted chromatin analyses using a cell fractionation approach based on nuclease accessibility (Fig. 5A). We isolated nuclei from wild-type, Dnmt1\textsuperscript{CC} and B9 cells, and infused them with micrococcal nuclease, which cleaves chromatin between nucleosomes. After nearly complete nuclease digestion was achieved, nuclease activity was quenched with EDTA and soluble chromatin was forced from nuclei into the supernatant by hypotonic treatment (extraction 1). We isolated the DNA from extraction 1 and found that it contained primarily mononucleosomes (Fig. 5B). A second hypotonic extraction was enriched in nuclease-resistant chromatin as judged by the presence of polynucleosome ladders (Fig. 5B, extraction 2). Chromatin from cells of each Dnmt1 genotype digested with similar efficiencies. Protein from each chromatin sample was analyzed by SDS-PAGE and Coomassie Blue staining. The majority of the protein in these samples consisted of histones (Fig. 5C), although the pattern of non-histone auxiliary bands differed between nuclease-sensitive and nuclease-resistant fractions. We subjected identical amounts of chromatin proteins from each fraction to western blotting for HP1 and
macroH2A (Fig. 5D). We found increased amounts of macroH2A in chromatin from CpG hypomethylated cell lines, and this effect was most pronounced in samples with highest resistance to micrococcal nuclease. In contrast, HP1 levels were insensitive to the state of DNA methylation. We therefore conclude that loss of DNA CpG methylation leads to an increase in macroH2A in demethylated, nuclease-resistant chromatin fractions.

Analysis of post-translational histone modifications in wild-type and demethylated ES cells

Because demethylation caused alteration of macroH2A distribution in ES cells, we performed experiments to investigate other histone features to see if they, too, would respond to reduced levels of DNA methylation. We first performed a series of western blots of total protein extracted from wild-type and Dnmt1C/C to screen for major changes in histone content or modification status of histones (Fig. 6). To do this, equal amounts of protein from wild-type (J1) and Dnmt1C/C cells were used. We confirmed that protein loading was equal both by Coomassie Blue staining of total protein and western blots of identically loaded lanes using an antibody specific for histone H2B. Similar blots showed that macroH2A protein is slightly more abundant in Dnmt1C/C cells than in wild-type cells. We found that trimethyl modifications of histones H3 at lysines 27, 9 and 4 exhibited little or no change in overall levels in response to demethylation.

We also investigated the distribution of methyl modifications at a subnuclear level through immunofluorescence analysis (Fig. 7). Trimethyl-H3K27 is a modified histone found predominantly in facultative heterochromatin (Cao et al., 2002; Plath et al., 2003). We found a finely particulate diffuse nuclear staining of trimethyl-H3K27 in both wild-type and Dnmt1C/C ES cells (Fig. 7A). A very diffuse nuclear distribution was observed for trimethyl-H3K4 (a modification enriched in euchromatin, (Santos-Rosa et al., 2002). The localization of trimethyl-H3K4 was also unperturbed by demethylation of DNA (Fig. 7B). Trimethyl-H3K9 is a modified histone found predominantly in constitutive heterochromatin, especially in pericentric chromatin (Maison et al., 2002; Peters et al., 2001). We assessed the localization of trimethyl-H3K9 in wild-type and Dnmt1C/C ES cells and observed little or no response to demethylation of DNA for this modification (Fig. 7C). We counted cells from each experiment and determined the proportion with diffuse nuclear versus punctate chromocenter staining (Table 1). Only macroH2A was redistributed from one class to another.

Demethylation-induced chromatin remodeling in somatic cells

Unlike somatic cells, ES cells remain viable even when CpG methylation is extensively reduced (Li et al., 1992), a feature that allowed the above studies to be performed on viable cells. However, we wished to investigate whether loss of CpG methylation affects macroH2A in differentiated cells. To do this, we conducted experiments using mouse embryonic
fibroblasts (MEFs) that harbor homozygous conditional alleles of \( \text{Dnmt1} \) in which exons 4 and 5 are flanked by \( \text{loxP} \) sites (Jackson-Grusby et al., 2001). T-antigen (T-Ag)-transformed derivatives of these MEFs remain viable after viral delivery of \( \text{cre} \) recombinase to induce recombination of the floxed \( \text{Dnmt1} \) exons. We exposed T-Ag-transformed conditional MEF lines to virus alone (Fig. 8A,C) or virus expressing \( \text{cre} \) recombinase (Fig. 8B,D). We found that conditional loss of \( \text{Dnmt1} \) in these MEFs led to a redistribution of the macroH2A signal similar to that observed in ES cells. Specifically, macroH2A formed foci coincident with demethylated pericentric DNA as indicated by intense DAPI and CREST staining (Fig. 6B,D). We observed this dramatic redistribution in two of four conditional MEF lines examined. The two responsive MEF lines proved to be male, as judged by PCR to detect \( \text{Y} \) chromosomes sequences (Jackson-Grusby et al., 2001). In contrast, a female MEF line called X17 failed to exhibit

Table 1. Chromatin patterns in wild type and \( \text{Dnmt1}^{c/c} \) ES cells

<table>
<thead>
<tr>
<th>Chromatin component</th>
<th>Wild type</th>
<th>( \text{Dnmt1}^{c/c} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. ( n )</td>
<td>Proportion ( \times 10^2 )</td>
</tr>
<tr>
<td>MacroH2A</td>
<td>13</td>
<td>0.064±0.017</td>
</tr>
<tr>
<td>Chromocenter (C)</td>
<td>189</td>
<td>0.936±0.017</td>
</tr>
<tr>
<td>Total (( n ))</td>
<td>202</td>
<td>1.000±0.000</td>
</tr>
<tr>
<td>Trimethyl-H3K27</td>
<td>207</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>Chromocenter (C)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total (( n ))</td>
<td>207</td>
<td>1.000±0.000</td>
</tr>
<tr>
<td>Trimethyl-H3K9</td>
<td>212</td>
<td>0.995±0.005</td>
</tr>
<tr>
<td>Chromocenter (C)</td>
<td>1</td>
<td>0.005±0.005</td>
</tr>
<tr>
<td>Total (( n ))</td>
<td>213</td>
<td>1.000±0.000</td>
</tr>
<tr>
<td>Trimethyl-H3K4</td>
<td>198</td>
<td>0.990±0.007</td>
</tr>
<tr>
<td>Chromocenter (C)</td>
<td>2</td>
<td>0.010±0.007</td>
</tr>
<tr>
<td>Total (( n ))</td>
<td>200</td>
<td>1.000±0.000</td>
</tr>
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Wild-type and \( \text{Dnmt1}^{c/c} \) ES cells were immunostained with antibodies recognizing the listed chromatin components and greater than 200 cells were visually examined to determine the pattern of localization of chromatin components.

*Two general chromatin patterns were observed: diffuse nuclear particulate distribution (D), or chromocenter-associated patterns (C).

†Number of cells counted.

‡Proportions were determined by dividing the number of cells counted with a given pattern (D or C) by the total (\( n \)). Standard deviations for proportions were determined with the equation \( \sigma = \sqrt{(1-p^2)/n} \), where \( p \) is the proportion under consideration (D/\( n \) or C/\( n \)) and \( n \) is the total number of cells counted.

Fig. 6. Western blot analysis of histone content in wild-type and demethylated ES cells. Equivalent amounts of cellular protein extracted from wild-type (J1) and \( \text{Dnmt1}^{c/c} \) (c/c) ES cells were loaded onto SDS polyacrylamide gels and stained with Coomassie Blue to confirm equal loading. Equivalently loaded lanes were transferred to membranes for western blot analyses using antibodies specific for the N-terminal tail of histone H2B, macroH2A and modification-state-specific antibodies that recognize histone H3 trimethylated at lysine 27 (methyl-H3K27), lysine 9 (methyl-H3K9) or lysine 4 (methyl-H3K4).

Fig. 7. Localization of post-translational methylation modifications of histone H3 in wild-type (J1) and \( \text{Dnmt1}^{c/c} \) (c/c) ES cells. (A) Immunostaining with an antibody specific for histone H3 trimethylated at lysine 27. (B) Immunostaining with an antibody specific for histone H3 trimethylated at lysine 4. (C) Immunostaining with an antibody specific for histone H3 trimethylated at lysine 9.
demethylation-induced redistribution of macroH2A (Fig. 8E,F), although these cells contained distinctive macrochromatin bodies corresponding to the positions of inactive X chromosomes (Costanzi and Pehrson, 1998) and lacked Y chromosomes, as judged by Y-specific PCR. A second female MEF line containing conditional genotype for Dnmt1 also failed to exhibit a redistribution of macroH2A in response to demethylation. Though this possible sex-dependent effect on chromatin dynamics is intriguing, these MEF cell lines may harbor other differences that we presently do not understand. Nonetheless, the results indicate that demethylation-induced chromatin reorganization involving macroH2A can occur in somatic cells.

Discussion

The results presented here show that ‘crosstalk’ exists that connects the DNA CpG methylation system with other aspects of heterochromatin structure. The constitutive heterochromatin of mammalian centromeres is ordinarily highly methylated at CpG dinucleotides. Our results show that pericentric heterochromatin is significantly altered when CpG DNA methylation is greatly reduced. Specifically, we find that CpG demethylation induces an apparent chromatin-remodeling event involving the histone variant macroH2A.

Two possible mechanisms might explain our findings. (1) Loss of DNA methylation may cause a decondensation of heterochromatin at pericentric regions, leading to the unmasking of macroH2A epitopes that are inaccessible in fully-methylated cells. (2) Loss of DNA methylation may induce a chromatin-remodeling event that causes increased macroH2A to become incorporated into demethylated pericentric heterochromatin. We cannot formally rule out the first mechanism, and indeed, several results suggest that chromatin remodeling may be involved. First, we found increased macroH2A signal on demethylated centromeres of metaphase chromosomes (Fig. 4). We also found that macroH2A protein is enriched in nuclease-resistant demethylated chromatin (Fig. 5D), and that other anti-histone antibodies detect no significant differences in chromatin patterns when wild-type and Dnmt1C/C cells are compared (Fig. 7). Together, these findings suggest that demethylation-induced unmasking of macroH2A epitopes is not likely to account for the observations. In either case, the results indicate that a close connection exists between DNA methylation and the structure of ES cell centromeres.

Heterochromatin is often classified into one of two types –
constitutive (obligately silenced, such as pericentric heterochromatin), and facultative (contextually silenced). Perhaps the best-studied example of facultative heterochromatin is the inactive X chromosome (Xi) of female placental mammals. In mice and humans, the chromatin of Xi is characterized by its association with the untranscribed Xist RNA and the presence of abundant macroH2A protein (Costanzi and Pehrson, 1998). Many studies have shown that CpG islands of X-linked genes subject to X-inactivation are highly methylated. However, promoter-proximal CpG islands make up only a small portion of available CpG dinucleotides on the X chromosome, and experiments that address global methylation levels on X chromosomes show that the inactive X chromosome is markedly undermethylated relative to the active X chromosome (Bernardin et al., 1996; Viegas-Pequignot et al., 1988).

Our results show that loss of CpG demethylation leads to the establishment of pericentric chromatin with features reminiscent of the inactive X chromosome, namely, low levels of CpG DNA methylation combined with a high content of macroH2A. This finding raises the interesting possibility that DNA methylation can act as a toggle between facultative and constitutive heterochromatin. This intriguing possibility led us to investigate the behavior of other chromatin constituents of euchromatin and facultative and constitutive heterochromatin by both western blot analysis and immunofluorescence. We found that trimethyl-H3K27, which is a marker of facultative heterochromatin (Cao et al., 2002; Plath et al., 2003), and found that trimethyl-H3K27, which is a marker of facultative heterochromatin (Cao et al., 2002; Plath et al., 2003), were essentially unaffected by loss of Dnmt1 function. We also found that trimethyl-H3K4, which is a marker of euchromatin (Santos-Rosa et al., 2002), was unaffected by loss of Dnmt1 function. These results show that macroH2A is relatively unique in its ability to respond to reductions in levels of DNA methylation. The finding that macroH2A but not trimethyl-H3K27 was affected in demethylated cells suggested that additional complexities may exist in the interplay between DNA methylation and facultative heterochromatin organization.

We thank Steve Doxsey for CREST anti-kinetochore serum, and Therese Doherty for proofreading. This research was supported in part by the Robert Leet and Clara Guthrie Patterson Trust and grant AG023687 from the National Institutes of Health, USA.

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