Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes

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Studies of histone methylation have shown that H3 can be methylated at lysine 4 (Lys4) or lysine 9 (Lys9)[1,2]. Whereas H3–Lys4 methylation has been correlated with active gene expression[3], H3–Lys9 methylation has been linked to gene silencing and assembly of heterochromatin in mouse and *Schizosaccharomyces pombe*[4–7]. The chromodomain of mouse HP1 (and Swi6 in *S. pombe*) binds H3 methylated at Lys9, and methylation at this site is thought to mark and promote heterochromatin assembly. We have used a well-studied model of mammalian epigenetic silencing, the human inactive X chromosome, to show that enrichment for H3 methylated at Lys9 is also a distinguishing mark of facultative heterochromatin. In contrast, H3 methylated at Lys4 is depleted in the inactive X chromosome, except in three 'hot spots' of enrichment along its length. Chromatin immunoprecipitation analyses further show that Lys9 methylation is associated with promoters of inactive genes, whereas Lys4 methylation is associated with active genes on the X chromosome. These data demonstrate that differential methylation at two distinct sites of the H3 amino terminus correlates with contrasting gene activities and may be part of a 'histone code' involved in establishing and maintaining facultative heterochromatin.

Dosage compensation of X-linked genes in mammals is mediated by transcriptional silencing of the majority of genes on one of the two X chromosomes in female cells[8]. This X-inactivation process is initiated by up-regulation of the noncoding *XIST* transcript and its association *in cis* with the chromosome to be inactivated. In addition, the inactive X chromosome acquires heterochromatic characteristics such as late replication[9], condensed appearance (Barr body) in interphase cells[10], DNA methylation of CpG islands at house-keeping genes[11], and association with altered nucleosomes that are composed of hypoacetylated histones and enriched for H2A variants macroH2A1 and macroH2A2 (refs 12–16). Although it is not clear how each of these characteristics contributes to the X-inactivation process, at minimum, they probably act together to maintain the stability of X inactivation through multiple rounds of cell division[17].

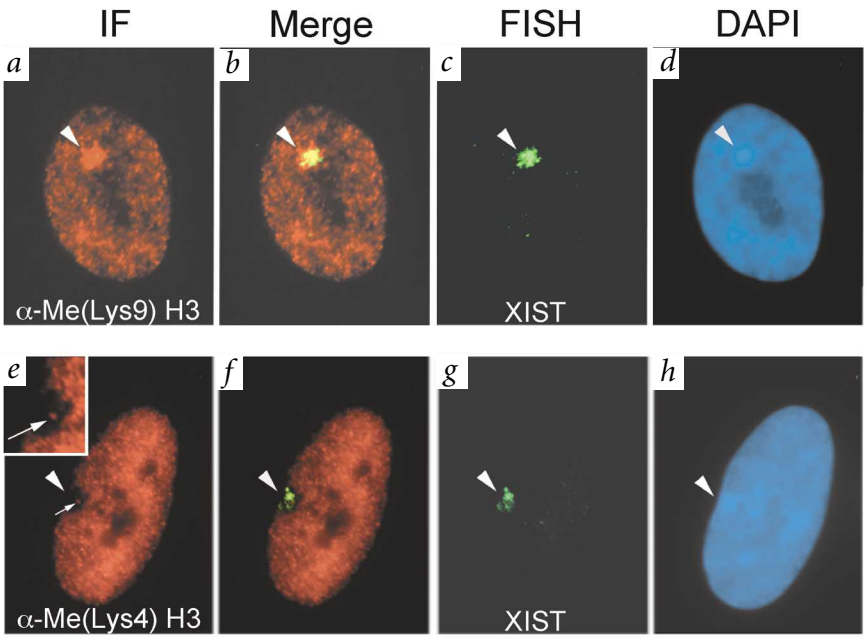

Fig. 1 *XIST* RNA FISH combined with immunofluorescence staining on human female IMR 90 cells. The arrowheads show the position of the inactive X chromosomes. ***a–d***, A representative human interphase nucleus stained with Lys9-methyl H3 antibody. *a*, Lys9-methyl H3 antibody staining detected by a Texas Red–conjugated secondary antibody (red). *b*, Merged image of *XIST* RNA FISH and immunofluorescence. *c*, *XIST* RNA FISH (*XIST* RNA detected in green). *d*, DAPI staining of DNA. ***e–h***, A representative human interphase nucleus stained with Lys4-methyl H3 antibody. *e*, Lys4-methyl H3 antibody staining detected by a Texas Red–conjugated secondary antibody (red). The inset is an enlargement of the inactive X domain showing a 'hot spot' of staining (indicated by small arrow) within this territory. *f*, Merged image of *XIST* RNA FISH and immunofluorescence. *g*, *XIST* RNA FISH (RNA detected in green). *h*, DAPI staining of DNA.

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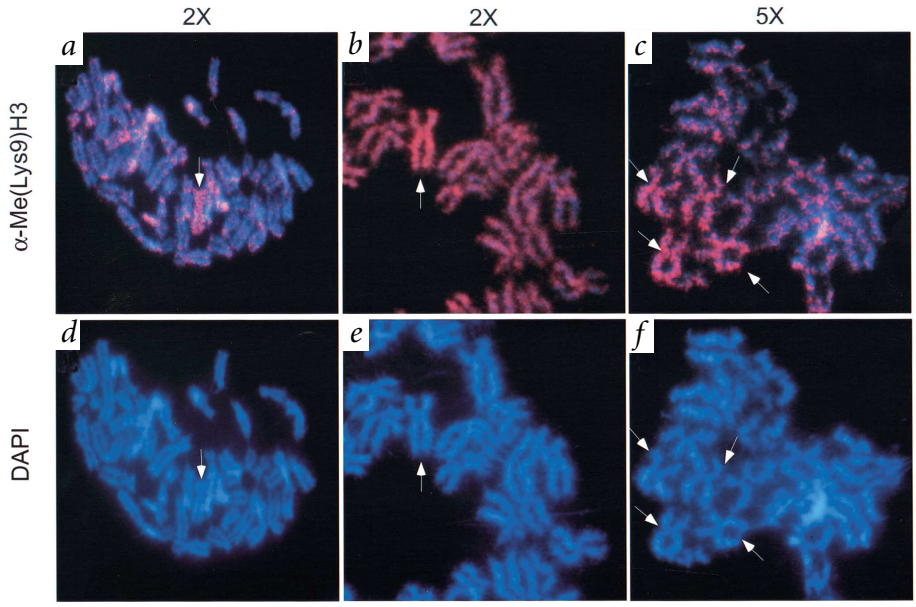

Fig. 2 Immunofluorescence patterns of human metaphase chromosomes stained with the Lys9-methyl H3 antibody. ***a***,***b***, Two different metaphase spreads from a normal female cell line showing preferential staining of one chromosome, as indicated by arrows. Localization of Lys9-methylated H3 was detected using Cy3-conjugated secondary antibody (red), and chromosomal DNA was stained with DAPI (blue). ***c***, Similar analysis of a metaphase spread from the cell line 6061B containing four inactive chromosomes; arrows point to the four chromosomes enriched for Lys9-methyl H3 staining. ***d–f***, Corresponding DAPI-stained images of the same metaphase spreads shown in *a–c*, respectively.

The inactive X chromosome is one of the best-studied examples of facultative heterochromatin in higher eukaryotes and offers an excellent model system for studying the link between histone methylation and epigenetic regulation of gene expression.To test whether histone H3 methylation at Lys9 is associated with facultative heterochromatin in a manner similar to its enrichment at constitutive heterochromatin, we examined human female IMR90 (normal diploid) interphase cells by a combination of indirect immunofluorescence and fluorescence *in situ* hybridization (FISH). The Lys9-methyl H3 antibody preferentially stained a nuclear region that colocalizes with the *XIST* RNA domain (Fig. 1*a*,*b*). This region was also heterochromatin-dense, as indicated by the stronger staining with DAPI (Fig. 1*a*,*d*). Together these findings suggest that it is the inactive X chromosome that is enriched for Lys9-methylated H3. In contrast, staining with an antibody specific for H3 methylated at Lys4 showed that the Lys4-methylated H3 was distributed throughout most of the nucleus, but conspicuously absent at the location of the inactive X chromosome (Fig. 1*e–h*). Notably, we consistently observed a single point of Lys4-methyl H3 staining within the otherwise negatively stained domain of the inactive X (Fig. 1*e*, inset), which may correspond to a region of chromatin containing Lys4-methylated H3 within the inactive X chromosome. The immunofluorescence/FISH analyses therefore indicate that the inactive X chromosome in female cells is globally enriched for Lys9-methylated H3, but largely lacks Lys-4 methylated H3 proteins.

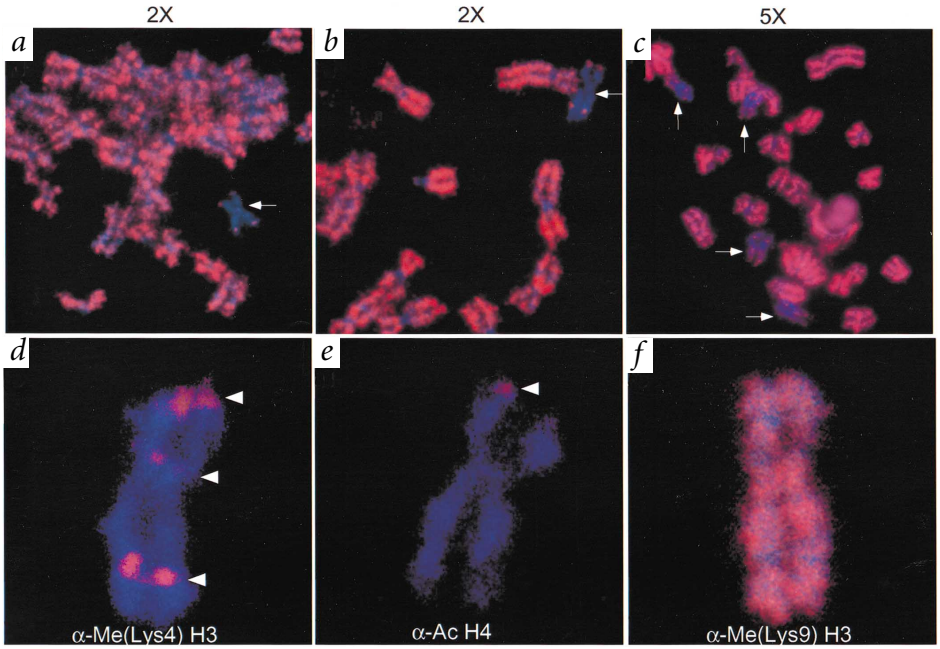

Fig. 3 Immunofluorescence patterns of human metaphase chromosomes. ***a***,***b***, Two separate metaphase spreads from a normal female lymphoblast cell line stained with Lys4-methyl H3 antibody. Antibody localization was detected with Cy3-conjugated secondary antibodies (red), and chromosomal DNA was stained with DAPI (blue). Arrows point to the chromosome in each metaphase spread that has a distinctly different antibody staining pattern from the other chromosomes. ***c***, Similar analysis of metaphase chromosomes from a cell line that contains four inactive X chromosomes. Arrows point to four chromosomes from the same metaphase spread that are deficient in antibody staining. ***d***, Enlarged image of an inactive X chromosome stained with Lys4 methyl H3 antibody. Arrowheads point to three distinct regions of intense staining with this antibody. ***e***, Enlarged image of an inactive X chromosome stained with an antibody against hyperacetylated H4. The arrowhead points to the enriched staining with this antibody at the p-telomeric region. ***f***, Enlarged image of an inactive X chromosome stained with the Lys9-methyl H3 antibody, showing intense staining covering nearly all of the chromosome.

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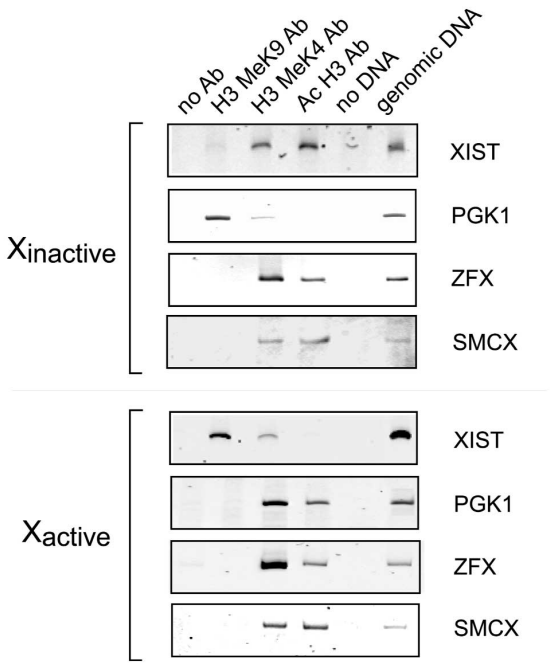

Fig. 4 ChIP analysis of somatic cell hybrid cell lines. Chromatin from CHO somatic hybrid cells containing the inactive ($X_{inactive}$) or active (X_{active}) human X chromosome was immunoprecipitated using the antibodies indicated. The presence of *XIST*, *PGK1*, *ZFX*, and *SMCX* promoter DNA sequences in the immunoprecipitated DNA was assayed by PCR. PCR products were separated on 15% polyacrylamide gels and imaged by a digital camera, and then the images were electronically inverted to facilitate visualization of the ethidium bromide–stained bands.

To better understand the distribution of the methyl-Lys9 and -Lys4 modifications along the inactive X, we also examined condensed metaphase chromosomes by indirect immunofluorescence. Staining of chromosomes from a normal female lymphoblast cell line showed that although all chromosomes have regions of Lys9-methylated H3, one chromosome in each metaphase spread was consistently more intensely and uniformly stained (Fig. 2*a*,*b*). This difference is not simply a reflection of a higher state of condensation, as there were no significant differences in this property between the metaphase chromosomes detected by DAPI staining alone (Fig. 2*d*,*e*). To confirm that the chromosome enriched for Lys9-methylated H3 is the inactive X chromosome, we also stained metaphase spreads from a cell line that contains five X chromosomes. In these cells, four of the five X chromosomes are expected to be inactivated[18] and, indeed, four chromosomes of equal size showed enriched staining by the Lys9-methyl H3 antibody (Fig. 2*c*). These findings support our earlier results (Fig. 1*a*–*c*) and suggest that the observed enrichment of Lys9-methyl H3 on the inactive X in interphase cells is not simply due to higher nucleosome densities of the Barr body[19]. Moreover, these results indicate that this methyl mark is retained during mitosis and chromosome condensation and could be a stably propagated epigenetic mark for the inactive X chromosome.

By contrast, in analyses of metaphase chromosomes using the antibody specific for Lys4-methylated H3, this antibody intensely stained all but one chromosome per cell (Fig. 3*a*,*b*). Staining of the metaphase spreads of the 5X cell line shows that four identical size chromosomes are understained using the Lys4-methyl H3 antibody (Fig. 3*c*), confirming that these chromosomes are the inactive X. Notably, staining is almost completely absent in the inactive X chromosome, except for three hot spots of H3–Lys4 methylation (Fig. 3*d*), at least one of which probably corresponds to the Lys4-methyl H3 signal within the inactive X domain observed in interphase nuclei (Fig. 1*e*). Based on the relative positioning of these hot spots along the length of the X chromosome[20], one region of H3 Lys4-methyl staining appears to be located at the pseudoautosomal region of the distal end of the p arm (Fig. 3*d*). Another region is located near Xq25–26 of the q arm, and fainter staining is frequently seen around Xp11 (Fig. 3*d*). The two regions of Lys4-methyl H3 observed on the p arm of the inactive X chromosome correspond to the locations of genes that escape inactivation[21]. The enrichment of Lys4-methylation at Xq25–26 is interesting, however, as this region is not known to contain genes that escape inactivation. Consistent with previous reports[12], staining of the inactive X chromosome with an antibody specific for hyperacetylated H4 showed that acetylated H4 was greatly depleted (Fig. 3*e*). In contrast to the three prominent areas of staining observed with the antibody against Lys4-methylated H3, only the telomeric region of the p arm on the inactive X routinely showed significant fluorescence with the hyperacetylated H4 antibody (Fig. 3*d*,*e*). These findings suggest that different regions along this chromosome may contain functionally distinct domains of chromatin with unique patterns of modified histones.

To determine whether methyl H3 marks the promoter regions of active and inactive genes on the X chromosome, we used two Chinese hamster ovary somatic hybrid cell lines that contain either a single active or inactive human X chromosome[22] in chromatin immunoprecipitation (ChIP) assays. We precipitated chromatin from each of these two cell lines using antibodies against Lys9-methylated H3, Lys4-methylated H3 or Lys9/14-acetylated H3, and carried out PCR amplification of the immunoprecipitated DNA using primers specific to the promoter regions of *PGK1*, *XIST*, *ZFX* and *SMCX*. *PGK1*, like most genes on the X chromosome, is transcriptionally silenced on the inactive X and is transcribed only from the active X chromosome. In opposite fashion, *XIST* is transcribed from the inactive but not from the active X chromosome. *ZFX* and *SMCX* are examples of genes that escape X-inactivation, as they are expressed from both the inactive and active X chromosomes. Consistent with previously published results[23] and with the known transcription status of these genes, the acetyl-H3 antibody immunoprecipitated *XIST* DNA only from the inactive X chromosome, and *PGK1* DNA only from the active chromosome, whereas *ZFX* and *SMCX* DNA were immunoprecipitated from both active and inactive X chromosomes (Fig. 4). Consistent with the data observed using the acetyl-H3 antibody, the Lys4-methyl H3 antibody only immunoprecipitated DNA corresponding to genes that are transcriptionally active (Fig. 4), regardless of whether they resided on the active or inactive X chromosome. In contrast, the Lys9-methyl H3 antibody preferentially precipitated DNA corresponding to genes that are transcriptionally silenced (*PGK1* from the inactive X and *XIST* from the active X chromosome; Fig. 4). Given that some genes, such as *XIST*, *ZFX* and *SMCX*, are preferentially immunoprecipitated by the Lys4-methyl H3 antibody from the inactive X chromosome, the identification of DNA sequences that correspond to the Lys4-methyl H3 hot spots seen on the metaphase spreads (Fig. 3) would be useful, as would determining the transcription status of these domains.

The observed association of Lys9-methylated H3 with the inactive X chromosome is also seen in the accompanying paper by Peters *et al.*[24] and suggests that this histone modification may participate in the transcriptional silencing process. Indeed, studies using mouse embryonic stem cells show that enrichment of Lys9-methylated H3 with the inactive X chromosome is an early event in the inactivation process[25]. Lys9-methylated H3 has been

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shown to bind the chromodomain of HP1 (refs 4–6), and its enrichment on the inactive X chromosome may provide binding sites for the recruitment of silencing factors. Our findings further show that reproducible association patterns for both Lys4- and Lys9-methylated H3 are retained during metaphase chromosome condensation, and therefore raise the possibility that these specific modifications may serve as stable epigenetic marks for the 'memory' of the inactive X during mitosis. Finally, our ChIP data demonstrate, at a molecular level, a reciprocal association of Lys9- or Lys4-methylated H3 with different genes, depending on the transcriptional status of the genes. Similar results have been observed in the chicken β-globin locus as well as the mating loci of *S. pombe*[26,27]. Collectively, these data show that differential methylation of H3 at two different sites may set up large domains of silenced or transcriptionally competent chromatin. In addition, they provide strong support for the concept of a 'histone code[2,28,' which suggests that specific modifications at different sites on the histone N-terminal tails impart distinct characteristics and carry out different cellular functions.

Methods

Interphase cell indirect immunofluorescence and FISH analyses. Normal female IMR 90 (ATCC) cells were grown on coverslips for 24–48 h and then fixed in 4% formaldehyde for 15 min at room temperature (RT). The cells were then permeabilized in PBS containing 0.5% Triton-X for 4 min on ice, washed in PBS and then washed in 2 × SSC before RNA FISH analysis. To preserve nuclear structures, cells were kept continuously hydrated. RNA FISH hybridization and washes were carried out essentially as described[29]. Briefly, we hybridized cells with an *XIST* probe comprising a pool of four exon-derived DNA fragments spanning a total of 4.5 kb of sequence, labeled by nick translation with Spectrum Red or Green dUTP (Vysis). After hybridization overnight at 37 °C, we washed cells three times in 50% formamide/2 × SSC, three times in 2 × SSC and then once with PBS/0.5% BSA before carrying out immunofluorescence staining. We incubated cells with primary and secondary antibodies for 1 h each at RT and washed them with PBS between the two incubation periods. We also counterstained nuclei with DAPI. Images were acquired using a Zeiss Axioplan 2 fluorescence microscope with an Orca 2 CCD camera (Hamamatsu) and Improvision software (IPLab).

Metaphase chromosome immunofluorescence. A normal human female lymphoblast cell line (HH) and a female lymphoblast cell line that contains five X chromosomes (6061B) were grown, harvested and collected onto microscope slides with a Cytospin 3 centrifuge. We detected modified histones by indirect immunofluorescence, essentially as described[16]. Briefly, we incubated cells for 1 h at 37 °C in a humid chamber with serial dilutions of either the primary Lys9- or Lys4-methyl H3 or control acetyl H4 antisera, and washed them in KCM (120 mM KCl, 20 mM NaCl, 10 mM TRIS-Cl, pH 8.0, 0.5 M EDTA, 0.1% Triton). We then added Cy3-conjugated, affinity-purified, donkey anti-rabbit IgG antibody (Jackson ImmunoResearch) diluted 1:40 in KCM, and incubated the mixture for 30 min at RT. Cells were once again washed with KCM and fixed in 4% formaldehyde for 10 min at RT. After a wash in sterile water, we counterstained chromosomes with DAPI, mounted the coverslips in antifade (Vectashield) and viewed the slides on a Zeiss Axiophot fluorescence microscope. We determined positions of immunofluorescent staining by comparison with DAPI-staining patterns and by relative distance measurements on chromosome arms[20].

Chromatin immunoprecipitation. We carried out chromatin immunoprecipitation assays as described previously[30]. In this case, formaldehyde-fixed chromatin was harvested from CHO somatic-cell hybrids containing either the active or inactive human X chromosome[22]. In each immunoprecipitation reaction, we used approximately 3×10^6 cells–worth of sonicated chromatin and the antibodies indicated in the text. After extensive washing, reverse cross-linking, RNase A and proteinase K digestions, we analyzed the immunoprecipitated DNA by PCR using primers specific for the promoter regions of human *XIST*, *PGK1*, *ZFX* and *SMCX*. Primer sequences for the first three genes were derived from previously described sequences[23]. We used oligonucleotides with the sequences 5′–AGTGCCGGTTTGACTCCA–3′ and 5′–TTACTCAAA AGTACCACCCC–3′ for *SMCX*.

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