Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin

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Post-translational modifications of histone amino termini are an important regulatory mechanism that induce transitions in chromatin structure, thereby contributing to epigenetic gene control and the assembly of specialized chromosomal subdomains[1,2]. Methylation of histone H3 at lysine 9 (H3–Lys9) by site-specific histone methyltransferases (Suv39h HMTases)[3] marks constitutive heterochromatin[4–6]. Here, we show that H3–Lys9 methylation also occurs in facultative heterochromatin of the inactive X chromosome (X_i) in female mammals. H3–Lys9 methylation is retained through mitosis, indicating that it might provide an epigenetic imprint for the maintenance of the inactive state. Disruption of the two mouse Suv39h HMTases abolishes H3-Lys9 methylation of constitutive heterochromatin but not that of the X_i. In addition, HP1 proteins, which normally associate with heterochromatin, do not accumulate with the X_i. These observations suggest the existence of an Suv39h-HP1-independent pathway regulating H3-Lys9 methylation of facultative heterochromatin.

The functional organization of chromosomes into euchromatic and heterochromatic subdomains has been correlated with distinct post-translational modifications of histone N termini, such as acetylation, phosphorylation and methylation[1,2]. Whereas acetylation and phosphorylation appear as transient marks, histone methylation may be a relatively stable modification that seems to be suited to the longer-term maintenance of epigenetic chromatin states[2]. One of the best examples of a stable higher-order chromatin structure is transcriptionally 'inert', constitutive heterochromatin that remains cytologically condensed in interphase and coincides with hypoacetylated[7–9] centromeric regions of metaphase chromosomes. The recent discovery of Suv39h HMTases[3] indicated that the selective methylation of H3–Lys9 is associated with pericentric heterochromatin. Disruption of Suv39h HMTase activities in mutant mice[10] and of Suv39h-homologous HMTase function in *Schizosaccharomyces pombe*[5,6] largely abolish pericentric H3–Lys9 methylation, indicating that this histone modification is an epigenetic imprint for constitutive heterochromatin.

Developmentally regulated, or facultative, heterochromatin is another relatively stable state of chromatin that is particularly important for dosage compensation in female mammals in which one of the two X chromosomes is selectively silenced[11]. The X_i is cytologically visible as the condensed 'Barr body' in female somatic cells[12]. X inactivation is initiated early in development by noncoding sense and antisense RNAs that emanate from the X inactivation center[13–16]. Accumulation of the X-inactive specific transcript, *Xist*, at the X_i triggers chromosome silencing through an as-yet undetermined pathway. Once established, the inactive state is maintained in an *Xist*-independent manner characterized by asynchronous replication and by chromatin modifications that include the hypoacetylation of histones H3 and H4 (refs 17,18) and the selective concentration of the histone variant macroH2A (refs 19,20). Because of the parallels between the higher-order chromatin structure of constitutive and facultative heterochromatin, we considered that H3–Lys9 methylation might also be a potential epigenetic imprint for the X_i.

Recently, we developed an antiserum (α-4x-methH3-K9; see Methods) that selectively recognizes H3–Lys9 methylation of heterochromatic subdomains[10]. Using these α-4x-methH3-K9 antibodies, we first examined female human primary cells from fetal

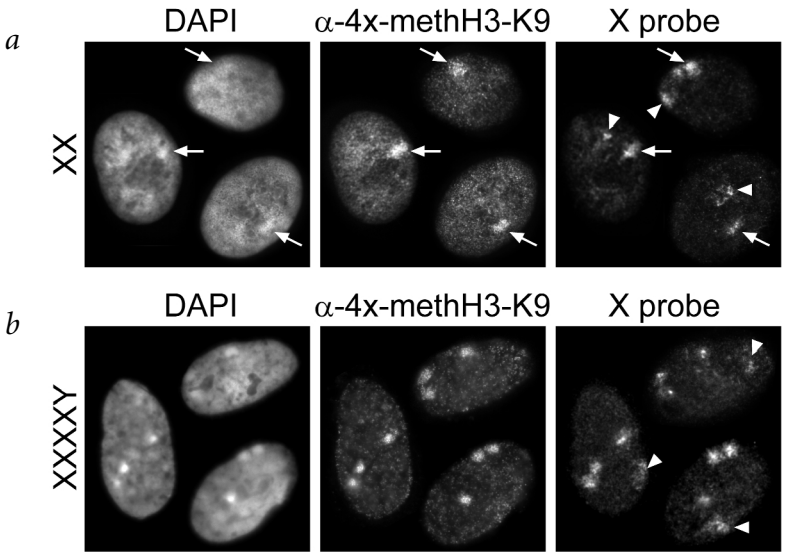

Fig. 1 H3–Lys9 methylation of the Barr body in human cells. ***a***, Immuno-FISH analysis of human female epithelial cells from fetal amniocenteses with α-4x-methH3-K9 antibodies and an X chromosome–specific DNA painting probe. DNA was counterstained with DAPI. Arrow indicates the Barr body comprising the X_i; arrowhead shows the active X chromosome. ***b***, Immuno-FISH analysis as above, showing the three Barr bodies present in the aneuploid (49,′XY) human cell line GM12013.

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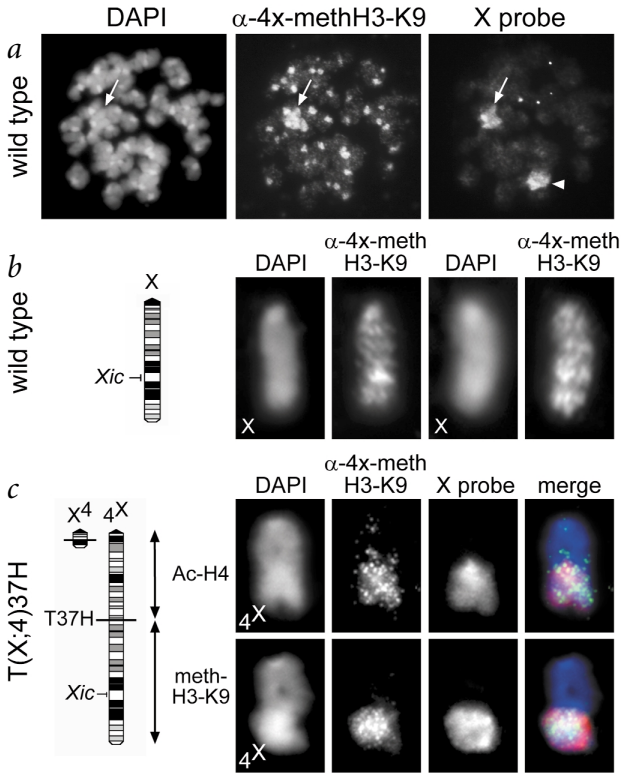

Fig. 2 H3–Lys9 methylation of the X_i in mouse metaphase chromosomes. ***a***, Immuno-FISH analysis as in Fig. 1 of metaphase chromosome spreads prepared from bone-marrow cultures of wildtype female mice. ***b***, Enlarged images of H3–Lys9–methylated X_i chromosomes showing nonuniform staining. ***c***, Immuno-FISH of metaphase chromosomes from XX female mouse T(X;4)37H cells that carry a reciprocal translocation between the X chromosome and chromosome 4 (see diagram at left). Shown are representative examples of the 4;X translocation product with selective H3–Lys9 methylation of X_i material.

amniocenteses (Fig. 1*a*), buccal smears and blood granulocytes (data not shown). Immunofluorescence *in situ* hybridization (FISH) analyses with the α-4x-methH3-K9 antibodies and an X chromosome–specific DNA painting probe detected a single area in the interphase nuclei of amniocentetic cells that coincides between α-4x-methH3-K9 staining and one of the two X chromosomes (Fig. 1*a*). In addition, the α-4x-methH3-K9 antibodies showed diffuse nuclear signals and the presence of some smaller heterochromatic foci. To confirm that H3–Lys9 methylation occurs on the X_i, we next analyzed an aneuploid (49,′XY) human cell line (GM12013) containing three inactive X chromosomes (J.E.M. and N.B., unpublished data). The α-4x-methH3-K9 antibodies detected three strongly stained nuclear areas that colocalize with the DAPI-dense Barr bodies and with the positions of three of the four X chromosomes (Fig. 1*b*).

To assess whether H3–Lys9 methylation of the X_i is a mitotically stable epigenetic imprint, we examined metaphase chromosomes prepared from bone-marrow cultures of female wildtype mice. Immuno-FISH analysis showed selective H3–Lys9 methylation of one X chromosome in the majority of metaphase spreads (Fig. 2*a*). In addition, we detected focal H3–Lys9 methylation signals that coincided with pericentric heterochromatin on all chromosomes. Higher magnification of the stained X chromosome showed that H3–Lys9 methylation is not uniform along the chromosome arms but is localized in a band-like pattern (Fig. 2*b*). We next investigated the distribution of H3–Lys9 methylation on metaphase chromosomes from T37H cells, which are derived from female mice carrying the T(X;4)37H reciprocal translocation. The 4;X rearrangement silences transcription of an autosomal locus approximately 20 cM from the breakpoint, but the silencing is not accompanied by significant encroachment of *Xist* RNA or by histone H4 hypoacetylation[21]. Accordingly, H3–Lys9 methylation was largely confined to the distal half of the 4;X translocation product, comprising X-chromosome material and including the X inactivation center (Fig. 2*c*). This indicates that H3–Lys9 methylation is specific for the heterochromatinized X_i.

The Suv39h HMTases are the major enzymes catalyzing H3–Lys9 methylation of pericentric heterochromatin. Combined disruption of both *Suv39h* loci in double-null (dn) mice results in loss of pericentric H3–Lys9 methylation and induces chromosomal instabilities[10]. To determine whether H3–Lys9 methylation on the X_i is also dependent on Suv39h HMTase function, we carried out immuno-FISH on metaphase chromosomes from bone-

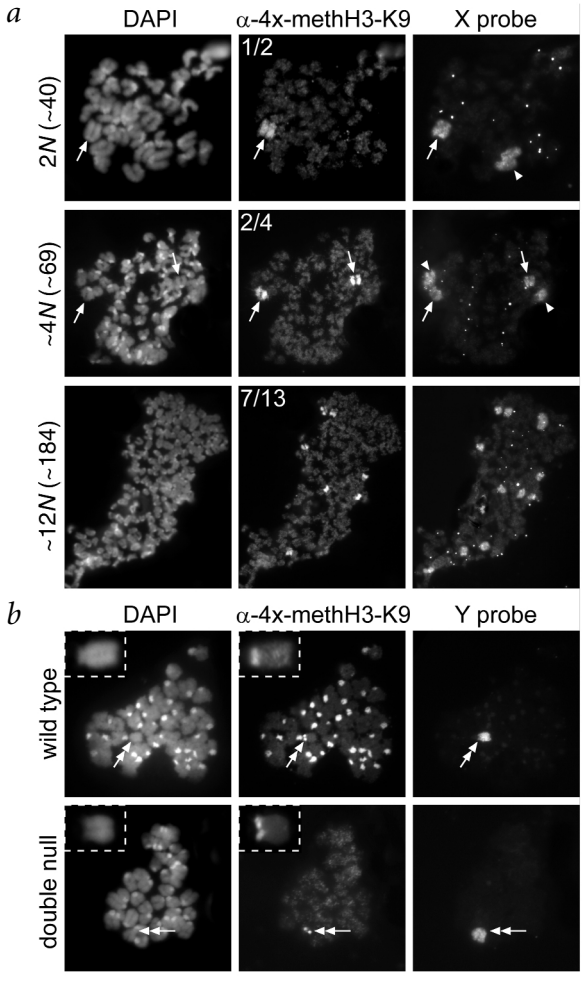

Fig. 3 Suv39h-independent H3–Lys9 methylation of the X_i and Y chromosomes in mouse bone-marrow spreads. ***a***, Immuno-FISH analysis as in Fig. 1 of metaphase chromosome spreads prepared from bone-marrow cultures of *Suv39h*-dn female mice. Examples show karyotypes with increasing ploidy, comprising diploid (2*N*), tetraploid (4*N*) and an approximately 12*N* chromosome complement. ***b***, Immuno-FISH analysis of metaphase chromosome spreads prepared from bone-marrow cultures of wildtype and *Suv39h*-dn male mice with α-4x-methH3-K9 antibodies and a Y chromosome–specific DNA painting probe . DNA was counterstained with DAPI. The double arrow indicates the Y chromosome and inserts show representative examples of enlarged Y chromosomes obtained from a different spread.

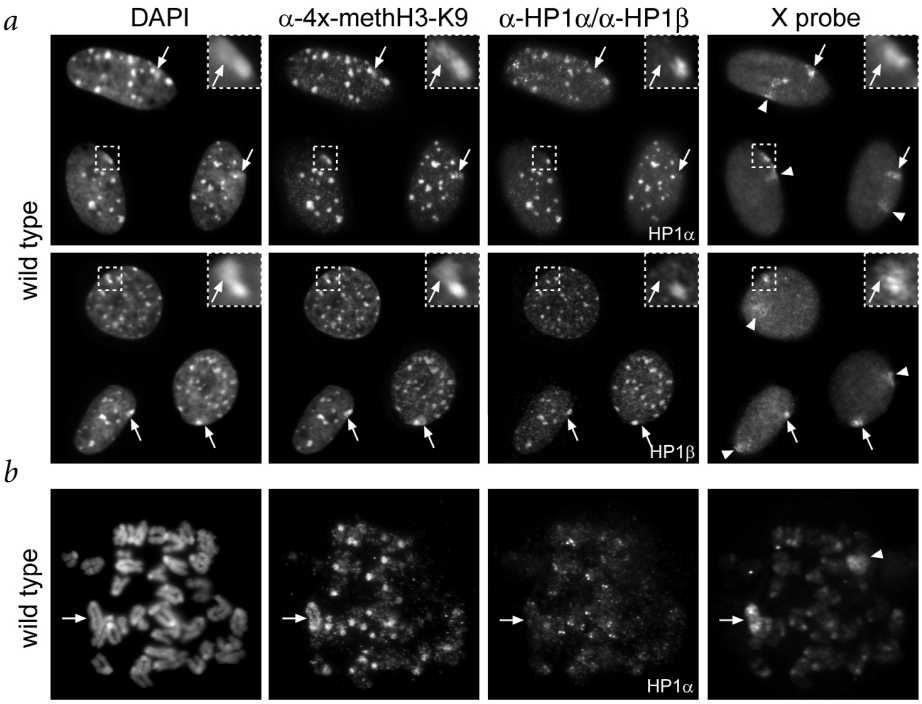

Fig. 4 Absence of HP1 localization in the Barr body and the X_i chromosome. ***a***, Double-labeling immuno-FISH of wildtype female PMEFs. DNA was counterstained with DAPI with α-4x-methH3-K9, α-HP1α or α-HP1β antibodies and an X chromosome–specific DNA painting probe. Arrows indicate the perinuclear Barr body; inserts show higher magnification of constitutive heterochromatin (intense DAPI staining) and the adjacent X_i (lighter DAPI staining). ***b***, Double-labeling immuno-FISH of metaphase chromosomes from wildtype female bone-marrow cells with α-4x-methH3-K9, α-HP1α antibodies and an X chromosome–specific DNA painting probe. Arrow indicates the inactive X chromosome.

marrow cultures derived from two separate *Suv39h*-dn female mice. Consistent with previous analyses[10], there was no pericentric H3–Lys9 methylation but, strikingly, one X chromosome did show α-4x-methH3-K9 signals (Fig. 3*a*, top panels). As *Suv39h*-dn cells often have increased ploidy[10], we were able to analyze H3–Lys9 methylation on the X_i in polyploid karyotypes (4*N* (≈69 chromosomes) and 12*N* (≈184 chromosomes)). We found that 2 of 4 and 7 of 13 X chromosomes showed H3–Lys9 methylation (Fig. 3*a*, bottom panels). Thus, H3–Lys9 methylation on the X_i is catalyzed by one or more enzymes distinct from the two Suv39h HMTases.

In addition, we also observed a two-dotted α-4x-methH3-K9 signal in the short arm of the Y chromosome in metaphase spreads from bone-marrow cultures of wildtype and *Suv39h*-dn male mice (Fig. 3*b*, insert). Suv39h-independent H3–Lys9 methylation occurs in pericentric heterochromatin and in the XY body during male gametogenesis[10]. It is not known whether the Suv39h-independent methylation of the X_i and the Y chromosome are transduced by the same or different HMTase activities.

H3–Lys9 methylation generates a high-affinity binding site for the heterochromatin-associated HP1 proteins[4–6]. We therefore sought to determine whether HP1 proteins[22] localize to the X_i. Female primary mouse embryonic fibroblasts (PMEFs) were co-labeled with α-4x-methH3-K9 and α-HP1α or α-HP1β antibodies and probed with an X chromosome–specific DNA painting probe. In wildtype PMEFs, the α-4x-methH3-K9 antibodies detected the heterochromatic foci and also a diffusely staining DAPI-rich area that often was localized to the nuclear periphery (Fig. 4*a*). Both HP1α and HP1β overlapped with the heterochromatic foci but were not concentrated in this perinuclear area. In addition, although a significant fraction of HP1α remained associated with pericentric regions of metaphase chromosomes, HP1α was not seen associated with the X_i (Fig. 4*b*). HP1 proteins also were not associated with the X_i of *Suv39h*-dn PMEFs. Although a single focus was visualized with the α-4x-methH3-K9 antibodies, HP1α and HP1β were not focally localized but dispersed across the entire nucleus (Fig. 5). Similar results were seen for HP1γ (data not shown). These data indicate that HP1 localization is strongly dependent on Suv39h HMTase function.

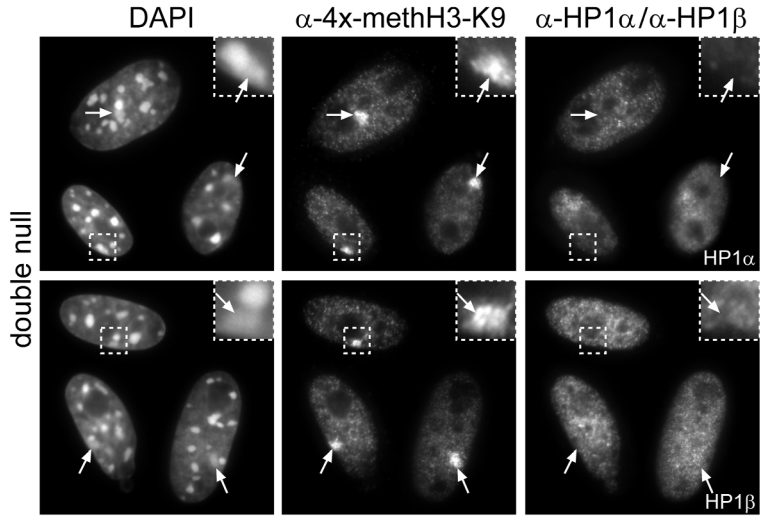

Fig. 5 HP1 fails to localize to Suv39h-independent H3–Lys9 methylation. Double-labeling immunofluorescence with α-4x-methH3-K9 and α-HP1α or α-HP1β antibodies of *Suv39h*-dn female PMEFs. DNA was counterstained with DAPI.

This work and the accompanying report by Boggs *et al.*[23] are the first to establish that H3–Lys9 methylation is an epigenetic imprint of facultative heterochromatin. Several key questions need to be addressed in future studies. For example, the developmental timing and the interdependence[24–27] of H3–Lys9 methylation and other epigenetic signals on the X_i, particularly expression of *Xist* RNA, should be determined. In addition, it will be crucial to identify the enzyme(s) that trigger H3–Lys9 methylation on the X_i and potentially in other regions of facultative heterochromatin. There are roughly 25 distinct gene sequences in the mouse genome that contain the evolutionarily conserved SET domain and

probably encode other HMTases (F. Eisenhaber, pers. comm.). Among those, G9a (ref. 28) and ESET (A. Kohlmaier and T.J, unpublished data) methylate histone H3 at lysine 9. It will be important to determine why HP1 proteins do not localize to the X_i. Both Suv39h and HP1 contain the chromo domain, although only the chromo domain of HP1 has been shown to recognize H3–Lys9 dimethylated N termini[4,5]. Notably, Suv39h enzymes and HP1 co-purify[29], suggesting that their direct or indirect interaction might facilitate their colocalization to pericentric heterochromatin. Alternatively, *Xist* RNA or DNA methylation[11] might induce a distinct higher-order chromatin structure or provide a secondary signal to induce recruitment of an X_i-specific HMTase complex, analogous to the RNA-mediated binding of a chromo domain–related protein with intrinsic histone acetyltransferase activity to the upregulated X chromosome in *Drosophila*[30].

Methods

Generation of α-4x-methH3-K9 antibodies. Hexameric peptides ($TARK(Me)_2ST$) containing a dimethylated lysine (Bachem) in the K9 position in the histone H3 N terminus were covalently linked at their carboxy termini by a lysine residue to generate a 'branched' peptide presenting four flexible 'fingers' of the above amino acid sequence. The structure of the branched antigen, generation of antisera, affinity purification and H3–Lys9 methyl specificity of the α-4x-methH3-K9 antibodies have been described[10]. The affinity-purified α-4x-methH3-K9 antibodies (concentration approximately 0.6 mg/ml) were used at a dilution of 1:1,000–1:5,000 for indirect immunofluorescence.

Cell lines and PMEFs. The T(X;4)37H translocation cell line[21] and primary mouse embryonic fibroblasts (PMEFs; at passage 4)[4,10] used in this study have been described. The presence of three inactive X chromosomes in the majority of cells from the 49,′XY human cell line (GM12013) has been confirmed by staining for macroH2A1.2. Human primary cells from fetal amniocenteses were provided by the St. Anna Hospital, Vienna.

Immunofluorescence and immuno-FISH. Analysis of interphase chromatin and of metaphase chromosomes by immunofluorescence and immuno-FISH was carried out as described[10,21].

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