

Histone H3 Lysine 9 Methylation Occurs Rapidly at the Onset of Random X Chromosome Inactivation

Jacqueline E. Mermoud,¹ Bilyana Popova,¹
Antoine H.F.M. Peters,² Thomas Jenuwein,²
and Neil Brockdorff^{1,3}

¹X Inactivation Group
MRC Clinical Sciences Centre
Imperial College School of Medicine
Hammersmith Hospital
London W12 0NN
United Kingdom

²Research Institute of Molecular Pathology
The Vienna Biocenter
Dr. Bohrgasse 7
A-1030 Vienna
Austria

Summary

In female mammals, a single X chromosome is stably and heritably silenced early in embryogenesis [1]. The inactive X is characterized by asynchronous DNA replication [2, 3] and epigenetic chromatin modifications, including DNA methylation [4], histone H3/H4 hypoacetylation [5–7], and incorporation of a variant histone macroH2A [8–10]. X inactivation is initiated by a *cis*-acting RNA molecule, the X-inactive specific transcript (*Xist*), which coats the chromosome [11–17]. However, the mechanism by which *Xist* induces chromosome silencing is poorly understood. An important approach towards answering this question has been to determine the temporal order of epigenetic chromatin modifications in an *in vitro* model system, differentiating XX embryonic stem (ES) cells, and thereby to identify candidate targets for *Xist* RNA [18–21]. To date, these studies have demonstrated that, following accumulation of *Xist* RNA [18, 20], the transition to late replication of the X chromosome is the earliest detectable event [19]. H4 hypoacetylation [19], macroH2A1.2 incorporation [21], and DNA methylation [19] all occur subsequently. Recently, it has been shown that chromatin of the inactive X is also characterized by methylation of histone H3 at lysine 9 (H3-K9) [22, 23]. Here we show that H3-K9 methylation is a very early event in the process of X inactivation, which closely parallels the onset of *Xist* RNA accumulation.

Results and Discussion

Chromatin structure plays a key role in setting up heritable states of gene activation and silencing. An important level at which this is achieved is through modifications of key residues at the N termini of the core histones. Recent attention has focused on histone methylation. H3 lysine 9 (H3-K9) methylation has been shown to be a feature of constitutive centromeric heterochromatin [24] and also to occur in silent chromatin domains at

the silent mating type locus in *S. pombe* [25] and in a developmentally regulated manner at the chicken β -globin locus [26]. Methylation of H3 lysine 4 (H3-K4) shows a reciprocal relationship, correlating with acetylated histones H3 and H4 in active chromatin domains [25, 26]. More recently, H3-K9 methylation has been found to be present on facultative heterochromatin of the inactive X chromosome [22, 23]. To better understand the role of this modification in X inactivation, we set out to determine the timing of H3-K9 methylation on the inactive X chromosome, using differentiating XX ES cells as a model system. In undifferentiated XX ES cells, both X chromosomes are active. X inactivation proceeds in a stepwise fashion as cells undergo differentiation in culture.

We used an antibody raised against a branched peptide containing four K9 dimethylated H3 amino termini [24] in indirect immunofluorescence studies. This allowed us to analyze temporal changes and to assess the extent of methylation over the whole chromosome. Metaphase spreads were prepared from undifferentiated PGK12.1 XX ES cells and at various times after induction of differentiation. Efc-1 XY ES cells were used as a control. In undifferentiated XX ES cells there was staining of pericentromeric regions of the chromosomes (Figure 1A), consistent with analyses in different cell types [24]. A similar pattern was seen in XY ES cells. Additionally, a single small chromosome was strongly stained in the majority of metaphase spreads (Figure 1B). DNA FISH analysis demonstrated that this was the Y chromosome. Methylation of the Y was also seen in differentiated Efc-1 cells (Figure 2A), indicating that it is not a developmentally regulated feature. The Y chromosome is largely heterochromatic, and H3-K9 methylation may simply reflect this. However, in mouse bone marrow cells [22] and also in an XY somatic cell line (data not shown), H3-K9 methylation occurs only on the Y chromosome short arm. The reason for this difference is unknown.

For analysis of methylation during X inactivation, we initially scored metaphases from cells differentiated for 0, 4, 8, and 12 days. These time points broadly encompass transitions for the other epigenetic markers of the inactive X observed in previous studies (see Figure 3 and associated text below for further detail). For each time point, data were compiled from a number of individual slides and in most cases from independent experiments carried out on different days. DNA FISH was used to verify that both X chromosomes were present. In undifferentiated XX ES cells, H3-K9 methylation of the X chromosome was undetectable in 99.5% of metaphase spreads, consistent with both X chromosomes being active. At day 4 of differentiation, 14% of spreads exhibited H3-K9 methylation of a single X chromosome. At later time points, day 8 and day 12, we observed levels of 44% and 68%, respectively. An example from day 8 differentiated cells is shown in Figure 1C. The scoring data are summarized in Figure 2B. These results demonstrate that there is a progressive methylation of H3-K9

³Correspondence: neil.brockdorff@csc.mrc.ac.uk

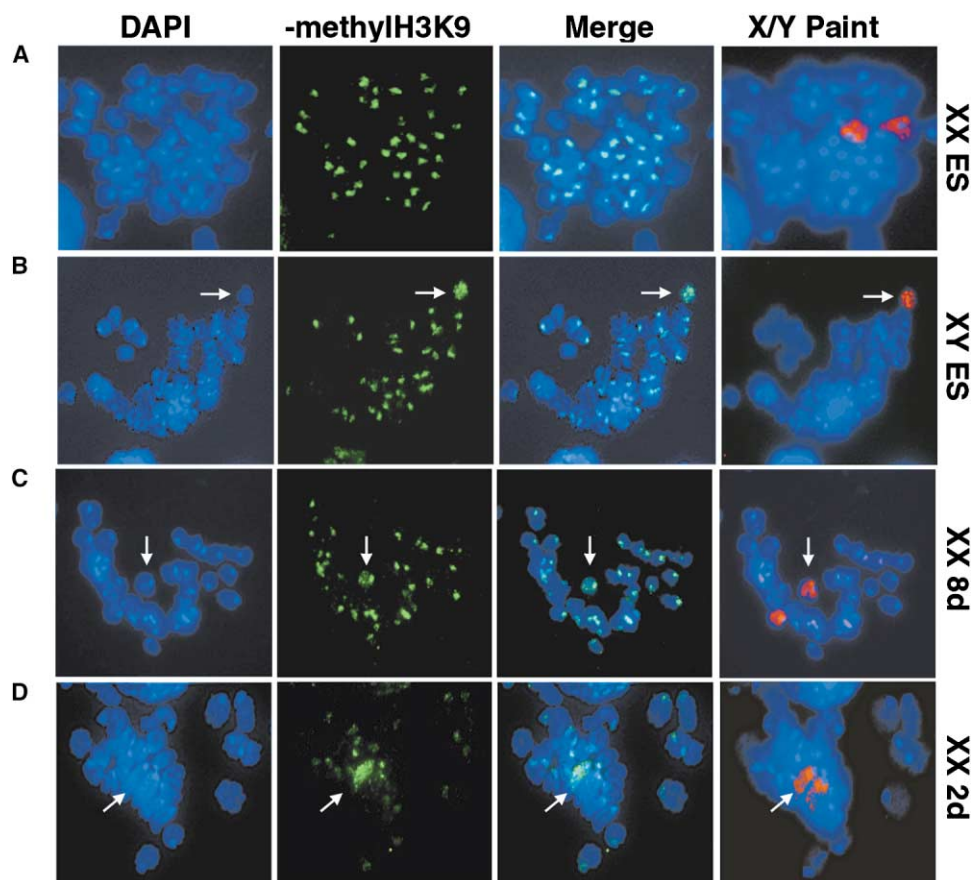


Figure 1. Indirect Immunofluorescence Detection of H3-K9 Methylation (FITC = Green) on Metaphase Spreads from Undifferentiated and Differentiated ES Cells

DNA was counterstained with DAPI, and the X or Y chromosome was identified by appropriate chromosome paint (Texas red; Cambio, Cambridge). (A) Example of a metaphase spread from undifferentiated PGK12.1 XX ES cells. H3-K9 methylation was seen in pericentromeric constitutive heterochromatin and at a low level, over the chromosome arms, but no overt staining of a single X chromosome was detected. (B) Example of a metaphase spread from undifferentiated Efc-1 XY ES cells. Pericentric heterochromatin is methylated and in addition there is extensive methyl H3-K9 staining of the Y chromosome (arrowed). (C) Example of a spread from 8 day (d) differentiated PGK12.1 XX ES cells showing methyl H3-K9 staining on one of the two X chromosomes (arrowed). (D) Example of a spread from 2 d differentiated PGK12.1 XX ES cells showing methyl H3-K9 staining on one of the two X chromosomes (arrowed). Methods: cells (PGK12.1, XX ES cells; Efc-1, XY ES cells) were as described [20]. Differentiated cultures were generated by the withdrawal of LIF [20]. Metaphase spreads were prepared by cytopinning, fixed in 2% formaldehyde for 10 min on ice, and processed as follows: after blocking in 3% BSA/0.1% TWEEN, slides were incubated for 2–3 hr at 37°C with primary antibody diluted 1/5000 (C) or 1/1500 (D), followed by detection with secondary antibody. Incubations were performed either in PBS/0.1% TWEEN (C) or in 4×SSC/0.1% TWEEN (D). Images were recorded and subsequently RNase treatment and DNA FISH with chromosome paints were performed.

on the inactive X chromosome in differentiating XX ES cells.

To determine exactly when H3-K9 methylation of the X chromosome begins, we analyzed time points between 1 and 4 days of differentiation. At day 1, methylation of the X chromosome was undetectable in 98.2% of metaphase spreads. This is not significantly different from the frequency in undifferentiated cells and therefore indicates that H3-K9 methylation has not begun at this stage. In marked contrast, by day 2 of differentiation there was a large increase, with 30% of spreads having H3-K9 methylation of the X chromosome (an example is illustrated in Figure 1D). Further increases to 48%, 46%, and 82%, respectively, were seen at days 3, 4, and 8. These data, summarized in Figure 2C, demonstrate that H3-K9 methylation is a very early event in the establishment of the inactive state.

In the latter experiment we used a modified immunofluorescence method with enhanced sensitivity (see legend to Figure 1). For this reason, the proportion of cells with H3-K9 methylation at days 4 and 8 was much higher than in our initial experiment (compare Figures 2B and 2C). In fact, the value at day 8 is similar to that seen in XX somatic cells (Figure 2C). We interpret this as signifying that within individual cells there is a progressive increase in total levels of X chromosome H3-K9 methylation during the differentiation time course. Thus, H3-K9 methylation could occur initially at a limited number of sites and then spread progressively to include the entire chromosome. This would be similar to the proposed spreading mechanism of H3-K9 methylation mediated by *Suv39h* and HP1 proteins in pericentromeric heterochromatin [27–29]. It should be noted that the progressive increase in the proportion of cells that

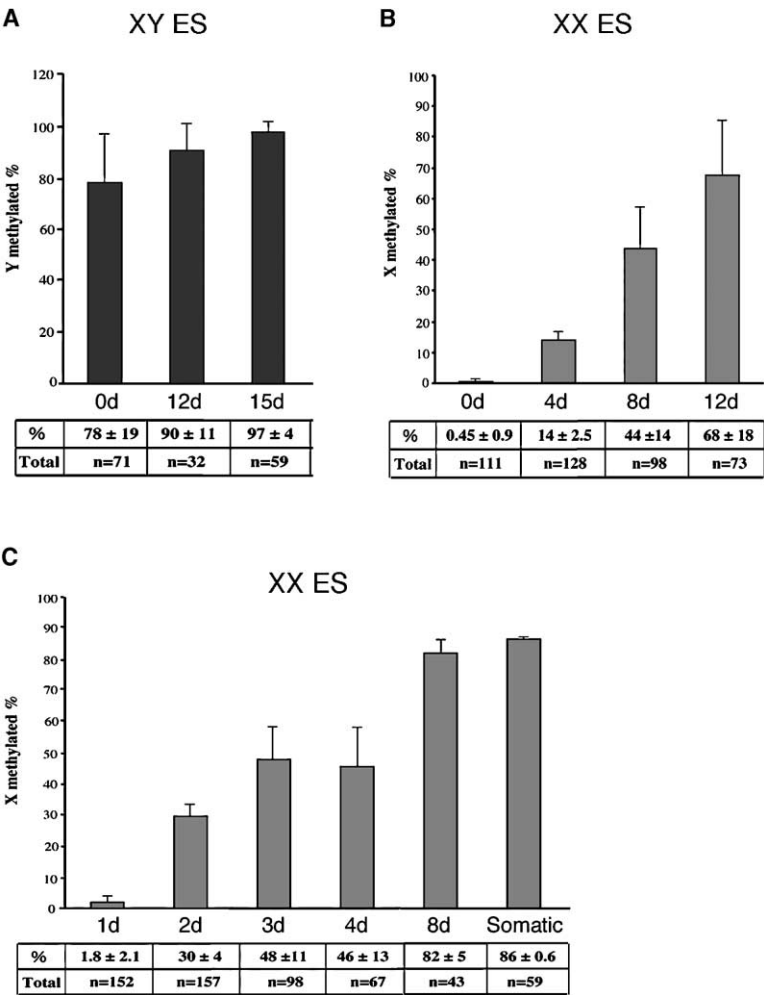


Figure 2. Quantitation of H3-K9 Methylation of the X and Y Chromosomes during ES Cell Differentiation

Summary of data indicating the percentage of X or Y methylated and the standard deviation as calculated from scoring individual slides and independent experiments. The table below indicates the total number (n) of metaphases scored for each time point. (A) Scoring for a fully methylated Y chromosome in Efc-1 cells differentiated for 0, 12, and 15 days. (B) Scoring for H3-K9 methylation of a single X chromosome in XX PGK12.1 ES cells differentiated for 0, 4, 8, and 12 days. Only metaphases with two X chromosomes were included in the analysis. (C) Scoring for H3-K9 methylation on one of the two X chromosomes in XX PGK12.1 cells differentiated for 1, 2, 3, 4, and 8 days using optimized conditions for immunofluorescence (see legend for Figure 1). Mouse XX fibroblast cell cultures (somatic) were analyzed to provide a positive control.

score positive could also, in part, be attributable to asynchronicity in the onset of H3-K9 methylation in the differentiating cell population. Indeed, the frequency of cells with accumulated *Xist* RNA during early stages of ES cell differentiation indicates that this is likely to be the case (see Figure 3 and associated text below).

Figure 3 summarizes our findings in the context of other characteristics of X chromosome inactivation. The initiating event, *Xist* RNA accumulation, begins at day 1.3 and reaches maximal levels by day 6 [20]. The transition to asynchronous replication of the inactive X chromosome begins at day 2 of differentiation and reaches maximal levels by day 7. Transcriptional silencing of X-linked genes was also reported to occur between days 2 and 4 [19]. In this study, we observe accumulation of H3-K9 methylation at day 2, synchronous with the latter two events, increasing progressively throughout the differentiation time course. Hypoacetylation of histone H4 begins later at day 4 of differentiation and reaches maximal levels after day 7 [19]. Similar kinetics have been observed for hypoacetylation of H3 lysine 14 (H. Spotswood and B.M. Turner p.communication). Incorporation of macroH2A1.2 begins much later at day 7 and reaches a maximal level by day 9 [21]. Thus, there is a sequential and possibly interdependent relationship between different chromatin modifications on the inactive X.

The frequency and kinetics of H3-K9 methylation of the inactive X closely mirrors the accumulation of *Xist* RNA observed in a previous study [20]. This is shown in Figure 3 (bottom). The close correlation is consistent with a scenario whereby the enzyme(s) responsible for methylating H3-K9 on the inactive X chromosome may be directly recruited by *Xist* RNA. Based on prevailing models that propose that H3-K9 methylation stabilizes the hypoacetylated state [27], it is likely that hypoacetylation, specifically at H3-K9, occurs prior to methylation. Thus, *Xist* RNA may target a complex comprised of H3-K9 deacetylase and methyltransferase activities. It should be noted that the *Suv39h* histone methyltransferase enzymes which catalyze H3-K9 methylation of centromeric heterochromatin, are not involved in X inactivation and that there are as yet unidentified enzymes that carry out H3-K9 methylation of facultative heterochromatin [22, 24, 30]. Discovery of the enzyme(s) responsible for this activity should allow the biochemical interactions between *Xist* RNA and chromatin silencing components to be elucidated.

Acknowledgments

We thank members of the X-Inactivation Group and Ros John for support and comments on the manuscript. This work was supported

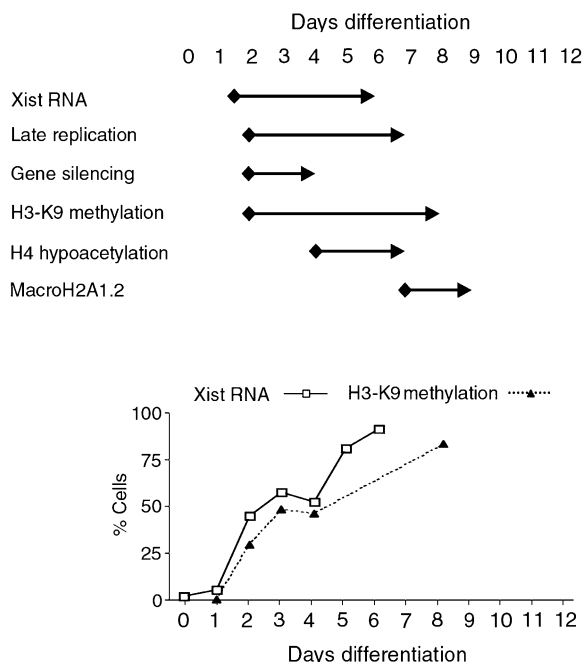


Figure 3. Model of the Onset and Progress of Different Characteristics of X Inactivation during Differentiation of PGK12.1 XX ES Cells (Top) Illustrated is a synopsis of previously published analyses and comparison of our data for H3-K9 methylation. Diamonds indicate the time of onset for the different characteristics, and the length of the arrow approximates the period during which maximal levels are attained. Data for *Xist* RNA accumulation are from Sheardown et al. [20]. Data for late replication, gene silencing, and H4 hypoacetylation are taken from a study by Keohane et al. [19]. Data for macroH2A1.2 are from Mermoud et al. [21]. DNA methylation on the X chromosome was detected only in cells differentiated for 21 days [19] and is therefore not included in this comparison. The data sets are directly comparable, as all studies used the PGK12.1 XX ES cell line and identical procedures for cell culture and differentiation. (Bottom) Comparison of the percentage of cells showing *Xist* RNA accumulation (open squares, solid line) and H3-K9 methylation (filled triangle, dotted line) at different times during differentiation.

by the Medical Research Council, UK (J.E.M. and N.B.) and by the IMP through Boehringer Ingelheim and the Austrian Research Promotion Fund and the Vienna Economy Promotion Fund to T.J.

Received: November 21, 2001

Revised: December 19, 2001

Accepted: December 19, 2001

Published: February 5, 2002

References

1. Lyon, M.F. (1961). Gene action in the X chromosome of the mouse (*Mus musculus* L.). *Nature* 190, 372–373.
2. Priest, J.H., Heady, J.E., and Priest, R.E. (1967). Delayed onset of replication of human X chromosomes. *J. Cell Biol.* 35, 483–487.
3. Takagi, N. (1974). Differentiation of the X chromosome in early female mouse embryos. *Exp. Cell Res.* 86, 127–135.
4. Mohandas, T., Sparkes, R.S., and Shapiro, L.J. (1981). Reactivation of an inactive X chromosome: Evidence for X-inactivation by DNA methylation. *Science* 211, 393–396.
5. Jeppesen, P., and Turner, B.M. (1993). The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene-expression. *Cell* 74, 281–289.
6. Belyaev, N.D., Keohane, A.M., and Turner, B.M. (1996). Differen-

7. Boggs, B.A., Connors, B., Sobel, R.E., Chinault, A.C., and Allis, C.D. (1996). Reduced levels of histone H3 acetylation on the inactive X chromosome in human females. *Chromosoma* 105, 303–309.
8. Costanzi, C., and Pehrson, J.R. (1998). Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. *Nature* 393, 599–601.
9. Chadwick, B.P., and Willard, H.F. (2001). Histone H2A variants and the inactive X chromosome: identification of a second macroH2A variant. *Hum. Mol. Genet.* 10, 1101–1113.
10. Costanzi, C., and Pehrson, J.R. (2001). MacroH2A2, a new member of the macroH2A core histone family. *J. Biol. Chem.* 276, 21776–21784.
11. Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tololrenzi, R., and Willard, F. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* 349, 38–44.
12. Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafreniere, R.G., Xing, Y., Lawrence, J., and Willard, H.F. (1992). The human *XIST* gene: Analysis of a 17kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* 71, 527–542.
13. Brockdorff, N., Ashworth, A., Kay, G.F., McCabe, V.M., Norris, D.P., Cooper, P.J., Swift, S., and Rastan, S. (1992). The product of the mouse *Xist* gene is a 15kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* 71, 515–526.
14. Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. *Nature* 379, 131–137.
15. Lee, J.T., Strauss, W.M., Dausman, J.A., and Jaenisch, R. (1996). A 450 kb transgene displays properties of the mammalian X inactivation center. *Cell* 86, 83–94.
16. Clemson, C.M., McNeil, J.A., Willard, H.F., and Lawrence, J.B. (1996). *XIST* RNA paints the inactive X chromosome at interphase: Evidence for a novel RNA involved in nuclear chromosome structure. *J. Cell Biol.* 132, 259–275.
17. Wutz, A., and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol. Cell* 5, 695–705.
18. Kay, G.F., Penny, G.D., Patel, D., Ashworth, A., Brockdorff, N., and Rastan, S. (1993). Expression of *Xist* during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell* 72, 171–182.
19. Keohane, A.M., O'Neill, L.P., Belyaev, N.D., Lavender, J.S., and Turner, B.M. (1996). X-Inactivation and histone H4 acetylation in embryonic stem cells. *Dev. Biol.* 180, 618–630.
20. Sheardown, S.A., Duthie, S.M., Johnston, C.M., Newall, A.E.T., Formstone, E.J., Arkell, R.M., Nesterova, T.B., Alghisi, G.-C., Rastan, S., and Brockdorff, N. (1997). Stabilisation of *Xist* RNA mediates initiation of X chromosome inactivation. *Cell* 91, 99–107.
21. Mermoud, J.E., Costanzi, C., Pehrson, J.R., and Brockdorff, N. (1999). Histone macroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X inactivation. *J. Cell Biol.* 147, 1399–1408.
22. Peters, A.H.F.M., Mermoud, J.E., O'Carroll, D., Pagani, M., Schweizer, D., Brockdorff, N., and Jenuwein, T. (2001). Histone H3 Lysine 9 methylation is an epigenetic imprint for facultative heterochromatin at the inactive X chromosome. *Nat. Genet.* 30, 77–80. Published online December 10, 2001. 10.1038/ng789.
23. Boggs, B.A., Cheung, P., Heard, E., Spector, D.L., Chinault, A.C., and Allis, C.D. (2001). Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes. *Nat. Genet.* 30, 73–76. Published online December 10, 2001. 10.1038/ng787.
24. Peters, A.H.F.M., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., et al. (2001). Loss of the *Suv39h* histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 107, 323–337.
25. Noma, K., Allis, C.D., and Grewal, S.I. (2001). Transitions in

- distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science* 293, 1150–1155.
26. Litt, M.D., Simpson, M., Gaszner, M., Allis, D., and Felsenfeld, G. (2001). Correlation between histone lysine methylation and developmental changes at the chicken β -globin locus. *Science* 293, 2453–2455.
 27. Jenuwein, T. (2001). Re-SET-ting heterochromatin by histone methyltransferases. *Trends Cell Biol.* 11, 266–273.
 28. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410, 116–120.
 29. Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C., and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromodomain. *Nature* 410, 120–124.
 30. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 406, 593–599.