A Chromosomal Memory Triggered by Xist Regulates Histone Methylation in X Inactivation

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We have elucidated the kinetics of histone methylation during X inactivation using an inducible Xist expression system in mouse embryonic stem (ES) cells. Previous reports showed that the ability of Xist to trigger silencing is restricted to an early window in ES cell differentiation. Here we show that this window is also important for establishing methylation patterns on the potential inactive X chromosome. By immunofluorescence and chromatin immunoprecipitation experiments we show that histone H3 lysine 27 trimethylation (H3K27m3) and H4 lysine 20 monomethylation (H4K20m1) are associated with Xist expression in undifferentiated ES cells and mark the initiation of X inactivation. Both marks depend on Xist RNA localisation but are independent of silencing. Induction of Xist expression after the initiation window leads to a markedly reduced ability to induce H3K27m3, whereas expression before the restrictive time point allows efficient H3K27m3 establishment. Our data show that Xist expression early in ES cell differentiation establishes a chromosomal memory, which is maintained in the absence of silencing. One consequence of this memory is the ability to introduce H3K27m3 efficiently after the restrictive time point on the chromosome that has expressed Xist early. Our results suggest that this silencing-independent chromosomal memory has important implications for the maintenance of X inactivation, where previously self-perpetuating heterochromatin structures were viewed as the principal form of memory.

Introduction

In mammals, dosage differences of X-linked genes between XX female and XY male cells are adjusted by transcriptional inactivation of one of the two female X chromosomes. X inactivation is a multistep process, in which the cell counts the number of X chromosomes, chooses one to be active, and silences all others. Initiation of silencing is triggered by accumulation of the 17-kb noncoding Xist RNA (Borsani et al. 1991; Brockdorff et al. 1991; Brown et al. 1991). Remarkably, Xist RNA attaches to chromatin and spreads from its site of transcription in cis over the entire inactive X chromosome (Xi), mediating transcriptional repression. Xist is essential for transcriptional silencing, but not for the maintenance of X inactivation (Penny et al. 1996; Marahrens et al. 1998; Csankovszki et al. 2001). Presently, the molecular nature of the silencing mechanism is not known. Previous studies have shown that X-chromosome inactivation involves the progressive recruitment of a variety of different factors and posttranslational modifications of lysine residues in the amino termini of histones (reviewed in Brockdorff 2002). The current view is that Xist expression initiates the formation of heterochromatin on the Xi, which can be perpetuated by redundant silencing mechanisms at later stages. Consistent with this view, it has been shown that the Xi in mouse embryonic fibroblasts is kept inactive in the absence of Xist by redundant mechanisms, including DNA methylation and histone H4 hypoacetylation (Csankovszki et al. 2001).

The Polycomb group proteins Ezh2 and Eed localise to the Xi in embryonic and extraembryonic tissues early in mouse development (Wang et al. 2001; Mak et al. 2002; Plath et al. 2003; Silva et al. 2003). The human EZH2/EED and its homologous E(z)/ESC complex in Drosophila melanogaster show intrinsic histone H3 lysine 9 (H3-K9) and lysine 27 (H3-K27) methyltransferase activity (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). Interestingly, H3-K27 methylation is one of the earliest chromosomal modifications on the Xi (Plath et al. 2003), and the requirement of Eed for histone methylation on the Xi has been demonstrated (Silva et al. 2003). However, analysis of Eed mutant embryos suggests that Eed is not required for initiation of silencing in trophoblast cells but is required for the maintenance of the Xi at later stages (Wang et al. 2001). Although data are consistent with the interpretation that Xist RNA recruits the Ezh2/Eed complex, thereby introducing histone H3 methylation, the significance of H3-K27 methylation for chromosomal inactivation is unclear. In flies, methylation on H3-K27 facilitates the binding of Polycomb to amino-terminal fragments of histone H3 (Cao et al. 2002; Min et al. 2003). Polycomb recruitment to the Xi has not been observed, and current models suggest that H3-K27 methylation in X-chromosome inactivation is indepen-
dent of classical Polycomb silencing (Mak et al. 2002; Silva et al. 2003).

We have previously shown that chromosomal silencing can be recapitulated in embryonic stem (ES) cells by expressing Xist RNA from cDNA transgenes integrated into autosomes and the X chromosome (Wutz and Jaenisch 2000), and this allowed for an uncoupling of Xist regulation from cellular differentiation. In this transgenic system, Xist expression is under the control of a tetracycline-responsive promoter, which can be induced by the addition of doxycycline to the culture medium. We showed that Xist RNA localisation and silencing can be separated by introducing specific mutations in Xist RNA (Wutz et al. 2002). Initiation of silencing depends on the repeat A sequence at the 5' end of Xist. Deletion of this element results in an RNA that localises to chromatin and spreads over the chromosome, but does not trigger transcriptional repression. Initial silencing in ES cells is reversible and dependent on Xist expression. At a later stage in differentiation this silent state becomes irreversible and independent of Xist, corresponding to the maintenance phase of X inactivation. We also showed that Xist expression must be induced early in ES cell differentiation to cause transcriptional repression (Wutz and Jaenisch 2000). Therefore, establishment of silencing is restricted to an initiation window in ES cell differentiation, and induction of Xist expression at a time point later than 24 h in differentiation no longer causes silencing. We found that Xist RNA loses its potential to initiate transcriptional repression roughly 24 h earlier in differentiation than the point at which silencing becomes irreversible. Notably, this left a gap of approximately one cell cycle in length between the initiation and maintenance phases. How silencing is maintained during this period and how the silent state becomes irreversible remained previously unexplained. In this report we perform kinetic measurements and quantification of histone H3 lysine 27 trimethylation (H3K27m3), revealing a novel chromosomal memory that is established by Xist expression at an early time point in ES cell differentiation independent of transcriptional repression. Our analysis suggests that this chromosomal memory might have an important role in the transition from the initiation phase to the maintenance phase of X inactivation.

Results

Profiling Histone Modification States at the Initiation of X Inactivation

We have previously reported that the initial steps of chromosomal silencing in mammalian X inactivation can be recapitulated in transgenic undifferentiated male ES cells (Wutz and Jaenisch 2000). Such ES cells are useful for studying the function of Xist RNA in the initiation of chromosomal silencing and for analysing the kinetics and relevance of chromosomal modifications. We aimed to delineate a pattern of histone methylation states that define the initial decision for facultative heterochromatin. To achieve this we performed immunofluorescence staining against the various modification states on histone H3 and H4 lysine residues in clone 36 ES cells, in which Xist expression can be induced from a transgene integrated on Chromosome 11 by addition of doxycycline to the culture medium (Wutz and Jaenisch 2000). We used highly specific antisera for a defined methylation state (mono-, di-, or tri-) at a particular lysine residue in the amino terminus of histone H3 and H4 (Peters et al. 2003; Perez-Burgos et al. 2004). Some cross reactivity of the H3K27m2 antiserum with H3K27m1 and H3K27m3, of the H3K4m3 antiserum with H3K4m2, and of the H4K20m2 antiserum with H4K20m1 and H4K20m3 was detected on peptide blots (Figure S1), but does not affect the conclusions drawn in this study. Our cytological experiments show a focal signal for H3K27m3 in the interphase nuclei of clone 36 ES cells upon Xist expression, which colocalises with Chromosome 11 in metaphase spreads and Xist RNA in interphase nuclei (Figure 1). In cells grown in the absence of doxycycline, a diffuse nuclear signal was observed. H3-K27 mono- and dimethylation were equally present on the inactivated chromosome and other autosomes (Table 1). Notably, we did not observe any specific enrichment for the H3K9m1, H3K9m2, or H3K9m3 signal on Chromosome 11 upon Xist induction (Figures 1C, 1G, and S2). H3K9m3 and H3K27m1 colocalised strictly with constitutive heterochromatin at pericentric regions and the Y chromosome (Figure 1G and 1H). H3K4m2 and H3K4m3 gave banded signals on chromosome arms that were reduced but not entirely erased on the transgenic chromosome, when Xist expression was induced (Figures 1E and S2). The heterochromatic Y chromosome completely lacked both H3K4m2 and H3K4m3 in the same metaphase spread. Thus, we conclude that the reduction of H3K4m2 and H3K4m3 on the Xist-expressing chromosome is consistent with a state of transcriptional repression (Santos-Rosa et al. 2002) and with earlier reports that implicate H3-K4 hypomethylation early in X inactivation (Heard et al. 2001; O’Neill et al. 2003). H3K4m1 was equally present on the Xist-expressing chromosome and other autosomes. Using antisera specific for methylation states of H4K20, we observed that H4K20m1 decorated Chromosome 11 upon Xist induction in undifferentiated clone 36 ES cells (in 46% of interphase nuclei; Figure 1B). H4K20m2 and H4K20m3 were not enriched on the Xist-expressing chromosome (Figure S2H and S2I; G. Schotta and M. Lachner, unpublished data). We also investigated the acetylation state of histone H4 in these cells using a sheep polyclonal antisera that preferentially recognises multiply acetylated H4 (Morrison and Jeppesen 2002). Using this antisera, we detected partial hypoacetylation of Chromosome 11 in metaphase spreads of clone 36 ES cells that were induced to express Xist (Figures 1F, 2K, and S2L). This observation is different from the global chromosome-wide hypoacetylation of H4 that was reported on the Xi later in differentiation (Keohane et al. 1996) and might reflect the absence of active promoters. We also detected a degree of hypoacetylation when a silencing-defective Xist RNA was expressed (Figure S3), making it likely to be the consequence of cross talk with H4-K20 methylation, which is mutually exclusive at least with H4-K16 acetylation (Nishioka et al. 2002). In conclusion, H3K27m3, H4K20m1, reduction of H3K4m2 and H3K4m3, and reduced multiple-lysine acetylation of histone H4 correlate with the inactive state of the chromosome in undifferentiated ES cells (Table 1).

Further confirmation of the cytological findings comes from chromatin immunoprecipitation (ChIP) experiments using antibodies specific for H3K27m3, H4K20m1, H3K4m3, H3K4m2, and H3K9m2 in both undifferentiated and differentiated clone 36 ES cells in the presence or absence of
**Figure 1. Epigenetic Imprints at the Initiation of X Inactivation**

(A–H) Indirect immunofluorescence and subsequent DNA FISH analysis on mitotic chromosomes prepared from undifferentiated clone 36 ES cells after 3 d of Xist induction. H3K27m3 (A), H4K20m1 (B), and Ezh2 (D) are enriched on the arms of Chromosome 11 upon ectopic Xist expression. H3K9m2 (C) is not enhanced upon Xist expression. H3K4m2 (E) is reduced on Chromosome 11 upon Xist induction (green box) and absent from pericentric heterochromatin and the Y chromosome (orange arrow). (F) Histone H4 multiple-lysine acetylation is partially reduced (green box, left panel). Hypoacetylation (red) is restricted to chromosomal regions which show high levels of H3-K27 trimethylation (green, right panel). H3K9m3 (G) and H5K27m1 (H) are enriched at constitutive heterochromatin of pericentric regions and the Y (orange arrows).

(I–K) Indirect immunofluorescence (upper panels) and subsequent Xist RNA FISH (red, Xist RNA; blue, DAPI) analysis of H3K27m3 (I), H4K20m1 (J), and Ezh2 (K) in interphase nuclei of undifferentiated clone 36 ES cells expressing Xist for 3 d.

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**Table 1. Histone Lysine Methylation States as Epigenetic Imprints during X Inactivation**

|       | H3-K4 |       | H3-K9 |       | H3-K27 |       | H4-K20 |       |
|-------|-------|-------|-------|-------|--------|-------|--------|-------|
|       | m1    | m2    | m3    | m1    | m2    | m3    | m1    | m2    |
| 0     | −     | −     | 0     | 0     | 0^b    | 0     | +      | +     |
| 0^a   | 0     | 0     | 0     | 0     | 0     | 0     | 0^b    | 0     |

^+^, chromosome-wide mitotically stable methylation marks recruited by Xist RNA; −, decreased levels due to initiation of X inactivation; 0, abundance and distribution independent of Xist (equal on all chromosomes); 0^a^, small regional increase during differentiation revealed by ChIP (see text); 0^b^, identified as epigenetic imprints of constitutive heterochromatin.

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doxycycline (Figure 2). We observed enhanced H3K27m3 and H4K20m1 in the cells expressing Xist regardless of the differentiation state on three microsatellite sequences on Chromosome 11 (Figure 2). A control microsatellite on Chromosome 15 did not show this effect (Figure 2F and 2K). Upon Xist expression, we also observed H3K27m3 on the puromycin marker gene cointegrated with the Xist transgene on Chromosome 11, compared to nearly undetectable levels in the uninduced sample (Figure 2B). This increase in H3K27m3 was paralleled by a marked decrease in H3K4m2 and H3K4m3, but no increase in H4K20m1 could be observed at this locus in undifferentiated ES cells. Upon differentiation, an increase in the H4K20m1 signal was observed when Xist was expressed on all sequences on Chromosome 11. A control tubulin gene located on Chromosome 15 showed no significant change upon Xist induction (Figure 2E and 2K). These data show that H3K27m3 and H4K20m1 are elevated by Xist RNA expression on the transgenic chromosome, in agreement with our cytological analysis. However, regional differences are revealed by the higher resolution of the ChIP experiment, showing that the two modifications do not display a completely overlapping distribution on the chromosome. Differentiation of the ES cells resulted in increased H4K20m1 signals dependent on Xist expression. H3K9m2 was also elevated on two loci on Chromosome 11.

H3K27m3 and H4K20m1 Are Triggered by Xist RNA Localisation and Are Independent of Silencing

In agreement with earlier studies (Plath et al. 2003; Silva et al. 2003), our results indicate that chromosome-wide histone H3K27m3 is efficiently triggered in undifferentiated ES cells and therefore is an early mark of X inactivation. We measured the kinetics of H3K27m3 following induction of Xist RNA expression in undifferentiated clone 36 ES cells (Figure S3A). At 6, 12, and 24 h after induction 0%, 12%, and 37% of the cells, respectively, showed a signal, and by 48 h a maximum of 70% was reached. Furthermore, the recruitment of Ezh2 protein to the transgenic Chromosome 11 upon Xist expression (see Figure 1D and 1K) is consistent with the idea that the Ezh2/Eed complex contains the enzymatic activity causing H3K27m3 in X inactivation (Mak et al. 2002; Plath et al. 2003; Silva et al. 2003).

To identify the Xist sequences that are required for the binding of the Ezh2/Eed complex and to trigger H3K27m3, we examined a panel of Xist RNA mutations (Figure 3A). In an earlier study we inserted Xist cDNA transgenes containing defined deletions into the Hprt gene locus on the single X chromosome in male mouse T20 ES cells and measured their ability to cause silencing (Wutz et al. 2002). We used deletions spanning the entire RNA that eliminate relatively large parts of Xist to analyse H3K27m3 by immunofluorescence in ES cells after induction of transgenic Xist expression (Figure 3). H3K27m3 staining was observed for all Xist mutations tested, with the exception of the ΔXSa deletion, where sequences required for localisation are deleted. The resulting XistΔXSa RNA did not localise well to chromatin and showed consequently greatly diminished potential to silence (Figure S4). We interpret the absence of detectable H3K27m3 in this case as a consequence of the failure of the RNA to localise. All other mutants analysed, including that containing a ΔXN deletion spanning a similar region, gave rise to RNA that localised well to chromatin and caused H3K27m3. A mutant with a deletion of repeat A (T20:ΔSX ES cells; Figure 3), which localises to chromatin but does not cause silencing, was able to induce H3K27m3, suggesting that methylation can be established independent of silencing, a finding consistent with the results of an earlier study (Plath et al. 2003). The expression of the silencing-deficient Xist RNA led to a significantly lower percentage of cells with H3K27m3 foci in

Figure 2. ChIP Mapping of H3K27m3, H4K20m1, H3K9m2, H3K4m3, and H3K4m2 on the Xist-Expressing Chromosome 11 during Differentiation of Clone 36 ES Cells

A genetic map of Chromosome 11 indicating the loci analysed is given on the left (Xist-TG, approximate integration site of Xist transgene; puro, PGKpuromycin marker). (A to F) Chromatin was prepared from undifferentiated clone 36 ES cells grown for 3 d in the presence (light bars) or absence (dark bars) of doxycycline. H3K27m3 and H4K20m1 were enriched at three intergenic microsatellite sequences at 18.0 (A), 45.5 (C), and 75.2 (D) cM. (B) H3K27m3 was established over the coding sequence of Xist given on the left (Xist-TG, approximate integration site of Xist transgene; puro, PGKpuromycin marker).

### References

- Plath et al. 2003
- Silva et al. 2003
- Mak et al. 2002
- Wutz et al. 2002
- Plath et al. 2003
interphase nuclei (3- to 4-fold reduction compared to wild-type Xist RNA; Figure 3D). Moreover, on metaphase chromosomes methylation appeared mostly as a single band (only 5% showed a wild-type pattern; Figure 3C). Since the transgene is integrated in the Hprt locus on the X chromosome and the endogenous Xist gene is still present in this cell line, the possibility exists that the transgenic RNA might have stabilised the endogenous Xist RNA or vice versa to effect H3K27m3. To address this point we made use of another cell line in which repeat A was deleted from the endogenous Xist gene and an inducible promoter was inserted by homologous recombination (J1:XistΔSX-tetOP; Wutz et al. 2002). Induction of Xist RNA expression caused H3K27m3 on the single X chromosome in these cells, confirming that H3K27m3 can be established by Xist expression in complete absence of repeat A sequences. However, in undifferentiated ES cells, expression of the silencing-deficient Xist RNA led consistently to lower numbers of cells (30%–35%) showing H3K27m3 staining compared to the wild-type Xist RNA (80%; Figure 3B and 3C). Mono- and dimethylation of H3-K27 were not visibly elevated in J1:XistΔSX-tetOP cells at the expense of the H3K27m3 signal (data not shown), suggesting that recruitment of the Ezh2/Eed complex was impaired in the absence of repeat A, and ruling out the possibility that repeat A would change the specificity of the complex to induce trimethylation activity. Consistent with this interpretation, Ezh2 was observed in only 9% of the J1:XistΔSX-tetOP ES cells compared to 76% of the clone 36 ES cells (see Figures 1K and 3D). We note that the lower methylation potential of Xist RNA lacking repeat A sequences was only observed in undifferentiated ES cells. When the cells were differentiated, methylation levels were elevated (see Figure S3C). We further determined the role of H4K20m1 in silencing. We detected H4K20m1 upon induction of Xist expression in 14% of the interphase nuclei in undifferentiated J1:XistΔSX-tetOP ES cells, showing that H4K20m1 can be established in the absence of repeat A (see Figure S3D). We conclude that H3K27m3 and H4K20m1 are independent of and not sufficient for silencing.

Efficient H3K27m3 Is Restricted to Early Stages of Differentiation

Xist-mediated transcriptional silencing is restricted in ES cell differentiation in that the potential of Xist to initiate repression diminishes 48 h after differentiation (Wutz and Jaenisch 2000). We investigated whether the ability to

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**Figure 3. Sequences of Xist RNA Required for H3K27m3 Establishment**

(A) Schematic representation of the Xist cDNA (top) indicating repeats A to E, restriction sites, and the locations of deletions (coloured bars) relative to the location of sequences required for localisation (black and hatched boxes; Wutz et al. 2002).

(B) Analysis of H3K27m3 on metaphase chromosome spreads from undifferentiated ES cells after 3 d of Xist induction (see text). The staining patterns (n > 100) were scored as chromosome-wide dense methylation (black), reduced methylation (grey), and a single band (open).

(C) Pattern of H3K27m3 triggered by different Xist mutants on metaphase chromosomes after 3 d of induction. Enlarged view of Chromosome 11 (clone 36) or the X chromosome (T20 lines, J1 knock-in line).

(D) Focal H3K27m3 staining in interphase nuclei (percentage given; n > 100) of undifferentiated ES cells expressing Xist constructs.

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It is established that H3K27m3 would be restricted to this initiation window in clone 36 ES cells. These cells carry a puromycin resistance gene (puro), which is cointegrated with the Xist cDNA transgene on Chromosome 11 and can be silenced by transgenic Xist expression. Xist expression was induced either from the beginning or at 24, 48, 72, 96, or 120 h after the onset of differentiation. The ability of Xist to initiate silencing at various time points was monitored by measuring puro expression, and H3K27m3 was analysed in parallel in all cultures at 12 d after differentiation (Figure 4A). When Xist was induced within 24 h of differentiation, H3K27m3 was observed in a large fraction of the cells. Induction of Xist after 24 h led to significantly lower methylation levels (10%–15% of cells; Figure 4A). The efficiency in H3K27m3 pattern establishment correlated at all time points with the potential of Xist to initiate silencing and Ezh2 recruitment (Figure 4B). Hence, an efficient H3K27m3 pattern establishment correlated at all time points with the potential of Xist to repress and silence the H3K27m3 enzyme at day 3 of differentiation and Ezh2 levels diminish more gradually towards even later time points. This demonstrates that the ability of Xist to induce efficient H3K27m3 is restricted at a time when both Eed and Ezh2 proteins are detected in similar amounts, as in undifferentiated ES cells, suggesting that the efficiency of methylation is not a function of the protein levels.

To confirm this finding, we assayed the effect of induction of Xist expression on H3K27m3 in embryonic fibroblasts. Fibroblasts were isolated from male, day 13.5 embryos carrying an insertion of the doxycycline-inducible promoter in the endogenous Xist locus (Xist-tetOP allele) and a homozygous insertion of the tetracycline-responsive transactivator in the ROSA26 locus (ROSA26-nlsrtTA allele; Wutz et al. 2002; F. Savarese, unpublished data). In these fibroblasts, expression of the endogenous Xist RNA from the single male X chromosome could be induced in 80% of the cells by addition of doxycycline (data not shown). In uninduced cultures and control male fibroblasts no H3K27m3 foci were detected by immunofluorescence in interphase nuclei. However, upon Xist induction 5% (after 48 h of Xist induction) or 15% (after 72 h) of the cells showed focal H3K27m3 staining (4K20m1 was established, as well; see Figure S3G). In control
female fibroblasts H3K27m3 staining was detected in 85% of the cells. This shows that Xist induction in embryonic fibroblasts leads to H3-K27 methylation in a low percentage of cells. We further examined histological sections of male embryos carrying the inducible Xist-tetOP allele and the ROSA26-nlsrtTA allele. Xist expression was induced by feeding doxycycline in drinking water to the mothers for 3 d starting either from day 9.5 or day 13.5 of gestation. Embryos were dissected 3 d later, on day 12.5 and 16.5, respectively. In the sections, 74% (day 12.5 embryos) and 52% (day 16.5 embryos) of the cells expressed Xist, as determined by RNA fluorescent in situ hybridization (FISH) analysis (Figure 4D and 4E). Focal H3K27m3 staining was detected in 61% of the cells in sections of the day 12.5 embryos but in only 18% of the day 16.5 embryos (Figure 4D and 4E), demonstrating a clear reduction in the number of cells showing H3K27m3 staining in response to Xist expression in the later-stage embryos. In summary, our data demonstrate that Xist has been able to effect H3K27m3 in all cell types tested. However, the efficiency of methylation is regulated in cellular differentiation and development. Our experiments show that Xist is not sufficient for efficient establishment of the H3K27m3 pattern in differentiated cells.

Reversibility of H3K27m3

Once efficient H3K27m3 is established by Xist expression in early ES cell differentiation, it can be maintained throughout differentiation. This would be consistent with the view that lysine methylation is a permanent epigenetic mark. To test whether H3K27m3 is stably maintained in the absence of continuous Xist expression, we tested H3K27m3 reversibility in undifferentiated clone 36 ES cells. Xist expression was induced from the transgenic Chromosome 11 in these cells for 3 d, and then the cells were washed and split into medium without doxycycline to shut off Xist expression. H3K27m3 levels and Xist RNA were determined by combined immunofluorescence RNA FISH at consecutive time points at 6, 12, 24, and 48 h. High levels of H3K27m3 persisted until 24 h after Xist was turned off, but H3K27m3 disappeared by 48 h (Figures 5A and S3B). Our data show that the Xist RNA signal disappeared by 12 h after the withdrawal of doxycycline, demonstrating that H3-K27 methylation is reversible in undifferentiated ES cells and is removed after a period of approximately two cell divisions following the turning off of Xist expression. We also analysed the reversibility of H4K20m1 and Ezh2 in undifferentiated clone 36 ES cells. The percentage of cells showing a signal went from 46% and 70% initially to 5% and 11% at 48 h after withdrawal of doxycycline for H4K20m1 and Ezh2, respectively.

To test whether H3K27m3 would become irreversible during ES cell differentiation, we turned off Xist expression in clone 36 ES cells at progressively later time points up to 6 d after initiation of differentiation. The H3K27m3 pattern was analysed in all cultures at day 12 of differentiation. In cells continuously expressing Xist during differentiation, methylation was detected in 60% of the cells at day 12. If Xist expression was turned off at any time points in the course of differentiation.
methyltransferase pattern during the initiation window early in ES cell differentiation. These observations could indicate that silencing enhances histone methylation in ES cell differentiation. To address this interpretation, we analysed the H3K27me3 pattern caused by expression of a mutant Xist RNA lacking repeat A, which cannot initiate silencing, in differentiating J1:XistΔSX-tetOP ES cells. When these cells were differentiated in the presence of doxycycline, focal H3K27me3 staining was observed in 78% of the cells at day 12 (Figure 6). This clearly indicated that methylation was maintained in a high number of these cells. Silencing is therefore dispensable for methylation in ES cell differentiation. Notably, we observed H3K27me3 staining in a high percentage of differentiated ES cells but in a significantly reduced percentage of undifferentiated ES cells expressing a silencing-defective Xist RNA (see Figure 3B, 3C, and S3C). Silencing or repeat A sequences are therefore required to sustain high H3K27me3 levels specifically in undifferentiated ES cells but are dispensable upon differentiation.

To test whether continuous Xist expression was required for maintenance of efficient H3K27me3, we induced Xist expression from the transgenic Chromosome 11 in undifferentiated clone 36 cells and from the X chromosome in J1:XistΔSX-tetOP ES cells for 3 d. The cells were then differentiated for 5 d in the presence of doxycycline followed by 5 and 7 d, respectively, without the inducer. At the end of this period H3K27me3 was analysed and could be detected in less than 20% and 10% of the cells, respectively (Figure 6). Parallel cultures were differentiated for 5 d in the presence of doxycycline followed by 4 d in the absence of doxycycline, and then doxycycline was added back for 1 or 3 more days. In these cells, in which Xist had been induced early, H3K27me3 was restored and detectable in 50%–65% of all cells. This level is significantly higher than the level in control cultures that had been induced de novo at day 6 or day 9 of differentiation (10% of all cells). In cells that had been continuously differentiated in the presence of doxycycline, methylation was detected in 75%–78% of the nuclei. Our data show that efficient methylation at late time points in differentiation did not require continuous Xist expression. Efficient remethylation occurred on a chromosome that had been exposed to Xist in early ES cell differentiation, consistent with the idea that Xist triggers a chromosomal change in early differentiation that is remembered until later time points to enhance H3K27me3 reestablishment. Importantly, the silencing-deficient Xist mutant RNA in J1:XistΔSX-tetOP ES cells gave identical results, showing that this memory is established independent of silencing.

We further determined at which time point in differentiation the chromosomal memory is established. For this, clone 36 ES cells were differentiated for 0, 12, 24, 36, 48, 60, or 72 h in the presence of doxycycline. Then Xist was turned off until day 8 of differentiation, when doxycycline was added back, and remethylation was assayed by immunofluorescence at day 13 in differentiation (Figure 7). In this experiment a transition occurred in a 24-h interval around 60 h if Xist was expressed for more than 48 h early in differentiation, allowing for efficient remethylation, a result consistent with the establishment of the memory in this interval. When Xist was turned off earlier than 60 h, remethylation was observed in only 10%–30% of the cells, demonstrating that the memory was not established. Turning off Xist at 72 h or later differentiation, the percentage of cells showing H3K27me3 was reduced to less than 10%, suggesting that methylation was reversible throughout differentiation and not stabilised (data not shown). We then analysed the kinetics of loss of methylation in differentiated ES cells. Clone 36 ES cells differentiated for 4 d in the presence of doxycycline were differentiated for 24, 48, and 72 more hours in the absence of doxycycline, and H3K27me3 was measured (Figure 5C). Focal H3K27me3 staining was initially observed in 97% of interphase nuclei and was reduced to 50% and 25% at 3 and 4 d, respectively, after Xist had been turned off. This shows that the decay of focal H3K27me3 was slower than in undifferentiated ES cells, possibly reflecting the slower cell cycle of differentiating cells.

**Early Xist Expression Triggers a Chromosomal Memory Independent of Silencing**

Detection of focal H3K27me3 staining persisted throughout ES cell differentiation when Xist was continuously expressed. Yet the methylation mark was reversible throughout ES cell differentiation, and Xist RNA could only establish an efficient memory was not established. Turning off Xist at 72 h or later

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**Figure 6. Early Xist Expression Imparts a Chromosomal Memory Independent of Silencing**

Transgenic Xist expression was induced from Chromosome 11 in clone 36 ES cells (black bars) or a silencing-deficient Xist RNA from the X in J1:XistΔSX-tetOP ES cells (open bars) at time points during differentiation (see text). The percentage of cells showing H3K27me3 staining is plotted (n > 250). Below, a scheme of Xist induction is given for all cultures, with arrows representing time of analysis.

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allowed remethylation in 85% of the cells. We also analysed the transition from Xist-dependent reversible to irreversible silencing in this experiment by Northern analysis of puro expression from the transgenic chromosome in differentiated clone 36 ES cells (Figure 7B). These data show that irreversible silencing was established in an interval between 48 and 72 h in ES cell differentiation, with puro expression levels dropping from 60% to 15% of the level in uninduced samples (Figure 7C), in agreement with our initial report (Wutz and Jaenisch 2000). The 24-h intervals for the transition can be explained by the asynchronous cell cycle states in the ES cell culture (doubling time, 21.4 h) at the time when differentiation was induced. We conclude that the establishment of the chromosomal memory is silencing independent and occurs at the time when X inactivation becomes irreversible and Xist independent.

Discussion

Our results identify H3K27m3 and H4K20m1 as specific modifications that mark the Xist-expressing chromosome in undifferentiated ES cells and contribute to the epigenetic histone code of the Xi (Table 1). We did not observe an enrichment of H3K9m2 or H3K9m3 signals on the Xist-expressing chromosome, which has been reported by other studies. This could be a shortcoming of our transgenic system, but we also did not detect the H3K9m2 or H3K9m3 signals in female mouse primary embryonic fibroblasts (less than 2% of the cells). We attribute the different observations in other studies to the various antisera used. We supply peptide blot analysis for our antisera that suggest that the antibodies are highly specific (see Figure S1). This is also supported by the specific staining patterns in immunofluorescence experiments. The lysine 9 methylation signal observed in other studies could potentially be a result of cross reactivity with H3K27m3, a fact we can exclude for our H3K9 antibodies based on the staining pattern and peptide blots. Alternatively, our antibody might not recognise the H3K9m2 modification in the context of the chromosome. However, this is unlikely since the H3K9m3 signals for the pericentric regions and the Y chromosome are clearly identified. The H3K9m2 antisera has been successfully used in ChIP analysis of the minor centromeric repeats (Yan et al. 2003) and reacts with these repeats in immunofluorescence, but does not show cross reactivity to H3K27m3. This suggests that our reagent is able to detect the modification in both ChIP and immunofluorescence experiments. Using highly specific antisera, we failed to see a strong signal for H3K9m2 in either ChIP or immunofluorescence experiments (see Figures 2 and S2E). In our ChIP analysis two chromosomal loci showed an enrichment of H3K9m2 upon Xist expression in differentiated ES cells, suggesting some enrichment for H3K9m2. We take these data to indicate that H3K9m2 is not a prominent mark of X inactivation but might be enriched locally to some degree upon differentiation.

Using Xist alleles that express a mutated version of Xist, which has a deletion of repeat A sequences and is unable to cause silencing, we showed that both H3K27m3 and H4K20m1 were established in the absence of transcriptional repression. This demonstrates that neither modification is sufficient to trigger silencing.

Xist expression led to rapid H3K27m3, which was complete
after 1 to 2 d of \textit{Xist} expression in both ES cells and differentiated cells (see Figure S3A and Figure 6, columns 1 and 2). This kinetics follows the localisation of \textit{Xist} RNA, which accumulates between 4 and 12 h after doxycycline addition in ES cells (Wutz and Jaenisch 2000), suggesting that H3K27m3 is an immediate effect. We have further shown that in undifferentiated ES cells no progressive accumulation of the histone modifications occurs over time by comparing the percentage of cells showing H3K27m3, H4K20m1, and Ezh2 staining after 3 and 10 d expressing either full-length \textit{Xist} RNA or a silencing-deficient mutant lacking repeat A (see Figure S3C). We have shown that H3K27m3 is a reversible modification throughout ES cell differentiation and depends on chromosome and not a function of the protein levels of Eed (Jaenisch 2000). The transient maintenance of H3K27m3 might be significant for the mechanism of \textit{X} inactivation. It could explain our observation that the inactive state will be locked in at 72 h of ES cell differentiation (Wutz and Jaenisch 2000).

Efficient methylation is established only when \textit{Xist} expression is induced early in ES cell differentiation. The window in which \textit{Xist} causes efficient methylation overlaps precisely with the initiation window, in which transcriptional silencing can be initiated. Yet methylation is independent of initiation of silencing. This would be consistent with the notion that H3K27m3 is necessary but not sufficient for silencing. However, this is unlikely, as a previous report has shown that in Eed mutant embryos, initiation of silencing is normal, but a defect in the maintenance of the inactive state leads to reactivation at later stages (Wang et al. 2001). Lower levels of Ezh2 and Eed could explain the restriction on the ability of \textit{Xist} to induce H3K27m3 efficiently in differentiated ES cells (Silva et al. 2003). We do not favour this interpretation, as this restriction is observed at day 2 in differentiation, when Ezh2 and Eed protein levels are still high (see Figure 4C). Our data further show that the ability to efficiently methylate a chromosome late in ES cell differentiation is a feature of the chromosome and not a function of the protein levels of Eed and Ezh2. This is also in line with our observation that chromosome-wide H3K27m3 in clone 36 ES cells, in which Eed messenger RNA was reduced to 10\%-15\% of wild-type levels by stable RNAi, was still detected in 45\%-60\% of cells compared to 80\% in control clone 36 cells (data not shown). Therefore, less abundant levels of Eed are sufficient to achieve efficient methylation. \textit{Xist} induction later in ES cell differentiation or in cells of embryonic origin establishes H3K27m3 in only a small percentage of cells. The significance of H3K27m3 in this small number of cells is unclear at present.

The restriction of efficient methylation to early ES cell differentiation and the finding that methylation is reversible logically require that a chromosomal memory exists that enables H3K27m3 maintenance during differentiation. Previous models have suggested that a lock-in of X inactivation is based on chromosomal silencing, arguing that self-maintaining heterochromatin structures establish the principal form of memory. Our data clearly demonstrate that H3K27m3 is maintained in the absence of transcriptional repression, suggesting a chromosomal memory independent of silencing on the Xi. Using the inducible \textit{Xist} expression system we have directly demonstrated the chromosomal memory (see Figure 6). A chromosome that had been exposed to \textit{Xist} and been H3-K27 trimethylated early could be remethylated later in differentiation, after a period where \textit{Xist} was turned off and methylation decayed, with significantly greater efficiency than a chromosome that had not expressed \textit{Xist} early (see Figure 6). We have further determined the time point in ES cell differentiation when the chromosomal memory is established and found that it overlaps with the transition from \textit{Xist}-dependent and reversible silencing to irreversible silencing. These data place the establishment of the memory in a critical phase of X inactivation. We note that the establishment of efficient H3K27m3 in the initiation window and the implementation of the memory are separated by a gap of approximately one cell division in ES cell differentiation. This parallels the gap between initiation of silencing and the maintenance of the silenced state independent of \textit{Xist}. Our kinetic measurements indicate that H3K27m3 would decay from the \textit{Xist}-expressing chromosome after two cell divisions; therefore, H3K27m3 could bridge the gap (critical window). We suggest that \textit{Xist} expression and H3K27m3 might be the signal to recruit a chromosomal memory mediating the lock-in of X inactivation (Figure 8). In this model, silencing would be specified by separate signals depending on repeat A of \textit{Xist}, which we predict would interact with the memory at the transition from reversible to irreversible and \textit{Xist}-independent repression. In this regard we note that silencing or repeat A sequences enhance the efficiency of H3K27m3 in undifferentiated ES cells (see Figure 3B). However, there is no requirement for repeat A when ES cells are induced to differentiate (see Figures 6 and S3C). This could point to interactions between the silencing machinery and the Ezh2/Eed methylation complex specifically in ES cells.

The molecular basis for the chromosomal memory is presently unknown. Our data rule out the possibility that continuous \textit{Xist} RNA expression or silencing is required for maintenance of the chromosomal memory and suggest that H3K27m3 is also not involved. The latter interpretation has to be treated cautiously, as it depends on the sensitivity of our assay to detect H3K27m3. Formally it is conceivable that low levels of H3K27m3 undetected by our assay could remain on the chromosome. Presently, it is also unclear what the role of H4K20m1 is and to what extent it interacts with H3K27m3. A H4-K20-specific histone methyltransferase has been identified (Fang et al. 2002; Nishioka et al. 2002; Rice et al. 2002), and we have performed in vitro functional analysis of the mouse Pr-Set7 protein (Figure S5; Protocol S1). Our results indicate that Pr-Set7 is a monomethylase for H4-K20. Its involvement in X inactivation and the function of H4K20m1 remain unclear at present. Future work is needed to identify
the components of the memory configuration and to determine its precise function in X inactivation.

**Materials and Methods**

**Cell lines, culture conditions, and histological sections.** Clone 36 ES cells (Wutz and Jaenisch 2000) and J1:XistSVx-terOT, T20Xist, and ES cells expressing Xist deletions (Wutz et al. 2002) were cultured in DMEM (Biochrome, Berlin, Germany), 15% fetal calf serum (Euroclone, Milan, Italy), and 250 U of LIF/ml as described in those references. ES cells were induced to differentiate in ES medium without LIF by addition of all-trans-retinoic acid to 100 nM as described previously (Wutz and Jaenisch 2000). Primary mouse embryonic fibroblasts were derived from day 13.5 embryos and grown in DMEM (Biochrome) and 10% fetal calf serum as described previously (Wutz and Jaenisch 2000). Xist expression was induced by the addition of 1 µg/ml of doxycycline to the culture medium or was administered in drinking water (100 mg and 100 g of sucrose per liter). For sections, embryos were sexed (Lambert et al. 2000) and fixed, and 10-µm-thick frozen sections were prepared. Mice were handled according to institutional guidelines.

**Immunostaining and Western blot.** For metaphase chromosome spreads, cells were incubated for 15 min at 37°C in RBS solution (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 5 mM MgCl₂) centrifuged for 10 min at 1,200 rpm onto Menzel SuperFrost slides (Roth, Karlsruhe, Germany) using a Cytospin 3 centrifuge (Thermo Shandon, Pittsburgh, Pennsylvania, United States) and processed as described above.

Phases of X inactivation are given relative to days of ES cell differentiation (bottom).

(A) In undifferentiated ES cells, efficient chromosome-wide H3K27m3 depends on both Xist RNA localization to the chromosome in cis and initiation of transcriptional silencing via the A repeat (black triangles).

(B) Early in differentiation, silencing becomes dispensable for high-level H3K27m3 (dotted arrow).

(C) The beginning of the critical window is specified in that Xist loses its potential to trigger H3K27m3 (dotted arrow) and transcriptional silencing. The critical window is negotiated by sustaining high levels of H3K27m3, which is thought to constitute—together with Xist RNA—the signal for the recruitment of the chromosomal memory (black oval). The memory is established on the Xi exactly when silencing becomes irreversible and Xist independent.

(D) During the maintenance phase of X inactivation the chromosomal memory allows Xist RNA to establish H3K27m3 efficiently.
Histone H3-K27 Trimethylation by Xist

Supporting Information

Figure S1. Specificities of H3-K9, H3-K27, H4-K20, and H3-K4 Monom-, Dim-, and Trimethyl Antibodies

Immunodot blot analysis (Peters et al. 2003) of the antisera used to detect specific methylation states of histone H3 on Lysine 9 (A), H3 on Lysine 27 (B), H4 on Lysine 20 (C), and H3 on Lysine 4 (D). IgG fractions of the methyl-lysine histone antibodies were tested at various dilutions, with the most optimal dilution being displayed. Dots blot contain 0.4, 2, 10, and 50 pmol of linear H3 (amino acids 1–20; amino acids 19–34; amino acids 25–45; amino acids 72–91) and peptides, either unmodified or mono-, di-, or trimethylated at the K4, K9, K27, K36, or K79 positions. In addition, a linear H4 (amino acids 12–51) peptide, mono-, di-, or trimethylated at the K20 position, was also used.

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Figure S2. Histone Modification Pattern of the Inactive X Chromosome

Immunofluorescence staining of metaphase spreads of clone 36 ES cells induced to express Xist for 3 d using HSR27m1 (A), HSR27m2 (B), HSR27m3 (C), HSR3m1 (D), HSR3m2 (E), HSR3m3 (F), HSR27m1 (G), HSR29m2 (H), HSR29m3 (I), HSR3m3 (J), and H4Ac (K) antisera. Chromosome 11 was identified by a DNA FISH probe (red; blue, DAPI) in (J) and (K). Clone 36 ES cells grown in the absence of doxycycline were used as a control for the H4Ac staining without Xist expression (L).

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Figure S3. Initiation and Maintenance of Histone Methylation during Differentiation

(A) The kinetics of H3K27m3 was measured in undifferentiated clone 36 ES cells. The percentage of cells showing H3K27m3 staining 6, 12, 24, and 48 h after induction of Xist expression is shown.

(B) The stability of H3K27m3 was determined in undifferentiated ES cells. The percentage of metaphase chromatides (n > 150) showing H3K27m3 staining was analysed in undifferentiated clone 36 ES cells, which expressed Xist for 3 d (lane 1) or were further grown without inducer for 24 h (lane 2) or 48 h (lane 3). This experiment complements data presented in Figure 5A and 5B providing a 'cell cycle synchronous' view of the H3K27m3 decay kinetics.

(C) Levels of H3K27m3 were measured in undifferentiated ES cells after 3 and 10 d of Xist expression. No progressive accumulation over time was observed, indicating that the steady state of H3K27m3 has been reached at 3 d of Xist expression. However, a marked increase in methylation is observed in J1:XistASX-tetOP ES cells upon differentiation for 2 d (hatched bar).

(D) Combined Xist RNA FISH (red) immunofluorescence analysis of EzH2 and H4K20m1 in undifferentiated J1:XistASX-tetOP cells expressing Xist for 3 and 10 d (percentage of nuclei showing a staining is given). Analysis of H3K27m3 and H4 acetylation using an antisemur specific for multiply acetylated forms of H4 in clone 36 and J1:XistASX-tetOP ES cells that were grown for 4 d in the presence of doxycycline and then shifted to differentiation conditions for 2 d more in the presence of doxycycline.

(E) Male primary mouse fibroblasts (PMFs) hemizygous for the inducible Xist-tetOP allele and homozygous for the tetracycline-inducible transactivator were induced with doxycycline for 2 d (lane 1) or 3 d (lane 2), and the number of cells showing H3K27m3 staining in interphase was analysed. Control female PMFs showed a methylation signal in the large majority of cells (lane 3); uninduced male PMFs were always negative.

(F) Representative indirect immunofluorescence of uninduced (top) and induced (bottom) male Xist-tetOP PMFs. The inducible Xist RNA triggers less pronounced and less dense foci of H3-K27 trimethylation (green) compared to the female wild-type control.

(G) Upon Xist expression, H4-K20 monomethylation (green) is observed in interphase Xist-tetOP PMFs (left). Focal enrichment colocalises with the site or Xist RNA clusters (red) on the X chromosome. Female wild-type PMFs (right).

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**Conflicts of Interest.** The authors have declared that no conflicts of interest exist.

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