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Establishment of Histone H3 Methylation on the Inactive X Chromosome Requires Transient Recruitment of Eed-Enx1 Polycomb Group Complexes

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Summary

Previous studies have implicated the Eed-Enx1 Polycomb group complex in the maintenance of imprinted X inactivation in the trophectoderm lineage in mouse. Here we show that recruitment of Eed-Enx1 to the inactive X chromosome (Xi) also occurs in random X inactivation in the embryo proper. Localization of Eed-Enx1 complexes to Xi occurs very early, at the onset of *Xist* expression, but then disappears as differentiation and development progress. This transient localization correlates with the presence of high levels of the complex in totipotent cells and during early differentiation stages. Functional analysis demonstrates that Eed-Enx1 is required to establish methylation of histone H3 at lysine 9 and/or lysine 27 on Xi and that this, in turn, is required to stabilize the Xi chromatin structure.

Introduction

A single X chromosome is transcriptionally silenced early in development in female mammals (Lyon, 1961). This process, termed X inactivation, equalizes the dosage of X-linked genes in XX females relative to XY males. Initiation and propagation of X inactivation occurs coincident with cellular differentiation (Monk and Harper, 1979) and involves a large nonprotein-coding RNA, the X inactive-specific transcript (*Xist*) (Brown et al., 1991, 1992; Borsani et al., 1991; Brockdorff et al., 1991, 1992). *Xist* RNA spreads over the X chromosome and is thought to induce chromosome inactivation through recruitment of as yet unidentified silencing factors (Clemson et al., 1996; Panning et al., 1997; Sheardown et al., 1997). A recent study demonstrated that the silencing function of *Xist* RNA maps to a short tandemly repeated element at the 5′ end of the transcript (Wutz et al., 2002).

In eutherian mammals X inactivation is initiated at random, with an equal probability for each cell that either the maternally (Xm)- or paternally (Xp)-inherited X is inactivated (Gardner and Lyon, 1971). In marsupials (Sharman, 1971) and also in certain extraembryonic lineages in (some) eutherians (Takagi and Sasaki, 1975), X inactivation is paternally imprinted. Both random and imprinted X inactivation are *Xist* dependent (Penny et al., 1996; Marahrens et al., 1997).

Following establishment of X inactivation, silencing is heritably maintained through all subsequent cell divisions. This is attributable to modifications of the chromatin structure that are hallmarks of developmentally regulated (facultative) and constitutive heterochromatin. Thus, heterochromatin on the inactive X chromosome (Xi) is characterised by hypoacetylation of histones H2A, H2B, H3, and H4 (Jeppesen and Turner, 1993; Belyaev et al., 1996; Boggs et al., 1996), methylation of histone H3 lysine 9 (H3-K9) (Peters et al., 2002; Boggs et al., 2002), loss of methylation at histone H3 lysine 4 (H3-K4) (Boggs et al., 2002), and incorporation of a variant histone H2A molecule (macroH2A) (Costanzi and Pehrson, 1998). Additionally Xi is replicated late in S phase (Priest et al., 1967; Takagi, 1974) and shows a high level of CpG island methylation (Mohandas et al., 1981; Norris et al., 1991).

One approach to identifying the proteins recruited by *Xist* RNA has been to analyze the temporal order of events leading to stable X inactivation after expression of *Xist* in differentiating XX embryonic stem (ES) cells. In this in vitro model system, global H4 hypoacetylation, recruitment of the variant histone, macroH2A, and CpG island methylation are all late events (Keohane et al., 1996; Mermoud et al., 1999), while transition to late replication was found to occur early (Keohane et al., 1996). Recently, deacetylation and methylation of histone H3 lysine 9 (H3-K9) and, also, loss of methylation on H3 lysine 4 (H3-K4) were found to be very early events (Heard et al., 2001; Mermoud et al., 2002). The proteins involved in these steps are therefore candidate targets for *Xist* RNA.

Establishment of X inactivation can only occur during a restricted window of opportunity early in development (Wutz and Jaenisch, 2000). Thus, if *Xist* expression is induced and maintained early during ES cell differentiation, stable chromosome silencing is established. If, on the other hand, induction of expression occurs later or in fully differentiated cells, chromosome silencing does not result (Clemson et al., 1998; Wutz and Jaenisch, 2000). Moreover, during the critical early stages, chromosome silencing is *Xist* dependent and reversible (Wutz and Jaenisch, 2000). In contrast to this, in differentiated cells, maintenance of silencing is *Xist* independent and (generally) irreversible (Brown and Willard, 1994; Csankovszki et al., 1999; Wutz and Jaenisch, 2000). These observations suggest that the silencing factors

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initially recruited by *Xist* RNA may be developmentally regulated. It should be noted that a recent report has demonstrated silencing in response to *Xist* transgenes in the HT-1080 human somatic cell line (Hall et al., 2002).

Similarities between the chromatin structure on Xi and other heritably silenced regions suggest that there may be common factors involved. Proteins involved in heritable silencing have been identified in genetic screens in *Drosophila*. These are the modifiers of position effect variegation (PEV) (reviewed in Weiler and Wakimoto, 1995) and the Polycomb group (PcG) proteins (reviewed in Pirrotta, 1997). The PcG proteins were identified initially as being required for maintenance of repression of homeotic genes. They are thought to have a wider role in heritable gene silencing, with homologs having been found in all multicellular organisms. Biochemical analysis indicates that PcG proteins belong to one of two multimeric complexes, PRC1 (Shao et al., 1999) or ESC-EZ (Sewalt et al., 1998; van Lohuizen et al., 1998; Jones et al., 1998; Tie et al., 1998). These complexes are thought to effect silencing by acting at the level of chromatin structure. Recent data indicates that the PRC1 and ESC-EZ complexes interact transiently during early embryogenesis in *Drosophila* (Poux et al., 2001).

The ESC-EZ complex includes the products of the *extra sex combs* (*esc*) gene (termed *eed* in mouse) and the *enhancer of zeste* [*e*(*z*)] gene (termed *Enx1* or *Ezh2* in mouse—the mouse ESC-EZ complex is referred to as the Eed-Enx1 complex throughout this study). Eed-Enx1/ESC-EZ complexes have been shown to interact with type 1 histone deacetylases (HDACs) (van der Vlag and Otte, 1999; Tie et al., 2001), and this has been suggested to be the basis for heritable silencing. More recent studies have demonstrated that the SET domain of the EZ protein catalyzes H3 lysine 9/27 methylation by ESC-EZ complexes (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002).

Mutations affecting ESC/Eed and EZ/Enx1 proteins have been shown to act very early in embryogenesis relative to other PcG mutants (Struhl and Brower, 1982; Schumacher et al., 1996; O'Carroll et al., 2001). In addition to its role in heritable silencing, EZ/Enx1 protein is required for maintenance of chromosome integrity during mitosis (Philips and Shearn, 1990) and, possibly related to this, in cell proliferation (Schumacher et al., 1996; O'Carroll et al., 2001; Beuchle et al., 2001).

In a recent study it was demonstrated that mutations in the mouse *eed* gene result in failure to maintain imprinted X inactivation, specifically in extraembryonic trophoblast cells (Wang et al., 2001). No effect was seen on random X inactivation in the embryo proper. Following on from this Mak et al. (2002) showed that, in trophoblast stem (TS) cells, a model system for imprinted X inactivation, Eed and Enx1 proteins are highly concentrated on Xi. No localization was seen in XX somatic cells, consistent with the observations of Wang et al. (2001). Taken together these studies suggested that there is a lineage-specific pathway for establishing X inactivation. Here we report that the recruitment of Eed-Enx1 complexes to Xi is in fact temporally regulated and not lineage specific. Localization of the complexes occurs rapidly at the onset of imprinted and random X inactivation, coincident with the onset of *Xist* RNA accumulation, both in vitro and in vivo. Analysis of *eed* mutant embryos demonstrates that the function of the Eed-Enx1 complex in X inactivation is to establish histone H3 N-terminal methylation at lysines 9/27.

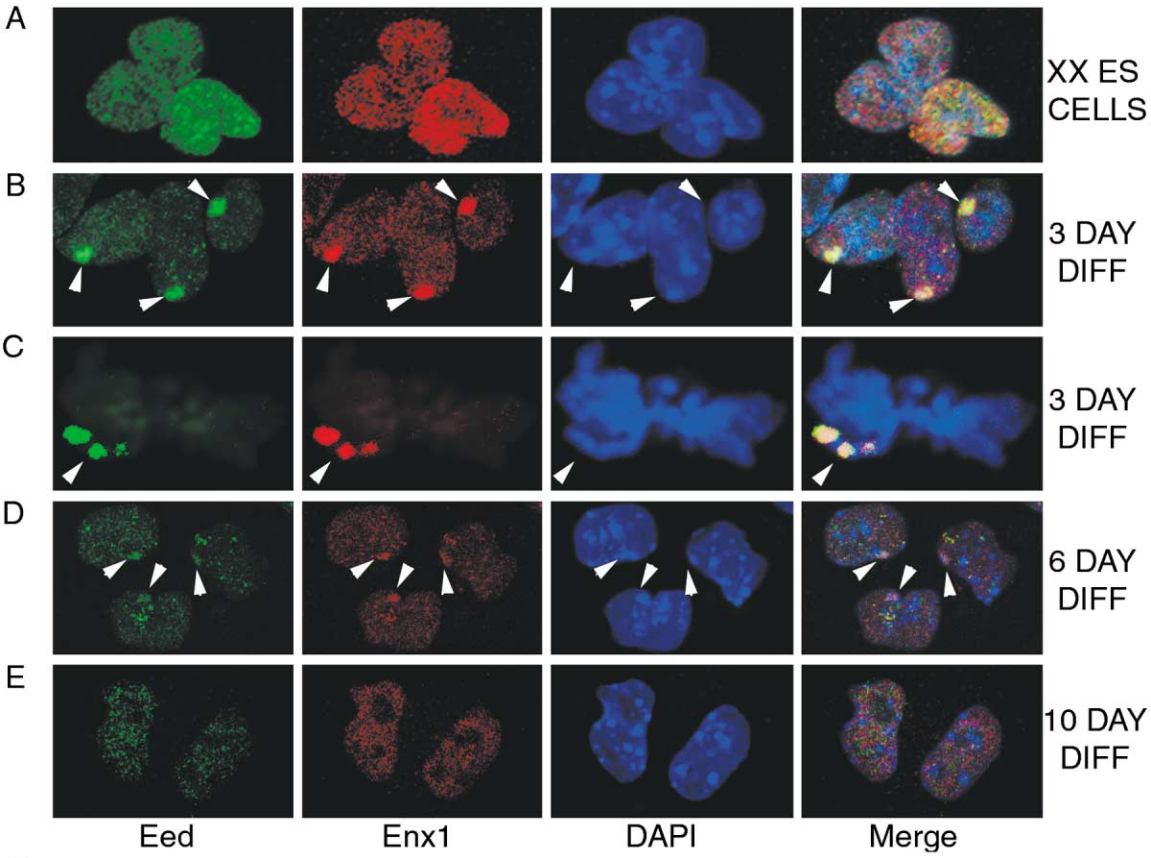
Results

Localization of Eed and Enx1 Proteins to Xi Is a Function of the Differentiation Status of ES and TS Cells

In a recent study we found that Eed-Enx1 PcG complexes localize to Xi in XX trophoblast stem (TS) cells (Mak et al., 2002). Localization was not observed in XX somatic cell lines, although nuclear staining for Eed and Enx1 proteins was detectable. These observations suggested either that Eed-Enx1 complex localization to Xi is lineage specific or, alternatively, that it is temporally regulated, occurring only early in development. To investigate this further we used indirect immunofluorescence to analyze undifferentiated and differentiated PGK12.1 XX ES cells. Previous studies have established that X inactivation in XX ES cells occurs in a progressive stepwise manner after initiation of differentiation in vitro (Keohane et al., 1996; Sheardown et al., 1997; Mermoud et al., 1999).

In undifferentiated ES cells, Eed and Enx1 proteins were seen to localize diffusely throughout the nucleoplasm (Figure 1A). In early stage differentiated cultures (1–4 days), however, a single strong focus of signal was present in addition to diffuse nuclear staining (referred to henceforth as the Eed/Enx1 body). An example of 3 day differentiated cells is illustrated in Figure 1B. On mitotic figures, Eed/Enx1 was seen to localize in a banded pattern on a single chromosome (Figure 1C). That this is the inactive X chromosome was verified by immunoRNA-FISH analysis of *Xist* RNA and Enx1 protein (see below). Interestingly, the Eed/Enx1 body was only detectable transiently. Thus, in 6 day differentiated cultures, staining of the body was weak (Figure 1D), and, after 10 days of differentiation, it was not detectable at all (Figure 1E). Scoring data obtained for a series of time points is shown in Figure 1F. The highest levels of Eed/Enx1 bodies (approximately 60% of cells) were seen at days 3 and 4 of differentiation. Similar results were obtained with a second XX ES cell line, LF2, analyzed at 0, 3, and 7 days differentiation (data not shown). Asynchronicity of differentiation in ES cell cultures accounts for the fact that Eed/Enx1 bodies are not seen in all cells at any given time point.

In addition to Eed/Enx1 bodies being reduced, overall intensity of nuclear staining for the proteins appeared to decrease at later differentiation time points (compare Figures 1A and 1B with Figures 1D and 1E). To examine this in more detail, we carried out Western blots on nuclear extracts prepared from undifferentiated and differentiated PGK12.1 ES cells (Figure 2A). Levels of both Eed and Enx1 proteins were found to be very high in undifferentiated ES cells and also during days 1–3 of differentiation. As differentiation progressed, however, levels fell markedly and, by day 10 of differentiation, were similar to those seen in the lymphocyte control. The reduction in overall protein levels was more pronounced for Eed than for Enx1. These observations suggest that the disappearance of Eed/Enx1 bodies may



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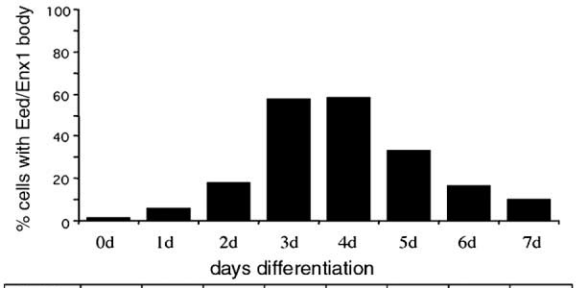


%Eed/Enx1 bodies	1.7	5.7	18.3	57.3	58.4	33.3	16.8	10
Number scored	179	176	279	122	77	132	125	131

Figure 1. Transient Recruitment of Eed-Enx1 Complexes to the Inactive X Chromosome in Differentiating XX ES Cells

(A–E) Immunofluorescence detection of Eed (green) and Enx1 (red) carried out on undifferentiated PGK12.1 XX ES cells (XX ES cells) and on cells differentiated (diff) for 3, 6, and 10 days, as indicated. DNA is counterstained with DAPI.

(B) Arrowheads indicate Eed/Enx1 bodies.

(C) Arrowhead indicates single chromosome with Eed/Enx1 localization in a cell undergoing mitosis.

(D) Arrowheads indicate weak Eed/Enx1 body staining (see text).

(F) Scoring data showing the percentage of PGK12.1 XX ES cells with Eed/Enx1 bodies during days (d) 0–7 of differentiation.

be attributable to the downregulation of Eed and Enx1 proteins during differentiation.

Levels of HDAC1 and HDAC2, which have been shown to be recruited by the ESC-EZ/Eed-Enx1 complex (van der Vlag and Otte, 1999), remained constant over a similar differentiation time course. They were, however, lower in lymphocytes (Figure 2B). The Bmi-1 and Hpc2 proteins, which are involved in the distinct PRC1 PcG complex (Shao et al., 1999), showed an opposite pattern of expression to Eed and Enx1, with highest levels detectable at late differentiation time points and in lymphocytes (Figure 2B). Immunofluorescence analysis of the HDAC and PRC1 proteins did not reveal specific localization to Xi at any of the differentiation stages analyzed (data not shown).

TS cells are multipotent precursors that can give rise to different cell types of the trophectoderm lineage after withdrawal of FGF4 from the culture medium (Tanaka et

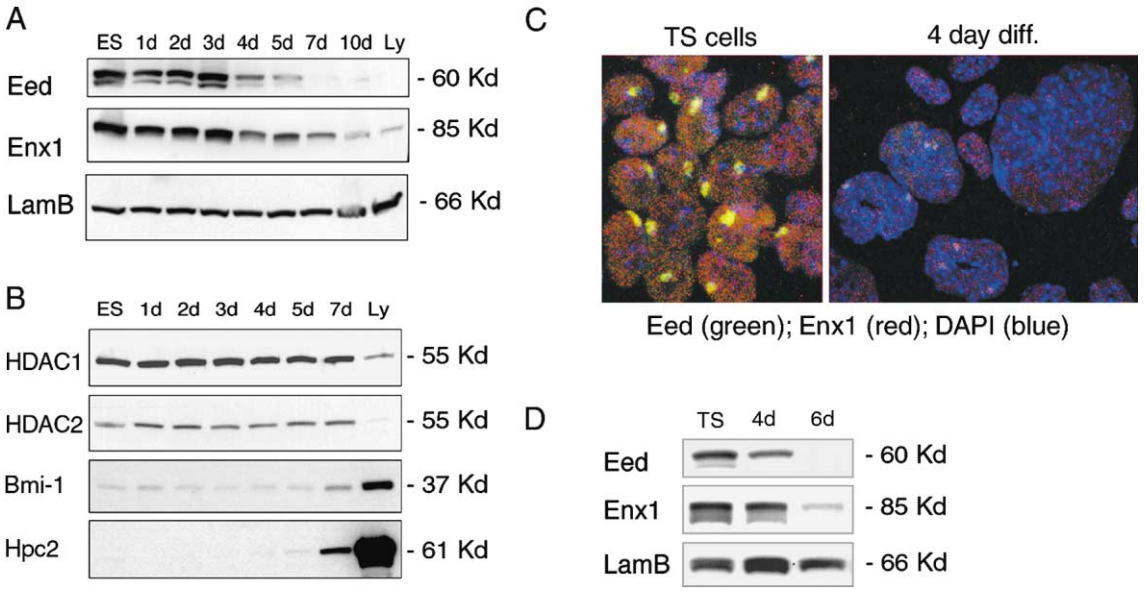


Figure 2. Eed and Enx1 Are Downregulated in Differentiated Cells

(A and B) Western blot analysis of nuclear extracts from PGK12.1 XX ES cells and cells differentiated for 1–10 days (d), as indicated. Lymphocyte (Ly) extracts were used as a somatic cell control. Proteins analyzed were Eed, Enx1, Lamin B (LamB), HDAC1, HDAC2, Bmi-1, and Hpc2. Molecular sizes are indicated. Note doublet for Eed protein is thought to be attributable to alternative RNA transcripts (Denisenko et al., 1998) or posttranslational modification (Ng et al., 2000).

(C) Immunofluorescence analysis of Eed and Enx1 proteins in undifferentiated B1 XX TS cells and in cells differentiated (diff) for 4 days. Note trophectoderm giant cell present in differentiated culture.

(D) Western blot analysis of Eed, Enx1, and LaminB (LamB) proteins in nuclear extracts from TS cells and from cells harvested 4 or 6 days after initiation of differentiation.

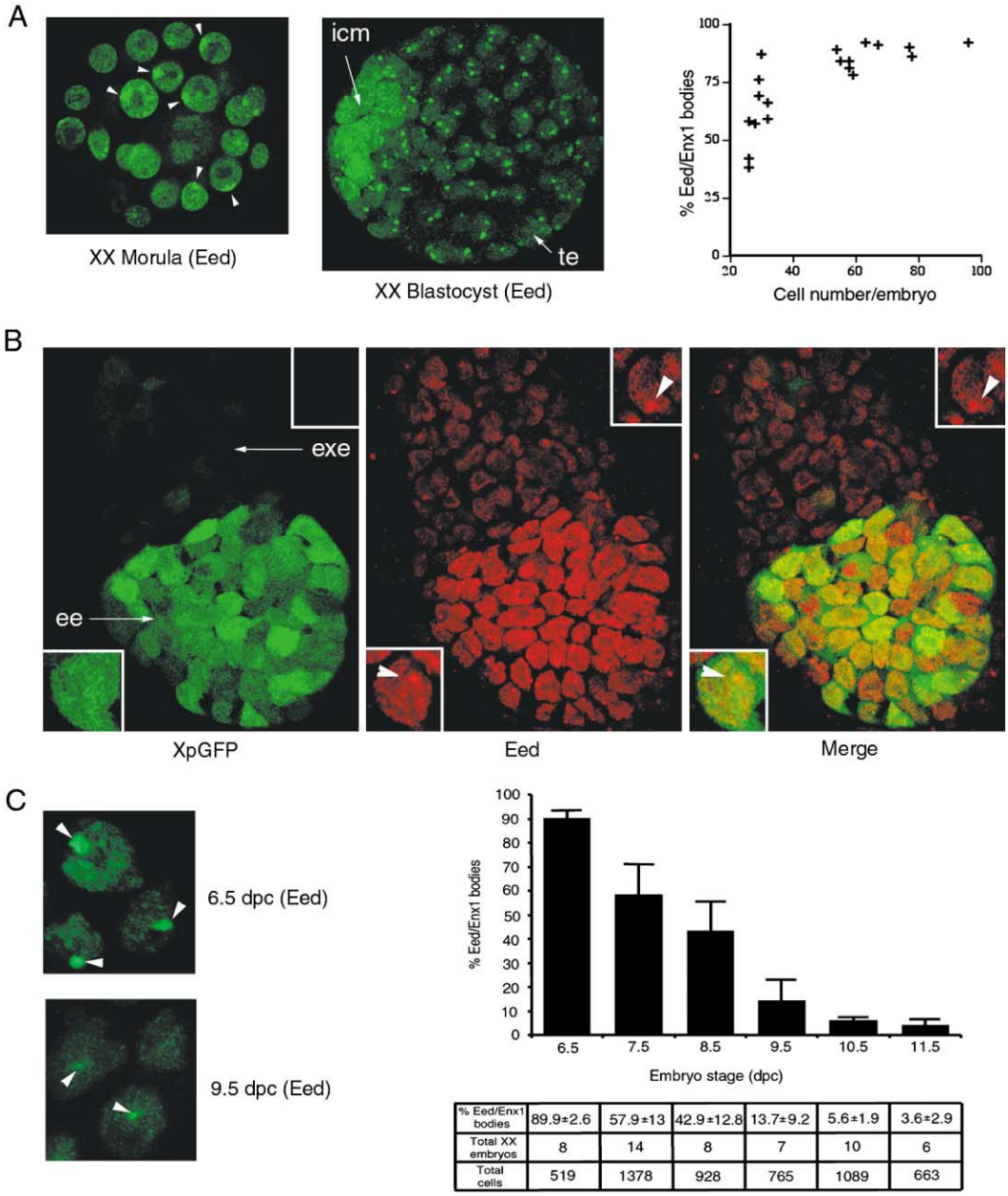
al., 1998). Previously, we analyzed Eed/Enx1 localization only in undifferentiated XX TS cells (Mak et al., 2002). In light of the above results, we decided to analyze differentiated TS cells. Immunofluorescence analysis revealed that the Eed/Enx1 bodies disappear after 4–6 days of differentiation (Figure 2C). Moreover, Western blot analysis revealed that there is a marked reduction in overall levels of Eed and Enx1 proteins (Figure 2D), as occurs in differentiating ES cells.

Taken together these results indicate that, with in vitro cell systems, transient localization of Eed-Enx1 complexes to Xi occurs as a function of the differentiation status of cells. This, in turn, appears to reflect downregulation of Eed/Enx1 proteins during differentiation from totipotent/multipotent precursors.

Transient Eed/Enx1 Localization on the Inactive X Chromosome In Vivo

We next analyzed Eed-Enx1 complex localization on Xi at different stages of mouse embryogenesis in order to confirm that our observations on ES and TS cells reflect events that occur in vivo. To analyze Eed/Enx1 body formation at the onset of imprinted X inactivation, we carried out immunofluorescence on preimplantation embryos at the late morula (16- to 32-cell) stage and the blastocyst stage, the time when the trophectoderm lineage first differentiates. At the morula stage single Eed/Enx1 bodies were clearly detectable in a proportion of cells in approximately 50% of embryos. An example illustrating Eed staining is shown in Figure 3A (left panel). Identical results were obtained for Enx1 (data not shown). Expression of the XGFP transgene, inherited on the Xp (see Experimental Procedures), or *Xist* expression detected in immunoRNA-FISH analysis (see below), confirmed that these were XX embryos. In blastocysts, Eed/Enx1 bodies were seen in most cells. Overall nuclear staining for Eed was much higher in the totipotent inner cell mass (ICM) cells than in differentiating trophectoderm (Figure 3A, center panel). Scoring data for a number of embryos demonstrate that the proportion of cells with an Eed/Enx1 body increases to maximal levels during the morula to blastocyst transition (Figure 3A, right).

We went on to analyze postimplantation embryos at 5.5 days post coitum (dpc), the time when random X inactivation is initiated in cells of the embryonic ectoderm. With the XGFP transgene transmitted on Xp, the embryonic (GFP-positive) and extraembryonic (GFP-negative) regions could be readily identified. In the example shown a mosaic GFP signal could be seen in the embryonic ectoderm, indicating that random X inactivation had initiated (Figure 3B, left panel). Eed/Enx1 bodies were detectable in both embryonic and extraembryonic cells at this stage. However, a clear difference in the overall levels of Eed protein was apparent, with markedly higher signal detected in the undifferentiated embryonic cells than in differentiated extraembryonic cells (Figure 3B, center panel).



% Eed/Enx1 bodies	89.9±2.6	57.9±13	42.9±12.8	13.7±9.2	5.6±1.9	3.6±2.9
Total XX embryos	8	14	8	7	10	6
Total cells	519	1378	928	765	1089	663

Figure 3. Eed/Enx1 Body Formation during Imprinted and Random X Inactivation In Vivo

(A) Examples of immunofluorescence analysis of Eed protein in XX morula (left panel) and blastocyst stage embryos (center panel). Eed/Enx1 bodies in the morula are indicated with arrowheads. The inner cell mass (icm), and trophectoderm regions (te) of the blastocyst are labeled. The scatterplot in the right panel records the percentage of cells with Eed/Enx1 bodies in XX embryos plotted against the total number of cells in individual embryos.

(B) Single optical section illustrating immunofluorescence analysis of Eed localization in a 5.5 dpc XX embryo with an X-linked GFP transgene present on the paternal X chromosome (Xp). Strong green fluorescence marks the embryonic ectoderm (ee) where random X inactivation is occurring (note mosaic pattern of GFP staining). Cells that have undergone imprinted X inactivation, notably extraembryonic ectoderm (exe), have silenced the GFP transgene on Xp. Insets indicate Eed/Enx1 bodies (arrows) in cells of exe (top right) or ee (bottom left) regions.

(C) Analysis of Eed/Enx1 bodies in cells of XX embryos at 6.5–11.5 dpc. Examples on the left show cells of early stages (6.5 dpc) with strong Eed/Enx1 bodies (top panel) and cells at a later stage (9.5 dpc) in which Eed/Enx1 bodies and general Eed staining are much weaker. The graph on the right shows scoring data from a number of experiments, giving the percentage of cells exhibiting Eed/Enx1 bodies at the stages indicated.

Localization of Eed-Enx1 complexes on Xi was subsequently monitored in XX embryos at time points after the onset of random X inactivation (Figure 3C). While most cells exhibited a single Eed/Enx1 body at 6.5 dpc, the frequency of detection began to decline thereafter. Overall levels of Eed and Enx1 proteins were also seen

to decline, as assessed by intensity of staining (see Figure 3C, 9.5 dpc example). By 11.5 dpc Eed/Enx1 bodies were undetectable in the majority of cells. These results demonstrate that transient localization of Eed-Enx1 complexes to Xi is a feature of X inactivation in vivo. As is the case for ES and TS cells, disappearance of Xi localization appears to be due to progressive down-regulation of Eed and Enx1 proteins during differentiation.

Eed/Enx1 Body Formation Occurs at the Onset of X Inactivation

We went on to assess the timing of Eed-Enx1 complex recruitment to Xi relative to other steps in the X inactivation process using the differentiating XX ES cell system. X inactivation is triggered by the accumulation of stable *Xist* RNA, and this is the earliest detectable step, occurring between 2 and 5 days differentiation in XX ES cells (Sheardown et al., 1997). Eed/Enx1 body formation was seen to closely mirror the accumulation of stable *Xist* RNA, being detectable in 80% of cells at 2 days differentiation and in nearly all cells thereafter (Figure 4A).

Low levels of *Xist* transcript and, also, antisense *Tsix* transcript occur on both alleles in undifferentiated cells and transiently on the active X chromosome allele during early differentiation stages (Panning et al., 1997; Sheardown et al., 1997; Lee et al., 1999; Debrand et al., 1999). This is detected as a fine pinpoint signal in RNA-FISH analysis (Figure 4A, left panel). We did not observe a concentration of Eed or Enx1 proteins colocalizing with these signals (Figure 4A). This may be attributable to the low level of *Xist* RNA, or, alternatively, coexpression of *Tsix* antisense RNA may preclude recruitment of the complex.

Recent studies have demonstrated that specific modifications on the N termini of histone H3, namely, deacetylation and methylation of H3-K9 (Heard et al., 2001; Mermoud et al., 2002), and loss of methylation at H3-K4 (Heard et al., 2001) are very early events in X inactivation. Global deacetylation of histone H4, on the other hand, occurs later (Keohane et al., 1996; Heard et al., 2001).

To analyze H3-K9 methylation relative to Eed/Enx1 body formation, we used antisera raised to a branched H3 peptide dimethylated at K9. This antibody detects di- and trimethylH3-K9 (meH3-K9) (T.J. and A.H.F.M.P., unpublished data) and, in previous studies, has been shown to highlight pericentromeric heterochromatin and the inactive X chromosome (Peters et al., 2001; 2002; Mermoud et al., 2002). We analyzed cells from 2, 3, and 6 day differentiated cultures, scoring first for the presence of a single Eed/Enx1 body and then for the presence or absence of underlying H3-K9 methylation (Figure 4B). MeH3-K9 staining of pericentric heterochromatin, as assessed by colocalization with DAPI dense-staining regions of the nucleus, was observed at all stages analyzed (Figure 4B). Strong meH3-K9 signal underlying Eed/Enx1 bodies was seen in 31% of cells at 2 days differentiation. A weaker signal, indicating partial methylation, was seen in a further 35% of cases. At later stages strong meH3-K9 staining was seen in the majority of cases. These results indicate that a progressive increase in detectable H3-K9 methylation on Xi occurs subsequent to recruitment of Eed-Enx1 complexes. Deacetylation of H3-K9 (Figure 4C) and loss of methylation of H3-K4 (Figure 4D) were also seen to occur rapidly at the time of Eed-Enx1 complex recruitment.

Recent studies have demonstrated that purified Eed-Enx1 (ESC-EZ) complexes catalyze methylation of histone H3-K27 and, to a lesser extent, H3-K9 (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002). We therefore analyzed H3-K27 methylation using antiserum specific for tri-meH3-K27. In immunofluorescene analysis this antibody highlights the inactive X chromosome, but not pericentromeric heterochromatin (A.H.F.M.P. and T.J., submitted). In differentiating XX ES cells, we observed strong meH3-K27 staining over Eed/Enx1 bodies in nearly all cells at all differentiation stages tested (Figure 4E). This result is consistent with Eed-Enx1 complexes catalyzing H3-K27 methylation on Xi (see below).

As a control for these experiments, we analyzed global deacetylation of H4, which has been reported to occur later than H3 modifications (Keohane et al., 1996; Heard et al., 2001). In agreement with these earlier studies, hypoacetylation of H4 in association with Eed/Enx1 bodies was only detected in a relatively small proportion of cells after 2 and 3 days differentiation, but increased to maximal levels after 6 days differentiation (Figure 4F).

Xist Expression Is Necessary and Sufficient to Recruit Eed-Enx1 Complexes

The above results demonstrate that localization of Eed-Enx1 complexes to Xi is a very early event, both during XX ES cell differentiation and in vivo. Thus, Eed and Enx1 are candidates for proteins recruited by *Xist* RNA. To investigate this idea further, we analyzed Eed-Enx1 complex localization in an XY ES cell line, LH1.2, which carries a multicopy *Xist* transgene integrated into an autsosome (Herzing et al., 1997). The transgene is expressed and accumulates in *cis* in undifferentiated cultures in approximately 30% of cells (see Figure 5A, left panel). Using immunoRNA-FISH analysis we found that Eed/Enx1 bodies are present and colocalize with the ectopic *Xist* RNA (Figure 5A). Thus, expression of *Xist* RNA in undifferentiated XY ES cells is sufficient to recruit Eed-Enx1 complexes. Scoring data showed colocalization in 58% of cells that had some degree of accumulation of *Xist* RNA ($n = 119$). This is slightly lower than that in differentiating XX ES cells (Figure 4A), probably reflecting the fact that transgene *Xist* RNA levels are sometimes relatively low. Analysis of H3/H4 modifications indicates that chromatin silencing occurs in response to ectopic *Xist* RNA expression in LH1.2 cells (data not shown).

We next examined Eed-Enx1 recruitment in response to ectopic *Xist* expressed in vivo in preimplantation embryos. The Tg15 line carries an *Xist* transgene integrated on the Y chromosome (Matsuura et al., 1996). The transgene is expressed in all cells in XY^{Tg15} preimplantation embryos (M. Ager and S.D.M. Brown, personal communication). To discriminate between XY^{Tg15} and XX blastocysts, Tg15 males were crossed with homozygous XGFP females. Blastocysts were then isolated from crosses between male F1 progeny ($X^{GFP}Y^{Tg15}$) and normal females (XX). XX^{GFP} and XY^{Tg15} embryos could then be distinguished on the basis of GFP expression. ImmunoRNA-FISH analysis was carried out, and Enx1 protein was

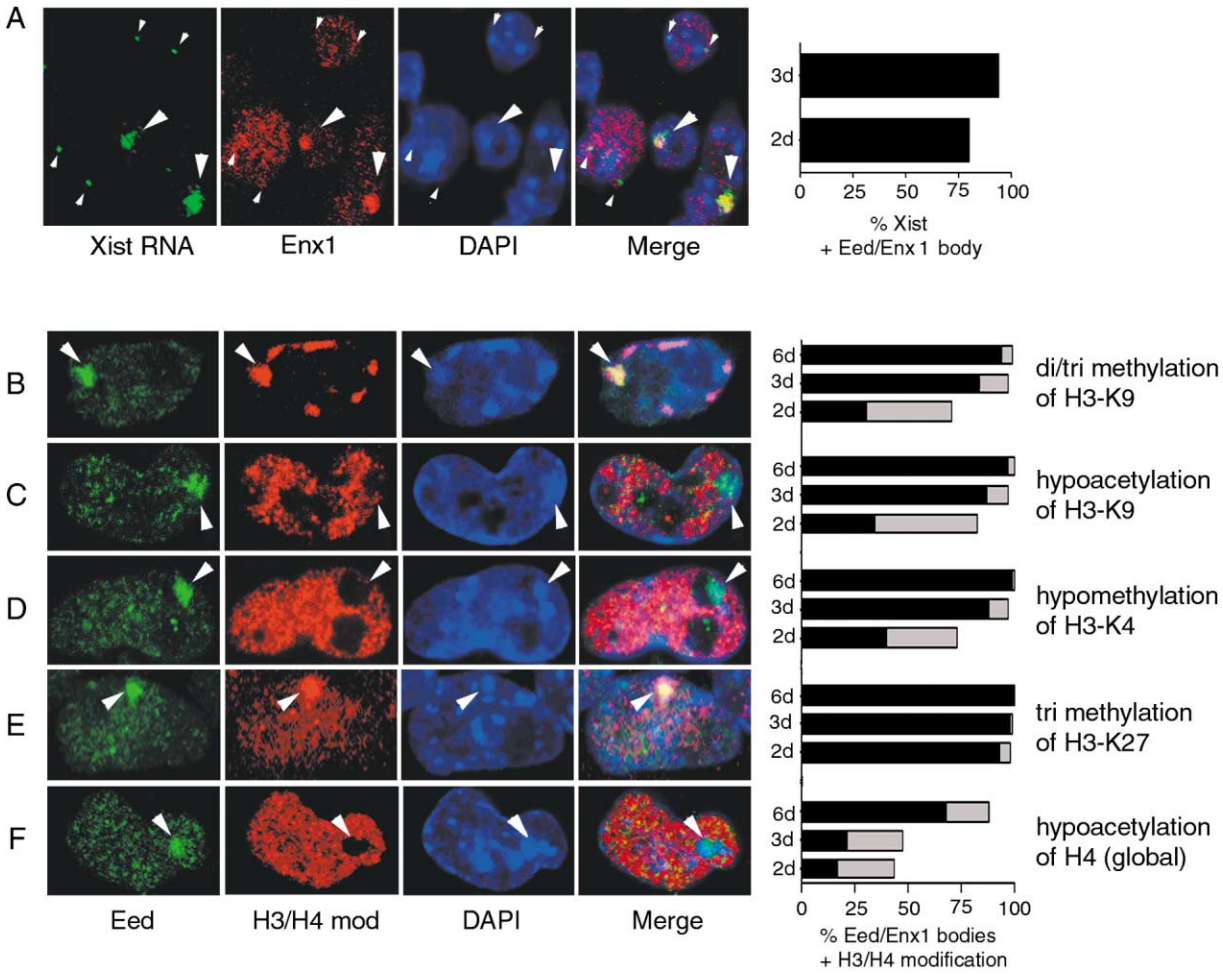


Figure 4. Recruitment of Eed-Enx1 Complexes Is an Early Event in Random X Inactivation

(A) Example showing immunoRNA-FISH detection of *Xist* RNA (green) and Enx1 protein (red) in 2 day differentiated PGK12.1 cells. DNA is counterstained with DAPI. Small arrowheads indicate two pinpoint *Xist*/*Tsix* signals seen in undifferentiated XX ES cells. Large arrowheads indicate accumulated *Xist* RNA and associated Enx1 staining. The chart on the right records the percentage of cells ($n > 100$) where *Xist* RNA signal has underlying Enx1 staining, after 2 and 3 days (d) differentiation.

(B–F) Examples of early stage differentiated PGK12.1 cells exhibiting histone H3/H4 modifications underlying Eed/Enx1 bodies. Graphs to the right illustrate scoring data ($n > 100$) at 2, 3, and 6 days differentiation. Filled areas represent the proportion of cells where histone modification was clearly detectable. Shaded areas represent cells where an intermediate level of histone modification was detected.

seen to colocalize with transgenic *Xist* RNA in a similar pattern to that seen in normal female blastocysts (compare Figure 5B with 5C).

Finally, we analyzed Eed-Enx1 recruitment in XX blastocysts carrying a mutant *Xist* RNA allele, *Xist*^{inv}. *Xist*^{inv} is a targeted mutation with a deletion of *Xist* exon IV and an inversion of sequences between 5.9 and 13.2 Kbp of the *Xist* genomic sequence (see Experimental Procedures). The *Xist*^{inv} allele is able to express a mutant RNA but fails to elicit X inactivation (N.B. and T.B.N., in preparation). XX preimplantation embryos in which *Xist*^{inv} is inherited on Xp express mutant RNA in all cells. As shown in Figure 5C, little or no Enx1 protein was seen to colocalize with *Xist*^{inv} RNA. Taken together these results demonstrate that expression of functional *Xist* RNA is both necessary and sufficient for the recruitment of Eed-Enx1 complexes.

Recruitment of Eed-Enx1 Complexes on Xi Is Required to Establish H3-K9/K27 Methylation

Biochemical studies have demonstrated that Eed interacts with type 1 histone deacetylases (HDACs) (van der Vlag and Otte, 1999; Tie et al., 2001). In addition Enx1 protein has a SET domain, the active site in histone methyltransferases (HMTases) (Rea et al., 2000), and recent studies have shown that ESC-EZ (Eed-Enx1) complexes catalyze methylation of H3-K9 and K27, with a strong preference for K27 (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002). Thus, the Eed-Enx1 complex is a good candidate for the enzyme activities catalyzing H3 N-terminal modification at the onset of X inactivation. To investigate this idea we set out to analyze H3 modifications in *eed* mutant embryos.

The *eed*^{3354SB} allele is a point mutation that disrupts

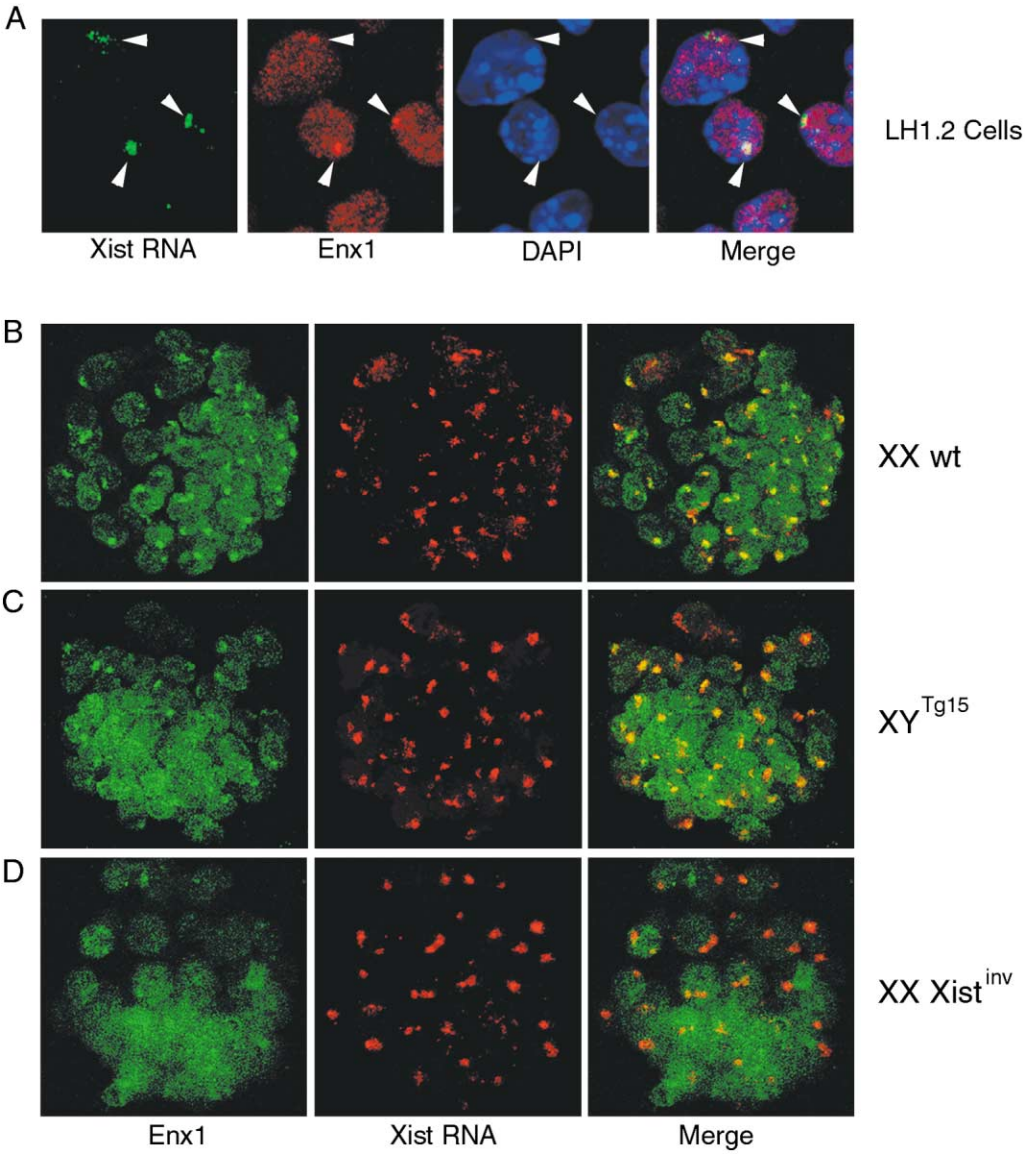


Figure 5. *Xist* Expression Is Necessary and Sufficient for Recruitment of Eed-Enx1 Complexes to Xi

(A) Example of immunoRNA-FISH analysis of undifferentiated LH1.2 XY ES cells showing individual cells expressing transgene *Xist* RNA and colocalizing staining for Enx1 protein.

(B–D) Examples of immunoRNA-FISH analysis of blastocyst stage embryos detecting Enx1 protein (green) and *Xist* RNA (red).

(B and C) Normal XX blastocyst (B) and XY^{Tg15} blastocyst expressing an *Xist* transgene integrated on the Y chromosome (C) show Eed/Enx1 bodies colocalizing with *Xist* RNA.

(D) XX blastocyst bearing the null $Xist^{inv}$ mutation on the paternal X chromosome. Mutant *Xist* RNA fails to elicit X inactivation, and Eed/Enx1 bodies are not detectable.

the structure of a WD40 domain required for the interaction of Eed with Enx1 (Schumacher et al., 1996). It appears to be a null allele, as the phenotype, embryo lethality at about 8.5 dpc, is indistinguishable from that caused by complete deletion of the locus. To study X inactivation in the embryo proper in *eed* mutants, we chose to analyze embryonic ectoderm of 7.5 dpc embryos, i.e., prior to the stage at which homozygous mutants die, but after the onset of random X inactivation.

We first analyzed localization of Eed and Enx1 proteins in $eed^{-/-}$ mutant embryos. Examples are shown in Figure 6A (center panel). Eed protein could not be detected at all, suggesting either that it is destabilized as a consequence of the mutation or, alternatively, that the epitope recognized by the antibody has been destroyed. Enx1 protein shows an abnormal localization, being diffusely distributed both in the nucleus and cytoplasm. An identical pattern was observed in male $eed^{-/-}$ embryos (data not shown). Eed/Enx1 bodies were not detected in mutant XX embryos. Furthermore, staining for Eed/Enx1 bodies was markedly reduced in heterozygous embryos (Figure 6A, compare first three panels).

We went on to examine *Xist* expression and Enx1 protein in mutant XX embryos using immunoRNA-FISH conditions. As shown in Figure 6A (right panels), Enx1 protein does not colocalize with *Xist* RNA domains. In fact, Enx1 staining in *eed*$^{-/-}$ embryos was nearly completely lost under these conditions. As immunoRNA-FISH involves a cell permeabilization step prior to fixation, the Enx1 protein, which is not bound up in a complex or associated with chromatin, probably diffuses out of the cells.

To determine the consequences of disruption of Eed-Enx1 complexes in *eed*$^{-/-}$ embryos, we carried out immunoRNA-FISH using either antisera to di/tri-meH3-K9 or tri-meH3-K27. Examples are shown in Figure 6B. In contrast to wild-type controls, the di/tri-meH3-K9 antibody failed to detect signal that colocalized with *Xist* RNA in the majority of cells (Figure 6B, left panels). MeH3-K9 at pericentromeric heterochromatin, however, was unaffected. This latter observation is expected as H3-K9 methylation at these sites is catalyzed by the Suv39h1/2 HMTases (Peters et al., 2001). The tri-meH3-K27 antisera also failed to detect a signal underlying *Xist* RNA (Figure 6B, right panels). General nuclear staining for tri-meH3-K27 was also much reduced, indicating that the Eed-Enx1 complex is the major H3-K27 HMTase present at this developmental stage.

As *eed*$^{-/-}$ embryos were previously shown to initiate, but not to maintain, imprinted X inactivation in trophectoderm (Wang et al., 2001), we went on to determine whether deacetylation at H3-K9 occurs on Xi following random X inactivation in the mutant embryonic ectoderm. In a large proportion of cells, hypoacetylation of H3-K9 was seen underlying *Xist* RNA signal, similar to wild-type cells (Figure 6C, top and center panels). However, in approximately 20% of cells, H3-K9 hypoacetylation was not detected. Moreover, in these cells, *Xist* RNA was diffusely distributed, consistent with the chromosome being relatively decondensed. Similar results were obtained with antisera to meH3-K4 (data not shown).

We went on to assay the inactivation status of two X-linked genes, *Brx* and *Pgk-1*, using RNA-FISH to detect nascent transcripts (Figure 6D). Cells obtained from 7.5 dpc *eed*$^{-/-}$ embryos and wild-type littermates were scored for the presence of either one or two nascent RNA foci. In wild-type and heterozygous embryos, we observed a single focus (Xa allele), generally located away from the *Xist* RNA domain, in the majority of cells. In *eed*$^{-/-}$ embryos, however, 10%–15% of cells were seen to have two signals, one of which localized close to the *Xist* RNA domain (Xi allele). Thus, the Xi allele is expressed in a proportion of cells from mutant embryos. Taken together these results indicate that X inactivation commences in *eed*$^{-/-}$ embryos but that, in the absence of H3-K9/K27 methylation, hypoacetylation of H3-K9 and loss of H3-K4 methylation are not stably maintained, with consequent reactivation of genes on Xi.

Discussion

In this study we have demonstrated that Eed-Enx1 complexes are transiently recruited to Xi at the onset of X inactivation, both in vitro and in vivo. At later developmental stages localization of Eed-Enx1 to Xi is no longer detectable, coinciding with downregulation of overall levels of these proteins. Recruitment to Xi is a very early event in X inactivation, occurring at the same time as *Xist* RNA accumulation. *Xist* expression is both necessary and sufficient to recruit Eed-Enx1 complexes. Analysis of *eed* mutant embryos demonstrates that Eed-Enx1 complexes are required to establish H3-K9/K27 methylation on the inactive X chromosome and, thereby, to stabilize the Xi chromatin structure.

Function of the Eed/Enx1 Complex in X Inactivation

In *eed*$^{-/-}$ XX embryos we found that Enx1 protein does not localize to Xi. This result is consistent with in vitro analysis demonstrating that the *eed*^{3354SB} mutation disrupts a WD40 domain required for the interaction of Eed with Enx1 (Denisenko et al., 1998). Thus, in the absence of functional Eed protein, the Enx1 HMTase cannot be directed toward specific targets. As recent studies have demonstrated that Eed/Enx1 complexes methylate H3-K9 and K27 in vitro, with a strong preference for K27 (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002), failure to localize Enx1 clearly accounts for the absence of H3-K9/K27 methylation on Xi in *eed*$^{-/-}$ embryos.

It should be noted that, while previous studies have identified H3-K9 methylation as an early mark of silent chromatin on the inactive X chromosome (Peters et al., 2002; Boggs et al., 2002), more recent data indicates that this could be attributable to crossreactivity of di/tri-meH3-K9 antisera toward di/tri-meH3-K27. New antisera highly specific for di/tri-meH3-K9 detect pericentromeric heterochromatin, but not Xi, while the tri-meH3-K27 antibody used in this study detects Xi, but not pericentromeric heterochromatin (A.H.F.M.P. and T.J., submitted). It is also possible that Xi is di/trimethylated both at K9 and K27 and that this configuration is not recognized by the novel di/trimethylH3-K9 antisera.

Our data suggest that H3-K9/K27 methylation serves to stabilize the Xi chromatin structure. Thus, in *eed*$^{-/-}$ embryos, H3-K9 hypoacetylation and loss of H3-K4 methylation on Xi are not seen in a significant number of cells. Moreover, both the Xa and Xi alleles of two X-linked genes were seen to be expressed in a similar proportion of cells. These observations provide a basis for explaining reactivation of the X-linked GFP transgene in trophectoderm cells of *eed*$^{-/-}$ embryos reported previously by Wang et al. (2001). However, Wang et al. (2001) did not observe reactivation of the GFP transgene in cells of the embryo proper, leading to the conclusion that Eed is required for the maintenance of imprinted, but not random, X inactivation. This difference can be accounted for by two factors. First, it is probable that reactivation of any given locus on Xi is sporadic and progressive. As embryos exhibit mosaic expression of the XGFP transgene because of random X inactivation, a relatively small increase in the proportion of cells expressing the transgene, as observed for endogenous X-linked genes in this study, would be difficult to quantify. A second factor, suggested by Wang et al. (2001), is that additional levels of epigenetic silencing, for example, DNA methylation (Sado et al., 2000), play a more significant role in maintenance of X inactivation in cells

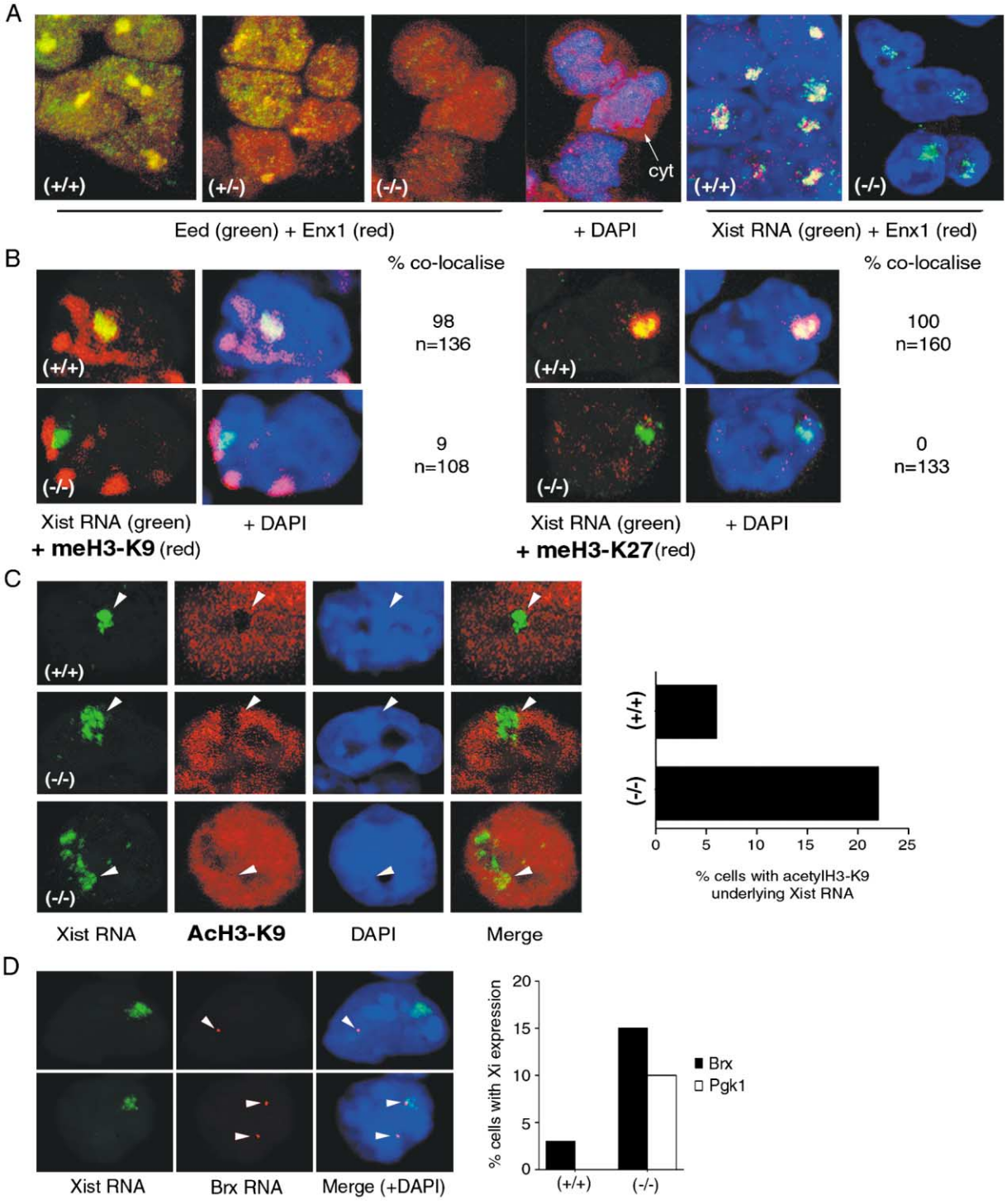


Figure 6. Eed-Enx1 Activity Is Required to Establish H3-K9/K27 Methylation on the Inactive X Chromosome

(A) Localization of Eed (green) and Enx1 (red) in XX *eed*^{3354SB} mutant embryos at 7.5 dpc. Examples of wild-type (+/+), heterozygote (+/−), and homozygous mutants (−/−) are illustrated (panels 1,2, and 3, respectively). Note that Eed/Enx1 bodies stain more weakly in heterozygotes than in wild-type cells. In homozygous mutant cells the Eed signal is nearly undetectable. Enx1 protein is found in the nucleus and cytoplasm (cyt), and there are no Eed/Enx1 bodies detectable. ImmunoRNA-FISH analysis detecting *Xist* RNA (green) and Enx1 (red) further demonstrates this finding (panels 4 and 5).

(B) Examples of immunoRNA-FISH analysis assessing association of di/tri-meH3-K9 (left panels) and tri-meH3-K27 (right panels) with *Xist* RNA domains in homozygous *eed* mutant embryos compared with wild-type littermates. Scoring data recording the percentage of cells with colocalizing signals are shown to the right of the panels.

(C) Examples of immunoRNA-FISH analysis assessing association of *Xist* RNA (green) and acetylH3-K9 (red). In wild-type XX embryos (top panels), the majority of cells show hypoacetylation of H3-K9 underlying *Xist* RNA. This is true in many cells from homozygous mutant embryos

of the embryo proper compared with the trophectoderm, potentially masking the effects of failure to establish H3-K9/K27 methylation.

Methylation of H3-K9 and H3-K27 on Xi are detected throughout development and in adult somatic cells (Heard et al., 2001; Mermoud et al., 2002; A.H.F.M.P. and T.J., submitted). As Eed-Enx1 localization on Xi is only detectable early in development, it is possible that there is a maintenance H3-K9/K27 HMTase that functions at later stages. Alternatively, low levels of Eed-Enx1 complex present in later development could be sufficient to maintain H3-K9/K27 methylation on Xi. A recent study has demonstrated that conditional deletion of Ezh2 (Enx1) in pro-B cells correlates with reduced levels of methylH3-K27 (Su et al., 2002).

Interaction of Eed-Enx1 Complexes and *Xist* RNA

The banded localization of Eed-Enx1 complexes on Xi parallels that observed for *Xist* RNA. We first noted this in TS cells and, on the basis of this observation, suggested that there could be an interaction, either direct or indirect, between *Xist* and the Eed-Enx1 complex (Mak et al., 2002). This view is further supported by observations reported in this study. First, recruitment of Eed-Enx1 complexes occurs extremely rapidly after the onset of stable *Xist* RNA accumulation in differentiating XX ES cells. Second, Eed-Enx1 recruitment occurs in response to expression of ectopic *Xist* RNA transgenes, both in undifferentiated XY ES cells and also in XY^{Tg15} blastocysts. Finally, Eed-Enx1 complexes are not recruited in response to expression of $Xist^{inv}$ mutant RNA, which fails to elicit X inactivation.

While our data support the idea that Eed-Enx1 complexes are recruited by *Xist* RNA, they do not prove that this interaction is direct. In fact, localization of Eed-Enx1 to Xi is first seen to occur at the morula stage, when imprinted X inactivation is initiated, whereas *Xist* RNA expression is detected earlier, in cleavage stage embryos (Nesterova et al., 2001). High levels of Eed-Enx1 complexes are available during the cleavage stages (N.B. and W.M., in preparation), suggesting that a factor(s) required for the interaction of the complex with *Xist* is absent.

Also relevant to the question of whether or not the Eed-Enx1 complex interacts directly with *Xist* RNA is the observation that $eed^{-/-}$ embryos are able to initiate X inactivation. This is, presumably, at least in part, attributable to recruitment of an HDAC complex that can deacetylate H3-K9. It is possible that components of the PcG complex other than Eed and Enx1 are still assembled in $eed^{-/-}$ embryos and that these confer H3-K9 HDAC activity. In the *Drosophila* ESC (Eed) protein, an equivalent mutation disrupts EZ (Enx1) recruitment but does not appear to ablate complex formation (Ng et al., 2000). An alternative scenario is that the complex responsible for H3-K9 deacetylation is a direct target of *Xist* RNA and that the Eed-Enx1 HMTase is then recruited by this complex. In support of this idea, heritable silencing at *Drosophila* homeotic genes is initiated by recruitment of the dMi2 protein, a component of an HDAC and chromatin remodeling complex, by hunchback (Kehle et al., 1998). Moreover, Eed protein can interact with type 1 HDACs, both in mammalian cells (van der Vlag and Otte, 1999) and in *Drosophila* (Tie et al., 2001). Thus, HDAC complexes may recruit Eed-Enx1 to target sites, including Xi, rather than vice versa.

Developmental Regulation of the Eed-Enx1 Complex

It is interesting to note that the time during which high levels of Eed-Enx1 complex are available corresponds closely to the window of opportunity during which cells are responsive to *Xist* RNA (Wutz and Jaenisch, 2000). On the basis of this consideration, we propose the model illustrated in Figure 7. We speculate that *Xist* RNA recruits HDAC and Eed-Enx1 complexes, which lead to establishment of a primary level of chromatin silencing. Only during early differentiation stages would levels of these complexes be sufficient to establish chromosome-wide primary silencing. This would explain why expression of *Xist* in more differentiated cell types cannot induce X inactivation (Wutz and Jaenisch, 2000). We further suggest that maintaining localization of the HDAC/Eed-Enx1 complexes is *Xist* RNA dependent. This would account for reversibility and *Xist* dependence of silencing in undifferentiated ES cells or during early differentiation stages (Wutz and Jaenisch, 2000). Extinguishing *Xist* expression would result in delocalization of HDAC/Eed-Enx1 complexes, loss of H3-K9/K27 methylation, increased H3-K9 acetylation, and, hence, reactivation of Xi.

To account for the fact that X inactivation does subsequently become stabilized and *Xist* independent, we suggest that the chromatin modifications induced by the HDAC and Eed-Enx1 complex provide a template for recruitment of other silencing components. These could be responsible for further histone N-terminal modifications, for example, global H4 deacetylation, and also for DNA methylation at CpG islands and recruitment of macroH2A1.2. It should be noted that proteins of the PRC1 PcG group complex are not localized to Xi at any stage (data not shown) and are therefore unlikely to be involved in maintaining X inactivation in late development.

A key finding from our experiments is that recruitment of Eed-Enx1 to Xi is temporally regulated, rather than lineage specific, and that this, in turn, appears to relate to temporal regulation of overall levels of Eed and, to a lesser extent, Enx1 proteins. A similar expression profile has been reported for ESC (Eed) protein in *Drosophila* embryogenesis (Ng et al., 2000). It is striking that these factors are expressed at highest levels in totipotent or multipotent precursors and during early stages of differentiation. One interpretation of this observation is that

(center panels). However, a significant proportion of homozygous mutant cells exhibit association of acetylH3-K9 signal and *Xist* RNA (lower panels). Quantitative analysis is shown to the right ($n > 100$).

(D) Expression of X-linked genes in $eed^{-/-}$ 7.5 dpc embryos. Examples show cells with Xa *Brx* expression (top panels) and with both Xa and Xi *Brx* expression (lower panels). The graph to the right shows quantitative data illustrating the percentage of cells expressing both Xa and Xi alleles for *Brx* and *Pgk1* loci in wild-type (+/+) or mutant (−/−) embryos.

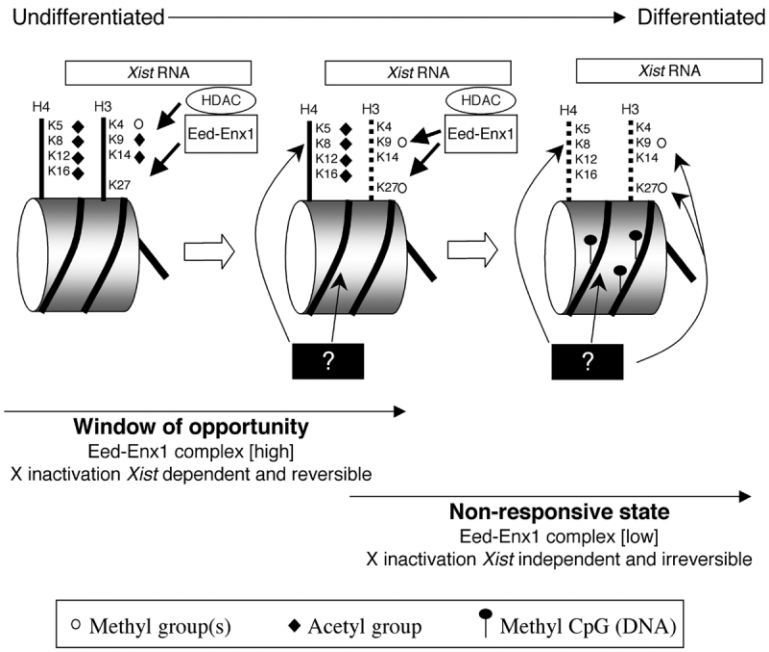


Figure 7. A Model for the Role of Eed-Enx1 Complexes in X Inactivation

The model depicts a histone octamer with H3 and H4 N termini acetylated (filled diamond) or methylated (open circle) at defined lysine (K) residues, as indicated. Recruitment of HDAC and Eed-Enx1 complexes by *Xist* RNA leads to deacetylation of H3-K9, methylation of H3-K9 and H3-K27, and a loss of methylation at H3-K4. The resultant change in H3 conformation (indicated by dashed line) is proposed to represent a primary level of chromatin silencing that is *Xist* dependent. Subsequently, it is proposed that the primary chromatin configuration recruits other modifying complexes, which lead, for example, to deacetylation of H4 and methylation of DNA. The resulting secondary heterochromatin structure (depicted by H4 dashed line) is proposed to be self-maintaining and, therefore, *Xist* RNA independent. See text for further details.

Eed-Enx1 complexes are components of the machinery required to confer genome plasticity. Thus, like X inactivation during the window of opportunity, silent chromatin at other Eed-Enx1 targets may be reversible if the primary signal (for example hunchback at homeotic loci in *Drosophila*) is removed. This would provide cells of the early embryo with the capacity to activate regions of the genome in response to specific differentiation signals, contrasting with the situation in differentiated cells, where heritable silencing is highly stable and is normally irreversible.

Experimental Procedures

Mouse Strains and Cell Lines

ES cell lines PGK12.1, LF2, Efc1, and LH1 were all grown, maintained, and differentiated as described previously (Norris et al., 1994; Sheardown et al., 1997; Herzing et al., 1997). B1 XX TS cells were grown and maintained as described by Mak et al. (2002). TS cell differentiation was carried out as described by Tanaka et al. (1998).

Tg(Cmu-GFP)1Nagy (XGFP) mice were from Jackson Labs. This strain carries a GFP transgene on the X chromosome, and, when inherited on Xp, it allows for XX and XY embryos to be distinguished (Hadjantonakis et al., 1998). The *Xist*^{inv} strain was produced by gene targeting (N.B. and T.B.N., in preparation) and maintained in house. The null mutation was induced by inserting a loxP site 5.9 Kb into the gene, in the opposite orientation to a loxP site retained after deletion of *Xist* exon IV. Deletion of exon IV by itself has no effect on X inactivation. *eed*^{3354SB} mutant mice were obtained from the Oak Ridge National Laboratory. To genotype adult animals we amplified by PCR a 570 bp fragment from genomic DNA using primers eed3 (tgtgcacctcatcagtctctttt) and eed4 (ggtttgcccccagccttatg) (annealed at 55°C). The *eed*^{3354SB} and wild-type alleles could then be distinguished after restriction digestion with Alu1.

Embryo Analysis

Normal preimplantation embryos were obtained from crosses between [C57Bl6 × CBA]F1 females and either [C57Bl6 × CBA]F1 or XGFP males. Embryos were harvested either at 0.5, 2.5, or 3.5 dpc. In some cases embryos isolated at 0.5 and 2.5 dpc were cultured in vitro in M16 medium, as described in Hogan et al. (1994), prior to subsequent analysis. Normal postimplantation stage embryos were obtained from crosses between CD1 or PGK strain females and either PGK strain or XGFP males. Embryos were dissected free of decidua, and, where appropriate, the Reicharts membrane was removed. Embryos from 8.5 dpc and later were dissected free of all membranes.

PCR for sexing/genotype analysis was carried out on DNA from extraembryonic ectoderm or embryo fragments. For analysis of the Tg15 line (Matsuura et al., 1996), Tg15 males were first crossed with XGFP females. Embryos were then obtained from male progeny crossed to [C57Bl6 × CBA]F1 females. Thus, X^{GFP}X females and XY^{Tg15} male embryos could be readily distinguished on the basis of GFP fluorescence.

Antibodies

Characterization of antibodies to Eed, Enx1, Bmi-1, and Hpc2 has been described elsewhere (Satijn et al., 1997; Sewalt et al., 1998; van der Vlag and Otte, 1999). Antisera to di/trimethylH3-K9 are described in Peters et al. (2001). The trimethylH3-K27 antibody is described in detail elsewhere (A.H.F.M.P. and T.J., submitted). Antisera to methylH3-K4 were a kind gift of D. Allis and are described in Boggs et al. (2002). Antisera to HDAC2 and Lamin B were from Santa Cruz Biotechnology. Antisera to acetylH3-K9, acetylH4, and HDAC1 were from Upstate Biotechnology. The following dilutions were used for immunofluorescence (IF) and Western blotting (WB): Eed mouse monoclonal, 1:200 (IF) and 1:1,000 (WB); Enx1 rabbit polyclonal, 1:200 (IF) and 1:2,000 (WB); Bmi-1 rabbit polyclonal, 1:10,000 (WB); Hpc2, 1:500 (WB); Lamin B goat polyclonal, 1:2,000 (WB); HDAC1 rabbit polyclonal, 1:2,000 (WB), HDAC2 rabbit polyclonal, 1:1,000 (WB); methylH3-K9 rabbit polyclonal, 1:1,000 (IF); trimethylH3-K27, 1:500 (IF); methylH3-K4 rabbit polyclonal, 1:1,000 (IF); acetylH3-K9 rabbit polyclonal, 1:200 (IF); acetylH4, 1:200 (IF). Secondary antibodies for immunofluorescence were from Molecular Probes, and those for Western blotting were from Amersham Life Science (anti-rabbit or anti-mouse Ig-HRP) or Dako (anti-goat Ig-HRP).

Western Blot Analysis

Whole-cell extracts were prepared from ES cells, TS cells, and lymphocytes as described by Mermoud et al. (1999). Proteins (approximately 20 μg) were electrophoresed by SDS-PAGE and then transferred to PVDF membranes by wet blotting. ECL detection (Amersham) was carried out according to manufacturer's recommendations.

Immunofluorescence and ImmunoRNA-FISH

Undifferentiated and differentiated ES cell samples were prepared for immunofluorescene as described previously (Mermoud et al., 1999). Immunofluorescence was carried out as described by Mak et al. (2002). For preimplantation embryos and for the 5.5 dpc postimplantation stage, immunofluorescence was carried out by transferring embryos in glass capillaries through wells in round bottom 96-well microtitre dishes. Steps were essentially as described by Mak et al. (2002). After incubation with antibodies, embryos were mounted by transferring into 2 μl drops of Vectashield antifade with DAPI (Vector Laboratories) and placed on glass chamber slides. For 6.5 dpc and later postimplantation stages, dissected embryos (or embryo fragments) were washed in PBS, transferred to 5 μl of trypsin solution, and dissociated into small cell clumps with a fine drawn-glass capillary. After the addition of 95 μl of EC10 medium (Mermoud et al., 1999), cells were placed onto Superfrost Plus glass slides (BDH) and allowed to adhere by incubation in a humidified chamber for approximately 3 hr. Immunofluorescence was then carried out as described in Mak et al. (2002).

ImmunoRNA-FISH was carried out as described by Heard et al. (2001). Details of variations used for specific probe/antibody combinations are available on request. Probes and conditions for detection of nascent transcripts for the *Brx* and *Pgk-1* loci have been described previously (Sheardown et al., 1997; Clerc and Avner, 1998;). Preimplantation embryos were cytospun onto glass slides as described by Costanzi et al. (2000).

The GFP signal in XGFP embryos was detected with a Leica M2FLIII dissection microscope. Slides were analyzed on a Leica TCS-Sp1 confocal microscope and processed with the Leica software and Adobe Photoshop.

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