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Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing

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In mammals X inactivation is initiated by expression of *Xist* RNA and involves the recruitment of Polycomb repressive complex 1 (PRC1) and 2 (PRC2), which mediate chromosome-wide ubiquitination of histone H2A and methylation of histone H3, respectively. Here, we show that PRC1 recruitment by *Xist* RNA is independent of gene silencing. We find that *Eed* is required for the recruitment of the canonical PRC1 proteins Mph1 and Mph2 by *Xist*. However, functional Ring1b is recruited by *Xist* and mediates ubiquitination of histone H2A in *Eed* deficient embryonic stem (ES) cells, which lack histone H3 lysine 27 tri-methylation. *Xist* expression early in ES cell differentiation establishes a chromosomal memory, which allows efficient H2A ubiquitination in differentiated cells and is independent of silencing and PRC2. Our data show that *Xist* recruits PRC1 components by both PRC2 dependent and independent modes and in the absence of PRC2 function is sufficient for the establishment of Polycomb-based memory systems in X inactivation.

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Introduction

Mammals equalise the dosage of X-linked genes between males and females by inactivation of one of the two female X chromosomes early in development. In female mice the paternal X chromosome is silenced in preimplantation embryos giving rise to the imprinted pattern of X inactivation in the extraembryonic lineages. In the cells forming the embryo, the inactive X (Xi) becomes reactivated at the blastocyst stage, followed by random inactivation of either the paternal or the maternal X before gastrulation (Huynh and Lee, 2003; Mak *et al*, 2004; Okamoto *et al*, 2004). Random X inactivation is recapitulated during the differentiation of mouse embryonic stem (ES) cells. The formation of an inactive X chromosome comprises an ordered series of chromatin modifications, including post-translational modifications of histones and the recruitment of Polycomb group (PcG) complexes (Plath *et al*, 2002). Initiation of silencing depends on the expression of the noncoding *Xist* RNA (Borsani *et al*, 1991; Brockdorff *et al*, 1991; Brown *et al*, 1991a, b). However, *Xist* is dispensable for the maintenance of the Xi at later stages of differentiation, when multiple pathways including DNA methylation and hypoacetylation of histone H4 stably propagate the inactive state (Csankovszki *et al*, 2001; Hernandez-Munoz *et al*, 2005). The silent state at the initiation of X chromosome inactivation is initially reversible (Wutz and Jaenisch, 2000) and is associated with chromosome-wide tri-methylation of histone H3 on lysine 27 (H3K27me3), mono-methylation of histone H4 on lysine 20 (H4K20m1) and ubiquitination of lysine 119 on histone H2A (H2AK119ub1) as well as the recruitment of the Polycomb repressive complexes 1 (PRC1) and 2 (PRC2; Cao *et al*, 2002; Plath *et al*, 2003; de Napoles *et al*, 2004; Fang *et al*, 2004; Kohlmaier *et al*, 2004).

PRC2 contains the Ezh2, Eed, Suz12 and RbAp46/48 proteins and has histone H3 specific lysine methylase activity (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002, 2004; Muller *et al*, 2002). Recruitment of PRC2 by *Xist* and appearance of H3K27me3 along the Xi are among the earliest events in X inactivation (Mak *et al*, 2002; Plath *et al*, 2003; Silva *et al*, 2003). This has led to the prevailing view that PRC2 and H3K27me3 have a crucial function in X inactivation. However, recruitment of the PRC2 complex and H3K27me3 also occur in the absence of transcriptional silencing (Plath *et al*, 2003; Kohlmaier *et al*, 2004). In differentiated cells *Xist* is necessary but not sufficient for recruitment of H3K27me3, and thus H3K27me3 also depends on epigenetic information residing on the chromosome (Kohlmaier *et al*, 2004). When *Xist* is expressed during an early time window in differentiation, a chromosomal memory is established that enables efficient histone methylation later in differentiation. This memory is maintained in differentiated cells independent of *Xist* and gene silencing (Kohlmaier *et al*, 2004). Establishment of the memory temporally coincides with the transition from reversible to irreversible silencing, consistent with a role in the maintenance of X inactivation. The observation that recruitment of PRC2 and H3K27me3 is strictly dependent on *Xist* RNA and is reversible excludes PRC2 as a stable component of the memory. However, this finding is compatible with a role of PRC2 in memory establishment.

PcG complexes are thought to maintain a transcriptional memory for several developmental control genes in flies and mammals (Ringrose and Paro, 2004). It has been proposed that PRC2 recruits PRC1 based on the specificity of the chromodomain of Polycomb towards H3K27me3 (Fischle *et al*, 2003; Min *et al*, 2003). *Eed* is required for the maintenance of the paternal Xi exclusively in differentiating extraembryonic trophoblast cells (Wang *et al*, 2001). However, no defect in the maintenance of imprinted X

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inactivation has been observed in *Eed* mutant trophoblast stem cells or extraembryonic endoderm tissue, which lack H3K27me3 (Kalantry *et al*, 2006). In trophoblast stem cells, *Eed* is necessary for *Xist* RNA stabilisation and reactivation of the Xi is observed only after onset of differentiation. The function of *Eed* in the initiation of random X inactivation in embryonic cells has not been studied and its significance in the embryo proper remains unclear.

Here, we test the idea that PRC2 acts to recruit PRC1 in random X inactivation. Contrary to the expectation we find that *Xist* recruits the PRC1 protein Ring1b independent of H3K27me3 and Ring1b acts independently in the establishment of memory systems for the maintenance of X inactivation. This suggests that the present models for PcG complex recruitment in X inactivation need to be revised.

Results

Xist mediated H2A ubiquitination is regulated by a memory in differentiated cells and independent of gene silencing

Biochemically purified mammalian PRC1 consists of several PcG proteins, including Ring1b, and its histone H2A lysine 119 specific ubiquitination activity has been shown (de Napoles *et al*, 2004; Wang *et al*, 2004). To investigate the function of PRC1 in X inactivation we have elucidated the kinetics of H2AK119ub1 in ES cells containing an inducible *Xist* expression system (Figure 1A). In the clone 36 ES cell line, an *Xist* cDNA transgene under control of the doxycycline inducible promoter is inserted into chromosome 11, and recapitulates chromosome-wide silencing (Wutz and Jaenisch, 2000). In ΔSX ES cells, the endogenous *Xist* locus has been modified by a targeted deletion of repeat A sequences of *Xist*, which are required for silencing, and concomitant introduction of an inducible promoter. This achieves inducible expression of a mutant *Xist* RNA, which does not cause gene silencing and thus circumvents the lethality associated with inactivation of the single X chromosome in this male ES cell line (Wutz *et al*, 2002). H2AK119ub1 was established rapidly upon *Xist* induction in undifferentiated clone 36 ES cells. Importantly, induction of the silencing-deficient *Xist* RNA in ΔSX ES cells was also able to establish H2AK119ub1 on the chromosome (Figure 1B), indicating that H2AK119ub1 is not sufficient for gene silencing in X inactivation.

We next studied the kinetics and stability of H2AK119ub1 during ES cell differentiation. We induced *Xist* starting at different time points in differentiating clone 36 ES cells and measured the levels of H2AK119ub1 and H3K27me3 at day 12 of differentiation (Figure 1C and D). In continuous presence of doxycycline, we detected a strong focal H2AK119ub1 signal in 69% of the nuclei, whereas no focus was observed if *Xist* was not induced. When *Xist* was turned off after 8 days of differentiation, focal H2AK119ub1 staining was observed in 7% of the cells on day 12 showing that H2AK119ub1 was reversible and *Xist*-dependent during differentiation. *Xist* induction starting from day 4 in differentiation resulted in low levels of H2AK119ub1 (16%) at day 12 compared to cultures where induction had occurred early. Therefore, in differentiated cells *Xist* is not sufficient for efficient imposition of H2AK119ub1, suggesting that H2A ubiquitination could be regulated by a chromosomal memory similar to H3K27me3. To test this, we induced *Xist* expression during the first 4 days of differentiation in clone 36 ES cells, subsequently turned off *Xist* for 4 days by withdrawing doxycycline and then measured H2AK119ub1 levels after re-induction of *Xist* for 4 more days. H2AK119ub1 staining was observed in 70% of these cells comparable to the percentage after 12 days of differentiation in continuous presence of doxycycline (Figure 1C). We conclude that *Xist* expression during an early time window in ES cell differentiation establishes a memory that is maintained independently of *Xist*. Reinduction of *Xist* in conjunction with this memory allows efficient H2AK119ub1 in differentiated cells. The recruitment of PRC1 mediated H2AK119ub1 therefore parallels the recruitment of PRC2 mediated H3K27me3 (Figure 1D) and could be a result of a dependency of PRC1 recruitment on H3K27me3.

Generation of ES cells lacking Eed

To directly investigate the function of the PRC2 complex in the recruitment of PRC1 at the initiation of X inactivation, we disrupted the *Eed* gene by targeting in clone 36 and ΔSX ES cells. The targeting vector replaced sequences encoding the first and second WD40 domains of the Eed protein with a stop cassette, which terminates transcription resulting in a null allele (Supplementary Figure 1A). After removal of the selection cassette from targeted ES clones by Cre-recombinase mediated deletion, the second allele of *Eed* was targeted using the same strategy. This yielded the cell lines $36^{Eed-/-}$ (clone 1 and 2) and $\Delta SX^{Eed-/-}$, derivatives of clone 36 and ΔSX ES cells, respectively. Northern analysis confirmed the absence of wild-type *Eed* transcripts in these cells (Figure 2A). Two truncated *Eed* RNA species were observed in $Eed^{-/-}$ ES cells consistent with the termination of transcription at the introduced stop cassette. Western analysis revealed that Eed protein was absent in the $Eed^{-/-}$ cell lines (Figure 2B), while in control clone 36 and ΔSX ES cell lines the four Eed isoforms were resolved. We further reconstituted *Eed* expression in $36^{Eed-/-}$ (clone 2) ES cells by introducing a transgene expressing an amino terminal fusion of the enhanced green fluorescent protein (EGFP) with the short Eed isoform. In these 36^{EedTG} ES cells, we observed one protein migrating with the expected molecular weight of the EGFP-Eed fusion protein and a faster migrating product likely due to proteolysis (Figure 2B). In *Eed* deficient ES cells, *Suz12* RNA levels were reduced whereas steady-state levels of the *Ezh2* transcripts remained unchanged compared to control cell lines (Figure 2A). Western analysis revealed that Ezh2 is drastically reduced below detection limit and Suz12 was found in reduced amount in $Eed^{-/-}$ cells (Figure 2B). In 36^{EedTG} cells, *Ezh2* and *Suz12* RNA and protein levels were rescued confirming that the effect was specific and caused by the lack of Eed (Figure 2B, and data not shown).

Eed deficient $36^{Eed-/-}$ and $\Delta SX^{Eed-/-}$ ES cells showed a reduced ability to form colonies compared to control 36 and ΔSX ES cells but proliferation and self-renewal of ES cells was largely independent of *Eed* (Supplementary Figure 1B and C). Furthermore, the plating efficiency is rescued in 36^{EedTG} ES cells showing that the defect is specific and due to lack of *Eed*. $Eed^{-/-}$ ES cells could be induced to differentiate with retinoic acid, but showed a reduced developmental potential indicated by the formation of irregular shaped embroid bodies and the absence of contractile structures indicative of cardiomyocytes in embroid body outgrowths (Supplementary Figure 1D and E, and data not shown).

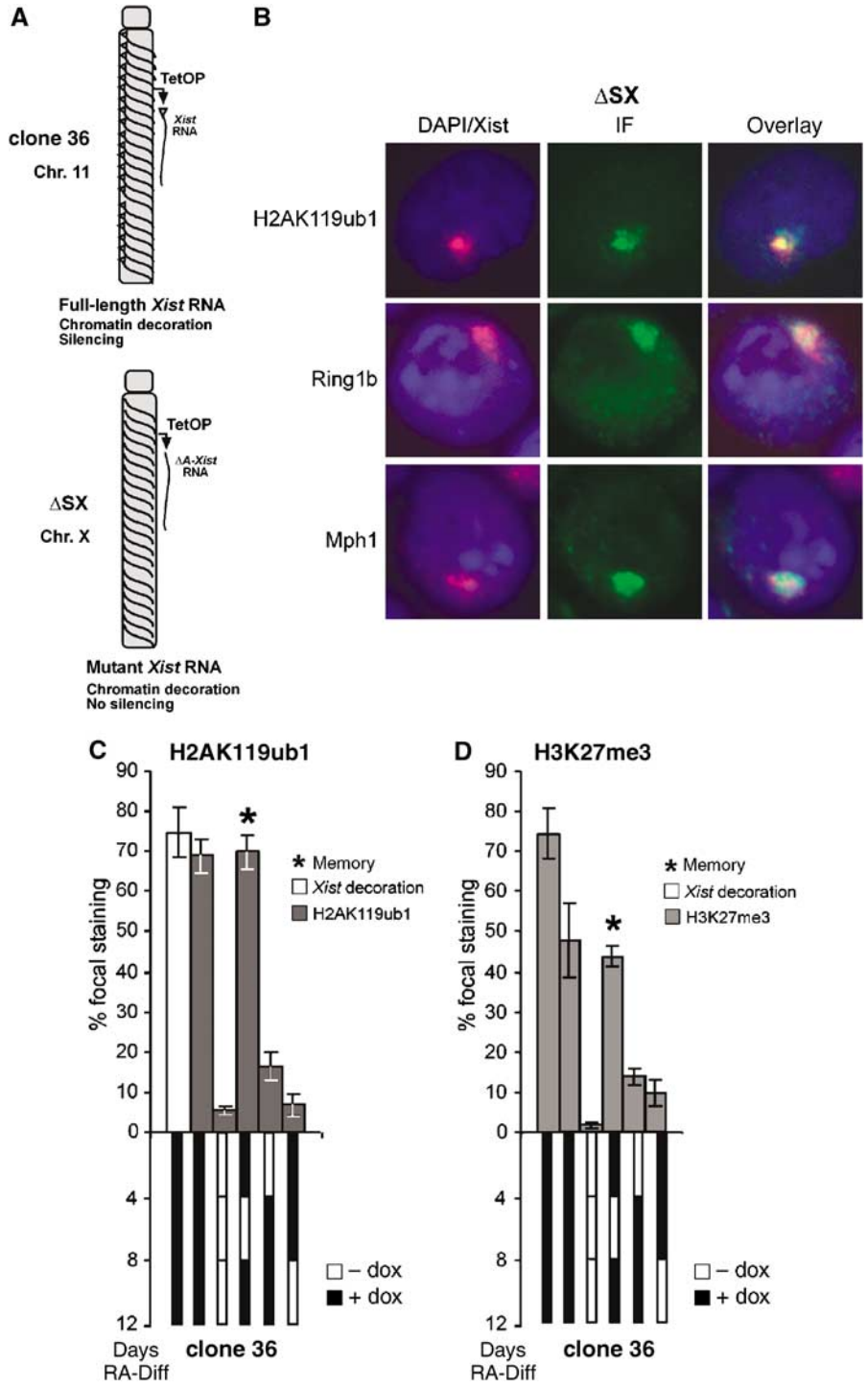

Figure 1 PRC1 recruitment by *Xist*. (**A**) Overview of the inducible *Xist* expression system (TetOP) on chromosome 11 and the X in clone 36 and ΔSX ES cells, respectively. In clone 36 ES cells, *Xist* induction silences a linked puromycin marker gene (puro). In ΔSX cells, the A repeat of *Xist* (triangle) is deleted. (**B**) Recruitment of the PRC1 components Ring1b and Mph1 as well as resulting H2AK119ub1 was observed by combined Xist RNA FISH (red) and immunofluorescence analysis (green) in undifferentiated ΔSX ES cells after 3 days *Xist* induction. (**C**) H2AK119ub1 is regulated by a chromosomal memory in differentiated cells. Bar graphs representing the percentage of nuclei with focal H2AK119ub1 signals (grey bars) and *Xist* RNA (white bars) is given (above). Error bars represent the standard deviation. Below a scheme of the ES cell differentiation time course showing the presence (black) or absence (white) of doxycycline. An asterisk marks the *Xist* induction scheme revealing the chromosomal memory. (**D**) Analysis of H3K27me3 in parallel cultures to (C).

Xist recruits Suz12 independent of functional PRC2

Western analysis of *Eed* deficient ES cells revealed reduced Suz12 protein levels compared to control 36 ES cells and a loss of Ezh2 protein (Figure 2B). This was verified by combined immunofluorescence *Xist* RNA fluorescence *in situ* hybridisation (FISH) analysis on ES cells, after 3 days of *Xist* induction with doxycycline. In control clone 36 ES cells, 89, 79 and 88% of cells showed colocalisation of *Xist* RNA with Eed, Ezh2 and Suz12, respectively (Figure 2C–E; Table I). In *Eed* deficient $36^{Eed-/-}$ ES cells *Xist* RNA showed

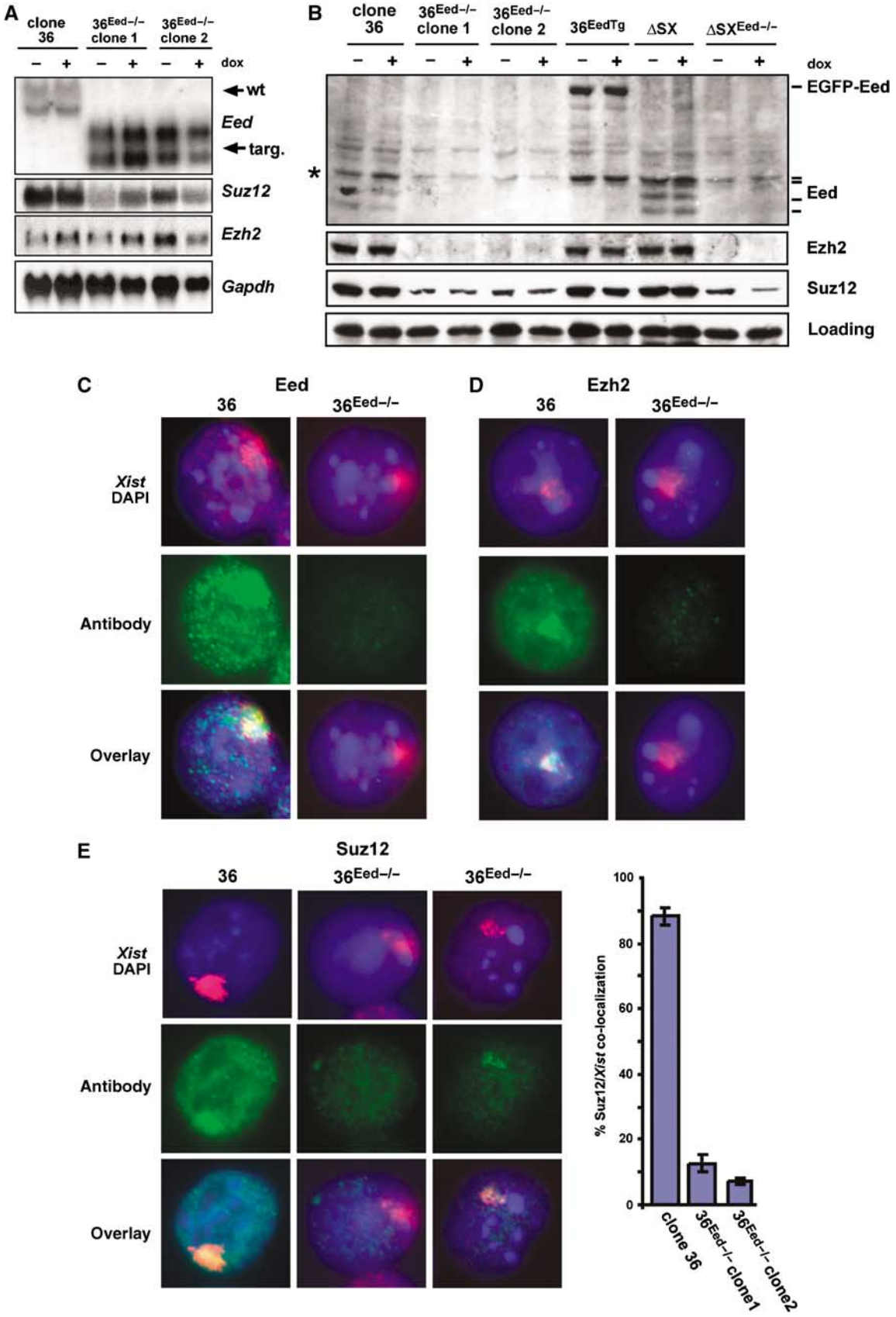

Figure 2 Generation of ES cells lacking *Eed*. (**A**) Northern analysis of *Eed*, *Suz12* and *Ezh2* in undifferentiated control clone 36 and Eed deficient 36Eed$^{-/-}$ ES cells after *Xist* was induced for 3 days (+) or not (−); *Gapdh* as loading control. (**B**) Western analysis of Eed, Ezh2 and Suz12 in nuclear extracts from uninduced ES cells (−) or induced for 3 days (+). hnRNP A as loading control, asterisk indicates a nonspecific band. (**C–E**) Indirect immunofluorescence (green) of Eed (C), Ezh2 (D) or Suz12 (E) and subsequent *Xist* RNA FISH (red) of representative nuclei of undifferentiated 36$^{Eed-/-}$ and control clone 36 ES cells after 3 days of *Xist* induction. DAPI (blue) stains DNA. Statistics of the number of nuclei showing colocalisation of Suz12 staining with *Xist* in 36 and 36$^{Eed-/-}$ ES cells. Error bars indicate standard deviation ($n > 600$).

normal localisation and no signal for Eed and Ezh2 was detected consistent with the loss of these proteins (Figure 2C and D). The Suz12 signal was markedly decreased in *Eed* deficient cells. However, we observed colocalisation of Suz12 with *Xist* RNA in 13 and 7% of $36^{Eed-/-}$ clone 1 and clone 2 ES cells, respectively (Figure 2E). This demonstrates that recruitment of Suz12 by *Xist* RNA can occur, at least in part, independent of Ezh2 and Eed suggesting a role for Suz12 in PRC2 recruitment in X inactivation.

Xist recruits PRC1 independent of Eed and H3K27me3 in ES cells

To study the chromosomal marks at the initiation of X inactivation in *Eed* deficient ES cells, we performed combined *Xist* RNA FISH immunofluorescence analysis on $36^{Eed-/-}$ and control ES cells (Figure 3 and Table I). After *Xist* induction for 3 days, we observed a strong focal H3K27me3 staining colocalising with *Xist* RNA in clone 36 ES cells. However, in $36^{Eed-/-}$ ES cells di- and tri-methylation of H3K27 were drastically reduced and no colocalisation with *Xist* was observed consistent with a loss of PRC2 function in these cells (Figure 3B and C). A faint H3K27me3 signal was still observed at pericentric heterochromatin possibly due to weak cross-reactivity of the antibody with H4K20me3 (Peters *et al*, 2003). We detected a robust H3K27me1 signal at pericentric heterochromatin in $36^{Eed-/-}$ cells comparable to controls (Figure 3A). In 36^{EedTG} ES cells transgenic expression of EGFP-Eed rescued H3K27me3 (Supplementary Figure 1F).

H2AK119ub1 and H4K20me1 are two marks associated with the initiation of X inactivation. H2AK119ub1 colocalised with *Xist* RNA in 97 and 98% of clone 36 and $36^{Eed-/-}$ ES cells, respectively (Figure 3E). A robust H4K20me1 signal colocalising with *Xist* RNA was detectable in 82% control clone 36 ES cells. In $36^{Eed-/-}$ ES cells, the H4K20me1 signal appeared less intense and was detected in 50% (clone 1) and 36% (clone 2) cells (Figure 3D). We conclude that ubiquitination of H2A on lysine 119 is independent of *Eed*, but PRC2 function supports the establishment of H4K20me1 by *Xist* (Table I).

We observed normal H2A ubiquitination upon *Xist* expression in *Eed* deficient cells, which in ES cells is thought to be mediated by Ring1b, a core component of PRC1 (Figure 3E). To assess if PRC1 was indeed recruited by *Xist* independent of PRC2, we performed immunofluorescence analysis using antisera specific for the PRC1 core components Ring1b, Mph1 and Mph2. Colocalisation of Ring1b with *Xist* RNA was observed in ES cells independent of *Eed* (Figure 4A). The Mph1 signal colocalised with *Xist* in 48% of control 36 ES cells, but no colocalisation was observed in $36^{Eed-/-}$ ES cells (Figure 4B). Colocalisation of Mph2 with *Xist* RNA was observed only in differentiated cells (Figure 4C), and was detected in 33% of clone 36 but not in Eed deficient $36^{Eed-/-}$ ES cells on day 8 of differentiation. We conclude that recruitment of Mph1 and Mph2 by *Xist* is dependent on PRC2 function, but Ring1b is recruited independently of PRC2, Mph1 and Mph2. Despite the lack of detectable Mph1 and Mph2 recruitment, the Ring1b protein is enzymatically active as shown by ubiquitination of H2A.

PRC2 is critical for H3K27me2 and H3K27me3 in ES cells

To assess if disruption of *Eed* in $36^{Eed-/-}$ and $\Delta SX^{Eed-/-}$ ES cells indeed caused a loss of PRC2 function, we performed an analysis of histone modifications. By Western analysis H3K27me2 and H3K27me3 were lost in $36^{Eed-/-}$ and $\Delta SX^{Eed-/-}$ ES cells, but we found mono-methylation of H3K27 only slightly reduced consistent with our immunofluorescence data (Figure 3F). The mono-, di- and tri-methylation states of histone H3 lysine 9 or of H4 lysine 20, and ubiquitination of histone H2A lysine 119 were not altered in *Eed* deficient ES cells (data not shown).

To further quantify the histone methylation marks, we performed a mass spectrometric analysis of nuclear extracts prepared from undifferentiated $36^{Eed-/-}$ and $\Delta SX^{Eed-/-}$ and control ES cells. In control 36 ES cells, 17% of bulk histone H3 were mono-methylated, 58% di-methylated and 14% tri-methylated on lysine 27 (Figure 3G) consistent with previous reports (Peters *et al*, 2003). In *Eed* deficient ES cells, H3K27me3 and H3K27me2 were dramatically reduced compared to controls, but only a moderate reduction in the H3K27me1 signal was observed (Figure 3G). The loss of H3K27 di- and tri-methylation in *Eed* deficient ES cells resulted in a concomitant increase in unmodified but not mono-methylated H3K27. Di- and tri-methylation of H3K27 was restored in 36^{EedTG} ES cells to 42 and 7%, corresponding to 72 and 50% of wild-type levels, respectively (Figure 3G). The methylation levels of H3K9 or H4K20 were unchanged by the absence of Eed (Supplementary Figures 2B and 3). However, H3K36me2 levels were significantly reduced from 50% in control clone 36 ES cells to 34% in $36^{Eed-/-}$ ES cells, and 31% in $\Delta SX^{Eed-/-}$ ES cells (Supplementary Figures 2A and 5). Restoration of H3K36me2 levels in 36^{EedTG} to 43% demonstrated that the PRC2 complex regulates global H3K36me2 marks.

We conclude that in ES cells PRC2 is crucial for H3K27 di- and tri-methylation, but has no detectable contribution to H3K9 methylation. The H3K27me1 mark on pericentric heterochromatin was unaffected in *Eed* deficient ES cells (Figure 3A).

Table I PcG proteins and histone modifications recruited by *Xist*

	Eed	Ezh2	Suz12	Ring1b	Mph1	Mph2*	H3K27me3	H4K20me1	H2AK119ub1
36	89±3% $n=624$	79±9% $n=346$	88±3% $n=629$	56±7% $n=368$	48±11% $n=502$	33±8% $n=470$	96±1% $n=346$	82±13% $n=410$	97±2% $n=488$
$36^{Eed-/-}$ Clone1	0 $n=510$	0 $n>600$	13±2% $n=624$	ND	ND	ND	0 $n>600$	50±10% $n=227$	98±0% $n=479$
$36^{Eed-/-}$ Clone2	0 $n=634$	0 $n>600$	7±1% $n=629$	53±8% $n=478$	0 $n=650$	0 $n=456$	0 $n>600$	36±7% $n=224$	ND

The percentage of focal signals colocalising with *Xist* RNA in ES cells treated with doxycycline for 3 days, or after 8 days of differentiation in the presence of doxycycline (*). Mean±s.d. of three independent slides and the total number of nuclei counted (n) are indicated.

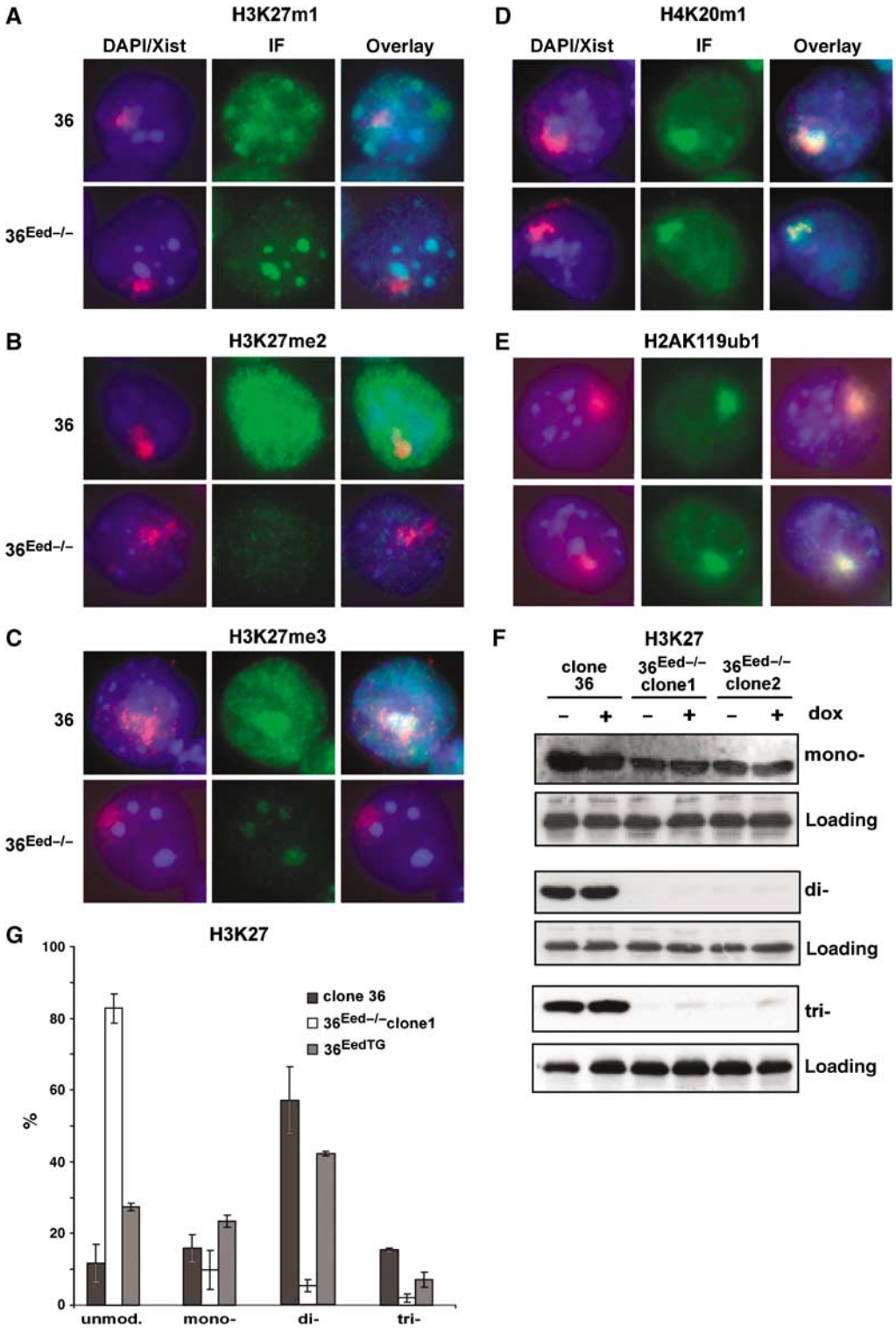

Figure 3 Histone modifications in *Eed* deficient ES cells. (**A–E**) Combined *Xist* RNA FISH (red) indirect immunofluorescence of indicated histone modifications (green) analysis on undifferentiated $36^{Eed-/-}$ and control 36 ES cells after 3 days *Xist* expression. Representative images are shown, statistics see Table I. (**F**) Western analysis of mono-, di- and tri-methylation of H3K27 in $36^{Eed-/-}$ clones 1 and 2 and control 36 ES cells after *Xist* induction for 3 days (+) or not (−); loading control hnRNP A. (**G**) Mass-spectrometric analysis of histone H3 lysine 27 methylation in clone 36, $36^{Eed-/-}$ and 36^{EedTG} ES cells. The percentage of the indicated modification state is given for three independent experiments; error bars indicate standard deviation.

Initiation of silencing by Xist is independent of PRC2

In clone 36 ES cells inducible *Xist* expression causes reversible silencing of a puromycin resistance gene, which was cointegrated with the *Xist* cDNA transgene on chromosome 11 (Wutz and Jaenisch, 2000). To establish whether *Eed* is required for initiation of silencing, we induced *Xist* expression in $36^{Eed-/-}$ ES cells for 3 days and analysed puromycin resistance gene expression by Northern (Figure 5A). Silencing

was equally efficient in control 36, 36^{EedTG} and *Eed* deficient ES cells, demonstrating that Eed and H3K27me3 are dispensable for initiation of silencing by *Xist*.

To investigate the role of *Eed* for the maintenance of silencing, we induced *Xist* in differentiating $36^{Eed-/-}$ and control 36 ES cells. In retinoic acid differentiated cells in the presence of doxycycline for 8 days or for 4 days followed by 4 days without *Xist* induction, we observed efficient maintenance of silencing of the puromycin gene compared to cultures, in which *Xist* had not been induced (Figure 5B). Notably, there was no difference between *Eed* deficient $36^{Eed-/-}$ and control 36 ES cells demonstrating that the shift from reversible to irreversible gene silencing had occurred in the absence of PRC2 function. To test the function of *Eed* for the maintenance of silencing in a more physiological differentiation model, we established embryoid body outgrowth cultures from *Eed* deficient $36^{Eed-/-}$ and control 36 ES cells in the presence or absence of doxycycline and measured expression of the puromycine marker gene after 4 weeks of differentiation. Northern analysis revealed that silencing was maintained in the absence of *Eed* (Figure 5C). Finally to establish that long-range silencing was maintained

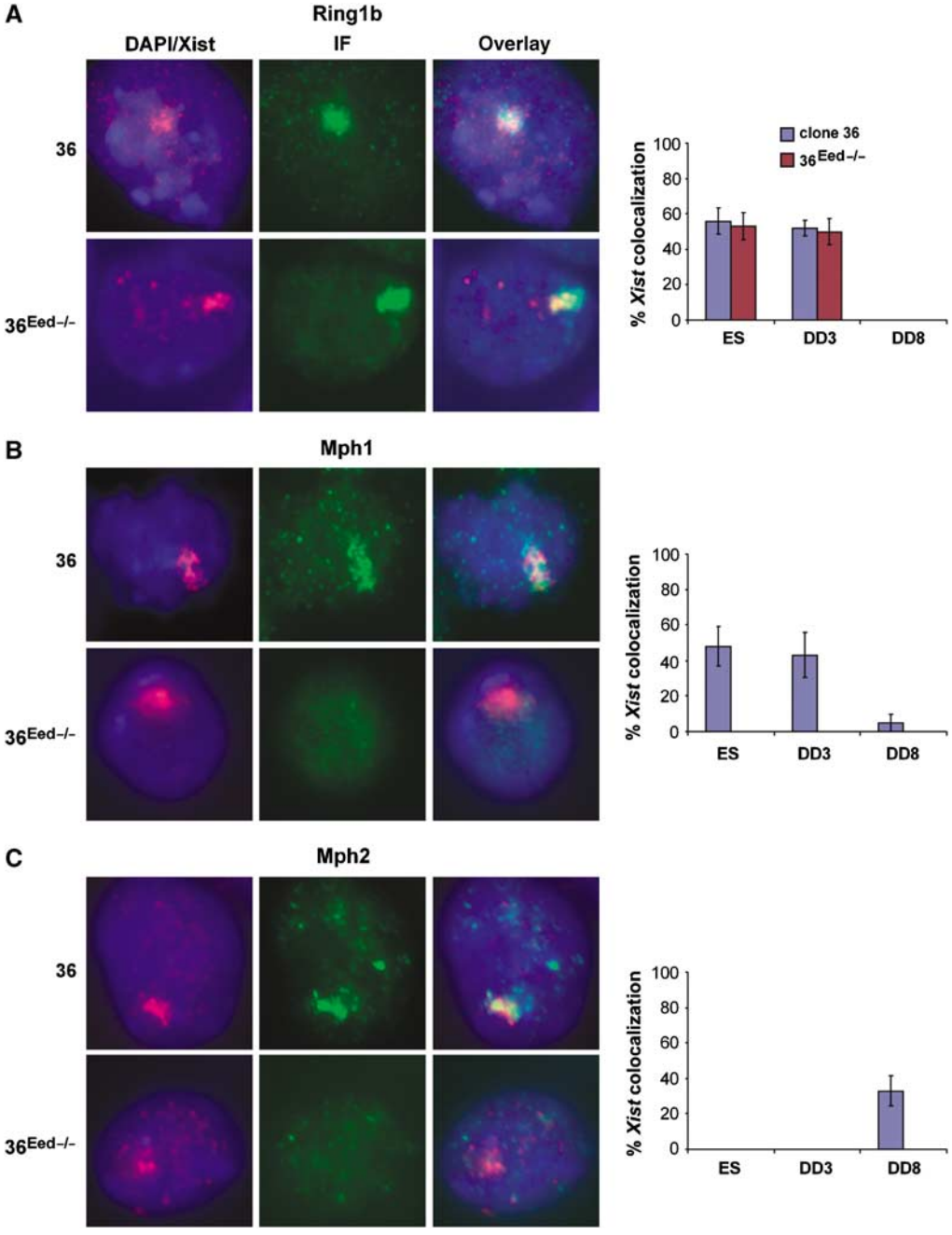

Figure 4 Recruitment of PRC1 components in the absence of Eed. (**A, B**) Indirect immunofluorescence (IF) of Ring1b (A), Mph1 (B) and subsequent *Xist* RNA FISH (red) analysis on undifferentiated $36^{Eed-/-}$ and control clone 36 ES cells after *Xist* expression for 3 days. (**C**) Analysis for Mph2 in ES cells differentiated for 8 days in the presence of doxycycline. The percentage of nuclei showing focal IF staining colocalising with *Xist* RNA is given for undifferentiated (ES), day 3 (DD3) and day 8 (DD8) of differentiation. Error bars represent standard deviation ($n > 350$).

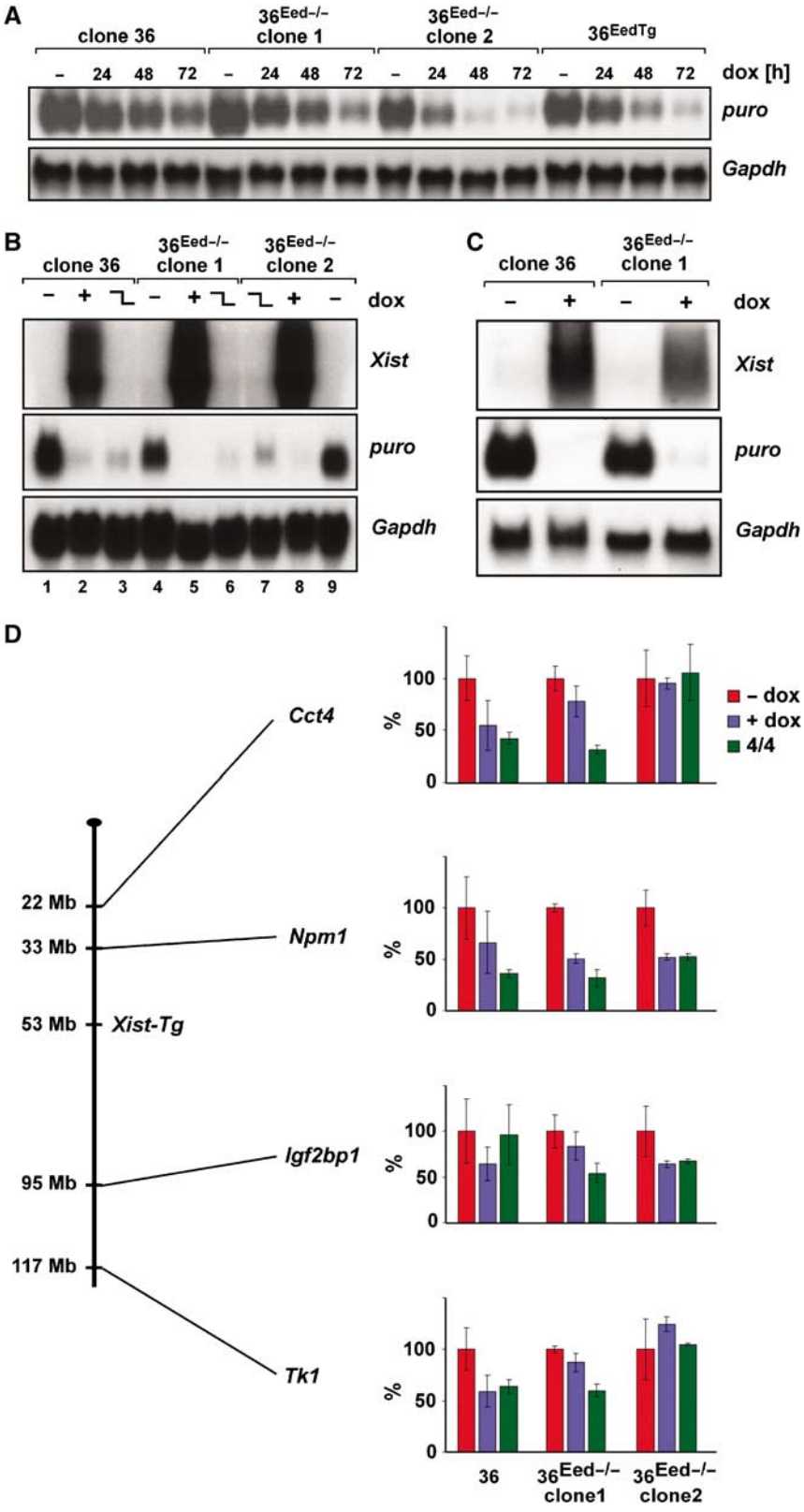

Figure 5 Initiation and maintenance of silencing independent of *Eed*. (**A**) Northern analysis of PGKpuromycin, (puro) silencing in $36^{Eed-/-}$ and control clone 36 cells after *Xist* induction for 24, 48 and 72 h; *Gapdh* as loading control. (**B**) Maintenance of puro silencing in cells differentiated in the presence (+; lanes 2, 5 and 8) or absence (−; 1, 4 and 9) doxycycline, or differentiated for 4 days in the presence followed by 4 days in the absence of doxycycline (lanes 3, 6 and 7). (**C**) Northern analysis of puro expression in embryoid bodies outgrowths established in the presence of doxycycline (+) or without (−) after 4 weeks. (**D**) Quantitative expression analysis of *Cct4*, *Npm1*, *Igf2b* and *Tk1* on chromosome 11 in control 36 and $36Eed^{-/-}$ ES cells at day 8 of differentiation in the absence (red bars), continuous presence (blue bars) of doxycycline, or presence of doxycycline for the first 4 days (green bars). Means of three independent measurements normalised to *Gapdh* are shown, error bars represent standard deviation. Scheme on the left shows the genes relative to the *Xist* transgene.

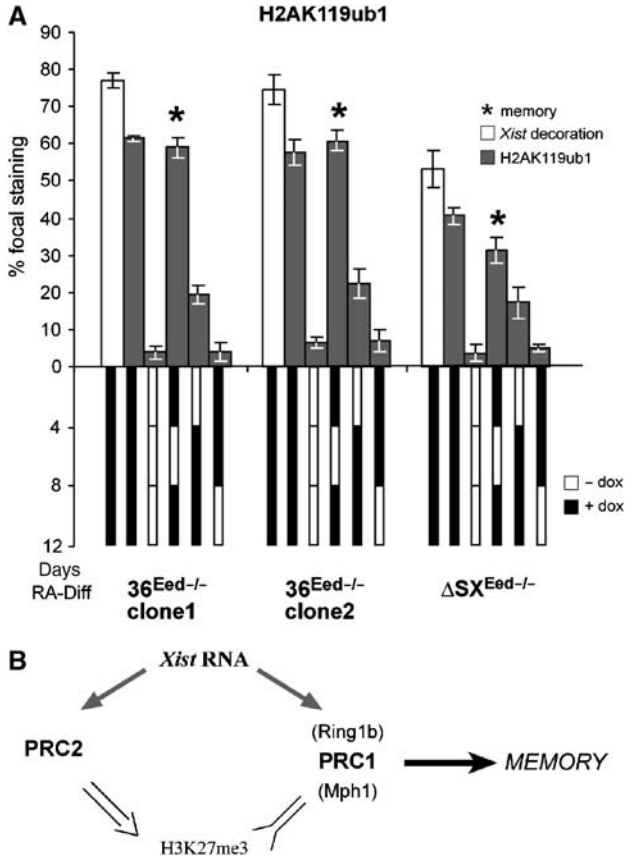

Figure 6 A chromosomal memory independent of silencing and *Eed*. (**A**) The percentage of nuclei showing focal staining for H2AK119ub1 or H3K27me3 (grey bars) and *Xist* RNA (white bars) is given (above). Error bars represent the standard deviation. Underneath the ES cell differentiation time course is depicted and the presence (black) or absence (white) of doxycycline is indicated. The *Xist* induction scheme revealing a chromosomal memory for H2AK119ub1 and H3K27me3 is marked by an asterisk. (**B**) Summary of recruitment of PRC1 and PRC2 by *Xist* in X inactivation.

as opposed to merely silencing of the marker gene in proximity of the *Xist* transgene, we established differentiated cultures by induction with retinoic acid and measured expression of genes on chromosome 11 by quantitative PCR analysis. The *Npm1* gene, which is located 20 Mb from the transgene integration site, was repressed by *Xist* on day 8 of differentiation in $36^{Eed-/-}$ as well as in control cells to approximately 50%. *Npm1* repression was also maintained in cells that were differentiated in the presence of doxycycline for 4 days followed by 4 days without (Figure 5D). The repression of three other genes *Cct4*, *Igf2b1* and *Tk1* in differentiated cells showed the same trend but was more variable, probably because of heterogeneous regulation in the differentiated cultures. We conclude that *Eed* is not required for the initiation of *Xist* mediated silencing, and that PRC2 function and H3K27me3 are dispensable for the maintenance of long-range silencing.

Memory recruitment for H2AK119ub1 is independent of PRC2 function

We observed that H2AK119ub1 is regulated by a chromosomal memory in differentiated cells. To investigate whether this memory would be established in *Eed* deficient ES cells and could still contribute in this context to *Xist*-mediated silencing, we analysed the establishment of H2AK119ub1 in *Eed* deficient $36^{Eed-/-}$ (clone 1 and 2) and $\Delta SX^{Eed-/-}$ ES cells (Figure 6A). The latter express a mutant *Xist* RNA that does not cause transcriptional repression, thus allowing us to follow memory establishment on an active chromosome. In all ES cell lines, *Xist* expression at early differentiation enabled efficient H2AK119ub1 at later time points comparable to control clone 36 ES cells (Figures 1C and 6A). We induced *Xist* expression for 4 days beginning at the start of differentiation, followed by withdrawal of doxycycline for 4 days. After this *Xist* RNA and H2AK119ub1 had been lost from the chromosome and then *Xist* expression was re-induced for 4 more days. In these cells we observed efficient re-ubiquitination (70, 59, 60 and 31% in 36, $36^{Eed-/-}$ clone 1, $36^{Eed-/-}$ clone 2 and $\Delta SX^{Eed-/-}$ ES cells, respectively). This is comparable to cells, which were differentiated in continuous presence of doxycycline (69, 61, 58 and 41%). Importantly, efficient re-establishment of H2AK119ub1 was observed in differentiated $\Delta SX^{Eed-/-}$ ES cells and was therefore independent of silencing. In contrast, *Xist* induction starting at day 4 in differentiation resulted in focal H2AK119ub1 staining in a low percentage of cells (16, 19, 22 and 17%). Moreover, when *Xist* expression was turned off by withdrawing doxycycline from the medium H2AK119ub1 was lost from the chromosome at all time points examined in ES cell differentiation (Figure 6, and data not shown). We conclude that *Xist* expression establishes a chromosomal memory independent of *Eed* and gene silencing suggesting a possible explanation for maintenance of X inactivation in *Eed* deficient embryonic cells.

Discussion

PRC1 recruitment in X inactivation is strictly dependent on Xist RNA

Using an inducible *Xist* expression system we have analysed the recruitment of PRC1 function in X inactivation. We find that *Xist* recruits Ring1b and concomitant H2AK119ub1 independent of transcriptional silencing. Recruitment of Polycomb complexes has been associated with heritable silencing of genes (Ringrose and Paro, 2004). We find that PRC1 and PRC2 also associate in the absence of gene silencing with the chromosome expressing *Xist*. Polycomb recruitment alone is therefore not sufficient for transcriptional repression in X inactivation. This is consistent with data in the fly where loading of PcG proteins onto Polycomb response elements (PREs) precedes the silencing of developmental control genes (Orlando *et al*, 1998). Polycomb binding and H3K27me3 on PREs has been observed independent of silencing (Ringrose *et al*, 2004) and loss of dRING function leads to derepression of genes despite of persistence of H3K27me3 (Wang *et al*, 2004). Alternatively, coordinate loading of PcG complexes on the promoter and a PRE could be required for repression. It is tempting to speculate that in X inactivation *Xist* repeat A acts as a signal to repress gene expression, thereby enabling recruitment of promoters to the PcG territory of the chromosome. In ΔSX ES cells, promoters would then be predicted not to associate with the repressive PcG territory established by the silencing deficient *Xist* RNA lacking repeat A.

We find that PRC1 recruitment is dependent on *Xist* RNA localisation and is reversible throughout ES cell differentiation when *Xist* is turned off. From this we conclude that PRC1 is not stable once loaded onto the chromosome, but depends on *Xist* and a chromosomal memory. Consistent with this, dynamic turnover of PRC1 has been observed on chromatin in the fly (Ringrose *et al*, 2004; Ficz *et al*, 2005). In striking contrast to the fly, where noncoding RNA transcription over PREs has been associated with gene activation (Schmitt *et al*, 2005; Sanchez-Elsner *et al*, 2006), in X inactivation the noncoding *Xist* RNA is associated with the repressed state. This suggests different mechanisms for Polycomb loading in the fly and in X inactivation and demonstrates a novel strictly RNA dependent recruitment mode for mammalian PRC1.

H2AK119ub1 activity of PRC1 does not require Mph1 or Mph2

Using *Eed*-deficient ES cells we show that the recruitment of the PRC1 core proteins Mph1 and Mph2 by *Xist* is dependent on *Eed*. This observation is consistent with the idea that PRC2 has a recruitment function for PRC1 components. A fly PRC1 core complex has been reconstituted containing the four components Psc, Pc, Ph and dRing (Francis *et al*, 2001). A similar composition has been proposed for mammalian PRC1 like complexes based on purification and reconstitution experiments (Lavigne *et al*, 2004; Wang *et al*, 2004). However, we observe in *Eed* deficient ES cells that Ring1b is not only recruited by *Xist* in the absence of the PRC1 core components Mph1 and Mph2 but also appears to be functional as demonstrated by the concomitant ubiquitination of lysine 119 on histone H2A. Thus, in X inactivation Ring1b is either functional alone and can be recruited independently of other PRC1 members by *Xist*, or is part of a distinct complex that is yet to be identified (Ogawa *et al*, 2002; Dou *et al*, 2005; Isono *et al*, 2005b).

Based on our data we therefore propose two mechanisms for recruitment of PRC1 function by *Xist* in X inactivation (Figure 6b). A PRC2 dependent mode involves the binding of the Polycomb chromodomain to the H3K27me3 mark and operates via Mph1 or Mph2. This is predicted from biochemical evidence that H3K27me3 acts as a affinity signal recognised by the chromodomain of mammalian homologues of Polycomb (Fischle *et al*, 2003; Min *et al*, 2003). Our data provide evidence for a second mode of recruitment for PRC1 function. In the absence of PRC2 function, *Xist* can recruit Ring1b independent of the PRC1 core proteins Mph1 or Mph2. Both recruitment modes for PRC1 by *Xist* act synergistically to mediate H2AK119ub1 in the initiation of X inactivation.

Ring1b and PRC2 are regulated by a chromosomal memory

Establishment of H2AK119ub1 is restricted to an early time window in ES cell differentiation such that little H2AK119ub1 is imposed if *Xist* is induced at late time points in ES cell differentiation. Thus, *Xist* expression during an early window in differentiation establishes a chromosomal memory that in differentiated cells is required for H2AK119ub1. This memory is established at the time when X inactivation becomes irreversible and is stably maintained independent of *Xist* expression. To date, the molecular nature of this chromosomal memory is unknown. Our data demonstrate that the memory regulating the imposition of H2AK119ub1 and H3K27me3 in differentiated cells is independent of silencing. Recruitment of both PRC1 and PRC2 is also dependent on *Xist* RNA localisation and both histone marks are lost from the chromosome when *Xist* is turned off. Hence, H2AK119ub1 and H3K27me3 are reversible modifications and depend on *Xist* expression. We conclude that PRC1 and PRC2 are not stably maintained on the Xi throughout ES cell differentiation and can be excluded as integral components of the memory. Yet, the recruitment of PcG proteins early in X inactivation is consistent with a role in the establishment of a special chromatin structure that functions as chromosomal memory. Importantly, our data demonstrate that once established this chromatin structure is self-perpetuating and stable in the absence of *Xist*, PRC1 and PRC2. We show that a chromosomal memory regulating H2AK119ub1 is established independent of PRC2, Mph1 and Mph2.

Eed and H3K27me3 are not crucial for X inactivation in embryonic cells

Disruption of *Eed* in ES cells caused a lack Eed and Ezh2 protein and reduced levels of Suz12 consistent with earlier reports (Pasini *et al*, 2004; Montgomery *et al*, 2005). In the absence of Eed the levels of Ezh2 protein, which contains the catalytically active SET domain required for PRC2 histone methylase function, are reduced below detection. This could be the result of impaired translation or enhanced turnover of Ezh2 protein in the absence of Eed. In support of this notion, the disruption of PRC2 function in *Eed* deficient ES cells is clearly demonstrated by the loss of H3K27me3 in *Eed* deficient ES cells. Interestingly, we find that *Xist* RNA can recruit Suz12 independent of a functional PRC2 complex. Suz12 is a core component of the biochemically purified PRC2 complex, suggesting that PRC2 might be recruited at least in part via Suz12 in X inactivation. Consistent with this Suz12 also has roles in position effect variegation in fly and thus can act independent of PRC2 (Birve *et al*, 2001).

Western, immunofluorescence and mass spectrometric analyses show that disruption of PRC2 function leads to a specific loss of di- and tri- but not mono-methylation of H3K27 *in vivo* without affecting global levels of H3K9 methylation. This finding is consistent with and extends data from *Suz12* deficient embryos (Pasini *et al*, 2004). Notably, the H3K27me1 marks at pericentric heterochromatin are not affected by loss of PRC2 function consistent with an independent regulation. In ES cells, *Xist* expression leads to rapid establishment of H3K27me3 along the chromosome, which requires PRC2 function. From mass spectrometric data we obtained a rough estimate that induction of *Xist* causes an approximately seven-fold increase in H3K27me3. Such an increase would require that 90% of the nucleosomes of the *Xist* expressing chromosome are tri-methylated on H3 lysine 27, compared to 14% total nuclear average (Supplementary Figure 2; Peters *et al*, 2003). Given that in bulk chromatin 60% of histone H3 is di-methylated on lysine 27, the effect of *Xist* is a shift from di- to a tri-methyl marks that could provide increased affinity for PRC1. Our observation that recruitment of Mph1 and Mph2 by *Xist* is abolished in the absence of PRC2 supports this view.

Reactivation of the paternal Xi was observed previously in differentiating trophoblast stem cells in *Eed* deficient embryos, indicating a role for PRC2 in maintenance of X inactivation (Wang *et al*, 2001). However, maintenance of the

Xi in trophoblast stem cells and extraembryonic endoderm is not affected by a mutation in *Eed* (Kalantry *et al*, 2006). Imprinted X inactivation is initiated very early in embryogenesis and a maternal contribution of Eed could possibly function early in the initiation of imprinted X inactivation in *Eed* mutant embryos. Using *Eed* deficient ES cells, we can rule out PRC2 function at the initiation of *Xist* mediated silencing in embryonic cells. *Xist* expression in ES cells lacking functional PRC2 fails to establish H3K27me3 and recruit Mph1 and Mph2. However, in the absence of *Eed*, stable X inactivation can still be achieved. This unexpected finding suggests that functionally redundant mechanisms compensate for the loss of PRC2 function to maintain *Xist* mediated silencing in ES cell differentiation. PRC1 and PRC2 function independently in gene regulation as indicated by the requirement of both *Eed* and *Ring1b* for embryonic development (Wang *et al*, 2002; Voncken *et al*, 2003). Our data show that Ring1b can be recruited by *Xist* independent of PRC2. This recruitment of PRC1 function provides a likely explanation for the lack of an obvious defect on Xi maintenance in *Eed* deficient embryonic cells. This is in contrast to PRC2 action in the regulation of other genes, where a recruitment function of PRC2 is essential (Zhang *et al*, 2004). The requirement of PRC2 for recruitment of some PRC1 components is also observed in X inactivation as *Xist* is unable to recruit Mph1 and Mph2 in the absence of Eed. In conclusion, we find that *Xist* can establish a chromatin structure that mediates a chromosomal memory in X inactivation independent of PRC2, suggesting the masking of a more dramatic defect in the maintenance of X inactivation in *Eed* deficient cells by an PRC2 independent mechanism for recruitment of PRC1 function by *Xist*. Future studies will be directed to establish the interplay between transcriptional silencing and the PcG complex mediated chromosomal memory during X inactivation.

Materials and methods

Cell culture and generation of ES cell lines

ES cells were cultured as described previously (Wutz and Jaenisch, 2000). *Xist* expression was induced by the addition of 1 μg/ml of doxycycline. Differentiation medium contained 100 nM all-*trans*-retinoic acid and no LIF. Embryoid bodies were generated by the hanging drop method in medium without LIF. After 2 days aggregates were pooled and cultured in suspension for 3 days and subsequently plated on gelatin-coated culture dishes for 3 weeks. Cell numbers were determined using a Casy 1 cell counter (Schaerfe System GmbH, Germany).

For construction of the *Eed* targeting vector, a 12 kb *Xho*I–*Cla*I genomic fragment was subcloned from a BAC isolated from the RPCII22 129 mouse BAC library (CHORI). The 2.8 kb *Sac*I–*Eco*RI fragment containing three exons coding for WD40 domains 1 and 2 of the Eed protein were replaced by a stop cassette containing the adenoviral splice acceptor and polyadenylation signal separated by a loxP-flanked hygromycin-thymidine kinase selection cassette. Finally, a diphtheria toxin A chain cassette was inserted for counter selection of random insertions (see Figure 1B). Targeted clones were identified after selection with Hygromycin B (130 μg/ml) by Southern analysis of *Eco*RV digested DNA using probe pEed by a 12 kb band (wild-type band runs at 23 kb). The targeting frequency was between 17 and 37%. After Cre recombinase mediated excision of the selection cassette, the second allele was targeted using the same strategy yielding $Eed^{-/-}$ cells. For pCAG–EGFP-Eed-IREShygPA the short Eed isoform, corresponding to the human isoform 3 (Kuzmichev *et al*, 2004), was tagged with EGFP at the N-terminus and cloned into pCAG-IREShygPA. $36^{Eed-/-}$ clone 2 ES cells were electroporated with 50 μg of pCAG-EGFP-Eed-IREShygPA to generate 36^{EedTG} cells.

Immunostaining and RNA FISH

ES cells were attached to poly-l-lysine coated coverslips or cytospun using a Cytospin 3 centrifuge (Thermo Shandon, USA). Differentiated cells were grown on Roboz slides (CellPoint Scientific, USA). Immunostaining was performed as described (Peters *et al*, 2003; Kohlmaier *et al*, 2004). Briefly, cells were fixed for 10 min at RT in 4% PFA in PBS, permeabilised for 5 min at RT in 0.1% Na Citrate/0.1% Triton X-100, blocked for 60 min at RT in PBS containing 5% (wt/vol) BSA, 0.1% Tween-20. For H2AK119ub1 immunostaining cells were pre-extracted in 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes pH 6.8 and 0.5% Triton for 2 min at RT before fixation.

RNA FISH probes were generated by random priming (Stratagene, USA) using Cy3-dCTP (Amersham). After immunostaining, cells were fixed in 4% PFA in PBS for 10 min at 4°C, de-hydrated, hybridised and washed as described (Wutz and Jaenisch, 2000). Images were obtained using a fluorescence microscope (Zeiss Axioplan) equipped with a CCD camera and the MetaMorph image analysis software (Universal Imaging, USA).

RNA and protein analysis

Northern analysis was performed using 20 μg of RNA (Trizol; Invitrogen) as described previously (Wutz and Jaenisch, 2000). Antibodies for histone lysine methylation states and Western analysis were previously described (Peters *et al*, 2003; Kohlmaier *et al*, 2004) and the following dilutions were used (immunostaining/Western blot): α-H3K9m1 (#4858, 1:1000/1:500); α-H3K9m2 (#4677, 1:1000/1:1000); α-H3K9m3 (#4861, 1:750/1:1000); α-H3K27m1 (#8835, 1:6,000/1:1000); α-H3K27m2 (#8841, 1:1000/1:2,000); α-H3K27m3 (#6523, 1:1000/1:7,000); α-H4K20m1 (#0077, 1:500/1:3,000); α-H4K20m2 (#0080, 1:1000/1:1000); α-H4K20m3 (#0083, 1:3,000/1:3,000). Additional antibodies were as follows: α-H2AK119ub1 (α-ubiquityl-Histone H2A, clone E6C5; #05-678 Upstate Biotechnology, Lake Placid, New York, USA), 1:50/1:400; α-Suz12 (# 07-379; Upstate), 1:1000/1:1000; α-Eed (rabbit polyclonal antiserum, AKS and AW, unpublished results); 1:1000 α-Ezh2 (rabbit polyclonal antiserum, M Busslinger unpublished results); 1:1000/1:1000); α-Ring1b (Atsuta *et al*, 2001), 1:100 for IF; α-Mph1 (Isono *et al*, 2005a), 1:5 for IF; α-Mph2 (Isono *et al*, 2005a), 1:100 for IF; α-hnRNP A1 (4B10 mouse monoclonal antiserum), 1:1000 for Western. Secondary antibodies: Alexa A-11034 Fluor 488 goat anti-rabbit IgG (H + L) and Alexa A-11034 Fluor 488 goat anti-mouse IgG (H + L) all at 1:500 (Molecular Probes, USA); HRP-conjugated AffiniPure goat anti-rabbit IgG (H + L), 1:10 000 and HRP-conjugated AffiniPure goat anti-mouse IgG (H + L), 1:5000 (Jackson ImmunoResearch Laboratories. Inc., USA).

Quantitative PCR expression analysis

Random primed cDNA was generated from 10 μg total RNA from clone 36 and $Eed^{-/-}$ ES cells using the Superscript II Reverse transcription kit (Invitrogen). Quantitative PCR using the Taqman method (Applied Biosystems) for *Tk1* (primers:

GCAACAGCTTCTCCACACATGA, GCGGAGCATGCAGGCT;
probe: CGGAACACCATGGACGCATTGC),
Npm1 (TGTAGAGGAAGATGCAGAGTCTGAA, CCTCCAGGAGCAGA TCGCT;
AGGAGGACGTAAAACTCTTAGGCATGTC),
Igfbp2 (CGGCAAAGGCGGCAA, TGGCACTACCACCTCAGCTG;
ACGGTGAATGAGCTGCAGAACTTGACC),
Cct4 (CCTACCAGGACCGCGACA, GCTTTGGCCGCGGAA;
CCAGCCCAGATCCGCTTCAGCAAT) and
Gapdh (CATGGCCTTCCGTGTTCCTA, TGTCATCATACTTGGCAGGT TTCT;
TCGTGGATCTGACGTGCCGCC)

on a ABI PRISM 7000 detection machine was performed in triplicate as described (Pauler *et al*, 2005). Quantification was achieved by the standard curve method using serial dilutions of cDNA generated from uninduced ES cells at day 8 of differentiation. Samples were normalised to *Gapdh* and the expression levels of uninduced clone 36 ES cells at day 8 of differentiation were set to 100 for each gene.

Nuclear extracts and mass spectrometry

ES cell cultures where harvested by trypsination and feeders were removed by plating on cell culture dishes twice for 30 min. Nuclear extracts were prepared as described (Peters *et al*, 2003). For mass

spectrometry 20 μg of nuclear extracts were separated by 15% SDS–PAGE and bands containing histone H3 and H4 were excised after Coomassie staining. Processing of the samples and quantitative mass spectrometric analyses were carried out as described (Peters *et al*, 2003).

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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