Developmentally regulated alterations in Polycomb repressive complex 1 proteins on the inactive X chromosome

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Polycomb group (PcG) proteins belonging to the polycomb (Pc) repressive complexes 1 and 2 (PRC1 and PRC2) maintain homeotic gene silencing. In *Drosophila*, PRC2 methylates histone H3 on lysine 27, and this epigenetic mark facilitates recruitment of PRC1. Mouse PRC2 (mPRC2) has been implicated in X inactivation, as mPRC2 proteins transiently accumulate on the inactive X chromosome (Xi) at the onset of X inactivation to methylate histone H3 lysine 27 (H3-K27). In this study, we demonstrate that mPRC1 proteins localize to the Xi, and that different mPRC1 proteins accumulate on the Xi during initiation and maintenance of X inactivation in embryonic cells. The Xi accumulation of mPRC1 proteins requires *Xist* RNA and is not solely regulated by the presence of H3-K27 methylation, as not all cells that exhibit this epigenetic mark on the Xi show Xi enrichment of mPRC1 proteins. Our results implicate mPRC1 in X inactivation and suggest that the regulated assembly of PcG protein complexes on the Xi contributes to this multistep process.

Introduction

Polycomb group (PcG) proteins assemble into two biochemically distinct complexes that are used to achieve developmentally regulated and tissue specific transcriptional silencing of *Hox* genes in flies and mammals (Lund and van Lohuizen, 2004). The enhancer of zeste 2 (Ezh2)/embryonic ectoderm development (Eed) complex in mammals and the homologous enhancer of zeste (E[z])/extra sexcombs complex in flies, also referred to as Polycomb (Pc) repressive complex 2 (PRC2), exhibit histone methyltransferase activity, methylating histone H3 lysine 27 (H3-K27) and to a lesser extent on lysine 9 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). The fly Pc protein contains a chromodomain that binds to methylated H3-K27 in vitro (Fischle et al., 2003; Min et al., 2003), and is a component of Pc repressive complex 1 (PRC1; Lund and van Lohuizen, 2004). In E(z) mutant embryos PRC1 proteins are not recruited to *Hox* genes, suggesting that E(z)-mediated histone methylation facilitates the recruitment of PRC1 (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002). PRC1 can mediate silencing of target genes by interfering with SWI/SNF chromatin remodeling machinery, blocking transcriptional initiation, or recruiting additional silencing activities (Shao et al., 1999; Francis et al., 2001; Dellino et al., 2004; King et al., 2002; Lavigne et al., 2004). In addition to Pc, *Drosophila* PRC1 contains four other PcG proteins, polyhomeotic (Ph), posterior sex combs (Psc), sex combs on midleg (Scm), and sex combs extra/dRING (Shao et al., 1999), and each of these has several mammalian homologues (Table I).

In addition to *Hox* gene silencing, PcG proteins belonging to mouse PRC2 (mPRC2) are implicated in X inactivation (Wang et al., 2001; Mak et al., 2002; Erhardt et al., 2003; Plath et al., 2003; Silva et al., 2003). A large, noncoding RNA, encoded by the X-linked *Xist* gene, coats the inactive X chromosome (Xi) to initiate X chromosome silencing (Penny et al., 1996; Marahrens et al., 1997; Wutz and Jaenisch,

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Abbreviations used in this paper: 293, transformed human embryonic kidney 293; Bmi-1, B lymphoma Mo-MLV insertion region 1; Cbx, chromobox homologue; Eed, embryonic ectoderm development; ES, embryonic stem; E(z), enhancer of zeste; Ezh2, enhancer of zeste 2; H3-K27, histone H3 lysine 27; H3-3mK27, histone H3 tri-methyl lysine 27; MEF, transformed mouse embryonic fibroblast; mPRC1, mouse PRC1; Pc, Polycomb; PcG, Pc group; Ph, polyhomeotic; Phc, Ph-like; PRC1, Pc repressive complex 1; PRC2, Pc repressive complex 2; Psc, posterior sex combs; Ring1, really interesting gene 1; Rnf, ring finger protein; Scm, sex combs on midleg; Scmh, sex combs on midleg homologue; TS, trophoblast stem; Xi, inactive X chromosome.

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2000). Two mPRC2 proteins, Eed and Ezh2, are transiently recruited to the Xi immediately after the initial chromosome coating by *Xist* RNA (Erhardt et al., 2003; Plath et al., 2003; Silva et al., 2003). The histone methyltransferase Ezh2 is responsible for the concomitant enrichment of the histone H3 tri-methyl lysine 27 (H3-3mK27) on the Xi (Erhardt et al., 2003). During later stages of development and in adult tissues, when the Xi is stably maintained, H3-3mK27 continues to be enriched on the Xi in many differentiated cell types (Gilbert et al., 2003). This enrichment is dependent on *Xist* RNA and Ezh2, although the latter no longer exhibits constitutive enrichment on the Xi (Plath et al., 2005).

The enrichment of subunits of mPRC2 and of H3-3mK27 on the Xi suggests that mouse PRC1 (mPRC1) also plays a role in X inactivation. As in the case of silenced *Hox* genes, Ezh2-mediated H3-K27 methylation could facilitate the recruitment mPRC1 to the Xi. However, mPRC1 proteins have not been detected on the Xi, suggesting that PcG proteins function differently during X inactivation and *Hox* gene silencing (Mak et al., 2002; Silva et al., 2003; Cao and Zhang, 2004). In this study, we have demonstrated mPRC1 proteins accumulate on the Xi. Chromobox homologue (Cbx) 2, a Pc homologue; B lymphoma Mo-MLV insertion region 1 (Bmi-1), a Psc homologue; and the Ph homologues Ph-like (Phc) 1 and Phc2, were enriched on the Xi in a stage-specific fashion during X inactivation. Phc1 accumulated on the Xi during initiation of X inactivation whereas Cbx2, Bmi-1, and Phc2 exhibited *Xist*-dependent Xi enrichment during the maintenance phase of X inactivation. In addition the combination of mammalian PRC1 proteins that accumulate on the Xi displayed cell type specific differences. Finally, mPRC1 components were not enriched in all cells exhibiting enrichment of H3-3mK27 on the Xi, indicating that the Xi accumulation of PRC1 cannot be regulated solely by the methylation of H3-K27 on the Xi.

Results

mPRC1 proteins are enriched on the Xi in somatic cells

To determine whether mPRC1 proteins are enriched on the Xi, we analyzed the distribution of Bmi-1, Cbx2, Phc1 and Phc2 in transformed mouse embryo fibroblasts (MEFs), which are tetraploid and contain two Xis. Immunostaining for these mPRC1 proteins was combined with FISH for *Xist* RNA or immunostaining for the histone variant macroH2A to mark the Xi (Fig. 1, a–d). When tested on MEFs overexpressing mPRC1 proteins, the antibody recognizing Phc2 showed some cross-reactivity for Phc1 and Phc3, whereas the other antibodies exhibited specificity and did not recognize closely related homologues (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200409026/DC1). All four mPRC1 proteins showed diffuse nuclear staining with exclusion from the nucleolus and pericentric heterochromatin and a variable number of speckles, and in a subset of cells these mPRC1 proteins showed Xi enrichment (Table II). Bmi-1 and Cbx2 exhibited Xi enrichment in 12.1% and 13.9% of cells, a proportion similar to that seen when tagged Bmi-1 or Cbx2 were expressed in MEFs (Fig. S2 and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200409026/DC1), suggesting that antibody staining accurately reflects the small proportion of cells with Xi enrichment of these mPRC1 proteins. Phc2 accumulated on the Xi in 3.4% of cells when assayed by immunostaining, whereas tagged Phc2

Table I. **The mammalian homologues of the *Drosophila* PRC1 proteins**

***Drosophila* proteins**	**Mouse proteins**	**Xi enriched**	**Human proteins**	**Xi enriched**
dPRC1	mPRC1		hPRC1	
Pc	**Cbx2**/M33	+[a,b]	**CBX2**/HPC1	+[a]
	Cbx4/Mpc2	−[b]	**CBX4**/HPC2	+[a]
	Cbx7	ND	**CBX7**	ND
	Cbx8	+[b]	**CBX8**/HPC3	ND
Ph[a]	**Phc1**/Edr1/Mph1/Rae28	+[a,b]	**PHC1**/EDR1/HPH1/RAE28	+[a]
	Phc2/Edr2/Mph2	+[a,b]	**PHC2**/EDR2/HPH2	+[a]
	Phc3/Edr3	+[b]	**PHC3**/EDR3/HPH3	ND
Scm	**Scmh1**	+[b]	**SCMH1**/SCML3	ND
	Scml2[c]	ND	**SCML2**	ND
	Scml4[c]	ND	**SCML4**	ND
			SCML1	ND
Psc[b]	**Bmi-1**	+[a,b]	**BMI-1**	+[a]
	Rnf110/Zfp144/Mel-18	+[b]	**RNF110**/MEL-18/ZNF144	ND
	Znf134/Mblr	ND	**ZNF134**	ND
dRing/Sce (Sex combs extra)	**Ring1**/Ring1a	+[b]	**RING1**/RNF1/RING1A	+[a]
	Rnf2/Ring1b	+[b]	**RNF2**	ND

Where applicable, the official name according to the Mouse Genomic or the HUGO Gene Nomenclature Committee is shown in bold.
[a]Flies contain two Ph homologues, Ph-proximal and Ph-distal.
[b]Flies encode three Psc homologues, Psc, Su(z)2, and Su(z)2(D).
[c]Scmh and SCMH are likely the bona fide Scm homologues as all three proteins contain a sterile alpha motif and MBT repeats, whereas Scm14/SCML4 and SCML1 each contain a sterile alpha motif homology domain and Scml2/SCML2 has MBT repeats.
+ indicates protein is enriched on the Xi (+[a] by immunofluorescence, +[b] using transiently transfected-tagged proteins).
− indicates protein is not detectable enriched on the Xi (−[a] by immunofluorescence, −[b] using transiently transfected-tagged proteins).
ND indicates the protein has not been assayed for Xi localization.

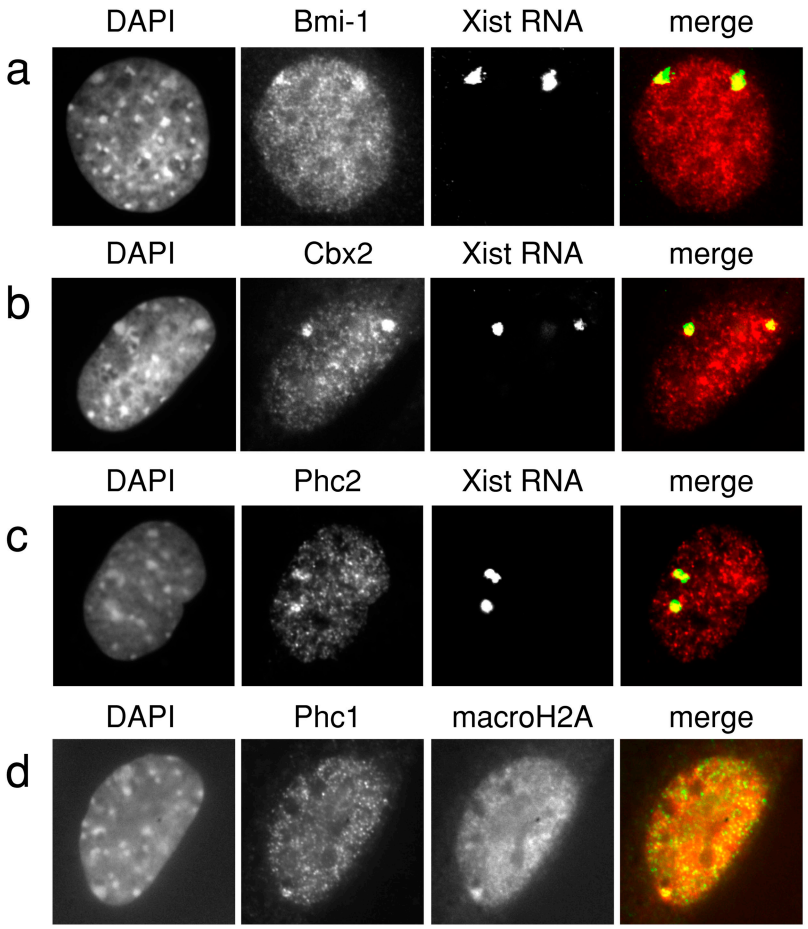

Figure 1. **Localization of mPRC1 proteins in somatic cells.** (a–c) MEFs were immunostained for Bmi-1, Cbx2, or Phc2 (second column) in combination with FISH for *Xist* RNA to detect the Xi (third column). DAPI delineates the nucleus (first column), and the merge presents *Xist* RNA in green and mPRC1 proteins in red (fourth column). (d) Transformed MEFs were stained for Phc1 (second column) and macroH2A (third column), to mark the Xi (Costanzi and Pehrson, 1998). Nuclei are visualized by DAPI staining (first column). The merge shows mPh1 in green and macroH2A in red (fourth column).

accumulated on the Xi in 27% of cells (Fig. S2 and Table S1), suggesting that possibility that more cells exhibit Xi enrichment of Phc2 than is indicated by antibody staining. Phc1 exhibited Xi enrichment in <1% of cells by immunostaining and expression of tagged protein, and Phc1 unusual in that in many instances it was detected on only one of the two Xis (unpublished data). In cells assayed for localization of macroH2A and Phc1, both proteins exhibited some overlapping sites of enrichment at regions other than the Xi. Similar results were observed when macroH2A was examined in combination with other mPRC1 proteins (unpublished data), suggesting that localization of mPRC1 proteins and this variant histone may be regulated by related mechanisms. These data demonstrate that Bmi-1, Cbx2, and Phc2 exhibit Xi localization in a significant proportion of cells, indicating a role for mPRC1 proteins in X inactivation.

Different mammalian PRC1 proteins accumulate on the Xi in different cell types

The distribution of mammalian PRC proteins has been analyzed in a number of female mouse and human cell types, and

Table II. **Percentage of cells exhibiting enrichment of PcG proteins or H3-3mK27 on the Xi in different cell types**

Cell type	Bmi-1/BMI-1	Cbx2/CBX2	Phc2/PHC2	Phc1/PHC1	H3-3mK27	Eed/EED[b]
MEF	12.1	13.9	3.4	0.3	95	1
ES, day 5 of differentiation	3.3	1.6	1.0	47.9	100	99
ES, day 10 of differentiation	6.7	19.3	32.1	0	99.5	0
ES + inducible	3.1	4.1	0	27.0	97.5	99
Xist, 24 h TS	99	99	99	69	99.5	99
293[a]	65.2	45.3	42.7	60.9	99.6	1

The percentages of MEFs with Xi enrichment of mPRC1 proteins and the standard errors are based on counts of 100–400 cells in each of three to nine independent experiments similar to that shown in Fig. 1. Percentages of cells with mPRC1 Xi enrichment in differentiating ES cells and undifferentiated ES cells ectopically expressing *Xist* are taken from Figs. 5 and 6. The percentages of TS and 293 cells with Xi enrichment of PRC1 proteins are based on counts of >75 cells in each of two or more experiments similar to those displayed in Figs. 2 and 3. H3-3mK27 percentages in all cell types are based on counts of at least 100 cells in two or more experiments. In all cases, only interphase cells were counted.
[a]293 cells also exhibited enrichment of CBX4 (65.5%) and RING1 (73.1%).
[b]Proportion of cells with Xi enrichment of the PRC2 protein Eed/EED is based on counts of at least 200 cells in all cell types.

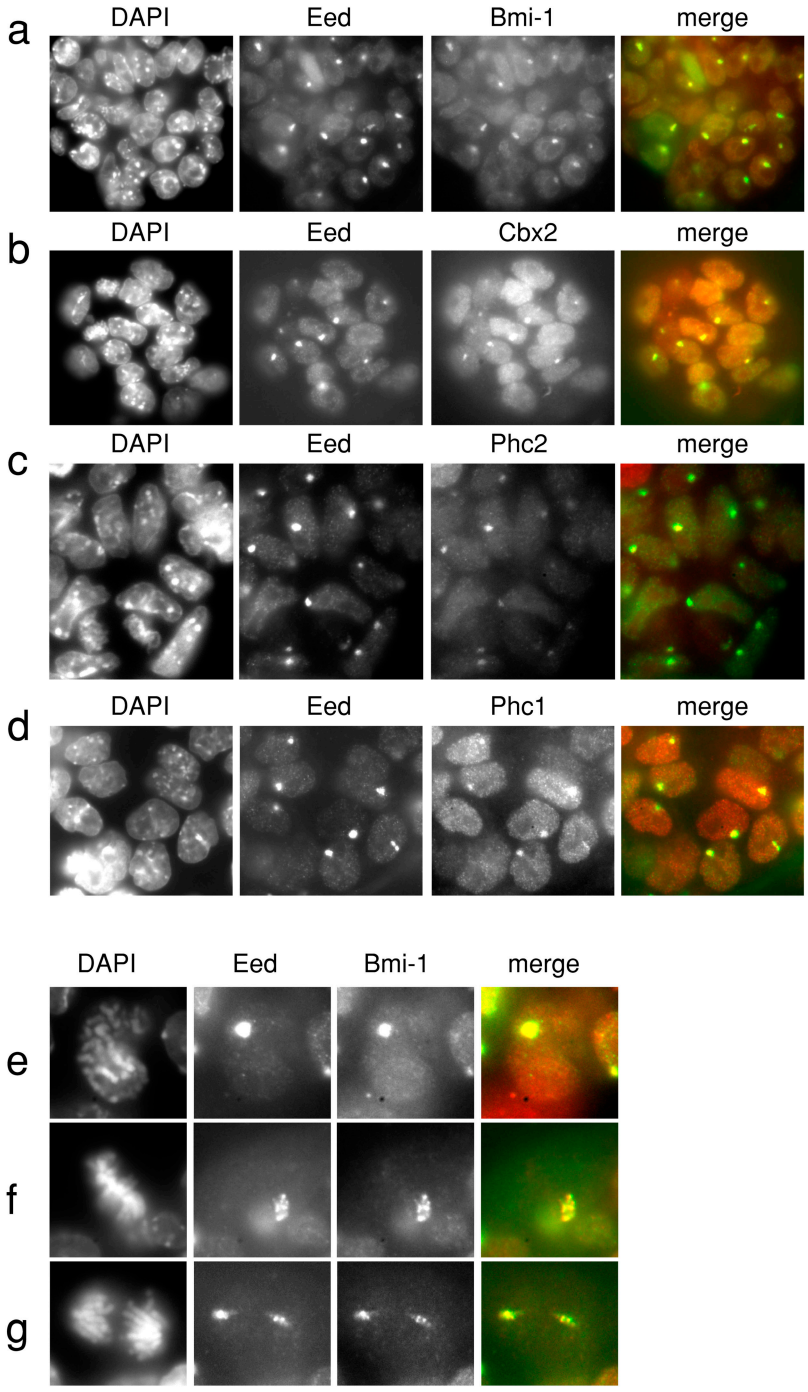

Figure 2. **mPRC1 protein localization in TS cells.** Immunostaining for Eed (second column) and mPRC1 proteins (third column) in female TS cells. (a–d) DAPI staining (first column) was used to mark the nuclei and the merge (fourth column) represents Eed in green and Bmi-1 (a), Cbx2 (b), Phc2 (c), or Phc1 (d) protein in red. (e–g) Bmi-1 shows mitotically stable association with the Xi, as enrichment of Bmi-1 can be detected on the Eed-marked Xi in prophase (e), metaphase (f), and anaphase (g). Phc1, Phc2, and Cbx2 also exhibit mitotically stable Xi enrichment (unpublished data).

in most instances these proteins accumulate in nuclear structures termed PcG bodies, and an Xi-like distribution has not been reported (Alkema et al., 1997; Gunster et al., 1997, 2001; Satijn et al., 1997b; Bardos et al., 2000; Mak et al., 2002). Mammalian PRC1 proteins exhibit variable expression in different tissues and cell types, suggesting that PRC1 proteins may not accumulate on the Xi in some mammalian cell types due to these differences in levels (Otte and Kwaks, 2003; Lund and van Lohuizen, 2004). To determine whether Xi accumulation of PRC1 proteins occurs in cell types other than MEFs, we analyzed the distribution of PRC1 proteins in female mouse trophoblast stem (TS) cells (Fig. 2), and in female human transformed embryonic kidney (293) cells (Fig. 3). TS cells show enrichment of the mPRC2 proteins Eed and Ezh2 on the *Xist* RNA-coated Xi in virtually all cells (Mak et al., 2002). When TS cells were stained for Eed and mPRC1 proteins, enrichment of Bmi-1, Cbx2, and Phc2 was detected on the Eed-marked Xi in >98% of cells, whereas Phc1 was enriched on the Eed-marked Xi in 69% of cells (Table II). Like Eed and Ezh2, both of which accumulate on the Xi in TS cells throughout interphase and mitosis, Bmi-1, Cbx2, Phc1, and Phc2 showed mitotically stable association with the Xi in TS cells (Fig. 2, e–k; unpublished data). Xi enrichment of mPRC1 proteins was not detected in TS cells in a previous re-

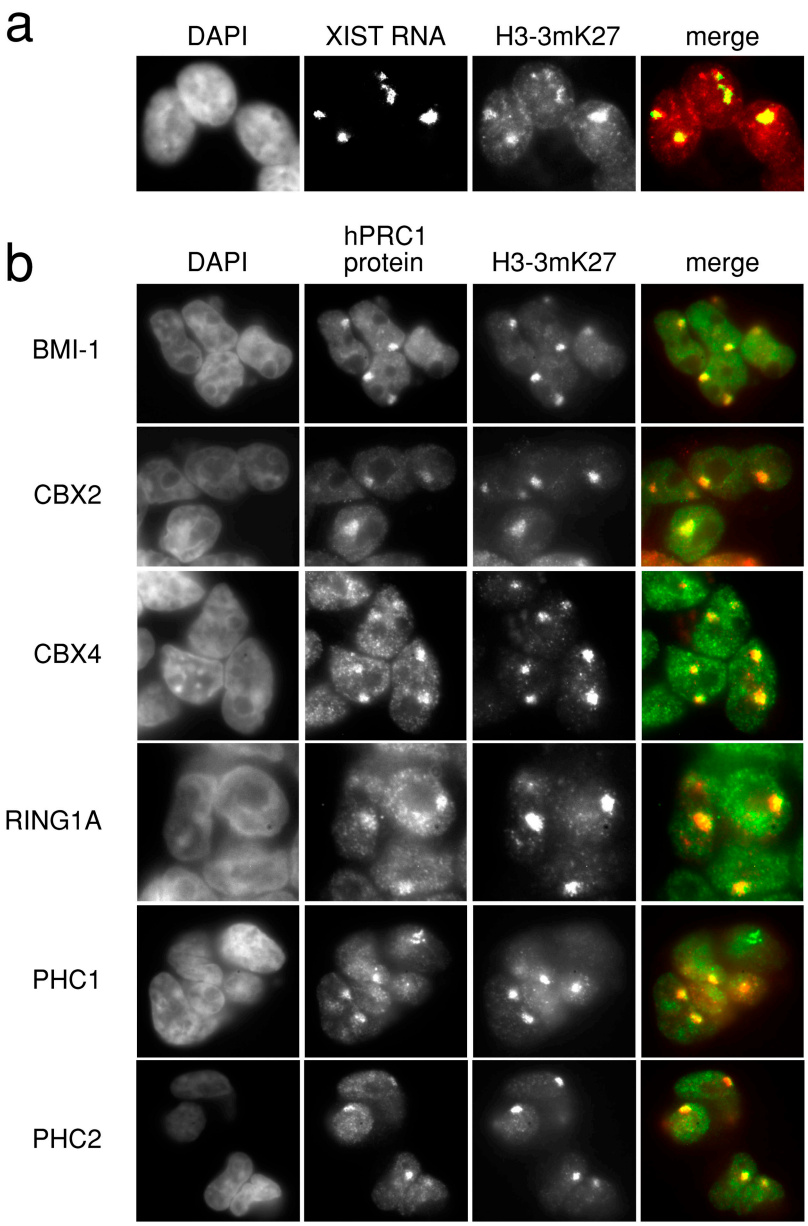

Figure 3. **Immunolocalization of human PRC1 proteins in 293 cells.** (a) 293 cells were stained for H3-3mK27 (third column) in combination with FISH for *XIST* RNA to detect the Xi (second column). Nuclei were stained with DAPI (first column) and merged image (fourth column) consists of *XIST* RNA in green and H3-3mK27 in red. 293 cells contain two Xis and virtually all cells showed Xi accumulation of H3-3mK27 (unpublished data). (b) Immunostaining for H3-3mK27 (third column) and hPRC1 proteins (second column) in 293 cells. DAPI staining (first column) was used to mark the nuclei and the merge (fourth column) represents H3-3mK27 in red and hPRC1 proteins in green. The fraction of cells with an H3-3mK27-marked Xi that also exhibited Xi enrichment for each mPRC1 protein assayed is indicated on the right. All percentages are based on counts of >75 cells.

port (Mak et al., 2002), which may be due to differences in experimental technique or antibodies.

To determine whether human PRC1 (hPRC1) proteins can be enriched on the Xi, we analyzed the distribution of hPRC1 proteins in 293 cells, which are highly enriched for most PcG proteins (Otte and Kwaks, 2003). Cells were stained for H3-3mK27 to mark the Xi and for the hPRC1 proteins BMI-1, CBX2, CBX4, really interesting gene 1 (RING1), PHC1, or PHC2 (Fig. 3). These hPRC1 proteins exhibited Xi enrichment in a variable proportion of cells, ranging from 40 to 75% of cells (Table II). We did not detect Ring1 and Cbx4 expression in mouse TS cells or MEFs (unpublished data), however these PRC1 antibodies were generated against human proteins (Satijn et al., 1997a) and may not necessarily efficiently detect the mouse proteins. Thus, the combination of mammalian PRC1 proteins that accumulates on the Xi appears to be dependent on cell type and may also be regulated in a species-specific manner.

Xi localization of Bmi-1, Cbx2, and Phc2 is *Xist* RNA dependent

mPRC2-mediated enrichment of H3-3mK27 on the Xi requires continued coating of the Xi by *Xist* RNA (Plath et al., 2005). Here, we tested whether the Xi localization of mPRC1 proteins also depends on *Xist* RNA, using female MEFs in which the *Xist* gene on the Xi is flanked by loxP sites. To delete *Xist*, cells were infected with adenovirus encoding Cre recombinase. Immunostaining for H3-3mK27 combined with FISH for *Xist* RNA confirmed that *Xist* RNA and the *Xist*-dependent Xi enrichment of H3-3mK27 were lost upon *Xist* deletion (Fig. 4, a and b). MEFs carrying the conditional *Xist* allele exhibited enrichment of Bmi-1, Cbx2, and Phc2 on the *Xist* RNA-coated Xi in an average of 11.2%, 17.3%, and 3.5% of cells respectively (Fig. 4 c). Upon deletion of *Xist*, an Xi-like focal accumulation of these proteins was no longer detected (Fig. 4 c). Thus, the Xi localization of the mPRC1 pro-

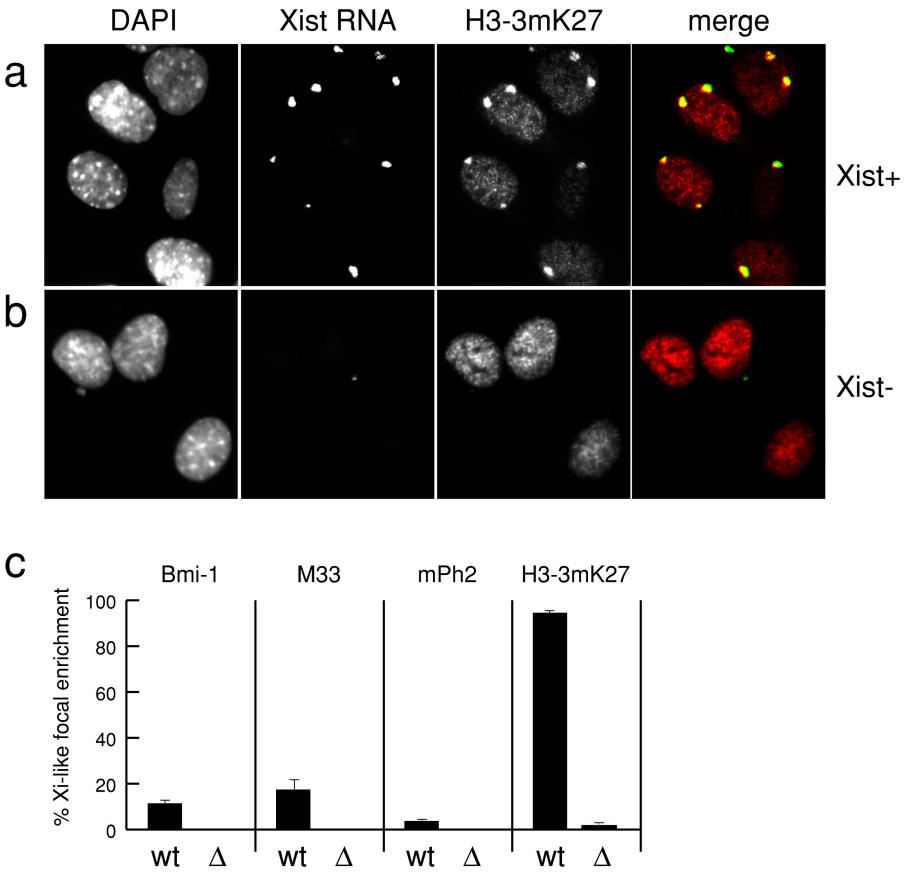

Figure 4. **Localization of mPRC1 proteins in cells lacking *Xist* RNA on the Xi.** (a and b) Detection of *Xist* RNA (second column) and H3-3mK27 (third column) in DAPI stained (first column) MEFs containing a conditional *Xist* allele on the Xi. The merge (fourth column) represents the overlay of H3-3mK27 (red) and the *Xist* RNA (green). (a) Most parental *Xist*+ cells contain two Xis, both of which are characterized by colocalization of *Xist* RNA and H3-3mK27. (b) After Cre-mediated deletion of *Xist* from the Xi, generating *Xist*− cells, no enrichment of H3-3mK27 or *Xist* RNA is detected on the Xi. (c) The proportion of cells with Xi or Xi-like enrichment of Bmi-1, Cbx2, Phc2, and H3-3mK27. In *Xist*+ cells the Xi accumulation of mPRC1 proteins or H3-3mK27 was confirmed by overlap with the area marked by *Xist* RNA. After addition of Cre, *Xist*− cells no longer expressed *Xist* RNA, and cells were scored for a pattern of mPRC1 or H3-3mK27 staining that overlapped with the DAPI-intense Barr body, which delineates the Xi. Percentages and standard errors are calculated from counts of >200 cells from three experiments.

teins Bmi-1, Cbx2, and Phc2, like the enrichment of H3-3mK27, depends on *Xist* RNA in somatic cells.

Phc1 associates with the Xi during initiation of X inactivation

To determine whether mPRC1 proteins accumulate on the Xi during initiation of X inactivation, when *Xist* is necessary for silencing, the distribution of Bmi-1, Cbx2, Phc1, and Phc2 was analyzed in cells that are trapped in the initiation phase of X inactivation. Ectopic *Xist* expression in undifferentiated embryonic stem (ES) cells results in initiation of X chromosome silencing and Xi enrichment of the mPRC2 proteins Ezh2 and Eed (Wutz and Jaenisch, 2000; Plath et al., 2003; Kohlmaier et al., 2004). X inactivation continues to be *Xist* dependent and does not progress into the maintenance phase as long as ES cells remain undifferentiated (Wutz and Jaenisch, 2000; Kohlmaier et al., 2004). *Xist* expression was induced in undifferentiated male ES cells, and localization of Eed and *Xist* RNA or Eed and mPRC1 proteins before and after 24 h of induction of *Xist* expression analyzed (Fig. 5). Uninduced cells did not exhibit Xi-like accumulation of *Xist* RNA or Eed. Upon induction Xi accumulation of Eed was detected in nearly 100% of *Xist* RNA expressing cells (Fig. 5 a and Fig. S3), confirming that Eed can be used to mark the Xi in undifferentiated ES cells that ectopically express *Xist*.

Although no Xi-like focal accumulation of Phc1 was observed in uninduced cells, an average of 27% of cells with an Eed-labeled Xi showed Xi enrichment for this mPRC1 protein after induction, though there was considerable variation between experiments (Fig. 5 b). Phc2 did not exhibit Xi-like focal enrichment in uninduced cells, and was not enriched on the Eed-marked Xi in induced samples (Fig. 5 b). Bmi-1 and Cbx2 accumulated on the Xi in a small proportion of undifferentiated ES cells that ectopically express *Xist* and this proportion varied between experiments (Fig. 5 b). The replicates that exhibited a larger proportion of cells with Phc1 enrichment exhibited a larger number of cells with Bmi-1 and Cbx2 enrichment (unpublished data), suggesting that there may be a correlation between the amount or duration of Phc1 Xi enrichment and the recruitment of Bmi-1 and Cbx2 during early stages of X inactivation. These data suggest that Phc1, but not Phc2, acts during the initiation of X inactivation. Cbx2 and Bmi-1 may be involved in the transition from initiation to maintenance of X inactivation, as they were detected on the Xi in very few undifferentiated ES cells that ectopically express *Xist* and exhibited Xi enrichment more frequently in MEFs.

Phc1 recruitment and silencing are separable functions of *Xist* RNA

Xist RNA contains several evolutionarily conserved sequence elements and the 5′-most of these conserved elements, termed the A-repeat, is required for *Xist* silencing function (Wutz et al., 2002). Although *Xist* RNA lacking the A-repeat is unable to initiate silencing, it can coat the chromosome and recruit Ezh2 and Eed (Wutz et al., 2002; Plath et al., 2003; Kohlmaier et al., 2004). To determine whether Phc1 can be recruited by *Xist*

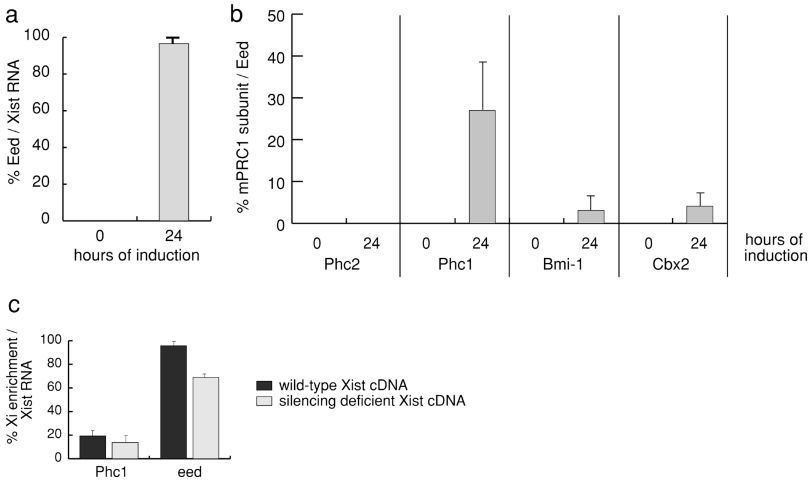

Figure 5. **Localization of mPRC1 proteins in undifferentiated male ES cells which ectopically express *Xist*.** (a) *Xist* expression was induced in undifferentiated male ES cells for 0 or 24 h and distribution of *Xist* RNA and Eed determined by immuno-FISH. The graph depicts the percentage of cells with an *Xist* RNA-coated chromosome that displayed overlapping accumulation of Eed. Percentages are based from counts of ~100 cells each from three independent experiments. (b) Graph displaying the proportion of cells that display Phc1, Phc2, Bmi-1, or Cbx2 enrichment on the Eed-marked Xi, at 0 and 24 h after induction. Percentages are calculated from six independent experiments in each of which ~100 cells with an Eed-marked Xi were counted. (c) The graph displays the fraction of cells that exhibit Xi enrichment of Eed or Phc1 on the *Xist* RNA-coated chromosome 24 h after induction of wild-type or A-repeat delete *Xist* cDNA expression in undifferentiated ES cells. The proportion of cells in which *Xist* expression was induced varied from 30 to 70%, in four experiments. Percentages are based on counts of >150 cells that exhibited *Xist* RNA coating. In all instances error bars indicate standard errors.

RNA that lacks the A-repeat, we assayed the distribution of Phc1 in undifferentiated male ES cells carrying an inducible wild-type or A-repeat mutant *Xist* cDNA transgene integrated into the X-linked *Hprt* locus (Wutz et al., 2002). Transgenic *Xist* expression was induced in both lines for 24 h, and immunostaining for Eed or Phc1 was performed in combination with *Xist* RNA FISH to detect the X chromosome coated by *Xist* RNA (Fig. 5 c). In cells expressing the wild-type *Xist* cDNA, 92.9% of cells with an *Xist* RNA-coated chromosome exhibited Xi enrichment for Eed, whereas 19.3% of cells containing an *Xist* RNA-coated Xi accumulated Phc1 on the Xi. The A-repeat mutant transgenic *Xist* transcript recruited Eed and Phc1 to the X chromosome in 68.9% of cells and 13.7% of cells, respectively, indicating that this mutant form of *Xist* is sufficient to recruit Phc1 to the Xi. The percentage of cells with X chromosome enrichment was reduced in equal proportions for both Phc1 and Eed, and this reduction may indicate that the A-repeat is required for efficient localization of these proteins, as has been suggested for Ezh2 (Kohlmaier et al., 2004). As the X chromosome coated with A-repeat mutant *Xist* RNA is not inactivated (Wutz et al., 2002), these results indicate that Phc1 recruitment is not sufficient to mediate transcriptional silencing.

Xi association of mPRC1 proteins alters during differentiation of ES cells

Next we analyzed whether there is a dynamic alteration in the combination of mPRC1 proteins that accumulate on the Xi when cells progress from the initiation to the maintenance stage of X inactivation, using differentiating female ES cells, which initiate X inactivation shortly after they are induced to differentiate and stably maintain the Xi after differentiation is complete. The proportion of cells that exhibited accumulation of mPRC1 proteins on the *Xist* RNA-coated Xi was assayed over a time course of ES cell differentiation (Fig. 6 and Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200409026/DC1). The mPRC2 protein Eed accumulates on the Xi during the earliest stages of X inactivation in differentiating ES cells (Plath et al., 2003; Silva et al., 2003). We assayed the proportion of cells that exhibited enrichment of Eed on the *Xist* RNA-coated Xi to compare the kinetics of the Xi accumulation of mPRC1 proteins with those of mPRC2 proteins. The proportion of cells with an Eed-enriched Xi peaked at day 5 of differentiation, dropped slightly throughout days 6, 7, and 8, and by days 10 and 11, Eed accumulated on the Xi in only a small subset of cells (Fig. 6 a). The mPRC1 proteins Bmi-1, Cbx2, Phc1, and Phc2 each accumulated on the *Xist* RNA-coated Xi with different kinetics of Xi enrichment from Eed. Phc1 was unique among the mPRC1 proteins in that it displayed kinetics of Xi accumulation most similar to those of Eed (Fig. 6 b). Like Eed, Phc1 was transiently enriched on the Xi during early time points of differentiation. In contrast to Eed, which was enriched on the Xi in all cells with an *Xist* RNA-coated Xi at day 5 of differentiation and declined abruptly from 84% to 14% between days 8 and 10, the fraction of cells with an Xi accumulation of Phc1 decreased gradually from 48% at day 5 to 0% at day 10.

The mPRC1 subunits Bmi-1, Cbx2, and Phc2 exhibited differentiation-induced Xi enrichment that was delayed when compared with that of Eed and Phc1 (Fig. 6, c–e). At day 5 of differentiation, when 100% of cells with an *Xist* RNA-coated Xi showed Xi localization of Eed, only a small fraction of cells contained an Xi enriched for Bmi-1, Cbx2, or Phc2 (1–3%). There was a gradual increase in the proportion of cells that exhibited Xi localization of these three mPRC1 proteins over time, and at day 8 the proportion of cells with Xi enrichment peaked at 20% for Bmi-1, 24% for Cbx2, and 34% for Phc2. The proportion of cells with Xi enrichment of Phc1 decreased between days 5 and 8, as the percentage of cells in which Bmi-1, Cbx2, or Phc2 accumulate on the Xi increased, suggesting that a transition in the mPRC1 proteins that localize to the Xi occurs during this time. These results are consistent with a regulated alteration in the mPRC1 proteins that accumulate on the Xi as cells progress from the initiation to the maintenance stage of X inactivation. The proportion of cells with Xi enrichment for Eed did not change dramatically from day 5 to day 8, when the transition in mPRC1 protein profiles on the Xi occurs, indicating that the alteration in mPRC1 pro-

teins that accumulate on the Xi is unlikely to be regulated by changes in Eed-enrichment on the Xi.

Enrichment of H3-3mK27 is not sufficient for mPRC1 accumulation on the Xi

The chromodomain of the Pc class of proteins binds methylated H3-K27 in vitro and disruption of E(z) activity results loss of H3-K27 methylation and of PRC1 protein binding to homeotic genes (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002; Fischle et al., 2003; Min et al., 2003), suggesting that H3-K27 methylation is necessary for PRC1 recruitment. To determine whether Xi enrichment of H3-3mK27 was sufficient for mPRC1 Xi accumulation, we analyzed the proportion of somatic cells, ES cells, and TS cells that exhibited Xi localization of H3-3mK27 and mPRC1 proteins (Table II). Approximately 95% of MEFs were characterized by Xi accumulation of H3-3mK27, whereas Bmi-1, Cbx2, and Phc2, accumulated on the Xi in a much smaller subset of these cells. In differentiating female ES cells, >99% of cells showed Xi enrichment of H3-3mK27 on the *Xist* RNA-coated Xi at all time points of differentiation, whereas Bmi-1, Cbx2, Phc1, and Phc2 were enriched on the Xi in only a subset of those cells. Similarly, in undifferentiated ES cells ectopically expressing *Xist* RNA, either from the endogenous locus or from X-linked transgenes, H3-3mK27 was enriched on the Xi in >95% of cells, whereas mPRC1 proteins showed Xi enrichment in a smaller proportion of cells. In undifferentiated TS cells, H3-3mK27, Bmi-1, Cbx2, and Phc2 accumulated on the Xi in nearly 100% of cells, whereas Phc1 was enriched in a smaller subset of cells. Finally, in 293 cells H3-3mK27 accumulated on the Xi in virtually 100% of cells, which hPRC1 proteins were Xi enriched a subset of cells. In combination, these results demonstrate that mPRC1 proteins are not always enriched on the Xi when H3-3mK27 is present, indicating that Xi enrichment of H3-3mK27 by itself is not sufficient to recruit mPRC1 proteins. When we costained for mPRC1 proteins and H3-3mK27 in MEFs or 293 cells, Xi localization of mPRC1 proteins without concomitant Xi enrichment of H3-3mK27 was never detected (unpublished data), suggesting that H3-3mK27 enrichment may be necessary for mPRC1 recruitment to the Xi.

Discussion

In this study, we have demonstrated that PRC1 proteins accumulate on the Xi in MEFs, TS cells, and 293 cells. These cell types all exhibited Xi enrichment of Cbx2/CBX2, Bmi-1/BMI-1, and Phc2/PHC2. TS cells also showed Xi enrichment of Phc1 and 293 cells also exhibited Xi accumulation of CBX4, PHC1, and Ring1. Thus, different cell types display Xi enrichment of different combinations of mammalian PRC1 proteins. This may be due to different combinations of mammalian PRC1 proteins that are expressed in each cell type, as there is considerable tissue-specific variation in expression of mammalian PRC1 proteins (Otte and Kwaks, 2003). There was also cell-type specific variation in the proportion of cells with Xi enrichment of these proteins, for example Cbx2/CBX2 accumulated on the Xi in ~14% of MEFs, 99% of TS cells, and

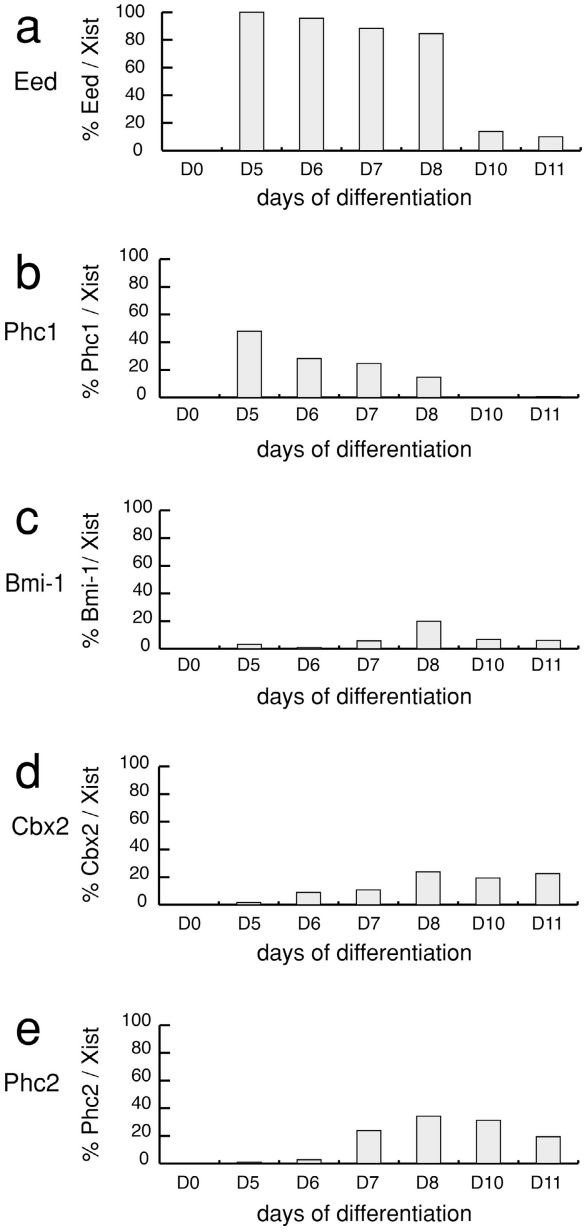

Figure 6. **Kinetics of Xi enrichment of mPRC1 proteins in differentiating ES cells.** The proportion of differentiating female ES cells exhibiting Xi enrichment for Eed (a), Phc1 (b), Bmi-1 (c), Cbx2 (d), and Phc2 (e) were determined over an 11 d time course. At each time point the percentage of *Xist* RNA-coated Xis that exhibited enrichment for the PcG protein indicated was calculated. At day 0 >100 cells were assayed and Xi enrichment of *Xist* or mPRC1 proteins was not detected. At each time point after day 0, percentages are based on counts of between 100 and 750 nuclei that exhibited *Xist* RNA coating of the Xi.

45% of 293 cells. This suggests that Xi recruitment of these mammalian PRC1 proteins is dynamic, and that the mechanisms that regulate changes in the Xi localization of these proteins may be different in each cell type. Many chromatin modifications exhibit cell cycle regulation of accumulation on the Xi (Chadwick and Willard, 2002; Kohlmaier et al., 2004; Plath et al., 2005), suggesting that the Xi enrichment of mPRC1 proteins may also be cell cycle regulated. Our preliminary results indicate that the majority MEFs that exhibit Xi enrichment of

Cbx2, Bmi-1, and Phc2 are in S phase (unpublished data), suggesting that mPRC1 proteins, like macroH2A and histone H3 lysine 9 and H4 lysine 20 methylation, show cell cycle–regulated enrichment on the Xi. It is possible that the other cell cycle–regulated epigenetic modifications that characterize the Xi may serve to modulate the Xi accumulation of PRC1 proteins. It is worth noting that mammalian PRC1 proteins may contribute to transcriptional silencing on the Xi even in the absence of their detectable enrichment. The PRC2 protein Eed is necessary for Ezh2-mediated H3-3mK27 accumulation on the Xi (Erhardt et al., 2003; Silva et al., 2003), however Eed/EED is not enriched on the Xi in a substantial fraction of MEFs or 293 cells (Table II), despite the Xi enrichment of H3-3mK27 in virtually all cells.

Different subsets of PRC1 proteins accumulated on the Xi in a different proportion of cells in each cell type. For example, Bmi-1, Cbx2, and Phc2 showed Xi enrichment in virtually all TS cells, whereas Phc1 accumulated on the Xi in 69% of TS cells. In contrast, BMI-1 and PHC1 were enriched on the Xi 60–65% of 293 cells and CBX2 and PHC2 accumulated on the Xi in ~45% of these cells. These data are inconsistent with a single complex containing these PRC1 proteins assembling on the Xi in all cells, and instead suggest that the composition of PRC1 complexes that accumulate on the Xi are subject to regulation, perhaps during the cell cycle or in response to additional epigenetic modifications on the Xi. Complexes consisting of different subsets of mammalian PRC1 proteins have been isolated in different cell types (Hashimoto et al., 1998; Levine et al., 2002; Wang et al., 2004), indicating that different PRC1 complexes can assemble and suggesting that it is possible that the composition of PRC1 complexes may be dynamic within cells.

When X inactivation is triggered in differentiating ES cells, *Xist* RNA is required for the Xi enrichment of mPRC1 proteins. *Xist* RNA is also necessary for Xi accumulation of Ezh2 and the resulting enrichment in H3-3mK27 (Plath et al., 2003; Silva et al., 2003). *Xist* RNA is required for Xi enrichment of mPRC1 proteins and for Ezh2-mediated accumulation of H3-3mK27 on the Xi in MEFs (Plath et al., 2005). In flies PRC1 recruitment to Hox genes is dependent on E(z) methyltransferase activity (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002), suggesting that the loss of mPRC1 Xi accumulation that is observed upon deletion of *Xist* is due to the loss of Ezh2-mediated H3-K27 methylation on the Xi. Therefore, these results are most consistent with a model in which *Xist* RNA recruits Ezh-2, which methylates H3-K27 and facilitates binding of mPRC1 proteins. mPRC1 proteins could then contribute to transcriptional silencing of the Xi by interfering with SWI/SNF chromatin remodeling machinery, blocking transcriptional initiation, mediating additional posttranslational histone modifications, or recruiting additional silencing activities (Shao et al., 1999; Francis et al., 2001; Poux et al., 2001; King et al., 2002; Dellino et al., 2004; Lavigne et al., 2004; Wang et al., 2004). However, we cannot rule out the possibility that *Xist* RNA also contributes more directly to recruitment of mPRC1 proteins, as the Ph proteins in flies, worms, and mammals contain a conserved RNA binding domain which is essential for homeotic gene silencing mediated by the Ph homologue SOP-2 in *C. elegans* (Zhang et al., 2004). Although enrichment of H3-3mK27 may be necessary for enrichment of mPRC1 proteins on the Xi, it is not sufficient, as H3-3mK27 was enriched on the Xi in almost all cells in each cell type examined, whereas mammalian PRC1 proteins were generally Xi enriched in a subset of cells.

mPRC1 proteins exhibited different patterns of enrichment on the Xi at different stages of X inactivation. During initiation of X inactivation in differentiating female ES cells and in undifferentiated ES cells ectopically expressing *Xist*, Phc1 accumulates on the Xi. In contrast, Bmi-1, Cbx2, and Phc2 exhibit Xi enrichment in a significant proportion of MEFs and of differentiating female ES cells at later stages of differentiation, both of which are in the maintenance stage of X inactivation. The proportion of differentiating ES cells exhibiting Xi enrichment of Phc1 declined as the proportion of cells exhibiting X-enrichment of Bmi-1, Cbx2, or Phc2 increased, suggesting that there may be an ordered series in the changes of mPRC1 proteins that accumulate on the Xi as cells progress from initiation to maintenance of X inactivation. As Phc1, Phc2, Bmi-1, and Cbx2 can exhibit simultaneous enrichment on the Xi in TS cells, the changes in Xi accumulation patterns of these four mPRC1 proteins that occur during the transition from initiation to maintenance stages of X inactivation in ES cells cannot be solely due to mutually exclusive recruitment of these proteins to the Xi. Overall levels of Phc2, Bmi-1, and Cbx2 appeared to be comparable in undifferentiated ES cells and differentiating ES cells when judged by intensity of immunostaining (unpublished data), suggesting that the increase in the proportion of cells with Xi enrichment of these mPRC1 proteins in differentiating ES cells is due to the stage-specific Xi enrichment of these proteins. Elucidating the molecular mechanisms that regulate the developmentally modulated accumulation of mPRC1 proteins on the Xi will be important in understanding how combinations of mPRC1 proteins are used to establish and maintain tissue-specific transcriptional silencing of the Xi, and Hox and other genes in mammals.

Our results implicate Phc1 in initiation of X inactivation. However, Xi accumulation of Phc1 during initiation of X inactivation in ES cells is not sufficient to initiate silencing, as *Xist* RNA lacking the A-repeat can recruit Phc1, but does not trigger silencing. Targeted disruption of Phc1 results in perinatal lethality in both sexes (Takihara et al., 1997). As disruption of X inactivation causes female-specific early embryonic death (Marahrens et al., 1997), the Phc1 mutant phenotype suggests that initiation of X inactivation is not affected in the absence of Phc1. It is possible that Phc2 or Phc3 can substitute for Phc1 in these mutant animals. Alternatively, maternal stores of Phc1 may be present in sufficient quantities to mask defects in the initiation of X inactivation, as has been suggested for Eed (Plath et al., 2003; Silva et al., 2003). In contrast to Phc1, Bmi-1, Cbx2, and Phc2 exhibited Xi accumulation more consistent with a role in maintenance of X inactivation. Deletion of the *Xist* gene, which results in loss of Xi enrichment of Bmi-1, Cbx2, and Phc2, results in a slight degree of reactivation of X-linked genes, and levels of re-

activation can be increased if cells are simultaneously treated with DNA demethylating agents and histone deacetylase inhibitors (Csankovszki et al., 2001). Thus, Bmi-1, Cbx2, and Phc2 may be among the redundant factors that contribute to stable X chromosome silencing in somatic cells.

Materials and methods

Cell culture, ES cell differentiation, and transfections

MEFs and 293 cells were cultured using standard conditions and grown on glass coverslips for analysis. MEFs carrying a conditional *Xist* allele on the Xi (Csankovszki et al., 2001) were infected with Cre-expressing adenovirus (Anton and Graham, 1995), as described previously (Plath et al., 2005). Female ES cells, male ES cells with a tetracycline-inducible endogenous *Xist* gene, male tetracycline-inducible *Xist* cDNA transgenic ES cells (Wutz et al., 2002), and TS cells were cultured under standard conditions. To generate tetracycline-inducible male ES cells the reverse tetracycline-controlled transactivator was targeted into the Rosa 26 locus in feeder-free E14 ES cells (Wutz and Jaenisch, 2000). The tetracycline responsive promoter was subsequently introduced into the *Xist* promoter region by homologous recombination. *Xist* expression from the endogenous locus or X-linked cDNA transgenes was induced in undifferentiated male ES cells by the addition of 1 μg/ml doxycycline to cells grown on gelatinized glass coverslips for 24 h before fixation. Differentiation of ES cells was achieved by embryoid body formation (Panning and Jaenisch, 1996).

Constructs

For targeting of the tetracycline-inducible promoter to the endogenous *Xist* gene, a construct was generated containing ~3 kb of sequence, from −4 to −1 kb relative to the P1 *Xist* transcriptional start site, and the first 3 kb of the *Xist* transcribed sequence in the vector pGEM-4Z (provided by S. Mlynarczyk-Evans and K. Worringer, UCSF). A hygromycin-thymidine kinase cassette followed by a tetracycline responsive element from the vector pTRE-d2EGFP (CLONTECH Laboratories, Inc.) was inserted between the homology arms.

Immunostaining and FISH

H3-3mK27 was detected using rabbit (Plath et al., 2003), mouse monoclonal (ABCAM), or chicken antisera. Chicken antibodies against H3-3mK27 peptides were generated, purified, and assayed for specificity as described previously (Plath et al., 2003). MacroH2A was detected using a human autoimmune serum. PcG proteins were detected using mouse mAb to Eed, and rabbit pAbs to mouse Phc1 and Cbx2 and human PHC2, BMI-1, CBX4, and Ring1 (Gunster et al., 1997; Satijn et al., 1997a,b; Sewalt et al., 1998).

For analysis by immunostaining and FISH cells were washed in PBS, and fixed for 10 min on ice in 1× PBS containing 4% PFA solution. Cells were then permeabilized by incubation for 5 min at RT with 1× PBS containing 0.5% Triton X-100, and stored in 1× PBS with 0.2% Tween 20. For immunostaining, cells were incubated for 30 min in blocking buffer (5% goat serum, 0.2% fish skin gelatin, 0.2% Tween in 1× PBS). Primary antibody incubations were performed for 2 h at RT in blocking solution, cells were washed in 1× PBS containing 0.2% Tween-20 (PBS/Tween), and incubated with FITC or Texas red anti–rabbit or anti–mouse antibodies or biotinylated anti–chicken antibodies (Vector Laboratories) in blocking buffer. The biotinylated chicken antibodies were detected with FITC or Texas red avidin DCS (Vector Laboratories) in blocking buffer. Cells were then washed with PBS/Tween, stained with DAPI, and mounted in Vectashield (Vector Laboratories). When FISH followed immunostaining, immunostaining was performed as described above with tRNA (Invitrogen) and RNase inhibitors (Promega) in the blocking buffer. After immunostaining cells were fixed with 4% PFA solution and *Xist* RNA FISH performed as described previously (Plath et al., 2003).

All images were gathered at RT on a epifluorescence microscope (model Eclipse E800; Nikon) using a100× oil (Plan Apo, 1.40 N/A) or a 60× oil (Plan Apo, 1.40 N/A) immersion lens. Images were acquired with a Princeton Instruments RTE/CCD-1317-K/S camera using Openlab 2.2 acquisition software. Images were optimized globally for contrast and brightness and assembled into figures using Adobe Photoshop 6.1.0.

Online supplemental material

Fig. S1 shows the specificity of Cbx2, CBX4, Phc1, PHC2, BMI-1, and Ring1 antisera. Fig. S2 shows localization of transiently expressed, tagged mPRC1 proteins. Fig. S3 shows localization of Eed in ES cells ectopically expressing *Xist*. Fig. S4 shows localization of mPRC1 proteins in differentiating ES cells. Table S1 illustrates localization of mPRC1 proteins in MEFs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200409026/DC1.

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Note added in proof. After submission of this manuscript, two groups also reported enrichment of PRC1 proteins on the inactive X chromosome (de Napoles, M., J.E. Mermoud, R. Wakao, Y.A. Tang, M. Endoh, R. Appanah, T.B. Nesterova, J. Silva, A.P. Otte, M. Vidal, H. Koseki, and N. Brockdorff. 2004. *Dev. Cell.* 7:663–676; Fang, J., T. Chen, B. Chadwick, E. Li, and Y. Zhang. 2004. *J. Biol. Chem.* doi:10.1074/jbc.C400493200).

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