Chromosoma (2005) 114: 183–192
DOI 10.1007/s00412-005-0008-6

RESEARCH ARTICLE

Cecile C. de la Cruz · Jia Fang · Kathrin Plath · Kathleen A. Worringer · Dmitri A. Nusinow · Yi Zhang · Barbara Panning

Developmental regulation of Suz12 localization

Received: 14 March 2005 / Revised: 3 May 2005 / Accepted: 9 May 2005 / Published online: 29 June 2005

Abstract Chromatin modifications are among the epigenetic alterations essential for genetic reprogramming during development. The Polycomb group (PcG) gene family mediates chromatin modifications that contribute to developmentally regulated transcriptional silencing. Trimethylation of histone H3 on lysine 27, mediated by a PcG protein complex consisting of Eed, Ezh2, and Suz12, is integral in differentiation, stem cell self-renewal, and tumorigenesis. Eed and Ezh2 are also implicated in the developmentally regulated silencing of the inactive X chromosome, as they are transiently enriched on the inactive X chromosome when X chromosome silencing is initiated. Here we analyze the dynamic localization of Suz12 during cellular differentiation and X-inactivation. Though Suz12 is a requisite member of the Eed/Ezh2 complexes, we found that Suz12 exhibits a notable difference from Ezh2 and Eed: while Ezh2 and Eed levels decrease during stem cell differentiation, Suz12 levels remain constant. Despite the differential regulation in abundance of Suz12 and Eed/Ezh2, Suz12 is also transiently enriched on the Xi during early stages of X-inactivation, and this accumulation is *Xist* RNA dependent. These results suggest that Suz12 may have a function that is not mediated by its association with Eed and Ezh2, and that this additional function is not involved in the regulation of X-inactivation.

Communicated by R. Paro

Electronic supplementary material Electronic supplementary material is available for this article at http://dx.doi.org/10.1007/s00412-005-0008-6 and accessible for authorised users.

C. C. de la Cruz · K. A. Worringer · D. A. Nusinow · B. Panning
Department of Biochemistry and Biophysics,
University of California,
San Francisco, CA, 94143, USA

J. Fang · Y. Zhang
Department of Biochemistry and Biophysics,
The University of North Carolina,
Chapel Hill, NC, 27599, USA

K. Plath
Whitehead Institute for Biomedical Research,
Cambridge, MA, 02139, USA

B. Panning (✉)
Genentech Hall, Room S372B, 600 16th Street,
San Francisco, CA, 94143-2200, USA
e-mail: bpanning@biochem.ucsf.edu
Tel.: +1-415-5140745
Fax: +1-415-5144080

Introduction

Epigenetic gene silencing is critical during development and plays a role in several cell fate processes, including maintenance of pluripotency, differentiation, and cancer progression. In each case, heritable gene silencing by chromatin modifications contributes to changes in gene expression patterns. Methylation of histone H3 on lysine 27, mediated by Polycomb group (PcG) proteins Eed, Ezh2, and Suz12, has been implicated in transcriptional silencing (Cao et al. 2002; Cao and Zhang 2004b; Czermin et al. 2002; Kirmizis et al. 2004; Kuzmichev et al. 2002; Muller et al. 2002; Pasini et al. 2004).

Dosage compensation in female mammals serves as an excellent model to study the effects of chromatin modifiers on gene expression, since alterations in the epigenetic landscape of the X chromosome result in the transcriptional silencing of one of the two X chromosomes. X-inactivation occurs twice during mouse embryonic development. The first wave of X-inactivation occurs exclusively on the paternally inherited X chromosome during preimplantation development, resulting in imprinted X-inactivation in extraembryonic tissues (Heard 2004). X-inactivation in the embryonic tissues occurs after implantation and is random, such that either the paternally or maternally inherited X chromosome has an equal probability of being silenced in each cell. Both forms of X-inactivation involve at least two steps: the initial silencing of the inactive X chromosome (Xi) and stable maintenance of the Xi throughout all subsequent cell divisions.

The *Xist* gene encodes a 17-kb noncoding RNA that remains in the nucleus to coat the Xi. When X-inactivation

is triggered, *Xist* RNA spreads in *cis* from its site of transcription to cover the Xi (Panning et al. 1997; Sheardown et al. 1997). X chromosomes bearing *Xist* deletions are not silenced, demonstrating that *Xist* is necessary for X-inactivation in *cis* (Marahrens et al. 1997; Penny et al. 1996). Expression of an inducible *Xist* cDNA transgene from autosomes results in *Xist* RNA coating and transcriptional silencing of autosomal genes lying in *cis*, indicating that *Xist* RNA coating is sufficient for silencing (Wutz and Jaenisch 2000). *Xist* continues to be transcribed exclusively from the Xi in differentiated female cells, where it plays a minor role in maintenance of the inactive state.

The Xi in somatic cells is characterized by a series of chromatin modifications that distinguish it from the active X chromosome and autosomes. The Xi exhibits enrichment of the variant histone macroH2A, altered levels of histone H3 and H4 methylation, decreased amounts of acetylation on histones H3 and H4, ubiquitination of histone H2A, and an increase in DNA methylation of CpG islands (de Napoles et al. 2004; Fang et al. 2004; Heard 2004; Smith et al. 2004). These changes in chromatin structure are set up sequentially during the early stages of X-inactivation, indicating that an ordered series of epigenetic modifications may be required to initiate and establish X chromosome silencing (Chaumeil et al. 2002).

The trimethylated form of histone H3 on lysine 27 (H3-3mK27) is enriched on the Xi during the earliest stages of X-inactivation, suggesting a role in initiation of X chromosome silencing (Plath et al. 2003; Silva et al. 2003). This modification persists on the Xi in some somatic cell types during the maintenance phase (Gilbert et al. 2003). H3-3mK27 accumulation on the Xi is dependent on the PcG proteins Eed and Ezh2, which are transiently enriched on the Xi in differentiating female embryonic cells (Erhardt et al. 2003; Plath et al. 2003; Silva et al. 2003). While *Eed* mutant females initiate X-inactivation normally, perhaps due to abundant maternal stores of Eed, a subset of extraembryonic cells reactivates their Xi, demonstrating a role for this complex in the maintenance of X-inactivation (Silva et al. 2003; Wang et al. 2001). Although Eed and Ezh2 play a vital role in the trimethylation of histone H3 on lysine 27 on the Xi, it is still unknown whether Suz12 is also required for this process.

To better understand the function of Suz12, we analyzed the alterations in its amount and distribution during stem cell differentiation. Eed and Ezh2 levels drop upon differentiation (Kuzmichev et al. 2005; Silva et al. 2003). In contrast, amount of Suz12 remained constant in differentiating stem cells, suggesting a role for Suz12 in addition to the regulation of Eed/Ezh2-mediated histone methylation. While Suz12 differed from Eed and Ezh2 in developmental regulation of abundance, it showed the same transient enrichment on the Xi in differentiating cells as Eed and Ezh2. Enrichment of Suz12 on the Xi required *Xist* RNA. As expected for an essential component of the H3-3mK27 histone methyltransferase complex, knockdown of SUZ12 in human cells results in loss of Xi-enriched and genome-wide H3-3mK27. These data indicate that Suz12 contributes to H3-K27 methylation on the Xi.

Materials and methods

Cell culture

Trophoblast stem (TS) cells were kindly provided by Uy et al. (2002) and cultured as described by Tanaka et al. (1998). To induce differentiation, TS cells were washed with PBS and then cultured in medium without FGF4, heparin, and EMFI-CM. Transformed mouse embryonic fibroblasts, transformed mouse fibroblasts, ES cells, and blastocysts were cultured as described previously (Panning et al. 1997). Analysis of ES cell differentiation was carried out as indicated in Plath et al. 2003. Male ES cells carrying a tetracycline inducible *Xist* gene on the X chromosome and expressing the reverse tetracycline-controlled transactivator from the *Rosa 26* locus (Plath et al. 2004) were incubated in the presence of 2 μg/μl doxycycline for 24 h. The IMR90 human female fibroblasts were purchased from American Type Culture Collection (ATCC, Cat # CCL-186) and cultured according to ATCC's handling procedures.

siRNA transfection

SUZ12 siRNA SMARTpool (M0069570050) and control GFP siRNA oligonucleotides (D0013000120) were obtained from Dhramacon Research Inc. siRNA duplexes were transfected into IMR90 cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfected cells were incubated with the siRNA for 72 h, at which time they were harvested and replated at the original starting density. Cells were then retransfected with the oligonucleotides and incubated for an additional 72 h. Six days after the initial siRNA transfection, the cells were fixed for immunofluorescence as detailed below.

Antibodies

Antibodies against Ezh2, Eed, H3-3mK27, and RbAp48 have been previously described (Cao et al. 2002; Hamer et al. 2002; Kirmizis et al. 2003, 2004). Two different rabbit polyclonal antibodies for Suz12 were used (Abcam; Cao et al. 2002). A chicken Eed serum (Abcam) gave results identical to the mouse monoclonal (Hamer et al. 2002) and was also employed in some instances. In some experiments, a mouse monoclonal H3-3mK27 antibody (Abcam) was used.

Immunofluorescence and fluorescence in situ hybridization

Immunofluorescence and combined FISH and immunofluorescence were carried out as described previously (Plath et al. 2003). *Xist* RNA was detected with a fluorescein-UTP (Roche) labeled single-stranded RNA probe antisense to mouse *Xist* exon 7. Human *XIST* RNA was detected with

a fluorescein-UTP (Roche) labeled single-stranded RNA probe antisense to *XIST* exon 1.

Western blotting

For Western blots, ES and TS cells were collected, washed, and lysed for 2 h in an appropriate amount of RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 1% NP-40, 0.25% sodium deoxycholate, 0.1 mM PMSF, 0.5 mM DTT, leupeptin, aprotinin, and pepstatin A, pH 7.4). The solution was then clarified by centrifuging at top speed for 15 min, and supernatant proteins were fractionated in a 12% SDS-PAGE. Blotting was carried out using standard procedures.

Results

Suz12 levels remain constant throughout stem cell differentiation

Eed and Ezh2 levels decrease upon differentiation of embryonic stem (ES) cells (Kuzmichev et al. 2004; Silva et al. 2003). To determine whether Suz12 was also down-regulated during differentiation, we analyzed the amounts of Eed, Ezh2, and Suz12 in undifferentiated ES cells and a transformed fibroblast cell line. Western blot analysis showed that levels of Eed and Ezh2 were very low to undetectable in the fibroblast line and abundant in the undifferentiated ES cells (Fig. 1a), consistent with the previously reported differentiation-induced decrease in abundance of these two PRC2 proteins (Kuzmichev et al. 2004; Silva et al. 2003). Suz12 was present in similar quantities in both cell types (Fig. 1a). These results were confirmed by immunostaining for Eed, Ezh2, and Suz12 in undifferentiated ES cells and fibroblasts (Supplementary Fig. 1). We next examined whether levels of Suz12 changed during differentiation of stem cells. During an ES cell differentiation time course, amounts of Ezh2 decreased rapidly upon differentiation, such that by day 6, no protein was detectable (Fig. 1b), consistent with the previously reported decrease in Ezh2 and Eed in differentiating ES cells (Kuzmichev et al. 2005; Silva et al. 2003). In contrast, levels of Suz12 remained constant throughout the time course. A stem cell type of the extraembryonic lineage, trophoblast stem (TS) cells, displayed similar dynamics of Ezh2 and Suz12 levels when differentiated (Fig. 1c). Ezh2 was virtually absent by day 6, and Suz12 levels remained uniform upon differentiation. These data indicate that developmental regulation of Suz12 expression differs from that of Ezh2 and suggests that Suz12 may carry out functions that are independent of its interaction with Eed and Ezh2.

Suz12 is transiently recruited to the Xi upon initiation of X-inactivation

Eed and Ezh2 are transiently enriched on the Xi during initiation of X chromosome silencing (Plath et al. 2003; Silva et al. 2003), and Suz12 has been detected in cells initiating X-inactivation (de Napoles et al. 2004). As levels of Suz12 remained constant throughout ES cell differentiation, while the levels of Eed and Ezh2 dropped, it suggested that Suz12 might show a temporally distinct pattern of Xi-enrichment during the progression of X-inactivation. To determine whether the kinetics of Xi-localization of Suz12 differed from that of Eed, we analyzed the localization of Suz12 and Eed in differentiating ES cells. Immunostaining for Suz12 was combined with

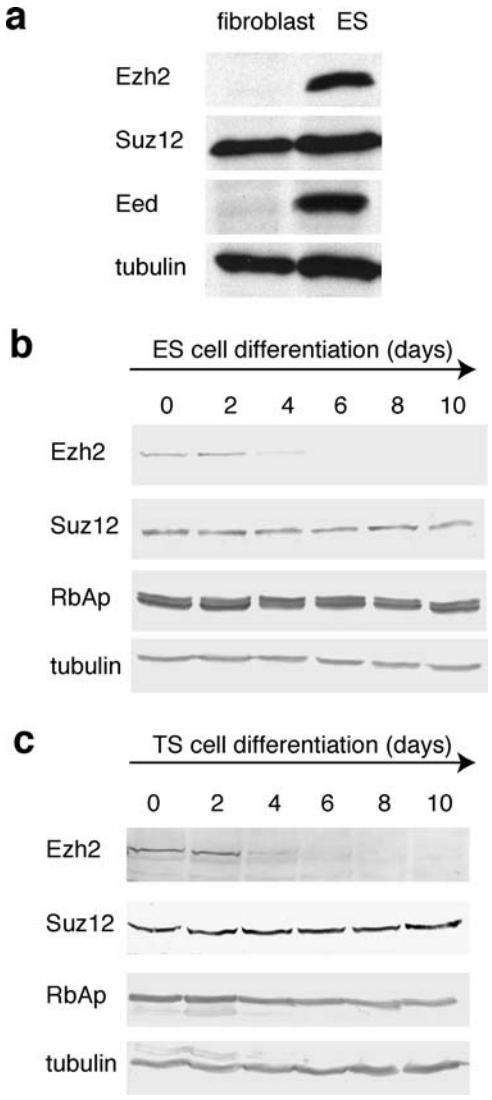

Fig. 1 Levels of Suz12 remain constant throughout stem cell differentiation. **a** Western analysis of Ezh2, Suz12, Eed, and tubulin levels in transformed mouse fibroblast cells and ES cells. Levels of Ezh2, Suz12, and Eed are abundant in ES cells, whereas only Suz12 is detectable in fibroblast cells. **b**, **c** Western analysis of Ezh2, Suz12, H3-3mK27, RbAp, and tubulin levels in differentiating ES (**b**) and TS (**c**) cells

fluorescence in situ hybridization (FISH) for *Xist* RNA or immunostaining for Eed to mark the Xi. In undifferentiated ES cells that had no *Xist* RNA coating or enrichment of Eed on either of active X chromosomes, Suz12 was distributed uniformly throughout the nucleus (Fig. 2a, c). When ES cells were induced to differentiate, Suz12 accumulated on the Xi, as marked by *Xist* RNA coating or Eed accumulation (Fig. 2b, d). By day 2 of differentiation, 97% of cells that had an *Xist* RNA-coated Xi showed Xi-enrichment of Suz12 (Fig. 2e). At day 6 of differentiation, 95% of cells that had an *Xist* RNA-coated Xi showed Xi-enrichment of Suz12 or Eed (Fig. 2e). The proportion of cells with an *Xist* RNA-coated Xi that displayed Suz12 or Eed enrichment remained steady until day 8 of differentiation and then dropped, so that by day 13, there were no cells with Suz12 or Eed Xi-enrichment. These results indicate that Suz12, like Eed, is transiently recruited to the Xi during initiation of X-inactivation in differentiating ES cells. Since Suz12 levels remain relatively high in differentiated cells, the lack of Xi-enrichment of Suz12 suggests that Suz12 can only accumulate on the Xi when it is present in a complex with Eed and Ezh2.

The enrichment of Eed and Ezh2 on the Xi is transient in extraembryonic cell types as well (Plath et al. 2003; Silva et al. 2003). To determine whether Suz12 showed the same transient enrichment on the Xi, we analyzed the distribution of Suz12 and Eed in cultured blastocysts. Trophoblast giant cells, differentiated extraembryonic cells derived from the trophectoderm, develop from cultured blastocysts. We observed progressive loss of Suz12 and Eed staining on the Xi in giant cells. Four days after plating, the giant cells of female blastocysts showed a strong Xi

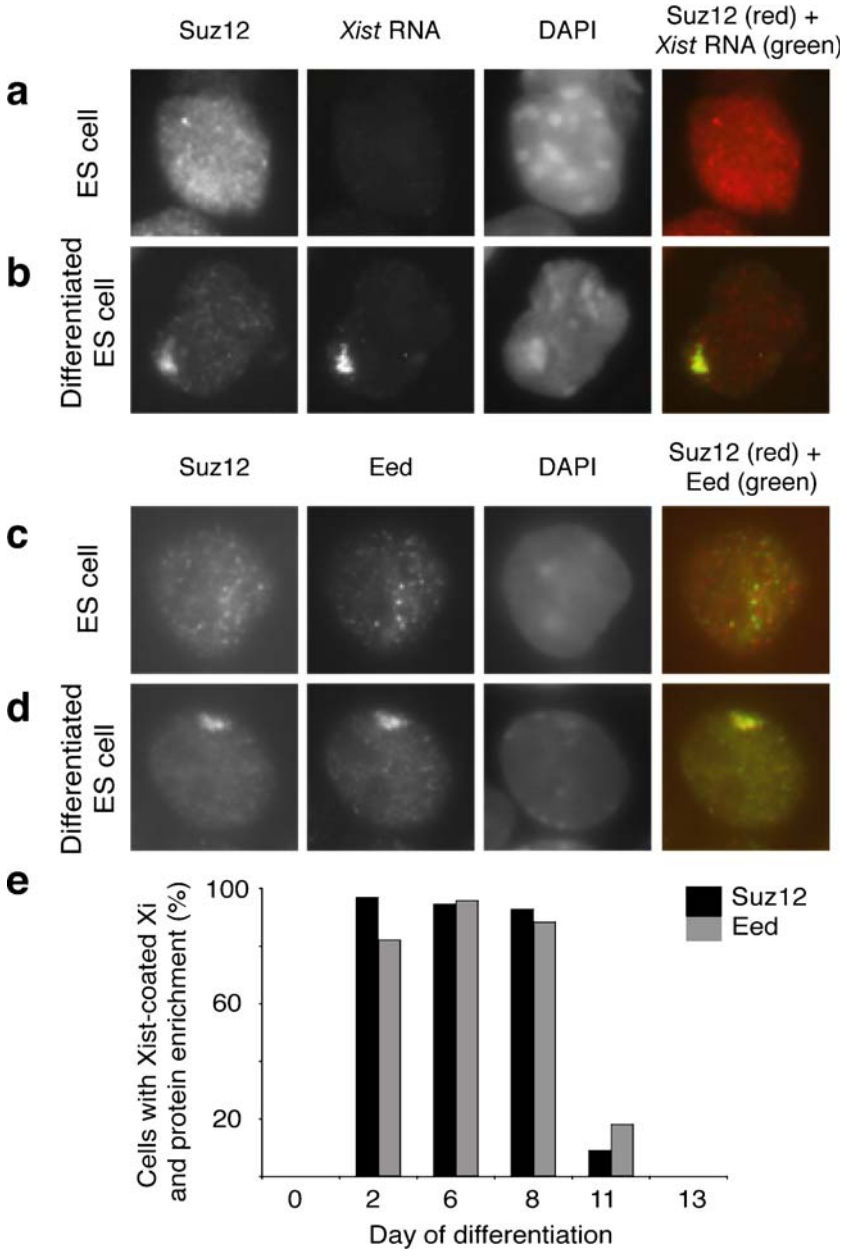

Fig. 2 Suz12 is enriched on the Xi in differentiating ES cells during the onset of X-inactivation. **a**, **b** Immunostaining for Suz12 (*first column*) was combined with FISH for *Xist* RNA (*second column*) in undifferentiated (**a**) and differentiated (**b**) ES cells. DAPI delineates the nucleus (*third column*), and the merged image (*fourth column*) consists of Suz12 (*red*) and *Xist* RNA (*green*). **a** In undifferentiated ES cells, X-inactivation has not occurred and *Xist* is not expressed. Suz12 is diffuse throughout the nucleus. **b** In differentiated ES cells, *Xist* is expressed from the Xi and this is accompanied by Xi-enrichment of Suz12. **c**, **d** Immunostaining for Suz12 (*first column*) and Eed (*second column*) in undifferentiated (**c**) and differentiated (**d**) ES cells. DAPI delineates the nucleus (*third column*), and the merged image (*fourth column*) is an overlay of Suz12 (*red*) and Eed (*green*). **c** Suz12 and Eed are diffusely distributed in undifferentiated ES cells. **d** Four days after induction of differentiation, Suz12 accumulates on the Xi, which is marked by Eed enrichment. **e** Graph indicating the percentage of ES cells with an *Xist* RNA-coated Xi accompanied by enrichment of Eed (*gray bar*) or Suz12 (*black bar*) during a differentiation time course (n>100)

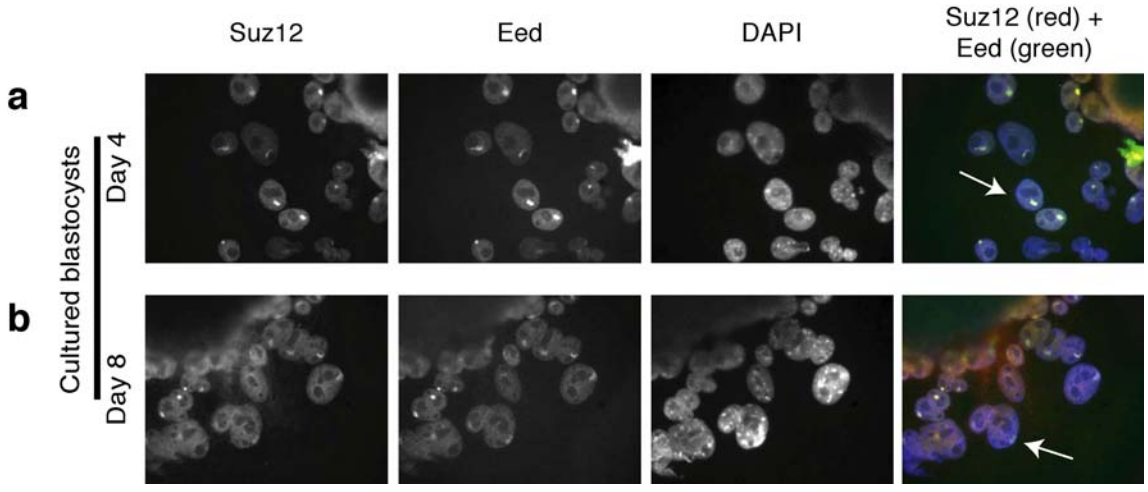

Fig. 3 Distribution of Suz12 in female blastocysts. **a**, **b** Immunostaining for Suz12 (*first column*) and Eed (*second column*) in cultured blastocysts. DAPI delineates the nuclei (*third column*), and the merged image (*fourth column*) consists of Suz12 (*red*) and Eed (*green*). **a** Female blastocysts cultured for 4 days. The Xi in differentiated extraembryonic cells that have migrated away from the ICM, marked by the accumulation of Eed, is also enriched for Suz12 (*arrow*). **b** Xi-enrichment of Eed and Suz12 is lost in most extraembryonic cells from blastocysts cultured for 8 days (*arrow*)

staining pattern of Suz12 and Eed (Fig. 3a). After 8 days in culture, the Xi staining of Suz12 and Eed were considerably weaker, and many giant cells lacked any detectable staining (Fig. 3b). These results demonstrate that Suz12 shows the same dynamic enrichment on the Xi that was previously observed for Eed and Ezh2.

TS cells are an extraembryonic stem cell type that is derived from the trophectoderm (Kunath et al. 2004; Tanaka et al. 1998). TS cells are unusual in that they are the only cell type that exhibits enrichment of Eed, Ezh2, and H3-3mK27 on the Xi in their undifferentiated state (Mak et al. 2002). When TS cells are differentiated, there is a decrease in Eed, Ezh2, and H3-3mK27 staining on the

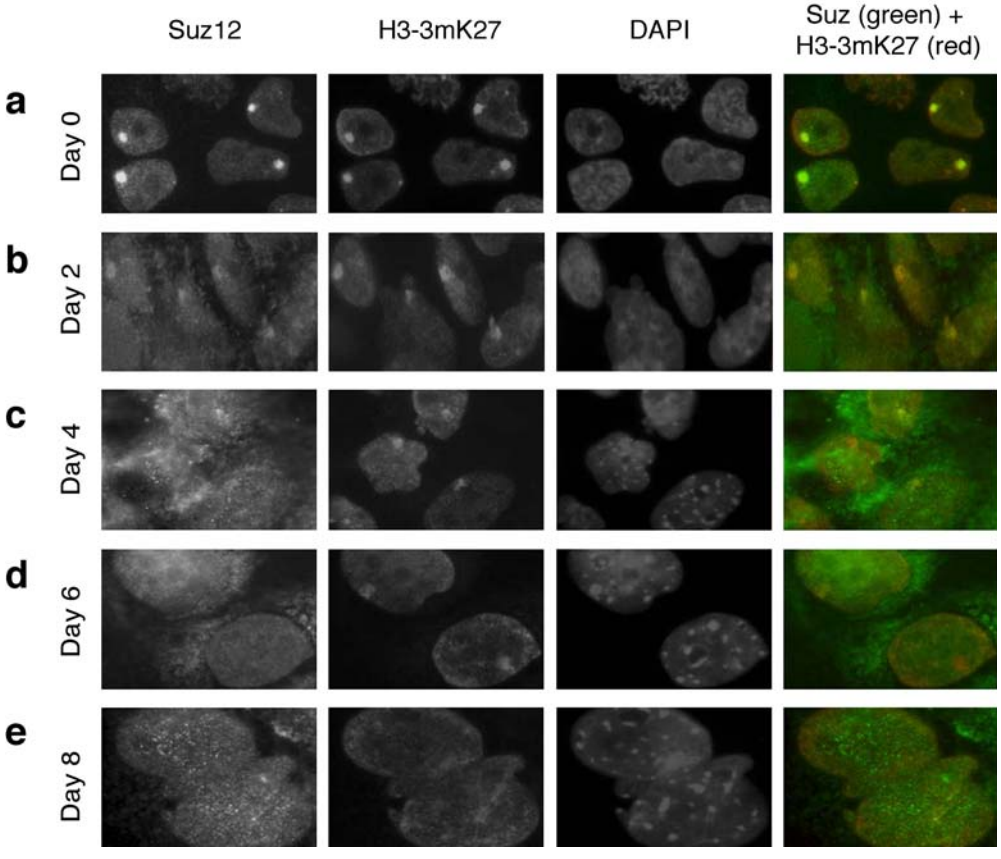

Fig. 4 Differentiating trophoblast stem (TS) cells exhibit a transient enrichment of Suz12. **a**–**e** Immunostaining for Suz12 (*green*, *first column*) and H3-3mK27 (*red*, *second column*) in TS cells throughout several time points during differentiation. DAPI (*third column*) delineates the nuclei, and the merged image (*fourth column*) consists of Suz12 and H3-3mK27. **a**, **b** At days 0 and 2 of TS differentiation, Suz12 is enriched on the Xi, as marked by H3-3mK27. **c** Suz12 Xi-accumulation is lost completely by day 4. **e** Loss of the histone methylation mark is observed by day 8

Xi, and after several days in differentiating conditions, Xi-enrichment of these marks is no longer detected (Plath et al. 2003; Silva et al. 2003). To determine if Suz12 showed the same progressive loss of Xi-enrichment, we performed immunofluorescence for Suz12 on differentiating female TS cells (Fig. 4). Undifferentiated TS cells exhibited enrichment of Suz12 on the Xi, shown by colocalization of Suz12 with H3-3mK27 (Fig. 4a). Differentiating TS cells showed clear localization of Suz12 and H3-3mK27 on the Xi in all cells at 2 days of differentiation (Fig. 4b). There was a gradual decrease in the proportion of cells showing Xi-localization of Suz12, such that by day 4, cells with Xi-enrichment of Suz12 were no longer detected (Fig. 4c). H3-3mK27 enrichment on the Xi also decreased over time and was no longer detectable by day 8 of differentiation (Fig. 4e). These results indicate that Xi-enrichment of Suz12, like Xi-enrichment of Eed, Ezh2, and H3-3mK27, is lost upon differentiation of TS cells. Suz12 redistributed from an exclusively uniform nuclear staining pattern with Xi-enrichment (Fig. 4a) to accumulate in many dispersed bright speckles within the nucleus (Fig. 4e), suggesting that developmental regulation of Suz12 localization at regions other than the Xi may occur. In addition, Suz12 was also detected in the cytoplasm of differentiating TS cells, a distribution that was unique to TS cells.

Suz12 recruitment to the Xi is dependent on *Xist* RNA

Xi-accumulation of Ezh2, Eed, and H3-3mK27 is dependent on initial coating of the Xi by *Xist* RNA (Plath et al. 2003; Silva et al. 2003). To determine whether Xi-enrichment of Suz12 was also *Xist* RNA dependent, we assayed whether induction of *Xist* expression in undifferentiated male ES cells was sufficient to recruit Suz12. We employed a cell line in which the *Xist* promoter was replaced by a tetracycline-inducible promoter (Plath et al. 2004). The distribution of *Xist* RNA and Suz12 was assayed in induced and uninduced cells. Uninduced cells did not express *Xist* RNA and did not show any localized enrichment of Suz12 (Fig. 5a). After 24 h of induction (Fig. 5b), *Xist* RNA coated the X chromosome and Suz12 was also enriched on the X, indicating that *Xist* RNA is sufficient to recruit Suz12 to the Xi. Given that *Xist* RNA is also sufficient to recruit Eed and Ezh2 (Plath et al. 2003), it is likely that Suz12 recruitment to the Xi occurs as a complex with Eed and Ezh2.

Differentiated cell types do not exhibit constitutive Xi-enrichment of Suz12

To determine if Suz12 is localized to the Xi in somatic cells in the maintenance stage of X-inactivation, we stained two female transformed clonal fibroblast cell lines for Suz12 and used FISH for *Xist* RNA to mark the Xi. The majority of these cells exhibited nuclear speckles of Suz12 that were not uniformly distributed (Fig. 6a). Suz12 was enriched on the Xi in a very small number of these female somatic cells (Fig. 6b). Therefore, we conclude that Suz12 functions in maintenance of X-inactivation by a mechanism that, at most, requires only transient enrichment on the Xi.

In transformed mouse fibroblasts, the DAPI-intense regions stained less intensely than the rest of the nucleus with Suz12 antibodies (Fig. 6a, b), suggesting that endogenous Suz12 does not accumulate on constitutive heterochromatin. This contrasts a previous study, which reported that tagged overexpressed SUZ12 colocalized with tagged, overexpressed HP1α in COS-1 cells, a transformed monkey kidney cell line (Yamamoto et al. 2004). These differing results may be due to differences in cell type or species or due to detection of endogenous vs overexpressed, tagged proteins.

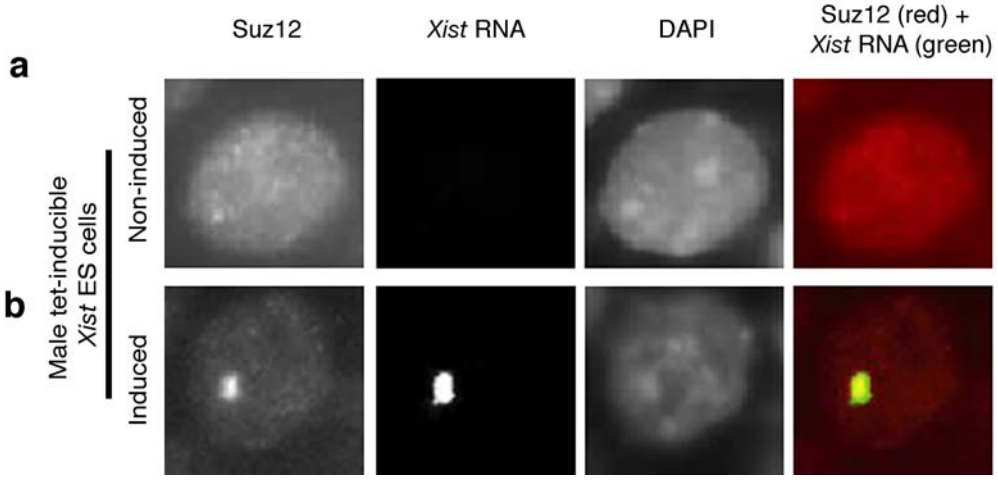

Fig. 5 Xi-recruitment of Suz12 is dependent on *Xist* and Eed. **a** *Xist* RNA coating is sufficient to recruit Suz12. Immunostaining for Suz12 (*first column*) with FISH for *Xist* RNA (*second column*) in a male ES cell with a tet-inducible promoter upstream of the single *Xist* gene. DAPI (*third column*) demarcates the nucleus, and the merged image (*fourth column*) is comprised of Suz12 (*red*) and *Xist* RNA (*green*). **a** Noninduced cells lack an *Xist*-coated body, and no enrichment of Suz12 is observed. **b** Induction of *Xist* expression results in coating of the single X chromosome and subsequent recruitment of Suz12

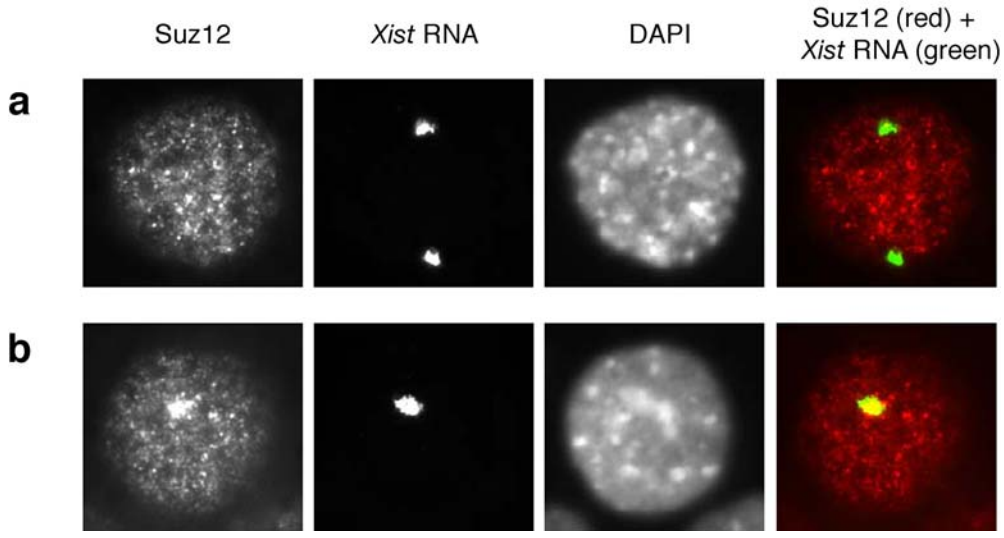

Fig. 6 Two patterns of Suz12 distribution in somatic cells. **a**, **b** Immunostaining for Suz12 (*first column*) with FISH for *Xist* RNA (*second column*) in mouse embryonic fibroblasts (MEFS)MEFS, which are aneuploid and, in some instances, have more than one Xi. DAPI demarcates the nucleus, and the merged image is comprised of Suz12 (*red*) and *Xist* RNA (*green*). **a** Staining pattern observed in a majority of cells. Suz12 is diffusely nuclear in somatic cells but do not exhibit an enrichment of Suz12 on the *Xist* RNA-coated Xi. **b** Staining pattern observed in a small proportion of cells (<1%, n>100). Suz12 shows colocalization with *Xist* RNA

Knockdown of SUZ12 in human somatic cells results in loss of H3-3mK27

Knockdown of SUZ12 in human somatic cells and a mutation of *Suz12* in mouse embryos cause a significant decrease in amounts of H3-3mK27, indicating that SUZ12 contributes to H3-K27 methylation (Cao and Zhang 2004b; Kirmizis et al. 2004; Pasini et al. 2004). We undertook a single cell analysis to determine if SUZ12 is required for EED/EZH2 complex-mediated H3-K27 methylation on the Xi, employing female human fibroblast IMR90 cells, which were significantly more efficiently transfected with siRNAs than mouse embryo fibroblasts. Immunostaining was used to assay the abundance and distribution of H3-3mK27 in IMR90 cells transfected with siRNA oligonucleotides directed against SUZ12 or GFP RNA.

Untransfected and GFP siRNA transfected IMR90 cells exhibited abundant nuclear staining for H3-3mK27 (Fig. 7a and data not shown). In 70% of interphase nuclei, there was enrichment of this methylated form of H3 on the Xi, as visualized by colocalization with *XIST* RNA (Fig. 7a). This result indicates that in IMR90 cells, Xi-enrichment of H3-3mK27 is maintained in a majority of cells, as previously reported for other somatic cell types (Gilbert et al. 2003; Plath et al. 2004). The remaining 30% of GFP siRNA transfected cells showed a similar intensity of uniform nuclear H3-3mK27 staining, without enrichment on the *XIST* RNA-coated Xi (Fig. 7b). GFP siRNA transfected IMR90 cells exhibited a uniform, speckled nuclear distribution of SUZ12 in 100% of cells with no apparent enrichment on the Xi, even in cells that showed Xi-accumulation of H3-3mK27 (Fig. 7c, d).

Transfection of cells with SUZ12 siRNA resulted in complete knockdown in half of the cell population (Fig. 7e, f). While a small proportion of cells lacking SUZ12 displayed normal H3-3mK27 staining either with or without Xi-enrichment (12% of cells; Fig. 7f), the majority of cells with no detectable SUZ12 did not accumulate H3-3mK27 (88% of cells; Fig. 7f). Cells lacking SUZ12 still retained an *XIST* RNA-coated Xi (Fig. 7g), demonstrating that H3-3mK27 enrichment on the Xi is not required for *XIST* RNA to associate with the Xi in these cells. Knockdown of Suz12 in transformed mouse embryo fibroblasts also resulted in complete loss of detectable Suz12 and H3-3mK27 staining in a fraction of cells (data not shown). These data indicate that complete knockdown of SUZ12 results in the global loss of H3-3mK27. Thus, SUZ12 is necessary for enrichment of H3-3mK27 on the Xi in somatic cells, even though it is not detectably enriched there. This result also demonstrates that a local enrichment of SUZ12 is not required for a local increase in H3-3mK27 histone methyltransferase activity during the maintenance phase of X-inactivation.

Discussion

Suz12 is enriched on the Xi during initiation of X-inactivation (de Napoles et al. 2004). Here we demonstrate that Suz12 Xi-enrichment is transient during early stages of X-inactivation in embryonic and extraembryonic cells. While Suz12, Eed, and Ezh2 showed very similar patterns of enrichment on the Xi during development, the total amount of Suz12 remained fairly constant throughout differentiation, whereas Ezh2 and Eed levels steadily decreased. Suz12 Xi-enrichment required *Xist* RNA, consistent with a role for *Xist* RNA in targeting Suz12 to the Xi as part of the Eed/Ezh2 complexes. Single cell analysis revealed that complete knockdown of SUZ12 resulted in loss of all detectable H3-3mK27 accumulation on the Xi and in other

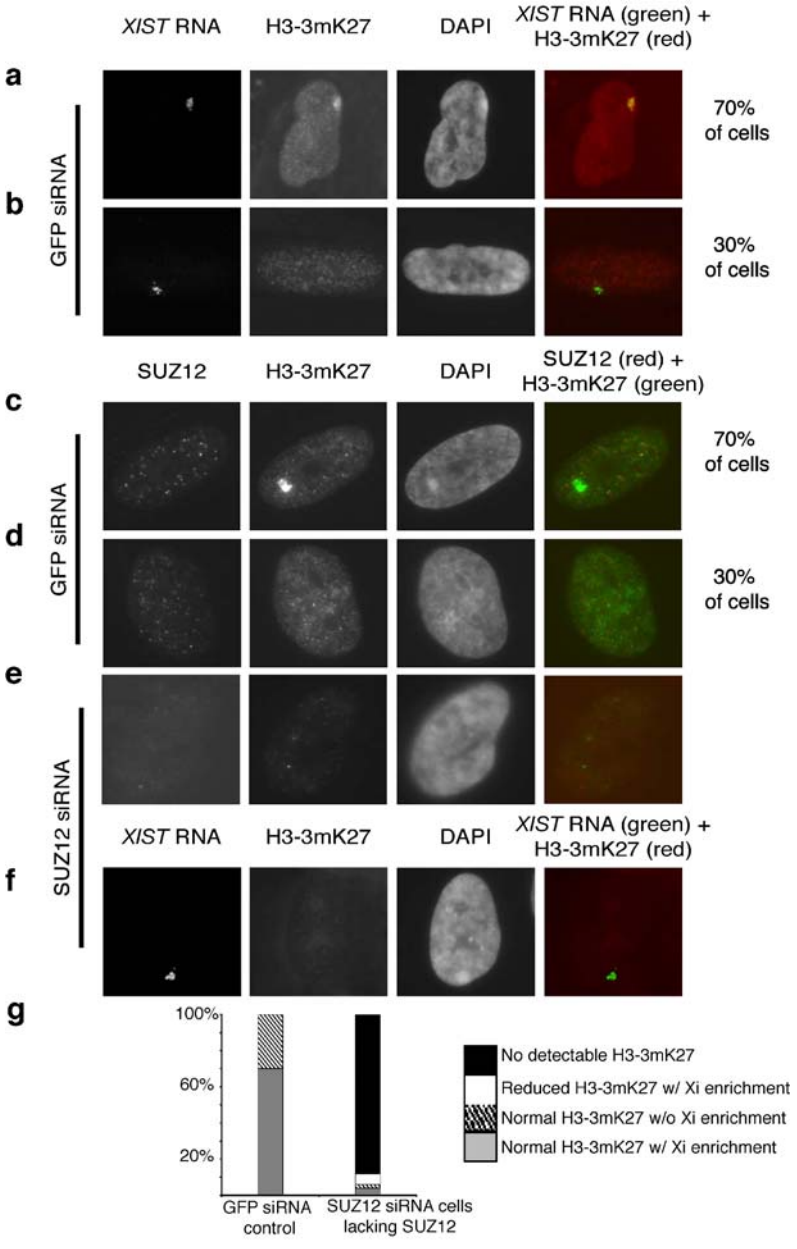

Fig. 7 Distribution of SUZ12 and H3-3mK27 in SUZ12 knockdown cells. **a**, **b** FISH for *XIST* RNA (*first column*) was combined with immunostaining for H3-3mK27 (*second column*) in IMR90 primary human fibroblasts. DAPI delineates the nucleus (*third column*), and the merged image (*fourth column*) consists of *XIST* RNA (*green*) and H3-3mK27 (*red*). Cells exhibit two patterns of H3-3mK27 distribution, fairly uniform nuclear distribution of fine speckles with (**a**) or without enrichment on the Xi (**b**). **c**–**e** Immunostaining for SUZ12 (*first column*) and H3-3mK27 (*second column*) in IMR90 cells treated with GFP siRNAs (**c**, **d**) or SUZ12 siRNAs (**e**). Nuclei are stained with DAPI (*third column*), and the merged image (*fourth column*) consists of SUZ12 (*red*) and H3-3mK27 in green. **c**, **d** SUZ12 is distributed uniformly throughout the nucleus with many larger speckles of more intense staining in GFP siRNA treated cells, the same distribution that is observed in mock transfected or untransfected cells (data not shown). **e** After SUZ12 siRNA treatment, half the cells exhibited little or no SUZ12 staining. The majority of these cells contained no H3-3mK27. **f** FISH for *XIST* RNA (*first column*) was performed with immunostaining for H3-3mK27 (*second column*) in IMR90 cells subjected to SUZ12 siRNA. DAPI delineates the nucleus (*third column*), and the merged image (*fourth column*) consists of *XIST* RNA (*green*) and H3-3mK27 (*red*). *XIST* RNA still coats the Xi in cells lacking H3-3mK27. **g** Graph indicating the proportion of nuclei in GFP siRNA-treated and SUZ12 siRNA-treated cells with different patterns of H3-3mK27 distribution (n>100 cells). Only SUZ12 siRNA-treated cells lacking SUZ12 were used for this analysis. *Diagonal striped bars* indicate proportion of cells with normal levels of H3-3mK27 and no enrichment on the Xi, *gray bars* indicate the proportion of cells with normal H3-3mK27 levels and Xi-enrichment, *white bars* indicate reduced nuclear staining with Xi-enrichment, and *black bars* indicate no detectable H3-3mK27 staining

genomic regions, consistent with a role for SUZ12 in H3-K27 methylation of the Xi.

The developmentally regulated decrease in abundance of Eed and Ezh2 but not Suz12 suggests one of two possibilities: Suz12 may be a nonstoichiometric component of the Eed/Ezh2 complexes in differentiated cells, or it may have functions outside its role as part of the Eed/Ezh2 complexes. Analysis of in vitro reconstituted EED/EZH2 complexes, as well as material purified from human somatic cells, indicates that SUZ12 is present in equimolar amounts with EED and EZH2 in active histone methyltransferase complexes, making it unlikely that Suz12 is required in excess in vivo (Cao et al. 2002; Kuzmichev et al. 2002, 2004). Thus, it seems likely that Suz12 may have activities that are independent of its role in the Eed/Ezh2 complexes.

Drosophila Su(z)12 is unique among PcG genes in that null and hypomorphic mutations are strong suppressors of position effect variegation, suggesting that in flies, Su(z)12 has functions beyond PcG-mediated facultative heterochromatin formation. The majority of mutations that affect position effect variegation alter the activity of proteins involved in constitutive heterochromatin formation. In mammals and flies, facultative and constitutive heterochromatin are characterized by histone H3 methylation at different lysine residues, mediated by the Eed/Ezh2 and Suv39h histone methyltransferases (Cao and Zhang 2004a; Jenuwein and Allis 2001). Additionally, RNAs are implicated in directing these histone methyltransferases to their genomic targets in mammals and fission yeast (Hall et al. 2002; Maison et al. 2002; Plath et al. 2003; Silva et al. 2003; Volpe et al. 2002, 2003). Therefore, an additional role for Suz12, as suggested by its differential developmental regulation from other PcG proteins, may be to function in constitutive heterochromatin formation, perhaps by affecting activity or targeting of additional histone methyltransferase complexes.

Acknowledgements We thank the members of the Panning Lab, Brian Biehs, and Uta Grieshammer for their critical reading of the manuscript. We are grateful to Susanna Mlynarczyk-Evans for assistance with cell culture and Hannah Cohen for generating the human *XIST* RNA probe. B.P. is a Pew Scholar and is funded by NIH and by grants from the Howard Hughes Medical Institute and the Sandler Family Foundation. K.P. was a Fellow of the Life Sciences Research foundation and is currently a Special Fellow of the Leukemia and Lymphoma Society. D.A.N. is a recipient of the University of California, Office of the President Dissertation Year fellowship. K.A.W. is an A.R.C.S. foundation fellow. C.C.C. is a recipient of a National Sciences Foundation predoctoral fellowship.

References

Cao R, Zhang Y (2004a) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 14:155–164

Cao R, Zhang Y (2004b) SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED–EZH2 complex. Mol Cell 15:57–67

Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298:1039–1043

Chaumeil J, Okamoto I, Guggiari M, Heard E (2002) Integrated kinetics of X chromosome inactivation in differentiating embryonic stem cells. Cytogenet Genome Res 99:75–84

Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V (2002) Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111:185–196

de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, Nesterova TB, Silva J, Otte AP, Vidal M, Koseki H, Brockdorff N (2004) Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell 7:663–676

Erhardt S, Su IH, Schneider R, Barton S, Bannister AJ, Perez-Burgos L, Jenuwein T, Kouzarides T, Tarakhovsky A, Surani MA (2003) Consequences of the depletion of zygotic and embryonic enhancer of zeste 2 during preimplantation mouse development. Development 130:4235–4248

Fang J, Chen T, Chadwick B, Li E, Zhang Y (2004) Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X-inactivation. J Biol Chem 279:52812–52815

Gilbert N, Boyle S, Sutherland H, de Las Heras J, Allan J, Jenuwein T, Bickmore WA (2003) Formation of facultative heterochromatin in the absence of HP1. EMBO J 22:5540–5550

Hall IM, Shankaranarayana GD, Noma K, Ayoub N, Cohen A, Grewal SI (2002) Establishment and maintenance of a heterochromatin domain. Science 297:2232–2237

Hamer KM, Sewalt RG, den Blaauwen JL, Hendrix T, Satijn DP, Otte AP (2002) A panel of monoclonal antibodies against human polycomb group proteins. Hybrid Hybridomics 21:245–252

Heard E (2004) Recent advances in X-chromosome inactivation. Curr Opin Cell Biol 16:247–255

Jenuwein T, Allis CD (2001) Translating the histone code. Science 293:1074–1080

Kirmizis A, Bartley SM, Farnham PJ (2003) Identification of the polycomb group protein SU(Z)12 as a potential molecular target for human cancer therapy. Mol Cancer Ther 2:113–121

Kirmizis A, Bartley SM, Kuzmichev A, Margueron R, Reinberg D, Green R, Farnham PJ (2004) Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 18:1592–1605

Kunath T, Strumpf D, Rossant J (2004) Early trophoblast determination and stem cell maintenance in the mouse—a review. Placenta 25(Suppl A):S32–S38

Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the enhancer of Zeste protein. Genes Dev 16:2893–2905

Kuzmichev A, Jenuwein T, Tempst P, Reinberg D (2004) Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol Cell 14:183–193

Kuzmichev A, Margueron R, Vaquero A, Preissner TS, Scher M, Kirmizis A, Ouyang X, Brockdorff N, Abate-Shen C, Farnham P, Reinberg D (2005) Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. Proc Natl Acad Sci U S A 102:1859–1864

Maison C, Bailly D, Peters AH, Quivy JP, Roche D, Taddei A, Lachner M, Jenuwein T, Almouzni G (2002) Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. Nat Genet 30:329–334

Mak W, Baxter J, Silva J, Newall AE, Otte AP, Brockdorff N (2002) Mitotically stable association of polycomb group proteins eed and enx1 with the inactive x chromosome in trophoblast stem cells. Curr Biol 12:1016–1020

Marahrens Y, Panning B, Dausman J, Strauss W, Jaenisch R (1997) Xist-deficient mice are defective in dosage compensation but not spermatogenesis. Genes Dev 11:156–166

Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, O'Connor MB, Kingston RE, Simon JA (2002) Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111:197–208

Panning B, Dausman J, Jaenisch R (1997) X chromosome inactivation is mediated by Xist RNA stabilization. Cell 90:907–916

Pasini D, Bracken AP, Jensen MR, Denchi EL, Helin K (2004) Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. EMBO J 23:4061–4071

Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N (1996) Requirement for Xist in X chromosome inactivation. Nature 379:131–137

Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, de la Cruz CC, Otte AP, Panning B, Zhang Y (2003) Role of histone H3 lysine 27 methylation in X inactivation. Science 300:131–135

Plath K, Talbot D, Hamer KM, Otte AP, Yang TP, Jaenisch R, Panning B (2004) Developmentally regulated alterations in Polycomb repressive complex 1 proteins on the inactive X chromosome. J Cell Biol 167:1025–1035

Sheardown SA, Newall AE, Norris DP, Rastan S, Brockdorff N (1997) Regulatory elements in the minimal promoter region of the mouse Xist gene. Gene 203:159–168

Silva J, Mak W, Zvetkova I, Appanah R, Nesterova TB, Webster Z, Peters AH, Jenuwein T, Otte AP, Brockdorff N (2003) Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed–Enx1 polycomb group complexes. Dev Cell 4:481–495

Smith KP, Byron M, Clemson CM, Lawrence JB (2004) Ubiquitinated proteins including uH2A on the human and mouse inactive X chromosome: enrichment in gene rich bands. Chromosoma 113:324–335

Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J (1998) Promotion of trophoblast stem cell proliferation by FGF4. Science 282:2072–2075

Uy GD, Downs KM, Gardner RL (2002) Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse. Development 129:3913–3924

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 297:1833–1837

Volpe T, Schramke V, Hamilton GL, White SA, Teng G, Martienssen RA, Allshire RC (2003) RNA interference is required for normal centromere function in fission yeast. Chromosome Res 11:137–146

Wang J, Mager J, Chen Y, Schneider E, Cross JC, Nagy A, Magnuson T (2001) Imprinted X inactivation maintained by a mouse Polycomb group gene. Nat Genet 28:371–375

Wutz A, Jaenisch R (2000) A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol Cell 5:695–705

Yamamoto K Sonoda M, Inokuchi J, Shirasawa S, Sasazuki T (2004) Polycomb group suppressor of zeste 12 links heterochromatin protein 1alpha and enhancer of zeste 2. J Biol Chem 279:401–406