

# Histone H3 Lysine 36 Trimethylation Is Established over the *Xist* Promoter by Antisense *Tsix* Transcription and Contributes to Repressing *Xist* Expression

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**One of the two X chromosomes in female mammals is inactivated by the noncoding *Xist* RNA. In mice, X chromosome inactivation (XCI) is regulated by the antisense RNA *Tsix*, which represses *Xist* on the active X chromosome. In the absence of *Tsix*, PRC2-mediated histone H3 lysine 27 trimethylation (H3K27me3) is established over the *Xist* promoter. Simultaneous disruption of *Tsix* and PRC2 leads to derepression of *Xist* and in turn silencing of the single X chromosome in male embryonic stem cells. Here, we identified histone H3 lysine 36 trimethylation (H3K36me3) as a modification that is recruited by *Tsix* cotranscriptionally and extends over the *Xist* promoter. Reduction of H3K36me3 by expression of a mutated histone H3.3 with a substitution of methionine for lysine at position 36 causes a significant derepression of *Xist*. Moreover, depletion of the H3K36 methylase *Setd2* leads to upregulation of *Xist*, suggesting H3K36me3 as a modification that contributes to the mechanism of *Tsix* function in regulating XCI. Furthermore, we found that reduction of H3K36me3 does not facilitate an increase in H3K27me3 over the *Xist* promoter, indicating that additional mechanisms exist by which *Tsix* blocks PRC2 recruitment to the *Xist* promoter.**

In mammals, X chromosome inactivation (XCI) provides dosage compensation between the sexes for X-linked genes (1). The noncoding RNA (ncRNA) *Xist* initiates chromosome-wide inactivation of one of the two X chromosomes of female cells. On the active X chromosome in males and females, *Xist* is repressed by several mechanisms. In mice, the *Tsix* ncRNA is transcribed over the *Xist* locus in the antisense orientation and functions as a repressor of *Xist* on the chromosome from which it is transcribed (2). The function of *Tsix* has been extensively studied in mouse embryonic stem (ES) cells, which constitute a model for studying the initiation of random XCI (1, 3–5). Disruption of *Tsix* leads to derepression of *Xist* whose extent varies with experimental details in a number of different studies (6–9). In mouse preimplantation development, imprinted XCI leads to inactivation of the paternally inherited X chromosome in female embryos. Overexpression of *Tsix* from the paternal X chromosome prevents XCI and causes lethality (10). Conversely, disruption of *Tsix* on the maternally inherited X chromosome in males and females causes lethality due to misregulation of imprinted XCI in the extraembryonic lineages (11, 12). However, in the embryonic lineages, the *Tsix* disruption-bearing X chromosome is fated to become the inactive X chromosome (Xi) (6, 12).

Mutation of *Tsix* causes death of male embryos due to initiation of X inactivation in extraembryonic tissues. This lethality can be prevented by complementing the extraembryonic lineages, suggesting that *Tsix*-independent mechanisms can act to repress *Xist* in the embryonic lineages (13). *Tsix*-independent mechanisms can also be inferred from other mammals, including humans, which lack a functionally conserved *Tsix* homologue (14). Our previous work linked *Tsix*-independent *Xist* repression to Polycomb repressive complex 2 (PRC2) (15). PRC2 contains the Polycomb genes *Eed* and *Suz12* and the SET domain histone H3

methyltransferase gene *Ezh2*. *Eed* is required for PRC2-mediated trimethylation of histone H3 lysine 27 (H3K27me3) (16). Combined mutations in *Tsix* and *Eed* lead to deregulation of *Xist* in male ES cells, leading to activation of *Xist* in a majority of the cells (15). Although it appears that *Tsix* and PRC2 act in parallel to repress *Xist*, the precise function of Polycomb complexes in repressing *Xist* remains to be established. Notably, transient enrichment of H3K27me3 on the *Xist* promoter has also been proposed as one of the sequential events for *Xist* activation (17). However, PRC2 is generally correlated with repression of genes, and no molecular mechanism for an activating function has been identified yet. Additional indirect effects of PRC2 disruption also cannot be ruled out.

Several regulators of *Xist* have been identified, including the X-linked *Rnf12*, *Ftx*, and *Jpx* genes. *Rnf12* inhibits *Xist* repression

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in part through targeting Rex1 protein for degradation (4, 18). Several transcription factors associated with ES cell pluripotency, including Oct4, Sox2, Nanog, and Rex1, have been proposed to be implicated in the repression of *Xist* in ES cells (3, 19, 20), but their precise function in the embryos remains to be resolved (21, 22). Recently, the activation of *Xist* during the progression from naive to primed pluripotency of mouse ES cells was examined in detail in chemically defined medium (5). *Ftx* and *Jpx* are ncRNA genes which are located upstream of *Xist* and positively regulate *Xist*. *Jpx* may function through evicting Ctf and changing chromatin conformation (23, 24). Mutation of *Ftx* leads to decreased *Xist* expression in ES cells (25), but *Ftx* is dispensable for imprinted XCI in embryos (26). Furthermore, a number of studies have suggested that changes in chromatin organization and pairing of the X chromosomes along the X chromosome inactivation center (*Xic*) regions contributes to the regulation of XCI (27–29). Taken together, these studies illustrate that multiple factors interact in the regulation of *Xist*.

Here, we investigated repressive mechanisms of *Xist* in male ES cells, which possess a single X chromosome, and thus, *trans* interactions and pairing are not expected to be relevant. We show that genetic disruption of *Eed* and *Tsix* leads to loss of *Xist* repression despite the presence of other regulators of *Xist*, including Rnf12, Nanog, and Oct4. Moreover, DNA methylation and PRC2 recruitment are not essential for *Xist* repression as long as *Tsix* transcription is unperturbed. We show that *Tsix* transcription induces trimethylation of histone H3 lysine 36 (H3K36me3) at the *Xist* promoter, which contributes to the repression of *Xist* expression, among other mechanisms.

## MATERIALS AND METHODS

**Cell culture and generation of ES cell lines.** Details of plasmid construction and *Setd2* knockdown are provided in the supplemental material. ES cells were cultured as previously described (30, 31). The *Dnmt* triple-knockout (*Dnmt* TKO) ES cells were a gift from Masaki Okano (Kumamoto University, Japan) (32). For generating  $R^{-/-}$   $\Delta Tsix$  cells, the conditions for electroporation, antibiotic selection, Southern hybridization, and removing the selection cassette by transient expression of Cre recombinase were described previously (33). The expression vector pPyCAG-EGFP-IZ (34) was a gift from Hitoshi Niwa (Kumamoto University, Japan) and provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. For generating enhanced green fluorescent protein (EGFP), H3.3wt, and K36M transgenic lines, 10  $\mu$ g of each expression vectors pPyCAG-EGFP-IZ, pPyCAG-H3.3wt-FH-IZ, and pPyCAG-K36M-FH-IZ was linearized by *Sall*, purified, and transfected into 2 million J1 ES cells with a Neon electroporator (Invitrogen, CA). The settings were as follows: 1,400 V; pulse width, 10 ms; 3 times. The cells were selected with 300  $\mu$ g/ml zeocin, and a pool of approximately 100 clones of transgene-expressing cells was used for the experiments.

**ChIP.** Three different chromatin immunoprecipitation (ChIP) methods were used: the H2AK119ub1-formulated method (see Fig. 4E) and the standard method without nuclear isolation (see Fig. 4D and 6C) and with nuclear isolation especially for improving the immunoprecipitation efficiency for nuclear transcription factors (see Fig. 2B and C, 3B and C, 5B, 6B, and 7C and D). ChIP for H2AK119ub1 was performed as previously described (35) with some modifications. The details of the procedures are provided in the supplemental materials and methods. The following antibodies were used: anti-H2AK119ub1 (05-678; Upstate Biotechnology, NY) and normal mouse IgM (M5909; Sigma-Aldrich, MO) as a mock-immunoprecipitated control (referred to as “mock”). A standard ChIP method without nuclear isolation was performed as previously described (36) except that a Covaris S1 system (Covaris Inc., MA) was used for DNA fragmentation, using the following settings: 20% duty, intensity of 10.0,

500 cycles/burst, 360-s duration, and 1 cycle. The following antibodies were used: anti-H3K27me3 (07-449; Upstate), anti-H3K36me3, (ab9050; Abcam, Cambridge, United Kingdom), and normal rabbit IgG (I5006; Sigma-Aldrich) as a mock control. The ChIP method with nuclear isolation was performed as previously described (37). The following antibodies were used: anti-Nanog (8822; CST, MA), anti-Oct4 (5677; CST), anti-H3K4me2 (CMA303; a gift from Hiroshi Kimura) (38), anti-H3K27me3 (CMA323; a gift from Hiroshi Kimura) (38), anti-H3K36me3 (ab9050; Abcam), and normal rabbit IgG (I5006; Sigma-Aldrich) as a mock control.

**Quantitative real-time PCR.** Total RNA was purified with RNeasy minikit (Qiagen, Hilden, Germany) and cDNA was generated by reverse transcriptase SuperScript II (Invitrogen, MA). Quantitative real-time PCR for gene expression and ChIP was performed using iQ SYBR green Supermix (Bio-Rad, CA) with a single-color detection MyIQ i cycler (Bio-Rad), SYBR green PCR master mix (Life Technologies, California, USA), or Thunderbird SYBR qPCR mix (Toyoko, Osaka, Japan) with a StepOne-Plus real-time PCR system (Life Technologies). Primer sequences covering the entire *Xist/Tsix* transcription unit (see Fig. 6A) were described previously (39), and those used for quantitative real-time PCR for both RNA expression and ChIP analyses are listed in Table S1 in the supplemental material.

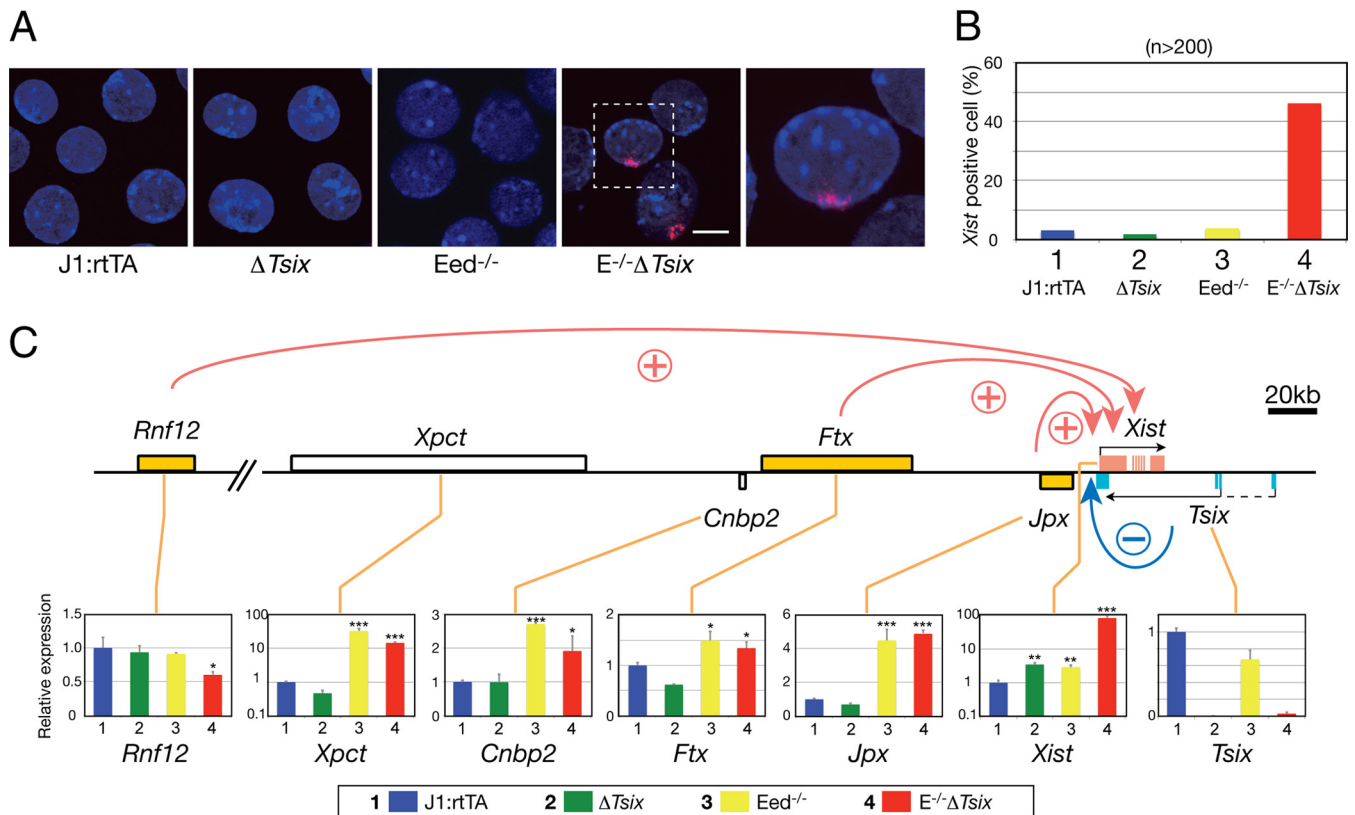
**RNA-FISH.** RNA fluorescence *in situ* hybridization (RNA-FISH) was performed according to the protocols described previously (10). The strand-specific RNA-FISH probe was generated as previously described (40).

**Immunostaining and Western blotting.** Methods for immunostaining and Western blotting were performed as described previously (33). The antibody for immunostaining was Ring1b (gift from Haruhiko Kosaki) (41). The antibodies for Western blotting were H3K36me3 (ab9050; Abcam), Flag (A8692; Sigma-Aldrich), H2AK119ub1 (05-678; Upstate), H3 (see Fig. S1G in the supplemental material) (ab1791; Abcam), H3 (see Fig. 4D) (39763; Active Motif), H3K27me3 (see Fig. S1G in the supplemental material) (07-449; Upstate), H3K27me3 (see Fig. 4D) (gift from Hiroshi Kimura; CMA323) (38).

**Bisulfite sequencing.** DNA methylation analysis by bisulfite sequencing was performed following the manufacturer’s protocol (Imprint DNA modification kit MOD50; Sigma-Aldrich) as described previously (10).

## RESULTS

**Eed is required for *Tsix*-independent *Xist* repression in naive ES cells.** Recently, the use of chemically defined culture conditions for investigating XCI has been explored (5). Naive pluripotent mouse ES cells (31) cultured in the presence of MAP kinase inhibitors and GSK3 kinase inhibitors (2i) have reduced promoter-specific Polycomb complex-associated histone modifications (37) and DNA methylation, which is likely to resemble the actual situation in the developing epiblast (42). We reasoned that under 2i culture conditions, the effects of chromatin on *Xist* repression could be discerned from indirect effects on the pluripotent state more readily. We analyzed *Xist* expression in naive male ES cells that harbor disruptions of *Eed* ( $Eed^{-/-}$ ) and *Tsix* ( $\Delta Tsix$ ) (see Fig. S1E in the supplemental material) (15) by RNA-FISH (Fig. 1A and B) and qRT-PCR (Fig. 1C). J1:rtTA ES cells were used as a parental control cell line (15). A significant derepression of *Xist* was observed in  $\Delta Tsix$  and  $Eed^{-/-}$  cells by semiquantitative PCR (Fig. 1C, *Xist* [ $\Delta Tsix$ ,  $3.47 \pm 0.46$ ;  $Eed^{-/-}$ ,  $2.93 \pm 0.39$ ;  $P < 0.01$ ]). However, the number of *Xist* clusters observed with *Xist* RNA-FISH was not significantly increased in  $\Delta Tsix$  and  $Eed^{-/-}$  cells over the control cell line J1:rtTA (Fig. 1B [J1:rtTA, 3.2%;  $\Delta Tsix$ , 1.9%;  $Eed^{-/-}$ , 3.9%]). In contrast, combined disruption of both *Eed* and *Tsix* resulted in the appearance of *Xist* clusters in 46.2% of  $E^{-/-}$   $\Delta Tsix$  ES cells (Fig. 1B) and high levels of *Xist* expression



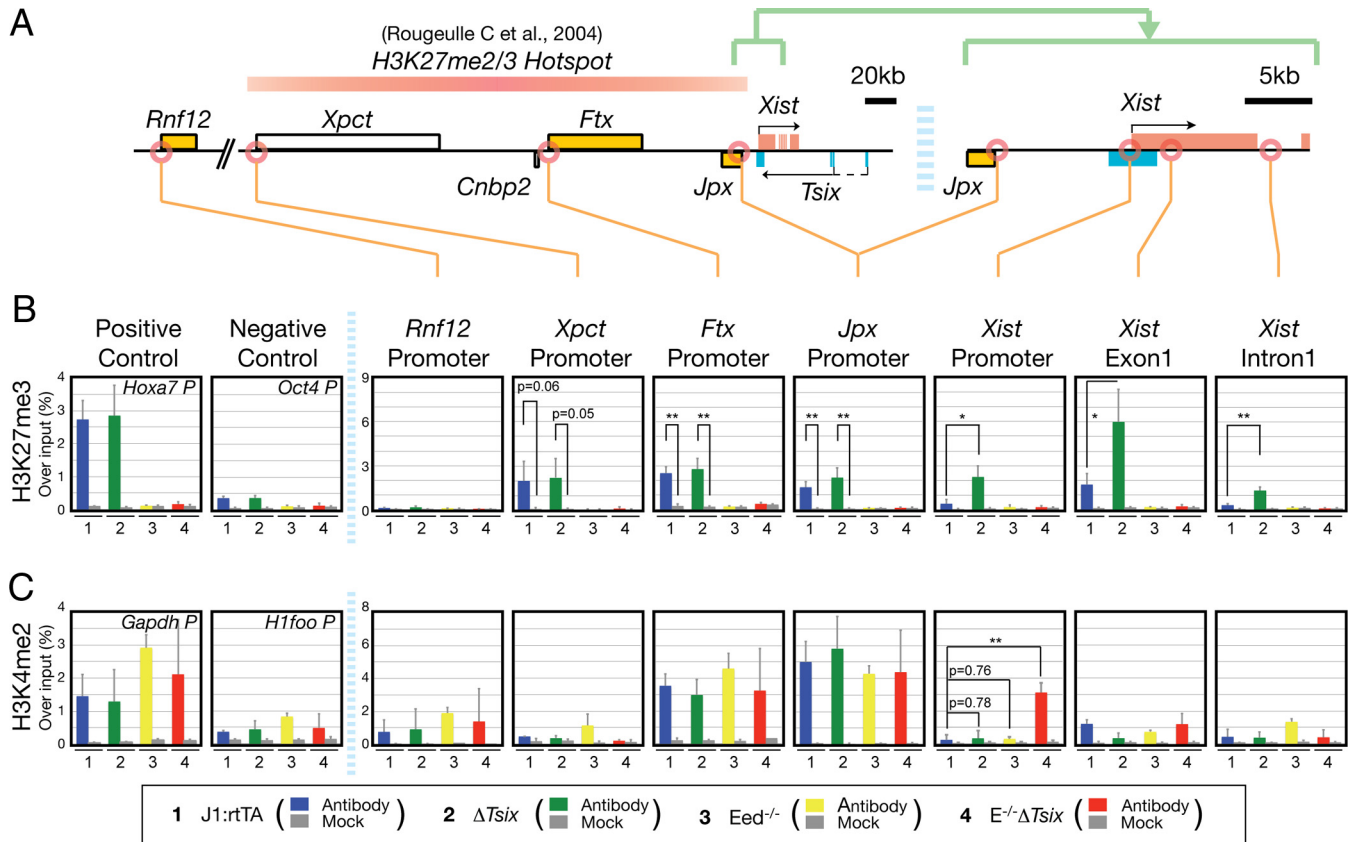
**FIG 1** H3K27me3 is required for *Tsix*-independent *Xist* repression in naive ES cells. (A) *Xist* RNA-FISH (red) in J1:rtTA control,  $\Delta Tsix$ ,  $Eed^{-/-}$ , and  $E^{-/-}\Delta Tsix$  ES cells (the boxed area is magnified in the rightmost panel). Nuclei were counterstained with DAPI (blue). Bar, 10  $\mu$ m. (B) Percent *Xist*-positive cells ( $n > 200$ ). (C) Map of the X chromosome inactivation center (*Xic*) and its linked genes *Rnf12*, *Xpct*, *Cnbp2*, *Ftx*, *Jpx*, *Xist*, and *Tsix*. *Rnf12*, *Ftx*, and *Jpx* are reported as *Xist* activators. Their expression was measured by qRT-PCR. Expression is shown relative to undifferentiated J1:rtTA ES cells and normalized to *Gapdh* ( $n = 3$ ). All cells were cultured with 2i medium. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (Student's *t* test).

(Fig. 1C, *Xist* [ $E^{-/-}\Delta Tsix$ ,  $81.73 \pm 15.33$ ;  $P < 0.001$ ]). Furthermore, the active histone mark H3K4me2 was strongly increased over the *Xist* promoter in  $Eed^{-/-}\Delta Tsix$  cells (Fig. 2C [ $E^{-/-}\Delta Tsix$ ,  $13.16 \pm 0.51$ ;  $P < 0.01$ ]), in contrast to cells with mutation of either *Tsix* or *Eed* (Fig. 2C [ $\Delta Tsix$ ,  $1.40 \pm 0.40$ ;  $Eed^{-/-}$ ,  $1.26 \pm 0.33$ ;  $P = 0.78$  and  $P = 0.76$ , respectively]). We conclude that PRC2 function is required for *Tsix*-independent *Xist* repression in naive pluripotent cells, a finding that is consistent with earlier observations in serum-LIF-cultured ES cells (15). We did not observe an increase of H3K4me2 on the *Xist* promoter, when *Tsix* was truncated in either in naive ES cells (Fig. 2C) or ES cells that were cultured in serum-LIF-based medium (15), contrasting a previous report on serum- and LIF-cultured ES cells (39). We attribute this apparent difference to the use of different ES cell lines and different strategies for mutating *Tsix*, where we have chosen an insertion of a gene trap cassette that truncates *Tsix* transcripts before the *Xist* gene locus.

**The *Xist* regulators *Ftx* and *Jpx* but not *Rnf12* are elevated in *Eed*-deficient ES cells.** To investigate the mechanisms of *Xist* activation in  $Eed^{-/-}\Delta Tsix$  cells further, we analyzed the expression of *Rnf12*, *Ftx*, and *Jpx* and two additional genes within the X chromosome inactivation center (*Xic*), *Xpct* and *Cnbp2*. Increased *Rnf12* expression is correlated with activation of *Xist* (18, 21) but was decreased in  $Eed^{-/-}\Delta Tsix$  cells (Fig. 1C, *Rnf12* [ $E^{-/-}\Delta Tsix$ ,  $0.60 \pm 0.05$ ;  $P < 0.05$ ]). This observation excludes upregulation of

*Rnf12* as a cause of *Xist* activation in  $E^{-/-}\Delta Tsix$  cells. The observed repression of *Rnf12* could likely be a consequence of the activation of *Xist* in  $Eed^{-/-}\Delta Tsix$  cells. *Ftx* and *Jpx* are ncRNAs that are located upstream of *Xist* and have also been proposed to positively regulate *Xist* RNA expression (23–25). We observed increased *Ftx* and *Jpx* expression in *Eed*-deficient ES cells (Fig. 1C, *Ftx* [ $Eed^{-/-}$ ,  $1.50 \pm 0.18$ ;  $E^{-/-}\Delta Tsix$ ,  $1.34 \pm 0.12$ ;  $P < 0.05$ ] and *Jpx* [ $Eed^{-/-}$ ,  $4.47 \pm 0.63$ ;  $Eed^{-/-}\Delta Tsix$ ,  $4.86 \pm 0.17$ ;  $P < 0.001$ ]). Similarly, *Xpct* and *Cnbp2* expression was also increased, showing that lack of *Eed* caused derepression of multiple genes within the *Xic* region (Fig. 1C, *Xpct* [ $Eed^{-/-}$ ,  $31.59 \pm 4.75$ ;  $E^{-/-}\Delta Tsix$ ,  $14.24 \pm 0.97$ ;  $P < 0.001$ ] and *Cnbp2* [ $Eed^{-/-}$ ,  $2.70 \pm 0.04$  ( $P < 0.001$ );  $E^{-/-}\Delta Tsix$ ,  $1.91 \pm 0.44$  ( $P < 0.05$ )]).

We next investigated the distribution of H3K27me3 across the *Xic* region (Fig. 2A). In J1:rtTA and  $\Delta Tsix$  cells, H3K27me3 could be detected at the *Xpct*, *Ftx*, and *Jpx* promoters, which are located within a 340-kb region 5' of *Xist* that has been previously characterized as a hot spot region harboring high levels of H3K27me3 and H3K27me2 (Fig. 2B, *Xpct*, *Ftx*, and *Jpx* promoters) (43). An H3K27me3 hot spot was prominent in ES cells cultured in 2i and serum-based medium and encompassed the *Xpct*, *Ftx*, and *Jpx* promoters. As expected, H3K27me3 was lost in  $Eed^{-/-}$  and  $E^{-/-}\Delta Tsix$  cells (Fig. 2B, *Xpct*, *Ftx*, and *Jpx* promoters). This finding suggests that the expression of *Xic*-linked genes *Xpct*, *Cnbp2*, *Ftx*, and *Jpx* is repressed (Fig. 1C) by PRC2 and H3K27me3 spreading



**FIG 2** *Tsix* prevents H3K27me3 invasion from the hot spot in naive ES cells. (A) Map of *Xic*. The H3K27me2/3 hot spot (43), in the 340-kb region 5' of *Xist*, is shown. (B and C) ChIP analysis of H3K27me3 (B) and H3K4me2 (C) across the *Xic* locus. *Hoxa7* and *Oct4* promoters for H3K27me3 and *Gapdh* and *H1foo* promoters for H3K4me2 were used as positive- and negative-control loci, respectively. Data are means and standard deviations from three independent experiments. All cells were cultured with 2i medium. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (Student's *t* test).

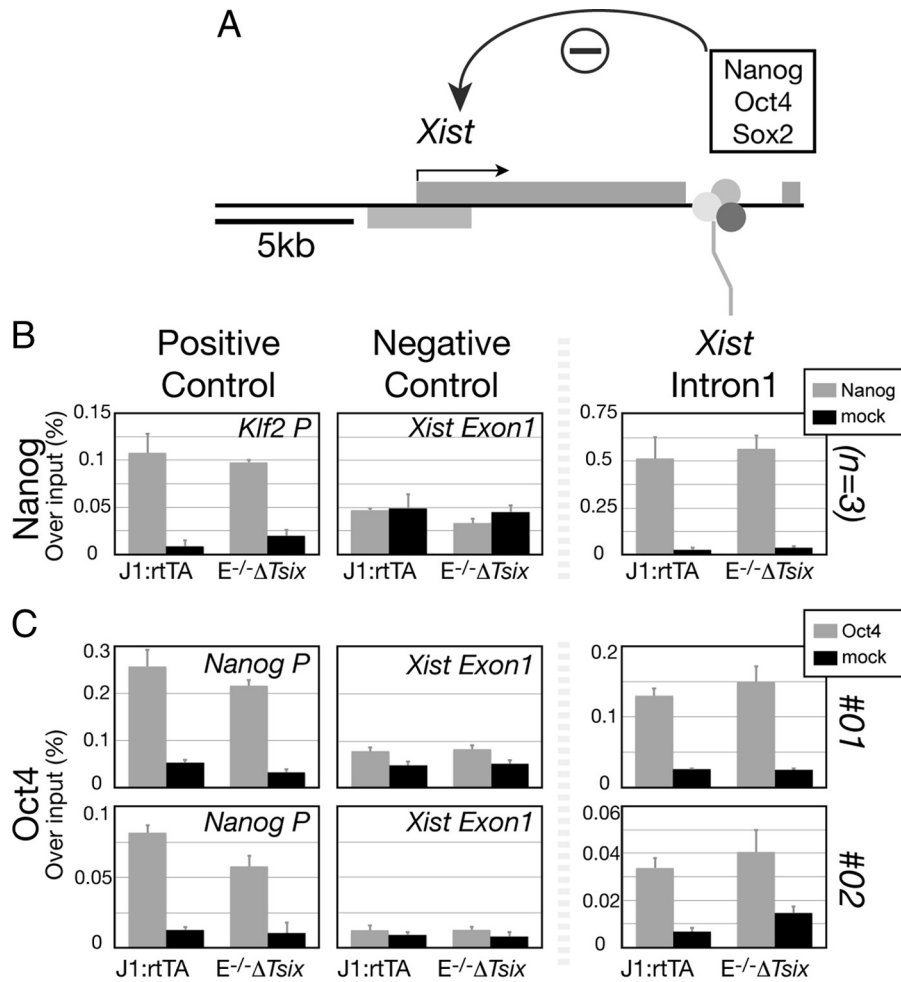
from the hot spot (Fig. 2B). Interestingly, the expression of the hot spot-linked genes *Xpct* and *Cnbp2* was decreased in *Eed*<sup>-/-</sup>  $\Delta Tsix$  cells compared with *Eed*<sup>-/-</sup> cells (Fig. 1C), suggesting that these genes are potentially also partially repressed by ectopically expressed *Xist* in cells lacking *Tsix* and *Eed*. However, this repressive effect of *Xist* activation is small compared with the derepression observed after loss of *Eed*, such that a net activation is observed between wild-type control and *Eed*<sup>-/-</sup>  $\Delta Tsix$  cells.

We next investigated whether *Tsix* prevented H3K27me3 spreading over the *Xist* promoter from the hot spot in J1:rtTA-naive ES cells. H3K27me3 was enriched at the *Xpct*, *Ftx*, and *Jpx* promoters (Fig. 2B) (*Xpct*, 1.99%  $\pm$  1.30% of input; *Ftx*, 2.49%  $\pm$  0.45% of input; *Jpx*, 1.51%  $\pm$  0.37% of input) but not at the *Xist* promoter (Fig. 2B) (0.41%  $\pm$  0.32% of input), suggesting a boundary of the H3K27me3 hot spot between the *Jpx* and *Xist* promoters. In  $\Delta Tsix$  ES cells, H3K27me3 extended over the *Xist* promoter and gene body (Fig. 2B [*Xist* promoter, 5.44-  $\pm$  1.77-fold change { $P < 0.05$ }; *Xist* exon 1, 3.49-  $\pm$  1.29-fold change { $P < 0.05$ }; *Xist* intron 1, 4.14-  $\pm$  0.81-fold change { $P < 0.01$ }] [enrichment is measured relative to J1:rtTA]). This observation showed that *Tsix* prevented the spreading of H3K27me3 from the hot spot into the *Xist* locus, which is consistent with earlier results in serum and LIF cultures (44).

**Nanog and Oct4 binding does not prevent *Xist* activation in *Eed*- and *Tsix*-deficient cells.** A number of recent studies have

implicated the binding of transcription factors, including Nanog and Oct4, in *Xist* repression through a prominent binding site within *Xist* intron 1 (Fig. 3A) (19). To assess whether these pluripotency factors remain bound when *Xist* is activated in *Eed*<sup>-/-</sup>  $\Delta Tsix$  cells under 2i conditions, we performed ChIP analysis. We observed that despite activation of *Xist* in *Eed*<sup>-/-</sup>  $\Delta Tsix$  cells, Nanog and Oct4 binding to *Xist* intron 1 remained unchanged from the pattern in control cells (Fig. 3B,  $P = 0.54$ ,  $n = 3$ ; Fig. 3C,  $P = 0.19$  and 0.50,  $n = 2$ ). This observation that Oct4 and Nanog binding is not sufficient for repressing *Xist* in 2i, where *Rex1* and *Nanog* are homogeneously expressed, is further consistent with previous reports that the binding site for Oct4 and Nanog in *Xist* intron 1 is dispensable for *Xist* regulation in embryos (22). Although a deletion of the binding site slightly skews the X chromosome for inactivation in female cells (21), we conclude that loss of pluripotency factor binding to *Xist* intron 1 does not explain the activation of *Xist* in *Eed*<sup>-/-</sup>  $\Delta Tsix$  cells.

**Ring1b is dispensable for *Tsix*-independent *Xist* repression.** PRC2-catalyzed H3K27me3 can act as a signal for recruitment of PRC1, consistent with observations of corecruitment of PRC1 and PRC2 to a large number of genes (45). PRC1 contains the RING finger domain proteins Ring1a and Ring1b and catalyzes monoubiquitination of histone H2A lysine 119 (H2AK119ub1) (46), which has been proposed to repress transcription by restraining poised RNA polymerase II (35). To investigate the potential func-

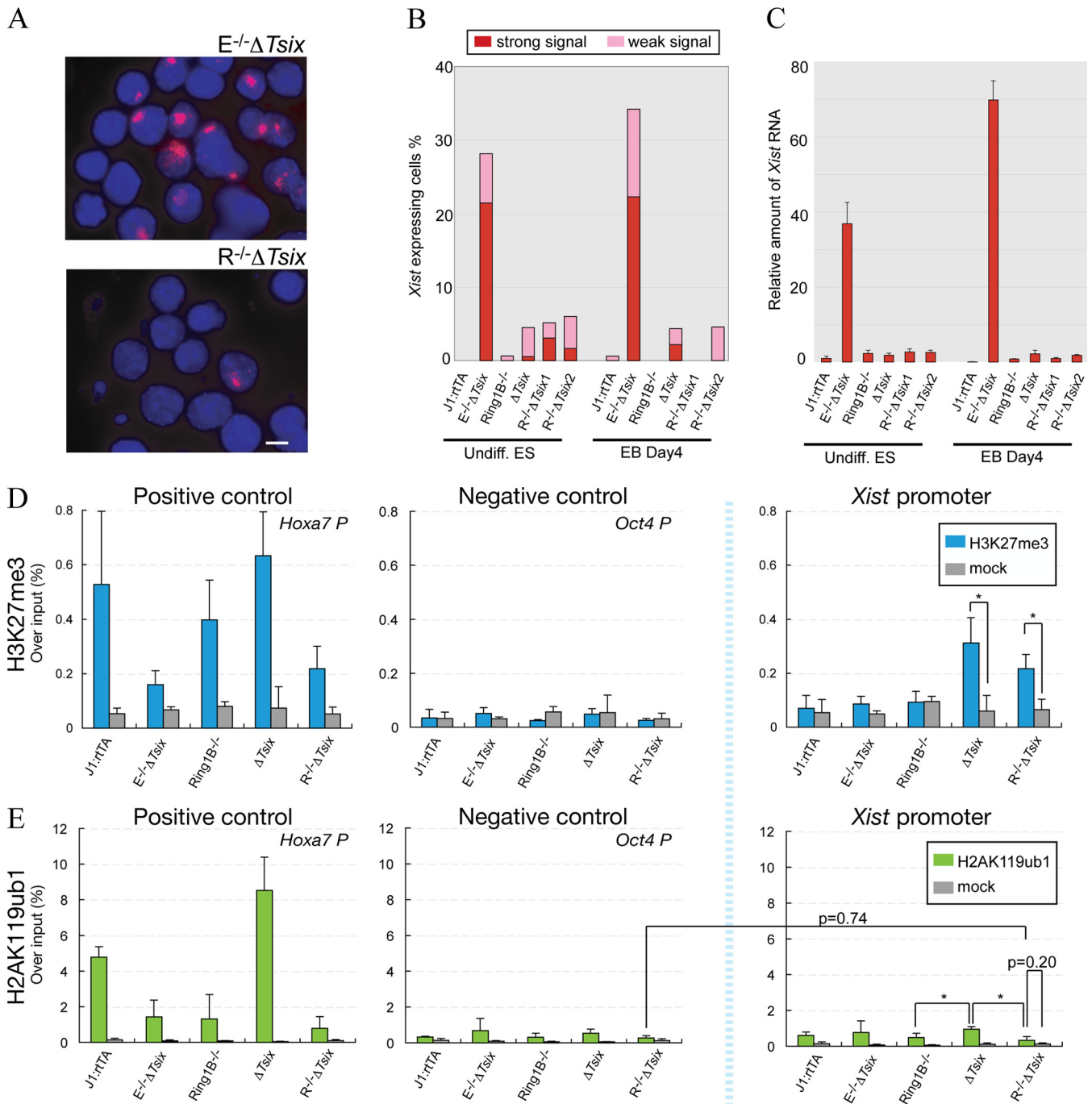


**FIG 3** Nanog and Oct4 enrichment is not changed in  $Eed^{-/-} \Delta Tsix$  cells. (A) Transcription factors associated with pluripotency, including Nanog, Oct4, and Sox2, are proposed as *Xist* repressors through a binding site within *Xist* intron 1. (B and C) Chromatin recruitment of the putative *Xist* repressors Nanog (B) and Oct4 (C) at its binding site at *Xist* intron 1, measured by ChIP. *Klf2* promoter and *Xist* exon 1 for Nanog and *Nanog* promoter and *Xist* exon 1 for Oct4 were used as positive- and negative-control loci, respectively. For Nanog, data are means and standard deviations from three independent experiments. For Oct4, data from two independent experiments are shown. All cells were cultured with 2i medium.

tion of PRC1 in *Xist* repression, we disrupted *Tsix* in ES cells that lack *Ring1b* ( $R^{-/-} \Delta Tsix$ ) (see Fig. S1 in the supplemental material). In contrast to  $Eed^{-/-} \Delta Tsix$  ES cells, which showed activation of *Xist* (Fig. 4A to C), *Xist* remained repressed in  $R^{-/-} \Delta Tsix$  ES cells (Fig. 4A to C). Although we could observe a few *Xist* clouds in  $R^{-/-} \Delta Tsix$  ES cells (Fig. 4A and B), there was no significant difference between  $R^{-/-} \Delta Tsix$  and  $\Delta Tsix$  ES cells (Fig. 4B). Quantitative PCR confirmed that *Xist* remained repressed in  $R^{-/-} \Delta Tsix$  ES cells (Fig. 4C). This observation demonstrated that *Ring1b* is not critical for *Tsix*-independent *Xist* repression.

ChIP analysis showed that H3K27me3 was enriched on the *Xist* promoter in  $R^{-/-} \Delta Tsix$  cells to a level comparable to that of  $\Delta Tsix$  cells (Fig. 4D, *Xist* promoter [ $\Delta Tsix$ , 5.27-  $\pm$  1.59-fold enrichment compared to the mock control ( $P < 0.05$ );  $R^{-/-} \Delta Tsix$ , 3.34-  $\pm$  0.81-fold enrichment ( $P < 0.05$ )). H2AK119ub1 was weakly enriched on the *Xist* promoter in  $\Delta Tsix$  cells and furthermore was reduced in *Ring1b* $^{-/-}$  ES cells (1.95-  $\pm$  0.31-fold change [ $P < 0.05$ ]) and  $R^{-/-} \Delta Tsix$  cells (Fig. 4E) (2.89-  $\pm$  0.46-fold change [ $P < 0.05$ ]). In *Ring1b* $^{-/-}$  ES cells, a very small amount of H2AK119ub1 could still be observed (see Fig. S1G in the supple-

mental material) that was not significantly increased upon deletion of *Tsix* in  $R^{-/-} \Delta Tsix$  cells (Fig. 4E, *Xist* promoter [2.48-  $\pm$  1.65-fold change ( $P = 0.20$ ) relative to the mock control]). We cannot fully rule out the possibility that Ring1a catalyzes low levels of H2AK119ub1 in the absence of Ring1b in  $R^{-/-} \Delta Tsix$  cells. However, in  $R^{-/-} \Delta Tsix$  cells, H2AK119ub1 enrichment on the *Xist* promoter was similar to the *Oct4* promoter, which served as a negative control (Fig. 4E, compare  $R^{-/-} \Delta Tsix$  data obtained with the *Xist* promoter to those obtained with the *Oct4* promoter; 1.19-  $\pm$  0.79-fold change [ $P = 0.74$ ]). Therefore, we conclude that establishment of H3K27me3 on the *Xist* promoter does not lead to efficient H2AK119ub1 recruitment, consistent with earlier results of low enrichment of H2AK119ub1 at the *Xist* promoter in serum-cultured ES cells (15). In addition, *Ring1b* and H2AK119ub1 appeared to be largely dispensable for *Xist* repression when *Tsix* was disrupted. Since H3K27me3 was still present at the *Xist* promoter, these observations pointed toward a PRC1-independent function of PRC2 in repressing the *Xist* promoter. This finding is surprising but consistent with observations of PRC1-independent PRC2 recruitment at a subset of genes in ES cells (45) as well as the idea of



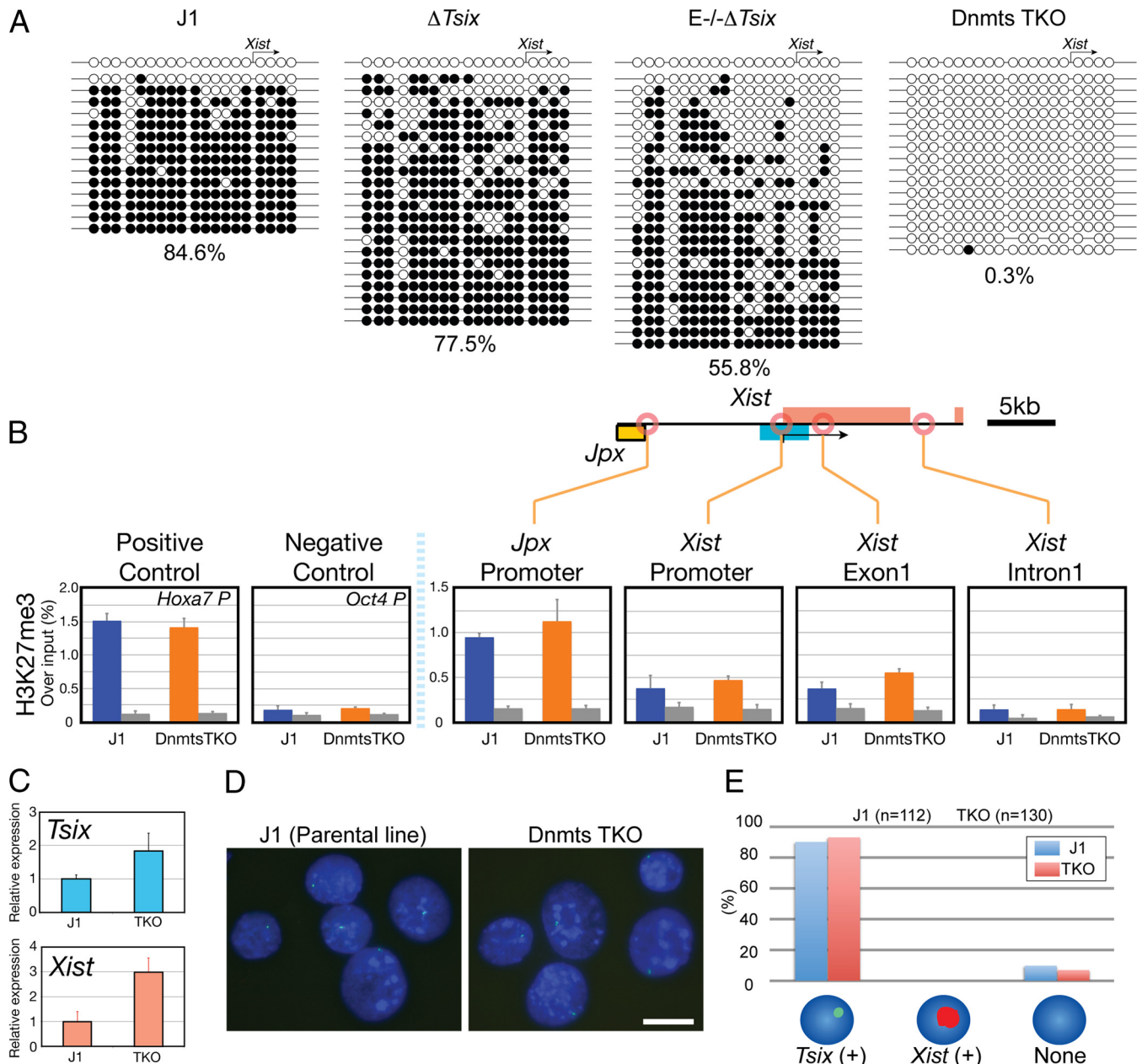
**FIG 4** *Xist* expression is largely repressed in  $R^{-/-} \Delta Tsix$  ES cells. (A) *Xist* RNA-FISH (red) in  $E^{-/-} \Delta Tsix$  and  $R^{-/-} \Delta Tsix$  cells differentiated for 4 days. Nuclei were counterstained with DAPI (blue). Bar, 10  $\mu$ m. (B) Number of *Xist*-positive nuclei measured by *Xist* RNA-FISH ( $n > 150$ ). (C) *Xist* expression (spliced product; exons 1 and 2) by qRT-PCR analysis is shown relative to undifferentiated J1:rTA ES cells and normalized to *Gapdh*. (D and E) ChIP analysis of H3K27me3 (D) and H2AK119ub1 (E). *Hoxa7* and *Oct4* promoters were used as positive- and negative-control loci, respectively. Values are means and standard deviations from three independent experiments. Cells were cultured in ES medium with (undifferentiated) or without (differentiated) LIF. \*,  $P < 0.05$  (Student's  $t$  test).

an evolutionarily older origin of PRC2, which is present in plants where PRC1 is not conserved (46).

#### DNA methylation is dispensable for *Xist* repression by *Tsix*.

Taken together, our results suggested that a repression mechanism based on PRC2-mediated H3K27me3 acts on genes within the *Xic* region. However, this mechanism is apparently disrupted

at the *Xist* promoter by *Tsix*, which prevents the spreading of H3K27me3 from the hot spot over the promoter and gene body of *Xist*. Since previous studies have shown that *Tsix* is important for DNA methylation at the *Xist* promoter (10, 17, 36, 39, 47, 48) and DNA methylation is inversely correlated with Polycomb recruitment (49), we investigated whether DNA methylation might ex-



**FIG 5** *Xist* expression is largely repressed in Dnmt TKO ES cells. (A) Cytosine DNA methylation was measured by bisulfite sequencing of the *Xist* promoter (filled circles, methylated; open circles, unmethylated). The percent methylation is given below the graphs. (B) ChIP analysis of H3K27me3 ( $n = 3$ ). *Hoxa7* and *Oct4* promoters were used as positive- and negative-control loci, respectively. (C) qRT-PCR analysis for *Xist* and *Tsix* expression in Dnmt TKO and parental J1 ES cells. Expression relative to undifferentiated J1 ES cells was normalized to *Gapdh* ( $n = 2$ ). (D) *Xist* (red) and *Tsix* (green) RNA-FISH in parental J1 (right) and Dnmt TKO (left) undifferentiated ES cells. Nuclei were counterstained by DAPI (blue). Bar, 10  $\mu$ m. (E) Percent *Tsix*- or *Xist*-positive nuclei, revealed by RNA-FISH ( $n > 100$ ).

plain the mechanism *Tsix* uses for blocking H3K27me3 spreading. DNA methylation was present on the *Xist* promoter in the naive ES cells and decreased after *Tsix* disruption in  $\Delta Tsix$  cells (Fig. 5A [J1, 84.6%;  $\Delta Tsix$ , 77.5%]). This observation confirmed that *Tsix* contributed to DNA methylation on the *Xist* promoter in naive male ES cells, similar to earlier findings in serum-cultured ES cells. To analyze the function of DNA methylation, we used ES cells harboring combined mutations of all three DNA methyltransferases *Dnmt1*, *Dnmt3a*, and *Dnmt3b* (32). In these *Dnmt* triple-

knockout (TKO) cells, DNA methylation at the *Xist* promoter was essentially absent (0.3%) (Fig. 5A). Despite the loss of DNA methylation, we observed that H3K27me3 remained unchanged and was excluded from the *Xist* promoter and gene body (Fig. 5B [Dnmt TKO], *Xist* promoter [ $0.47 \pm 0.05$ ], *Xist* exon 1 [ $0.55 \pm 0.04$ ], and *Xist* intron 1 [ $0.14 \pm 0.05$ ; values are relative to input values], and (Fig. 2B [ $\Delta Tsix$ ], *Xist* promoter [ $2.23 \pm 0.73$ ], *Xist* exon 1 [ $6.01 \pm 2.23$ ], and *Xist* intron 1 [ $1.32 \pm 0.26$ ]). We further observed that *Tsix* transcription was slightly increased in Dnmt

TKO cells (Fig. 5C, *Tsix*) (1.87- ± 0.66-fold change). In Dnmt TKO cells, DNA methylation was lost (Fig. 5A) and spreading of H3K27me3 from the hot spot to the *Xist* promoter was blocked by *Tsix* (Fig. 5B), leaving the *Xist* promoter unaffected by these two repressive modifications. This prompted us to investigate whether, in this situation, reactivation of *Xist* could be observed, similar to that in *Eed*<sup>-/-</sup>  $\Delta$ *Tsix* cells, in which DNA methylation at the *Xist* promoter was also significantly decreased (Fig. 5A, *E*<sup>-/-</sup>  $\Delta$ *Tsix* [55.8%]). We observed only a moderate upregulation of *Xist* in Dnmt TKO cells (Fig. 5C, *Xist*) (3.01 ± 0.33), comparable to the increased *Xist* expression in single *Eed*<sup>-/-</sup> and  $\Delta$ *Tsix* mutant ES cells (Fig. 1C). Furthermore, no *Xist* clusters were observed in Dnmt TKO cells by *Xist* RNA-FISH (Fig. 5D and E), demonstrating that *Tsix* represses *Xist* through additional mechanisms, when neither DNA methylation nor H3K27me3 is present at the *Xist* promoter.

***Tsix* recruits H3K36me3 to the *Xist* promoter.** To identify such additional mechanisms, we considered histone marks associated with coding and noncoding transcription. H3K36me3 is correlated with transcriptional elongation and repression of inappropriate initiation of transcription within the gene body (50–54). We performed ChIP to investigate whether H3K36me3 was established by *Tsix* transcription over the *Xist* gene body and promoter (39). We detected a strong enrichment of H3K36me3 over the entire *Xist* locus that, importantly, also included the *Xist* promoter (Fig. 6A). The enrichment of H3K36me3 over *Xist* is also observed in genome-wide data sets of serum-cultured ES cells (see Fig. S2 in the supplemental material). We observed H3K36me3 in both J1 and Dnmt TKO cells at the *Xist* promoter (Fig. 6B, *Xist* promoter [J1, 10.35% ± 0.15% of input; Dnmt TKO, 7.17% ± 0.09%]) and exon 1 (Fig. 6B [J1, 10.52% ± 2.34%; Dnmt TKO, 7.57% ± 0.69%]). H3K36me3 was not detected upstream (Fig. 6B, *Jpx* promoter [J1, 1.98% ± 0.24% of input; Dnmt TKO, 1.81% ± 0.16%]) or far downstream of *Xist* (Fig. 6B, *Xist* intron 1 [J1, 1.98% ± 0.24% of input; Dnmt TKO, 1.00% ± 0.06%]), where we observed levels of H3K36me3 that are comparable to the negative-control *Sox2* promoter (Fig. 6B [J1, 1.27% ± 0.10% of input; Dnmt TKO, 1.24% ± 0.11%]). An enrichment of H3K36me3 on the *Xist* promoter was also observed in J1:rtTA, *Eed*<sup>-/-</sup>, and *Ring1b*<sup>-/-</sup> ES cells (Fig. 6C, *Xist* promoter [J1:rtTA, 8.55% ± 1.89% of input; *Eed*<sup>-/-</sup>, 9.20% ± 0.59%; *Ring1b*<sup>-/-</sup>, 11.09% ± 2.53%]), demonstrating that H3K36me3 is enriched at the *Xist* promoter and does not require PRC function. Importantly, H3K36me3 was strongly reduced to near background levels in ES cells that lack *Tsix* (Fig. 6C, *Xist* promoter [ $\Delta$ *Tsix*, 1.69% ± 0.73%; *E*<sup>-/-</sup>  $\Delta$ *Tsix*, 0.99% ± 0.67%; *R*<sup>-/-</sup>  $\Delta$ *Tsix*, 1.63% ± 0.31%]). Taken together, these findings show that *Tsix* transcription is required for establishing H3K36me3 at the *Xist* promoter.

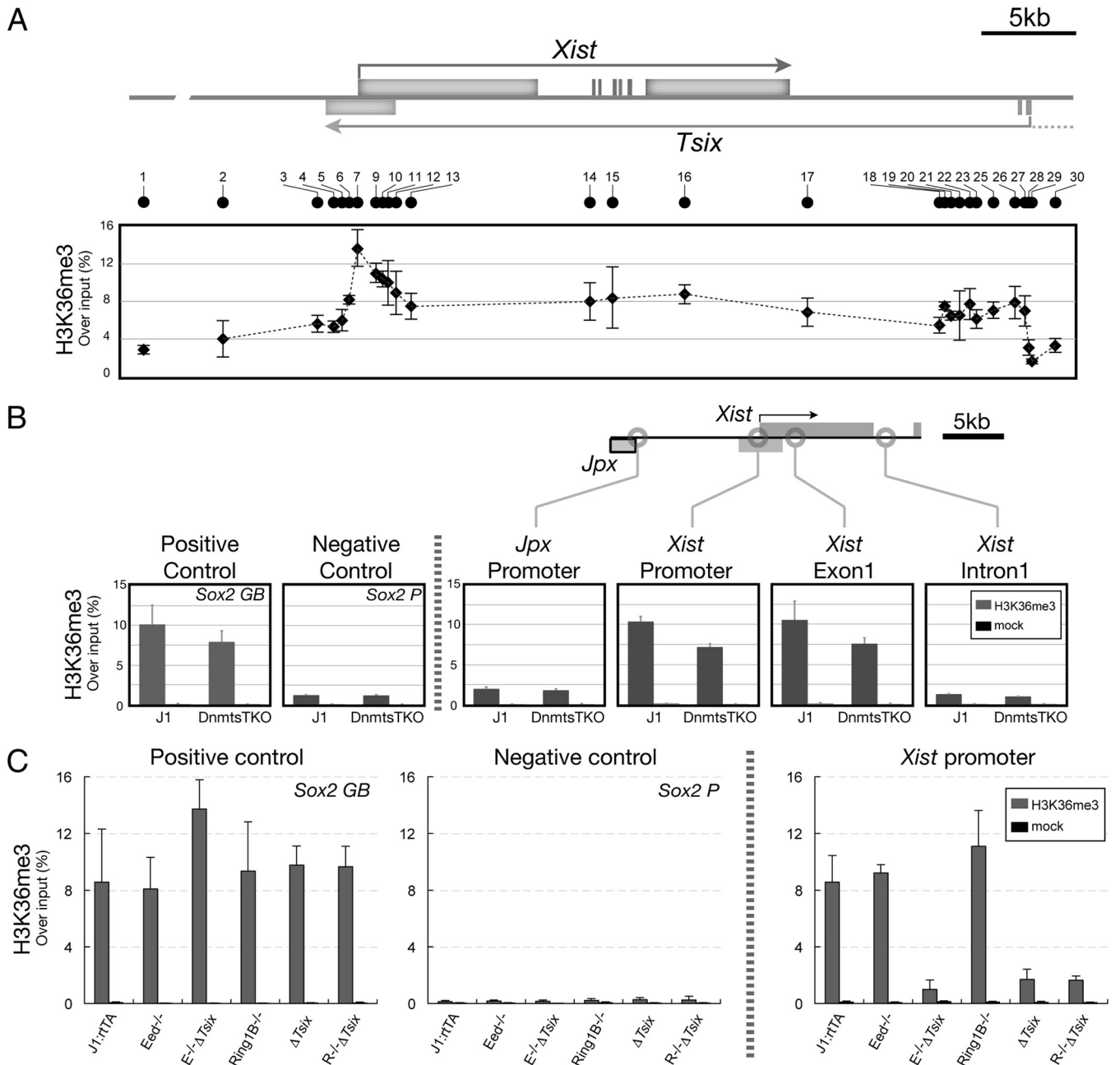
**H3K36me3 contributes to repression of *Xist* transcription.** H3K36me3 recruitment on the *Xist* promoter via *Tsix* transcription prompted us to investigate whether it could exert a repressive function on the *Xist* promoter in *cis*. In mammals, to date, at least eight histone H3 lysine K36 methyltransferases (H3K36 HKMTs) have been reported, including those encoded by the *NSD1*, *NSD2*, *NSD3*, *SETD2*, *SETD3*, *ASH1L*, *SETMAR*, and *SMYD2* genes (55). The number of H3K36 HKMTs and their potential redundancy make it difficult to eliminate H3K36 methylation. To address this issue comprehensively and also to consider potential cell viability issues, we selected two complementary strategies. First, we aimed to disrupt H3K36 methylation through expression of a mutant

histone protein, H3.3-K36M (K36M), that has a substitution of methionine for lysine at position 36. This mutant protein has been previously shown to reduce endogenous K36 methylation through binding and sequestering H3K36 HKMTs (56). We established stable transgenic J1 ES cell pools that express wild-type and mutated histone H3.3 proteins (Fig. 7A). Anti-Flag immunoblotting (Fig. 7B, Flag) and long exposure of anti-H3 (see Fig. S3 in the supplemental material) confirmed the expression of H3.3wt and H3.3-K36M. The total amount of endogenous H3K36me3 was only slightly increased by transgenic expression of H3.3wt (Fig. 7B, H3K36me3) (1.22-fold change compared to H3.3wt with EGFP cells, normalized to H3). In contrast, in K36M-expressing cells, H3K36me3 was decreased to approximately half the level of control cells (Fig. 7B, H3K36me3) (0.40-fold change for K36M compared with H3.3wt, normalized to H3). Importantly, H3K36me3 was also reduced over the *Xist* promoter (Fig. 7C) (0.48- ± 0.07-fold change for the K36M mutant compared with H3.3wt [ $P < 0.05$ ]) and *Xist* exon 1 (Fig. 7C) (0.31- ± 0.07-fold change for the K36M mutant compared with H3.3wt [ $P < 0.01$ ]). To test for specificity, we also measured H3K27me3, which remained unchanged globally (Fig. 7B, H3K27me3) (1.08-fold change for the K36M mutant compared with H3.3wt, normalized to H3) and locally on the *Xist* promoter (Fig. 7D) (1.04- ± 0.18-fold change for the K36M mutant compared with H3.3wt [ $P = 0.82$ ]) and *Xist* exon 1 (Fig. 7D) (1.35- ± 0.56-fold change for the K36M mutant compared with H3.3wt [ $P = 0.34$ ]). We did not measure an increase in the number of *Xist* clusters by RNA-FISH (see Fig. S4 in the supplemental material), but we did observe a significant derepression of *Xist* in K36M-overexpressing cells by qRT-PCR (Fig. 7E, *Xist*) (3.95- ± 1.49-fold change [ $P < 0.05$ ]), whereas the expression of *Tsix* remained unchanged (Fig. 7E, *Tsix*) (1.15- ± 0.04-fold change [ $P = 0.39$ ]). To obtain independent evidence by a second method, we aimed to reduce H3K36me3 using an RNA interference-mediated depletion of *Setd2*. The product of *Setd2* is considered a major H3K36 methylase responsible for H3K36me3 (55). We observed a significant derepression of *Xist* in J1 ES cells after RNA interference-mediated depletion of *Setd2* (see Fig. S5 in the supplemental material). Importantly, under our experimental conditions, the expression of *Tsix* remained unaffected. Taken together, our data suggest that H3K36me3 is established in a *Tsix*-dependent manner over the *Xist* promoter and contributes to a repressive effect on the *Xist* promoter.

## DISCUSSION

In our study, we addressed the function of chromatin modifications regulating the *Xist* promoter. Our results suggest two independent repressive mechanisms: one is mediated by *Tsix* transcription, and a second and independent mechanism is mediated by PRC2. Although this is a relatively simple scenario, which accurately predicts the derepression of *Xist* when both repressive mechanisms are impaired in *Tsix*- and *Eed*-deficient cells, further insights into the molecular interactions are needed. Here, we dissected the functions of several known and one novel chromatin modification in this system. Combined disruption of *Tsix* and *Eed* caused activation of *Xist* in naive ES cells. Notably, *Xist* was activated, although the binding of Nanog and Oct4 in *Xist* intron 1 was preserved, demonstrating that Nanog and Oct4 binding is not sufficient for *Xist* repression. This observation is consistent with a recent report showing that the intron 1 binding site is not essential



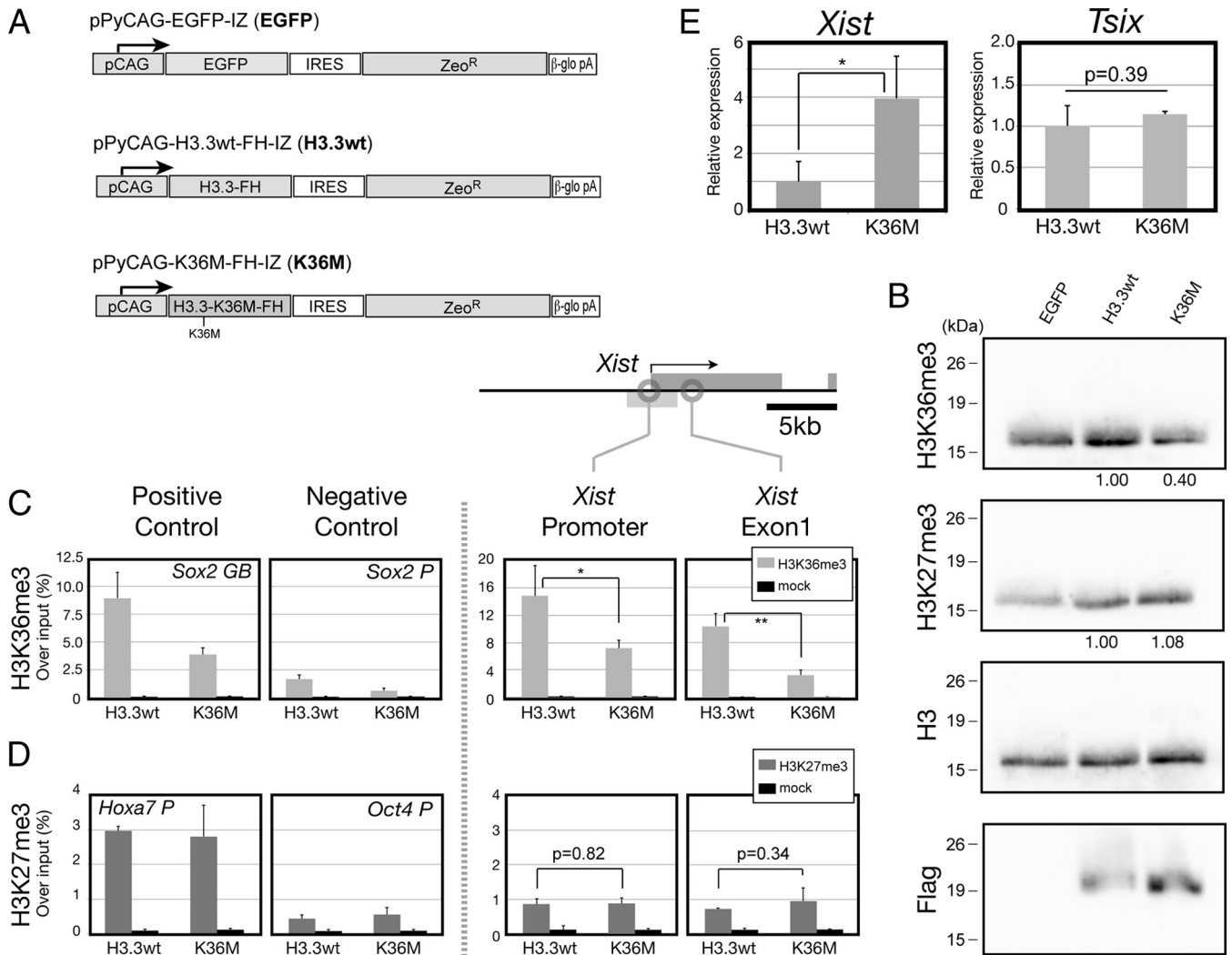


**FIG 6** H3K36me3 is accompanied by *Tsix* transcription. (A) ChIP analysis of H3K36me3 in J1 cells covering the entire transcription unit of *Tsix* (39). (B and C) ChIP analysis of H3K36me3 in Dnmt TKO cells and its parental line, J1 (B), and J1:rtTA, *Eed*<sup>-/-</sup>, *E*<sup>-/-</sup> Δ*Tsix*, *Ring1B*<sup>-/-</sup>, Δ*Tsix*, and *R*<sup>-/-</sup> Δ*Tsix* cells (C). The *Sox2* gene body and its promoter were used as positive- and negative-control loci, respectively. Data are means and standard deviations from three independent experiments.

for *Xist* repression (21, 22). However, our data do not rule out the possibility that Oct4 and Nanog repress *Xist* through recruitment of PRC2. Indeed, a previous study reported a synergistic effect between the loss of the Oct4 binding site in *Xist* intron 1 and *Tsix* on *Xist* activation (57). Furthermore, Oct4 could possess independent modulatory functions in the repression of *Xist* that are not critical in male cells.

In our system, repression of *Xist* by *Tsix* required neither DNA methylation nor Polycomb recruitment, suggesting that additional mechanisms exist by which *Tsix* exerts its repressive func-

tion. We find that H3K36me3 is recruited to the *Xist* promoter in naive as well as serum-cultured ES cells. We show that H3K36me3 recruitment at the *Xist* promoter and gene body depends on *Tsix* transcription but requires neither the PRC complex proteins *Ring1b* and *Eed* nor DNA methylation. H3K36me3 is associated with transcriptional elongation and a function in repression of inappropriate transcription (50–54). To address the function of H3K36me3 in repressing *Xist*, we performed two experiments aiming at reducing H3K36me3 globally. The first approach is based on a mutated form of histone H3.3 that is able to seques-



**FIG 7** H3K36me3 is functionally involved in *Xist* repression. (A) Schematic representation of expression constructs. (B) Western blot analysis of histone modifications H3K36me3 and H3K27me3 in J1 cells expressing the indicated transgenes. H3 was used as a loading control, and Flag was used to confirm expression of a Flag-tagged transgene. The relative amount of histone modification, with the amount of H3.3wt set to 1 and normalized to histone H3, is given beneath the lanes in the top two panels. (C and D) ChIP analysis of H3K36me3 (C) and H3K27me3 (D) in J1 cells expressing H3.3wt and K36M transgenes. The *Sox2* gene body and its promoter for H3K36me3 and the *Hoxa7* and *Oct4* promoters for H3K27me3 were used as positive- and negative-control loci, respectively. Data are means and standard deviations from three independent experiments. (E) qRT-PCR analysis for *Xist* and *Tsix* expression in H3.3wt-expressing (H3.3wt) and K36M-expressing (K36M) J1 ES cells. Expression relative to H3.3wt-expressing J1 ES cells normalized to *Gapdh* ( $n = 3$ ) is shown. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (Student's *t* test).

ter histone methylases specifically (56). Expression of a histone H3.3 carrying a substitution of methionine for lysine at position 36 led to a reduction of endogenous H3K36me3 to half the amount observed in control cells. Reduced H3K36me3 caused a marked derepression of *Xist* but had little effect on *Tsix* and a control gene. Our second and independent attempt at reducing H3K36me3 was based on RNA interference-mediated depletion of *Setd2*. *Setd2* is considered the major histone methyltransferase catalyzing H3K36me3. This approach was less efficient in reducing H3K36me3 but also resulted in *Xist* upregulation. Taken together, our experiments support the view that H3K36me3 recruitment contributes to *Xist* repression. However, we were not able to eliminate H3K36me3 entirely and thus may have observed a partial effect on *Xist* expression. In addition, depletion of H3K36me3 might also have indirect

effects through perturbation of transcription units of other genes. We did not observe *Xist* clusters after depletion of H3K36me3 by RNA-FISH, despite the fact that H3K27me3 and H3K36me3 were reduced at the *Xist* promoter, suggesting that additional mechanisms exist through which *Tsix* represses *Xist*.

In the absence of *Tsix*, H3K27me3 is recruited to the *Xist* promoter through PRC2. H3K27me3 apparently spreads from a hot spot that is located 5' to *Xist*. When *Eed* is mutated, H3K27me3 is lost and *Xist* is activated. We find that loss of H3K27me3 enrichment also leads to derepression of the *Ftx*, *Jpx*, *Cnbp2*, and *Xpct* genes, suggesting that multiple genes within the *Xic* are repressed by PRC2. The expression of *Ftx* (25) and *Jpx* (23) is elevated in differentiating female ES cells and correlates with *Xist* activation. Currently, the function of *Cnbp2* in XCI is unknown, and overexpression of *Xpct* does not lead to *Xist* expression (58).

Notably, H3K27me3 at the *Xist* promoter does not efficiently recruit H2AK119ub1. H2AK119ub1 is reduced in *Ring1b*-deficient cells to near background levels, but simultaneous loss of *Ring1b* and *Tsix* did not result in activation of *Xist*. This observation could suggest that at the *Xist* promoter H3K27me3 exerts a repressive function that is largely independent of H2AK119ub1. A PRC1-independent function of PRC2 is presently not regarded as a prominent silencing mechanism. However, genes have been identified in ES cells that are targets of PRC2 but not PRC1, suggesting that this mode of regulation is not entirely specific to the *Xist* gene promoter (45).

In cells carrying an intact *Tsix* gene, H3K27me3 appears to spread from a hot spot upstream of *Xist* over several genes within the *Xic* region but is prevented from entering the *Xist* locus. We aimed to clarify whether known chromatin modifications can explain how *Tsix* prevents the spreading of H3K27me3. Previous reports have shown that DNA methylation is recruited by *Tsix* to the *Xist* promoter (10, 17, 36, 39, 47, 48). Notably, DNA methylation and H3K27me3 are inversely correlated in undifferentiated ES cells (49). Using ES cells that lack DNA methylation, we demonstrated that DNA methylation is not required for restricting the spreading of H3K27me3. H3K27me3 also remained excluded from the *Xist* promoter after depletion of H3K36me3. This observation suggests that H3K36me3 is not critical for restricting spreading of H3K27me3 over the *Xist* promoter. However, we cannot rule out the possibility that we have not been able to reduce H3K36me3 to low enough levels. Blocking of H3K27me3 spreading by *Tsix* might involve functional redundancies between H3K36me3 and DNA methylation. Alternatively, additional, as-yet-identified mechanisms might exist.

Our data implicate H3K36me3 as a chromatin modification that is established cotranscriptionally by *Tsix* over the *Xist* promoter and contributes to *Xist* repression. This modification can now be considered in future analyses of *Xist* regulation and will facilitate progress in understanding the chromatin-based mechanisms that contribute to the initiation of XCI. It will also be interesting to see if H3K36me3 is also relevant for gene regulation by noncoding transcription at other gene loci.

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We have no conflicts of interest to declare.

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