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Alteration of Histone Tail Modifications in the Xist *Locus in Wild-Type and* Tsix*-Mutant Male Embryonic Stem Cells during Differentiation*

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Abstract: *The non-coding RNA* Xist *is indispensable for X chromosome inactivation. Transcriptional control of* Xist *gene depends on its antisense partner gene* Tsix *which prevents* Xist *up-regulation in* cis*. Previous studies proposed* Tsix *acts by regulating chromatin structure. Although histone modifications in the* Xist *locus during differentiation have been described in female embryonic stem (ES) cells, they remain unclear in males. Here we addressed histone modifications in the* Xist *locus in wild-type and* Tsix*-mutant male ES cells during differentiation. Their active and repressive modifications were attenuated upon differentiation, while the histone modification profile in males resembled that of females in an undifferentiated condition. These results provide implications in understanding the regulation of* Xist *gene, as well as other developmentally regulated genes, through chromatin structure.*

Key words: *antisense genes, histone tail modification,* Xist

Embryonic stem (ES) cells are undifferentiated pluripotent cells derived from the inner cell mass of blastocysts [14]. ES cells can differentiate to generate all types of cells in an embryo or an adult, and they are useful not only in making knockout mice but also for regenerative medicine, tissue engineering, and animal cloning. It has been shown that epigenetic mechanisms including DNA methylation, histone tail modifications, and the regulation of higher-order chromatin structure by chromatin-associated factors, participate in the maintenance of pluripotency in ES cells [8]. Notably, recent findings highlight the roles of two representative histone modifications, the histone H3-dimethyl lysine 4 (H3K4m2) known as a transcriptionally active chromatin mark and the histone H3-trimethyl lysine 27 (H3K27m3) as a repressive chromatin mark, in the control of developmental regulator genes [1, 2]. These studies demonstrated co-occupation of these two modifications in lineage-specific gene loci. This finding points out the crucial roles of the two histone modifications in the maintenance of ES cell pluripotency; i.e., the H3K27m3 modification prevents ectopic ES cell differentiation by repressing lineage-specific genes and the H3K4m2 maintains ES cells' ability to differentiate to any tissue by retaining developmental genes ready for activation.

The H3K4m2 and H3K27m3 modifications have also been implicated in the regulation of the *Xist* gene that is

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indispensable for X chromosome inactivation (XCI) (for XCI review refer to [3]). Because *Xist* is developmentally regulated, i.e., *Xist* transcription is extremely low in undifferentiated embryonic cells and is immediately upregulated upon differentiation, knowledge of the molecular mechanism of *Xist* regulation by the two histone modifications would be useful for understanding the maintenance of ES cell pluripotency as well as the XCI mechanism itself. XCI is a manner of sex chromosome dosage compensation employed by female mammals, in which one of the two female X chromosomes is inactivated during early development. *Xist* is an X-linked gene and functions as non-coding RNA. *Xist* is specifically transcribed from females' inactive X and the X to be inactivated. *Xist* RNA is believed to recruit Polycomb group proteins in *cis,* thereby achieving long-range chromosomal silencing.

Transcriptional control of the *Xist* gene depends on its antisense partner gene *Tsix* which prevents *Xist* upregulation in *cis* in females, while male embryonic cells can keep the *Xist* gene repressed without *Tsix* [5, 6, 11, 12]. Previous studies proposed *Tsix* acts by regulating chromatin structure [7, 9, 15]. Navarro *et al*. indicated *Tsix* transcription represses *Xist* by attenuating H3K4m2 modification in the *Xist* locus [7]. In addition, Sun *et al*. demonstrated that *Tsix* transcription prevents H3K27m3 modification in the *Xist* promoter and gene body in female ES cells in which XCI processes can be replicated *in vitro* [15]. They proposed immediate *Tsix* downregulation on the future inactive X chromosome induces a transient heterochromatic state in the *Xist* locus, which preempts XCI choice. However, the biological significance of the repressive H3K27m3 modification induced by *Tsix* downregulation is unclear, because in females it paradoxically results in transcriptional activation of the *Xist* gene. Sun *et al*. indicated that the H3K27m3 modification in the *Xist* locus disappeared after XCI establishment, but during the XCI initiation phase, active *Xist* transcription and H3K27m3 modification coexisted [15]. *Tsix* truncation results in elevated H3K27m3 modification in the *Xist* locus in male ES cells as well as in females [7], but the alteration of the modification during the course of differentiation has not been addressed yet. The male X chromosome is not a subject of XCI, and it would be beneficial to know how H3K27m3 modification in the *Xist* locus is regulated in males during differentiation. In order to gain insights into the regulation of the *Xist* gene as well as other developmentally important genes through chromatin structure, we generated a *Tsix*-trap male ES cell line and investigated H3K4m2 and H3K27m3 modifications in undifferentiated and differentiating conditions.

We targeted the E14.1 male ES cell line and newly generated a *Tsix*-trap mutant cell line (Figs. 1A–C). The targeting vector and genomic PCR screening were described previously [12]. Southern blot was also done as previously described except that genomic DNA was digested with *Spe* I instead of *Xho* I. Truncation of *Tsix* transcription in the mutant cell line was confirmed by allele-specific RT-PCR (Fig. 1D) [12].

We investigated the H3K27m3 and H3K4m2 histone tail modifications in the *Xist*/*Tsix* locus in wild-type and *Tsix*-trap male ES cells by chromatin immunoprecipitation (ChIP). The results were quantified by TaqMan PCR and the positions of PCR amplicons are shown in Fig. 2A. The Taqman probes and PCR primers are described elsewhere [13]. In an undifferentiated condition, the H3K27m3 modification was clearly elevated in both *Xist* promoter and gene body in the mutant, while the wild-type cell was almost devoid of the modification (Fig. 2B). This result agrees with a previous report [7]. In contrast, the H3K4m2 modification in the mutant was attenuated in the *Xist* gene body compared to the wild-type cell (*Xist*GB2 and *Xist*GB3, Fig. 2C). We could not find any differences in the H3K4m2 level in the *Xist* promoter between the wild-type cell and the mutant. These data basically agree with a previous report [7], though our results were less pronounced, which may be due to the difference of ChIP-PCR amplicons and/or the ES cell line used.

Subsequently, we examined the two histone modifications in differentiating embryoid bodies (EB). In order to prepare EB, ES cells were cultured without feeders or leukemia inhibitory factor for upto 11 days. On day 4, which corresponds to the initiation phase of XCI in female EB, the H3K4m2 modification in the *Xist* promoter was elevated in the mutant male EB, while there was no difference in the gene body (Fig. 2C). The result likely reflects the ectopic *Xist* activation found in a minor population of *Tsix*-mutant male ES cells upon differen-

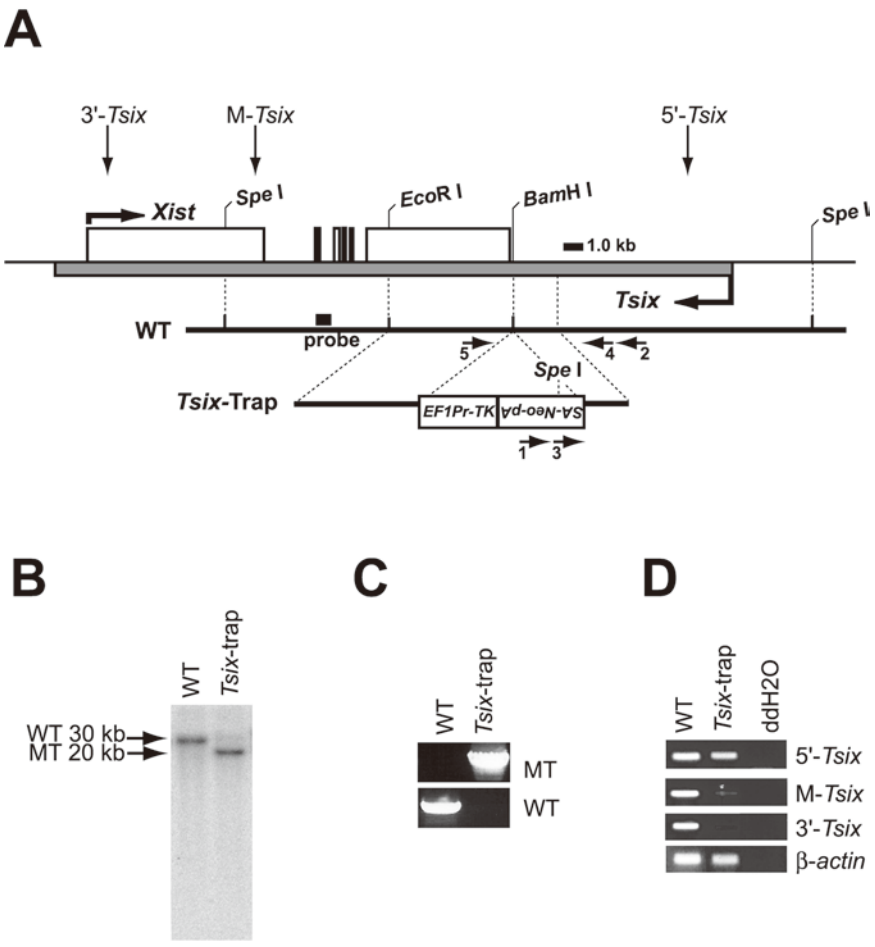

Fig. 1. Generation of a *Tsix*-trap male embryonic stem (ES) cell line. (A) The restriction map of the *Xist*/*Tsix* locus and the targeting construct. Positions of genomic PCR primers are indicated as numbered arrows. Upper open rectangles represent *Xist* exons and the lower grey one shows the *Tsix* gene. WT: wild-type, EF-1Pr: Elongation factor-1 promoter, TK: thymidine kinase gene, SA: splice acceptor, Neo: neomycin-resistant gene, pA: polyadenylation signal. (B) Southern blot of *Spe* I digested genomic DNA confirming proper recombination of the long arm. Position of the probe is shown in (A). MT: mutant. (C) Genomic PCR confirming proper recombination of the short arm. Results from nested PCR using primer sets [1-2] and [3-4] for MT, and [5-2] and [5-4] for WT are shown. (D) Strand-specific RT-PCR for *Tsix* transcript in WT and *Tsix*-trap ES cells verifying its truncation in the mutant. Positions of *Tsix* PCR amplicons are indicated in (A).

tiation [10]. On the other hand, the H3K27m3 level in the mutant was persistently higher than that in the wild-type EB (Fig. 2B). Hence, it is tempting to speculate that the elevated H3K27m3 repressive modification in the *Tsix*-trap EB may be inhibiting ectopic *Xist* activation in developing male cells. In this context the H3K27m3 modification would be a redundant way for a cell to protect the single male X chromosome from inactivation in the physiological condition. On day 11, which corresponds to the XCI establishment phase, both H3K4m2 and H3K27m3 modifications almost disappeared in the *Xist* promoter and gene body. The absence of H3K4m2 is likely to reflect the silencing of *Xist* transcription and the loss of H3K27m3 mirrors the depletion of Ezh2 and Eed proteins, which are histone methyltransferases of H3K27m3, in differentiated cells with less developmen-

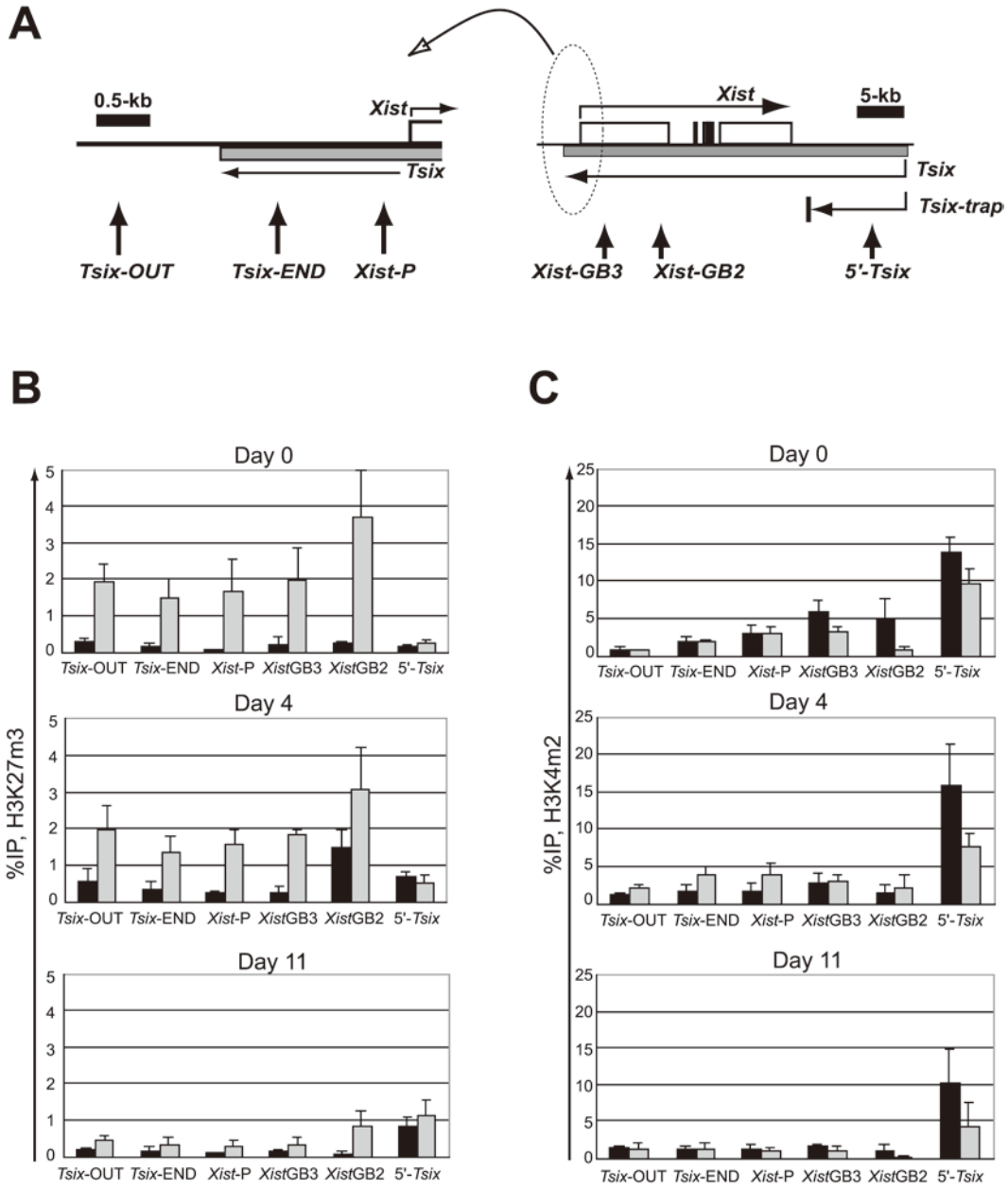

Fig. 2. Chromatin immunoprecipitation (ChIP) in wild-type and *Tsix*-trap male ES cells in undifferentiated or differentiating conditions. (A) Positions of ChIP-PCR amplicons. The left panel represents an enlarged view of the *Xist* promoter, while the right panel shows the entire *Xist/Tsix* locus. Upper open rectangles represent *Xist* exons, and the lower grey one shows the *Tsix* gene. (B) ChIP for histone H3-trimethyl lysine 27 (H3K27m3) in undifferentiated ES cells (Day 0) and in embryoid bodies differentiated for 4 (Day 4) or 11 (Day 11) days. Black columns represent results in the wild-type and grey ones those in the *Tsix*-trap cells. Error bars show SD. All results were from three independent experiments. (C) ChIP for histone H3-dimethyl lysine 4 (H3K4m2).

tal plasticity [4].

In this brief paper we have firstly reported on the change of H3K4m2 and H3K27m3 modifications in male wild-type and *Tsix*-trap ES cells during differentiation. We propose a new role for H3K27m3 modification in the *Xist* locus that prevents ectopic *Xist* activation during

differentiation, thereby protecting the single male X-chromosome from inactivation. Our results have implications for the understanding of developmental gene regulation by chromatin structure and antisense genes.

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