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Minireview

X-chromosome inactivation: the molecular basis of silencing

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Abstract

X-chromosome inactivation occurs randomly for one of the two X chromosomes in female cells during development. Inactivation occurs when RNA transcribed from the *Xist* gene on the X chromosome from which it is expressed spreads to coat the whole X chromosome. In the first issue of *Epigenetics and Chromatin*, Nesterova and colleagues investigate the role of the RNA interference pathway enzyme Dicer in DNA methylation of the *Xist* promoter.

X-chromosome inactivation is the transcriptional silencing of one X chromosome in female mammalian cells that equalizes dosage of gene products from the X chromosome between XX females and XY males [1-3]. X-chromosome inactivation in the embryo proper occurs early in development. The two X chromosomes have an equal probability of being silenced [4]. Silencing, once established, is stable: the same X chromosome remains inactivated in all subsequent cell generations. As a result, each female is a mosaic of cells in which either the maternally inherited or the paternally inherited X is silenced. Nesterova and colleagues in the first issue of *Epigenetics and Chromatin* shed new light on how this process is regulated [5].

An antisense pair of non-coding RNAs, encoded by *Xist* and *Tsix* (Figure 1), is important in the regulation of the random inactivation of mouse X chromosomes. Before the signal that initiates random X-chromosome inactivation is received, *Xist* and *Tsix* are transcribed from all active X chromosomes in each male and female cell [6]. Once inactivation is initiated, *Xist* and *Tsix* are differentially regulated on the X that will become the active X chromosome (X_A) and the one that will become the inactive X chromosome (X_I). On the X chromosome that will become the X_I, *Xist* transcripts spread *in cis* from their site of synthesis to coat the entire X chromosome and establish transcriptional silencing. Concomitant with *Xist* RNA coating, *Tsix* is silenced on the X_I. The expression of *Xist* and *Tsix* persists on the X_A for a brief period after silencing of the X_I is complete, and is eventually extinguished. *Xist* RNA continues to coat the X_I throughout all subsequent cell divisions, where it contributes to the maintenance of silencing. These patterns of *Xist* and *Tsix* expression are also seen in mouse female embryonic stem (ES) cells, which have two X_As and which undergo X-chromosome inactivation when they are induced to differentiate *in vitro*. Thus, ES cells provide a useful model system to study X-chromosome inactivation.

Mutations in *Xist* or *Tsix* can cause non-random X inactivation

Heterozygous mutation of *Xist* or *Tsix* causes non-random X-chromosome inactivation in female cells. When *Xist* expression is increased from one X chromosome in pre-X-chromosome-inactivation cells, that X chromosome always becomes the X_I and the wild-type X always becomes the X_A [7]. In female ES cells or embryos in which *Xist* is disrupted on one X chromosome, the mutant X chromosome always becomes the X_A and the wild-type X chromosome always becomes the X_I [8-10]. Disruption of *Tsix* has the opposite

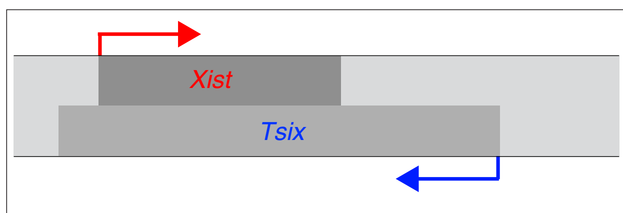

Figure 1
Transcription of *Xist* and *Tsix* on the X chromosome. The coding sequences of *Xist* and *Tsix* overlap on opposite strands of the X-chromosome DNA.

effect: the mutant X chromosome becomes the X_I and the wild-type X chromosome is always the X_A [11-13]. This is known as primary non-random X-chromosome inactivation because the X chromosomes are chosen as the X_A and X_I before silencing is initiated. A second cause of non-random X-chromosome inactivation is the selective death of cells that inactivate the incorrect number of X chromosomes: because the fates of the X chromosomes are not determined before silencing, this is known as secondary non-random X-chromosome inactivation [14]. Because *Xist* and *Tsix* mutations cause primary non-random X-chromosome inactivation, it is likely that these non-coding RNAs function in the choice of the X_A and X_I before silencing is initiated. Understanding how *Xist* and *Tsix* are regulated in pre-X-chromosome-inactivation cells is central to understanding how one X chromosome is randomly selected as the X_A and the other as the X_I in each cell.

In addition to having opposing roles in random choice, *Xist* and *Tsix* also negatively regulate each other in ES cells. *Xist* and *Tsix* are transcribed from overlapping regions on opposite strands of the X-chromosome DNA (Figure 1). Deletion of *Tsix* promoter sequences or a mutation that blocks *Tsix* transcription before it reaches *Xist* RNA coding sequences abolishes *Tsix* transcription and causes a roughly ten-fold increase in *Xist* RNA levels from the mutant X chromosome [11-13]. Thus, transcription of *Tsix* across *Xist* is necessary for *Tsix* to negatively regulate *Xist*. In the *Tsix* truncation mutant the *Tsix* promoter has histone modification patterns that are generally associated with transcriptional silencing [15]. These epigenetic marks also characterize the X_I, and their recruitment to the X_I requires transcription of *Xist* [16-18]. Together, these results suggest that the increase in *Xist* RNA that occurs on *Tsix* mutant chromosomes represses *Tsix*. Consistent with the possibility that *Xist* negatively regulates *Tsix*, *Tsix* RNA levels are increased from *Xist* mutant X chromosomes [10,19]. Insights into the nature of factors that are involved in the mutual regulation of *Xist* and *Tsix* in pre-X-chromosome-inactivation cells are likely to be important in developing an understanding of how these non-coding RNAs ensure that the two X chromosomes have an equal probability of being silenced in each cell.

The role of DNA methylation

The mechanisms underlying the mutual regulation of *Xist* and *Tsix* in pre-X-chromosome-inactivation cells are not well characterized. An interesting new study by Nesterova and colleagues suggests that DNA methylation may be involved in this mutual negative regulation [5]. Nesterova *et al.* demonstrate a correlation between *Xist* promoter DNA methylation and *Xist* expression in ES cells. In XY ES cells (in which the single X chromosome remains active), two regions flanking the *Xist* transcription start site show high levels of DNA methylation. Two XY ES cell lines bearing *Xist* promoter mutations that result in increased *Xist* expression showed DNA hypomethylation at these sites. In addition, a mutation that truncates *Tsix* transcription before it traverses *Xist* also resulted in increased *Xist* expression and DNA hypomethylation at these sites. These results establish a clear correlation between the levels of DNA methylation at *Xist* and expression of *Xist* in ES cells. It remains to be established whether the increase in *Xist* expression triggers demethylation or vice versa. In addition, *Xist* and *Tsix* negatively regulate each other, raising the possibility that *Tsix* also has a role in regulation of *Xist* DNA methylation.

Tsix has also been implicated in the direct regulation of DNA methylation. The *de novo* DNA methyltransferase Dnmt3a can be immunoprecipitated with *Tsix* RNA using an RNA-chromatin immunoprecipitation procedure [20]. Furthermore, Dnmt3a can *de novo* methylate *Xist* [21,22]. Together, these data suggest a model in which *Tsix* RNA directs Dnmt3a to *Xist* in ES cells (Figure 2a). Thus, the hypomethylation of *Xist* DNA in the *Tsix* truncation line may occur because Dnmt3a cannot act on *Xist* when *Tsix* RNA is not present to recruit it there.

This model explains the hypomethylation of *Xist* DNA in the *Tsix* truncation line, but how does it account for the hypomethylation in the *Xist* promoter mutation lines? As in the *Tsix* truncation line, the *Xist* promoter mutation lines show increased *Xist* expression. In contrast to the truncation line, which does not produce *Tsix* RNA, the *Xist* promoter mutation lines continue to express *Tsix* RNA. However, *Tsix* RNA levels have not been quantitated in these cell lines, so it is not possible to establish a correlation between *Tsix* expression levels and *Xist* DNA methylation. One possibility is that the increase in *Xist* expression causes a decrease in *Tsix* RNA levels and a corresponding decrease in Dnmt3a activity at *Xist* DNA. There is also an alternative possibility: it may be that *Xist* RNA (or an epigenetic modification induced by *Xist* RNA) interferes with the activity of Dnmt3a

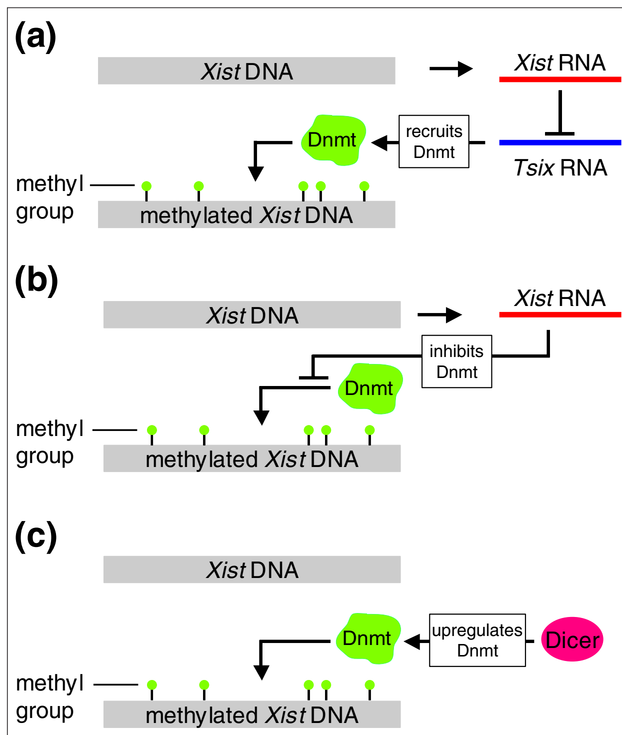

Figure 2
Models for the coordinate regulation of *Xist* DNA methylation and expression by *Tsix*, *de novo* DNA methyltransferases and Dicer. *De novo* DNA methyltransferases (Dnmt) promote methylation of *Xist* DNA. Increased *Xist* expression, as is seen in the *Xist* promoter mutants, could trigger *Xist* DNA hypomethylation **(a)** indirectly by affecting *Tsix* RNA levels, if *Tsix* is necessary to direct *de novo* DNA methyltransferases to the *Xist* gene, or **(b)** directly, if *Xist* RNA can interfere with *de novo* DNA methyltransferase activity locally. **(c)** Because Dicer deficiency causes a global decrease in levels of *de novo* DNA methyltransferases, Dicer must lie directly upstream of the *de novo* DNA methyltransferases and need not function through either *Xist* or *Tsix* to regulate *Xist* DNA methylation. (The DNA is shown as methylated in a, b and c (bottom), although in a and b if the inhibitory interactions between *Xist* and *Tsix* RNA (a) or Dmt (b) prevail, the DNA will be hypomethylated.)

or other *de novo* methyltransferases (Figure 2b). Indeed, the *Xist* RNA-coated X_I shows overall lower levels of DNA methylation than the X_A, consistent with *Xist* RNA interfering with DNA methylation [23]. Because *Xist* RNA accumulates only locally in ES cells, this activity would be restricted to the *Xist* locus and perhaps nearby genes. Analysis of *Xist* DNA methylation in *Xist* and combined *Xist* + *Tsix* mutant ES cells will be required to distinguish between these possibilities.

X inactivation and Dicer deficiency

Nesterova and colleagues have further investigated the role of *de novo* methyltransferases in regulation of *Xist* expression in an analysis of *Dicer* mutant male ES cells. Dicer is an RNAse III enzyme that is central to the RNA interference (RNAi) pathway. RNAi regulates many aspects of gene expression and involves the production of antisense RNA complementary to sequences in the mRNA of the gene that is being regulated [24]. The formation of sense-antisense double-stranded RNA can trigger transcriptional or post-transcriptional gene silencing. Given that *Tsix* RNA contains sequences complementary to *Xist* RNA, an obvious question is whether the RNAi pathway has a role in X-chromosome inactivation. Nesterova *et al.* show that several independently derived Dicer-deficient male ES cell lines show *Xist* DNA hypomethylation and upregulation of *Xist* expression. They also find that the two imprinted loci *H19* and *Igf2rAir* show hypomethylation in Dicer-deficient cells. Hypomethylation of *Xist*, *H19* and *Igf2rAir* seems to be the consequence of changes in the levels of the *de novo* methyltransferases Dnmt3a, Dnmt3b and DnmtL, all of which were downregulated upon deletion of *Dicer*. This decrease in *de novo* methyltransferase activity in Dicer-deficient cells was also seen in two other studies of independently derived *Dicer* mutant ES cell lines [25,26]. In these studies *Dicer* mutant ES cells show hypomethylation of subtelomeric repeats or of *Oct4*, *Tsp50* and *Sox30* promoters, which are normally methylated. The downregulation of the *de novo* methyltransferases could be attributed to an increase in levels of the repressor Rbl2, which is negatively regulated by the miR-290 microRNA cluster [25,26]. Together, these results provide a compelling argument that the change in *Xist* DNA methylation seen in *Dicer* mutant ES cells is an indirect consequence of the loss of *de novo* methyltransferase activity (Figure 2c).

Does the change in *Xist* DNA methylation in pre-X-chromosome-inactivation cells affect the fate of the X chromosomes after inactivation is initiated? To answer this question Nesterova *et al.* analyzed *Dicer* mutant embryos. *Dicer* mutants die shortly after implantation, between embryonic day (E)7.5 and E8.5. X-chromosome inactivation is initiated at approximately E5.5, providing a brief window in which X-chromosome inactivation can be assayed in *Dicer* mutants. The cells of male and female Dicer-deficient E6.5 embryos and their wild-type littermates did not show any appreciable difference in either *Xist* or *Tsix* expression. These results indicate that one X chromosome can be selected as the inactive X and *Xist* RNA can coat that X chromosome in *Dicer* mutant embryos. Thus, X-chromosome inactivation seems unaffected by *Dicer* deficiency *in vivo*.

The results of Nesterova *et al.* contrast with those from another study of the role of Dicer in X-chromosome inactivation. Ogawa *et al.* [27] examined X-chromosome inactivation in *Dicer* mutant female ES cells and found that *Xist* RNA could not coat and silence an X chromosome on

differentiation. These results indicate that Dicer is necessary for X-chromosome inactivation *in vitro*. Why do female ES cells and embryos differ in their requirements for Dicer during X-chromosome inactivation? One possibility is that maternal stores of Dicer persist long enough to promote X-chromosome inactivation in female *Dicer* mutant embryos. However, the homozygous *Dicer* mutant female ES cells used by Ogawa *et al.* contained a *Dicer* transgene that was expressed at less than 5% of wild-type levels (this was deployed to overcome the block to differentiation in *Dicer* mutants that would have otherwise interfered with analysis of X-chromosome inactivation), suggesting that small amounts of Dicer are not sufficient to promote random inactivation. A second possibility is that *Dicer* mutant female embryos fail to reverse imprinted X-chromosome inactivation in their embryonic compartment. In mice, the extra-embryonic tissues undergo imprinted X-chromosome inactivation, in which there is exclusive silencing of the paternal X chromosome [28]. Imprinted X-chromosome inactivation is initiated in pre-implantation development and seems to occur in all cells of the early embryo. Imprinted X-chromosome inactivation is reversed in the cells that will go on to form the embryo proper, and these cells subsequently undergo random X-chromosome inactivation after implantation [29,30]. Determining whether *Dicer* mutant female embryos show random or imprinted X-chromosome inactivation will establish whether Dicer is important to erase imprinted X-chromosome inactivation. Clearly much work remains to be done to determine how Dicer regulates *Xist* expression during development.

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