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Xist gene regulation at the onset of X inactivation

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The large non-coding RNA *Xist* is the master regulator of X inactivation. *Xist* is negatively regulated by its antisense transcript *Tsix*. This repressive antisense transcription across *Xist* operates at least in part through the modification of the chromatin environment of the locus. However *Tsix* is not sufficient to repress *Xist* in pluripotent cells and there is emerging evidence that transcription factors associated with pluripotency are involved in *Tsix*-independent repression. This review focuses on recent advances in this area and discusses the implications for our understanding of *Xist* gene regulation at the onset of X inactivation.

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Introduction

X inactivation is a dosage compensation strategy employed by mammals to ensure that females (XX) and males (XY) transcribe equal levels of X-linked genes [1]. A large non-coding RNA termed *Xist* (X inactive specific transcript) is the master regulator of this process [2,3]. In females, at the onset of X inactivation, *Xist* is upregulated from the presumptive inactive X chromosome, coats the chromosome in *cis* and triggers the onset of transcriptional silencing via the modulation of chromatin structure [4–7]. The other X chromosome remains active.

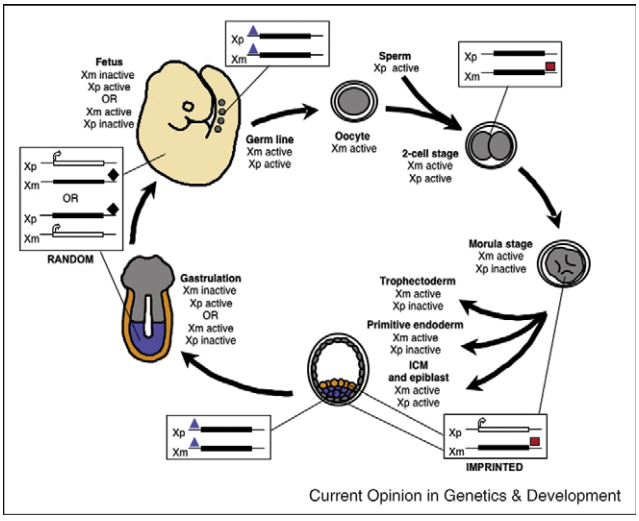
Throughout embryonic development *Xist* expression, and thus X inactivation, is dynamic (Figure 1). Early in embryogenesis, at around the two to four cell stage, *Xist* transcripts are detected from the paternally inherited X chromosome, which is consequently inactivated in all cells. This imprinted X inactivation persists in the trophectoderm and primitive endoderm lineages specified at the blastocyst stage. However, in the pluripotent inner cell mass *Xist* expression is downregulated and the inactive X is reactivated [8,9]. As these cells begin to differentiate, *Xist* is again upregulated, this time randomly from either the maternally or paternally inherited X chromosome. *Xist* downregulation and X reactivation occurs yet again during the life cycle of females in maturing XX primordial germ cells (PGCs) [10–13] (Figure 1). Here we review the recent advances in our understanding of how the dynamic pattern of *Xist* expression is co-ordinated.

Tsix modulates the chromatin modification status of the *Xist* promoter in ES cells

Tsix, a large non-coding RNA, is transcribed antisense to *Xist* (Figure 2) [14]. It is well established that *Tsix* functions in *cis* to negatively regulate *Xist*. A number of publications have reported that when *Tsix* transcription is ablated on one allele in XX ES cells and XX embryos, the choice of which X chromosome to inactivate is skewed towards the mutant allele, presumably because *Xist* is not efficiently repressed by *Tsix* [15–18]. Interestingly, the upregulation of *Xist* and X inactivation even occurs in some [19–21], although not all [15,22] differentiating XY and XO ES cells bearing *Tsix* mutations. A possible reason for the discrepancy in this data is discussed below.

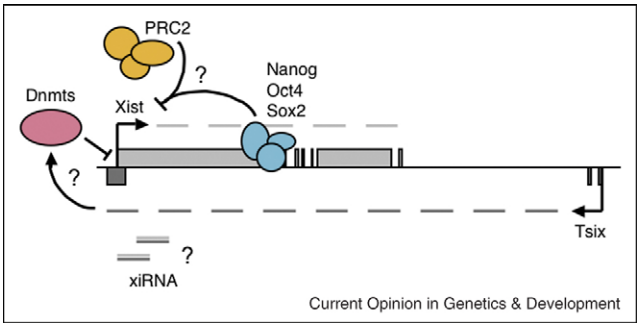
A key question in the field is to understand the mechanism by which *Tsix* modulates *Xist* activity in *cis*. This is thought to involve the modification of the chromatin environment of the locus. In undifferentiated cells *Tsix* expression appears to maintain an open chromatin structure through the body of the *Xist* locus [23,24]. However, there are discrepancies between reports describing effects on the *Xist* promoter. Navarro *et al.* found that mutants in which *Tsix* transcription is ablated the *Xist* promoter shows an increase in H3K4me2 characteristic of active chromatin [23]. Subsequently they reported that active marks H3K4me3 and H3K9 acetylation are also enriched at the *Xist* promoter, whereas features of repressed chromatin, such as H3K9me3 and DNA methylation, are reduced [25]. In contrast to this, Sun *et al.* reported that *Tsix* mutants show an increase in the repressive modification H3K27me3 at the *Xist* promoter [24]. A possible explanation is that the parental cell line used in the experiment by Sun *et al.* more efficiently utilises a *Tsix*-independent mechanism for *Xist* repression which may have been further selected for following deletion of *Tsix*. Such a mechanism for *Xist* repression could also explain the aforementioned discrepancies regarding whether or not *Xist* gene upregulation occurs following the differentiation of *Tsix*-deficient XY/XO ES

Figure 1



The X inactivation cycle. *Xist* has a dynamic expression pattern throughout embryogenesis. *Xist* is not expressed in sperm or oocytes and the X chromosome is active. The Xm allele has a repressive imprint (red square) so that *Xist* expression is upregulated at the two to four cell stage on the paternally inherited X chromosome only and imprinted X inactivation is established. The imprint is erased in the cells of the inner cell mass, *Xist* is downregulated and the inactive X is reactivated. *Xist* repression is maintained by the pluripotency programme (blue triangles). As epiblast cells differentiate *Xist* is upregulated from either of the two X chromosomes and random X inactivation is established. The *Xist* allele on the active X chromosome is shut down (black diamonds). As primordial germ cells migrate from the hindgut to the genital ridges *Xist* is downregulated and the inactive X is reactivated.

Figure 2



Factors involved in regulation of the Xist promoter. *Xist* (light grey) is negatively regulated by its antisense transcript *Tsix* (dark grey). The balance of sense and antisense transcription across the promoter influences methylation of the *Xist* promoter by Dnmts (pink), through an as yet uncharacterised mechanism. It has been reported that *Xist* and *Tsix* RNAs form duplexes that are processed by RNAi enzyme Dicer to small xiRNAs. Whether or not xiRNAs regulate *Xist* expression is subject to debate. A *Tsix*-independent pathway also negatively regulates *Xist*. The pluripotency factors Nanog Oct4 and Sox2 (blue) have been shown to repress *Xist* and this is suggested to result from a direct interaction between binding sites for these factors in intron 1 of the gene and the *Xist* promoter. Polycomb repressor complex 2 (PRC2) (orange) has been shown to repress transcription from the *Xist* promoter in the absence of *Tsix* transcription, indicating that it may contribute to the *Tsix*-independent pathway.

cells [15,18–22]. Direct evidence supporting this idea is discussed in a subsequent section.

Xist promoter repression by *Tsix*

Evidence that *Tsix* modulates chromatin modification of the *Xist* promoter still leaves open the issue of how? What is the mechanism? *Tsix* transcription in an antisense direction through the *Xist* locus, rather than the antisense transcript itself, mediates *Xist* promoter repression. This conclusion stems from the results of an experiment where the *Tsix* transcript was truncated before it reached the *Xist* gene body, and yet had the same effect as completely ablating *Tsix* transcription [26]. A more recent publication confirmed that antisense transcription must proceed through the *Xist* promoter in order for *Xist* to be repressed: truncating *Tsix* to 93% of its full wildtype length resulted in a failure to establish repressive chromatin at the *Xist* promoter, and hence *Xist* silencing in mutant embryos [27].

Just as ablating *Tsix* transcription skews the choice of inactive X chromosome towards the mutated allele, in ES cell lines carrying mutant *Xist* alleles where sense transcription is increased, there is skewing of choice towards the mutated allele [28]. Together these studies suggest that the balance of sense and antisense transcription is important in defining which *Xist* allele is upregulated in XX cells. A recent study shows that this skewing correlates with the hypomethylation of the *Xist* promoter on the mutant allele [29••].

The importance of the levels of sense and antisense transcripts suggests a possible involvement of double stranded RNA and the RNAi pathway. This hypothesis has been tested in two recent studies. Ogawa *et al.* report that *Xist* and *Tsix* form duplexes that are processed by the RNAi enzyme Dicer during the onset of X inactivation into small ~30 nt RNAs, termed xiRNAs [30••]. Given the extensive literature demonstrating that Dicer cleaves dsRNA to yield 20–24 nt siRNAs, it is not clear what mechanisms could account for the 30 nt RNAs. Regardless, the authors postulate that these xiRNAs are involved in repressing *Xist* on the presumptive active X chromosome. This was suggested as depleting Dicer to 5% of the normal endogenous level, reduced the levels of xiRNAs and led to a 5–10-fold increase in *Xist* in undifferentiated ES cells.

The second study, by Nesterova *et al.*, comes to a very different conclusion. X inactivation occurs normally in *Dicer*−/− female embryos and while the *Xist* promoter is hypomethylated and *Xist* RNA is upregulated in *Dicer*−/− XY ES cells, this is a secondary effect due to the down-regulation of Dnmt3 enzymes [29••]. This conclusion is in

line with other studies demonstrating co-ordinate downregulation of *de novo* methyltransferases and global hypomethylation in Dicer-deficient ES cells. These effects are attributed to increased levels of the Rbl2 repressor that in turn is regulated by the miR-290 microRNA cluster [31,32]. If indeed there is no direct role for the RNAi pathway, the question of how *Tsix* transcription influences chromatin at the *Xist* promoter remains unanswered.

Xist repression and pluripotency factors

As discussed above, there is evidence for *Tsix*-independent repression of *Xist* in pluripotent cells. The downregulation of *Xist* in the inner cell mass of the late blastocyst [8,9], in PGCs [13] and during reprogramming experiments involving the fusion of somatic cells with pluripotent cells [33], points to a possible role for pluripotent cell transcription factors such as Nanog and Oct4, either directly or indirectly repressing *Xist*.

The pluripotency factor Nanog has been hypothesised to play an important role in *Xist* downregulation [34]. Nanog can function either as an activator or repressor of downstream targets in pluripotent cells [35], and displays a reciprocal expression pattern relative to *Xist* [36–38]. Strikingly, X reactivation is seen only in the Nanog-expressing cells of the inner cell mass [8]. Further to this, Nanog has been shown to enhance reprogramming of neural stem cells in fusion experiments as assayed by *Xist* expression [39].

Recently, evidence for the direct regulation of *Xist* by Nanog and other pluripotency factors, Oct4 and Sox2 has been described [40••]. Chromatin immunoprecipitation experiments revealed the binding of all three proteins in intron 1 of *Xist* in undifferentiated ES cells. Interestingly this region has been identified as a hypersensitive site and is predicted to constitute a matrix attachment region [41]. The functionality of this binding was tested by depleting XY ES cells of Nanog or Oct4. Loss of binding coincided with an upregulation of *Xist*, suggesting these factors do repress *Xist* expression. *Xist* upregulation preceded downregulation of *Tsix*, indicating that this mode of repression is independent of *Tsix*. However the authors note that even in cells where Oct4 had been depleted for 96 hour, and consequently Nanog and Sox2 were also depleted, only 10% of cells had an *Xist* RNA domain on one X chromosome, indicating that alternative repression mechanisms, most likely *Tsix* transcription, help maintain *Xist* repression. An important experiment for the future will be to delete the intron 1 Nanog/Oct4/Sox2 binding element in ES cells and thereby formally prove that Oct4 and Nanog repress *Xist* directly. Then, comparing *Xist* upregulation both in the presence and the absence of *Tsix* expression should determine the extent to which these two repression mechanisms contribute to *Xist* gene regulation at the onset of X inactivation.

A recent study reported that the polycomb-group (PcG) repressor protein Eed, an essential core component of the histone methyltransferase responsible for H3K27me3, is involved in repressing *Xist*. In double mutant *Eed*−/−*Tsix*-XY ES cells there is not only a loss of H3K27me3 but also a reduction of CpG methylation and an increase in H3K4me2 at the *Xist* promoter. This is accompanied by the hyperactivation of *Xist* upon differentiation [42]. These findings point to an involvement of PcG repressors in mediating *Tsix*-independent repression of *Xist*. Assuming that the binding of pluripotency factors to the *Xist* intron 1 element does directly repress *Xist*, it will be important to establish the link with PcG-mediated repression of *Xist*.

In addition to the repression of *Xist* by the pluripotency programme, pluripotent cells may also lack an activator of *Xist* required for full upregulation. Indeed the presence of an X-linked activator which would itself be upregulated upon differentiation has been postulated [43]. According to the model, each X chromosome in a cell has an individual probability of upregulating *Xist* in response to the activator. Once one chromosome becomes inactive the X-linked activator is downregulated. It is proposed that cells that inappropriately express both *Xist* alleles are rapidly selected against and are therefore rarely seen beyond the very earliest stages of differentiation. Cells containing only one X chromosome do not inactivate their X, as levels of the activator do not reach a critical threshold level. While most of the data is obtained from experiments using tetraploid cells, the model is convincingly supported by the fact that diploid XX cells heterozygous for a large deletion spanning *Xist*, *Tsix* and *Xite* (an enhancer element for *Tsix*) still inactivate one X chromosome.

X reactivation in PGCs

Once random X inactivation has taken place in the epiblast, the inactive X chromosome is reactivated specifically in the PGC lineage. During PGC maturation major chromatin changes occur that reprogram the genome back to pluripotency [44]. Early studies analysing X chromosome reactivation in PGCs concluded that this process occurs once they have occupied the genital ridges, with *Xist* expression being downregulated at around 11.5dpc and consequent reactivation of genes occurring between 11.5 and 13.5dpc [10–13]. However, three recent publications demonstrate that X reactivation initiates earlier than previously thought. PGCs were analysed for *Xist* expression throughout their development. A small but significant proportion of 7.75dpc nascent PGCs were found to be *Xist* negative (indicative of X reactivation) and this proportion increased steadily as the PGCs migrated and finally entered the genital ridges [45••,46••]. H3K27me3, a marker of X chromosome inactivation also diminished throughout this time period [47••].

Discrepancies with previously published data can be easily explained by the fact that more sophisticated methods were used to ensure that somatic cells were not included in the analysis, such as the use of antibodies that are more highly specific to PGC markers than those used previously, or fluorescent proteins under the control of a promoter active in PGCs. Further to this, previous studies analysed reactivation of genes at the protein level which may be delayed in comparison to the appearance of transcripts. Indeed, one study, despite seeing depletion of H3K27me3 on the inactive X chromosome in PGCs between 7.5 and 10.5dpc did not see reactivation of an X-linked GFP transgene until between 11.5 and 13.5dpc [47••]. While the mechanism for X reactivation in PGCs is unclear, Nanog expression is detectable in PGCs from 7.75dpc, consistent with its putative role in *Xist* repression [38].

Conclusion

There is increasing evidence that the regulation of *Xist* in mouse involves interplay between at least two major pathways: *Tsix*-mediated repression of the *Xist* promoter via chromatin modulation, and repression of *Xist* either directly or indirectly by factors expressed only in pluripotent cells. Key challenges for the future are to understand how *Tsix* transcription brings about chromatin changes over the *Xist* promoter, to formally prove that the intron 1 element directly represses *Xist* in pluripotent cells, and to understand the interplay of the different regulatory pathways, most importantly in the context of maintaining a single active X chromosome in XY and XX cells, both in imprinted and random forms of X inactivation.

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