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REVIEW

Mechanistic concepts in X inactivation underlying dosage compensation in mammals

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Mammals inactivate one of the two female X chromosomes to compensate for the unequal copy number of X-linked genes between males and females. This process of X inactivation entails the silencing of one X chromosome in a developmentally regulated manner. In this work, we review recent findings in X inactivation and discuss how these advance the mechanistic understanding. Recent results provide an insight how the cell counts and chooses the appropriate number of X chromosomes to inactivate, how chromosome-wide gene repression is coordinated and how a stable inactive X chromosome is established. Key components of this complex regulatory system have now been identified and provide entry points for understanding epigenetic regulation in mammals. A majority of the data has been obtained from studying mice. It is presently not clear how general these findings can be applied to other mammalian species. We try to assess this aspect from data, which has become available.
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Introduction

In mammals one of the two X chromosomes is transcriptionally silenced during early female development. Thereby, either the paternally or the maternally inherited X chromosome can be inactivated randomly making the developing female embryo a genetic mosaic of cells with opposite X inactivation patterns. The prospect of identifying one X chromosome among all DNA within the nucleus seems to be outside the domain explained by current knowledge and stretches the imagination of molecular biologists. For random X inactivation, a particular system has evolved that relies on the large non-coding *Xist* RNA that is expressed from and localizes specifically to the inactive X chromosome (Xi). *Xist* expression is controlled by a mechanism that ensures that one X chromosome remains active in a diploid cell. Regulation involves sequences around the *Xist* gene, which make up the complex genetic locus of the X inactivation center (*Xic*). *Xist* RNA has been identified in human (Brown *et al*., 1991) and mouse (Borsani *et al*., 1991; Brockdorff *et al*., 1991) and is apparently conserved among placental mammals (Nesterova *et al*., 2001; Chureau *et al*., 2002). *Xist* has not been detected in marsupials or the more distant vertebrates (Duret *et al*., 2006). Presently, it is thought that the mammalian *Xist* gene may have been derived from a protein-coding gene, *Lnx3* (Duret *et al*., 2006), in a process that involved the insertion of transposable elements (Elisaphenko *et al*., 2008). Interestingly, the genomic region around the *Xic* shows different arrangements in placental mammals and marsupials and is autosomal in monotremes (reviewed in Deakin *et al*., 2009). The *Xic* region has undergone repeated genetic rearrangements during the evolution of mammals. In marsupials dosage compensation is achieved by inactivation of the paternally inherited X chromosome (Graves, 1996; Koina *et al*., 2009). As marsupial *Xist* has not been identified it is likely that an alternative mechanism of imprinted X inactivation exists.

In mice, *Xist* RNA forms a domain over the non-genic chromatin of the core of the X chromosome territory (Chaumeil *et al*., 2006). Also on the human Xi *XIST* seems to overlap the central region of the chromosome territory, which contains mostly genomic repeats (Clemson *et al*., 2006). This indicates that the primary targets of *Xist* are not genes but sequences in the non-genic regions of the X chromosome. On the *Xi* genes are positioned at the periphery of the *Xist* domain irrespective if they are silenced or not (Clemson *et al*., 2006). The situation on the Xi is complex. A number of genes on the human Xi and a few genes on the mouse Xi escape gene silencing and are expressed from the Xi and the active X chromosome (Xa). What determines if a gene is silenced or remains active on the Xi is not entirely clear. Recent results show that at least one of the escape genes in mice, *Jarid1c*, contains elements that determine its active status independent of its chromosomal position on the Xi (Li and Carrel, 2008). This indicates that chromosomal sequences or properties of the gene locus modulate the effect that *Xist* exerts on expression. It is also unknown how *Xist* represses genes from its primary non-genic binding sites, albeit, evidence for nuclear compartmentalization has been obtained (Heard and Bickmore, 2007; Chow and Heard, 2009).

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Chromosome-wide gene repression and epigenetic modification of the Xi

In female somatic cells, the Xi forms a stable heterochromatic structure and can be visualized by a variety of staining techniques. Reactivation of genes on the heterochromatic Xi can hardly be achieved unless changes in cell fate are triggered during reprogramming to an early embryonic type (Takagi *et al*., 1983). Also during the generation of induced pluripotent stem cells from female mouse somatic cells reactivation of the inactive X has been observed (Maherali *et al*., 2007). Experiments blocking defined epigenetic pathways in somatic cells, including histone deacetylation and DNA methylation, have obtained partial reactivation of the Xi in a small proportion of cells (Csankovszki *et al*., 2001). This has led to the conclusion that gene silencing on the Xi is mediated by a number of epigenetic factors that act together for maintaining the inactive state of the chromosome. The Xi is subject to several modifications and is enriched for factors that have been associated with silent chromatin (Figure 1). Such a factor is the histone variant macroH2A that is distributed nearly uniformly over the entire Xi in somatic cells (Mietton *et al*., 2009). This could indicate that once stable inactivation has been achieved genes and non-genic sequences are covered with similar epigenetic modifications on the Xi.

The chromatin composition of the Xi differs markedly from that of other chromosomes in somatic cells. The Xi is enriched in several histone modifications such as histone H3 lysine 27 methylation and ubiquitination of histone H2A (Plath *et al*., 2003; de Napoles *et al*., 2004; Fang *et al*., 2004). This is paralleled by the recruitment of Polycomb group complexes (Figure 1a), which have been shown to catalyze these histone modifications (Plath *et al*., 2004). Furthermore, histone H4 is hypoacetylated and histone H3 lysine 4 di- and tri-methylation are strongly reduced on the Xi (Chaumeil *et al*., 2002). These histone modifications have been generally correlated with gene expression and a reduction of these chromatin modifications on the Xi could be interpreted as a consequence of its transcriptional inactivity. The function of Polycomb group proteins and histone modifications as well as other components of the Xi, which include the histone variant macroH2A as well as the nuclear scaffold protein SAF-A (Fackelmayer, 2005), remain to be characterized. The current view is that several chromatin features contribute to the maintenance of repression on the Xi (Csankovszki *et al*., 2001; Hernandez-Munoz *et al*., 2005).

A genetic screen for epigenetic modifiers in mice has identified a gene that is specifically required for maintenance of gene repression on the Xi (Blewitt *et al*., 2005). The SMC-hinge domain containing protein SmcHD1 (structural maintenance of chromosomes flexible hinge domain containing 1) has been shown to be required for DNA methylation of Xi-linked gene promoters and silencing on the Xi in mice (Blewitt *et al*., 2008). A homozygous mutation of SmcHD1 in female mice does not affect the localization of *Xist* RNA to the Xi and the

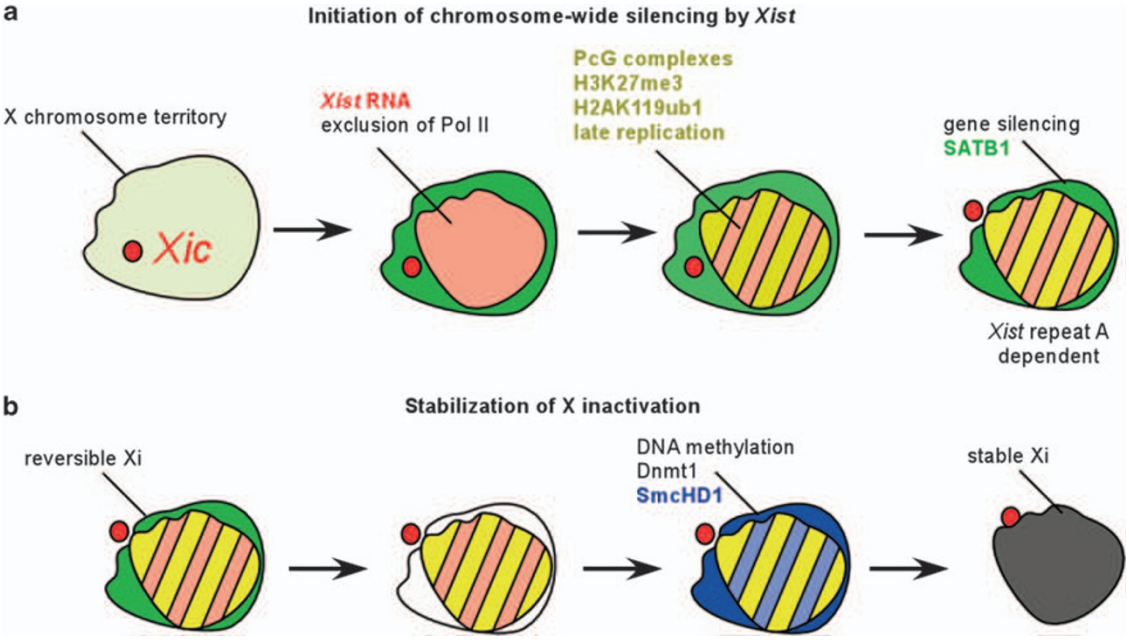

Figure 1 Initiation and maintenance of X inactivation. (**a**) X inactivation is initiated by expression of *Xist* RNA. *Xist* initially overlaps the repeat rich core of the X chromosome territory. RNA polymerase II and components of the transcription machinery are excluded from the *Xist* domain, whereas genes are located in the periphery of the chromosome territory. Polycomb group (PcG) complexes are recruited to the Xi and mediate tri-methylation of histone H3 lysine 27 (H3K27me3) and H2A mono-ubiquitination (H2AK119ub1). The chromosome also becomes late replicating in S-phase. Gene repression is then initiated and genes associate with the repressive compartment of the *Xist* domain on silencing. Initiation of gene repression depends on the repeat A sequence of *Xist* and on the presence of factors such as SATB1. At this stage chromosome-wide gene repression is entirely reversible and depends on the continued expression of *Xist*. (**b**) On cell differentiation the X chromosome attracts additional modifications and X inactivation becomes stable. In differentiated cells, SATB1 expression is lost and *Xist* loses its ability to induce gene repression. SmcHD1 and DNA methylation are essential for the maintenance of X inactivation. Genes on the Xi are reactivated in female mouse embryos with a disruption of *SmcHD1* or the DNA methyltransferase *Dnmt1*. In somatic cells further epigenetic modifications contribute to stable silencing leading to Xi that is largely resistant to reactivation.

initiation of gene silencing (Blewitt *et al*., 2008). This shows that SmcHD1 has a role in maintaining the Xi silent. DNA methylation of promoters on the Xi has been previously linked to gene repression. A mutation of the DNA methyltransferase Dnmt1 has been shown to lead to a failure in maintaining repression on the Xi (Sado *et al*., 2000). These observations strongly argue for a crucial role of DNA methylation in stabilizing the Xi (Figure 1b). However, blocking of DNA methylation in somatic cells either by using conditional mutagenesis of *Dnmt1* or by chemical inhibition does not lead to an efficient reactivation of genes on the Xi (Csankovszki *et al*., 2001). This indicates that SmcHD1 and DNA methyltransferases are required for a crucial intermediate step in stabilizing gene repression on the X inactivation. The Xi is likely further stabilized by other epigenetic changes with progressing differentiation of the cells, which ultimately make it refractory to reactivation. The discovery of SmcHD1 as a novel protein represents an important entry point for deciphering the contribution of DNA methylation to gene silencing in mammals.

The gene silencing mechanism of the non-coding *Xist* RNA

X inactivation is normally initiated during early female embryogenesis in mice. The conversion of an active into a transcriptionally silent X chromosome is also observed in differentiating mouse embryonic stem cells. This provides a powerful cell culture system for studying the mechanism of chromosome-wide silencing (Payer and Lee, 2008). X inactivation is triggered by the accumulation of *Xist* RNA over the future Xi. A conserved sequence on the 5′ of *Xist*, which has been termed repeat A, is required for gene silencing (Wutz *et al*., 2002). Expression of *Xist* lacking repeat A does not cause gene repression but results in localization of *Xist* and recruitment of most chromatin modifications to the Xi (Chaumeil *et al*., 2006). The repeat A RNA motif is predicted to fold into a stem loop structure, which could function as a binding site for silencing factors (Wutz *et al*., 2002). Recently, the structure of repeat A RNA has also been experimentally examined (Duszczyk *et al*., 2008). This study confirmed the first stem loop with the additional finding that the RNA motif might dimerize via a sequence, which was previously suggested to form a second loop. Deletion of repeat A in mice has also uncovered a function for repression of the antisense *Tsix* gene within the *Xi*, suggesting additional functions for the repeat A region in regulating X inactivation (Hoki *et al*., 2009).

Chromosome-wide gene repression by *Xist* is restricted to certain cell types. Normally X inactivation is initiated in cells of the early embryo. In addition, an appropriate cellular context for the initiation of silencing has been shown in specific progenitors in the blood system (Savarese *et al*., 2006). This was shown by experiments using an inducible expression system for activation of *Xist* in male mice. *XIST* expression can also cause gene repression in certain human somatic cell lines with varying extents of chromatin modification (Chow *et al*., 2007). Recently, it has been shown that *Xist* can initiate chromosome-wide silencing in tumor cells derived from a mouse lymphoma (Agrelo *et al*., 2009). This finding brings up the question if epigenetic pathways normally active during X inactivation in the embryo can be accessed by tumor cells and if these pathways contribute to tumorigenesis.

Using the lymphoma model a component of the *Xist* silencing pathway has been identified. It was shown that the special AT-rich sequence binding protein SATB1 is required for initiation of gene repression by *Xist* in lymphoma cells (Agrelo *et al*., 2009; Brockdorff, 2009). SATB1 is known as a chromatin organizer with important functions for coordinating gene expression in T cells (Alvarez *et al*., 2000). In T cells, SATB1 forms a cage-like nuclear network that overlaps with the base of chromatin loops in which genes reside (Cai *et al*., 2006). SATB1 is also expressed in mouse embryonic stem cells at the time when X inactivation is initiated (Agrelo *et al*., 2009). However, SATB1 is not essential for initiation for X inactivation as female mice with a disruption of the *SATB1* gene can develop (Alvarez *et al*., 2000). A potential redundancy with other factors such as the closely related gene *SATB2* has been offered as an explanation, but this issue remains to be analyzed further. Interestingly, at the initiation of X inactivation SATB1 does not to colocalize with *Xist*, but is observed in the periphery of the *Xist* domain (Agrelo *et al*., 2009), in which it might be associated with genes (Figure 1a). The specific expression pattern of SATB1 suggests that it might change the responsiveness of genes to modulatory signals of gene repression at certain cellular differentiation states. SATB1, thus, might provide a potential entry point for unraveling a developmentally controlled gene regulatory pathway of mammals. It will be interesting to decipher the relationship between *Xist* repeat A and SATB1. Both of which are required for the initiation of silencing, yet they do not appear to interact directly.

Regulation of the X inactivation process

Before chromosome-wide gene silencing commences and *Xist* is expressed, a complex process determines if X inactivation should be initiated and ensures that one X chromosome remains active per diploid chromosome set (Figure 2). The elements that identify the presence of an X chromosome in the cell are either located within the *Xic* or are closely associated with the *Xic* (Lee *et al*., 1999; Monkhorst *et al*., 2008). At the onset of X inactivation, a physical interaction of the two X chromosomes along the *Xic* regions has been observed (Bacher *et al*., 2006; Xu *et al*., 2006). The precise sequences that induce the pairing of the *Xic* regions within a brief developmental interval are not known. Interestingly, a deletion of all known elements within the *Xic* has been shown to be compatible with counting of X chromosomes indicating that an additional activator might be present outside the *Xic* region (Monkhorst *et al*., 2008). This could involve a novel pairing region, which was recently identified upstream of the *Xist* gene (Augui *et al*., 2007). The mechanism of *Xic*–*Xic* pairing is poorly understood and it remains unclear how two X chromosomes move and position themselves toward each other in the short developmental interval before *Xist* upregulation (Figure 2a). Recently, CCCTC-binding factor (CTCF) has been suggested as a crucial factor for pairing (Xu *et al*., 2007). As CTCF is also expressed before and after

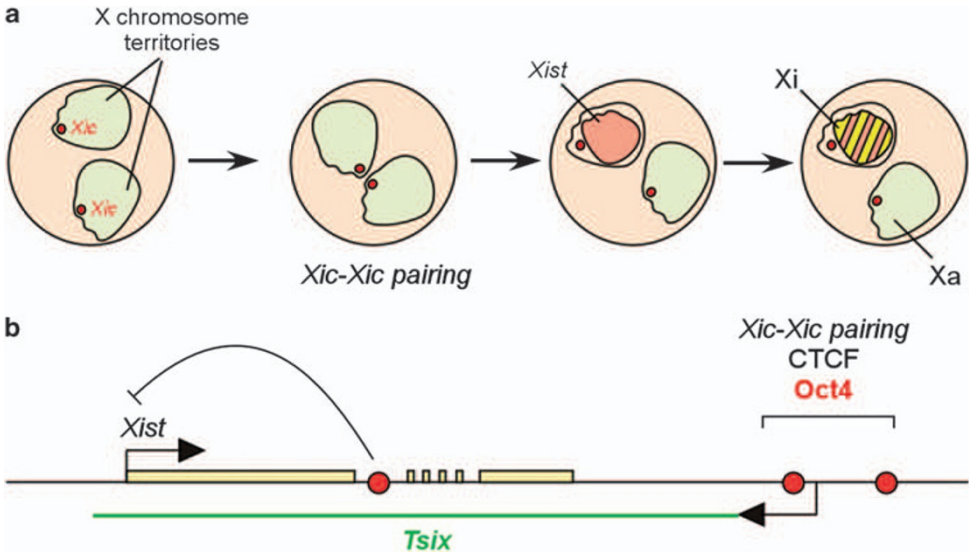

Figure 2 Regulation of X inactivation. (**a**) X inactivation is initiated in a developmentally controlled manner. All but one X chromosome are inactivated in a diploid cell. Presently, the view is that this is achieved by regulation of *Xist* expression. Elements for *Xist* regulation make up the *Xic* region. At the onset of X inactivation it is believed that *trans*-chromosomal pairing of the *Xic* regions triggers *Xist* expression of one X chromosome. This leads to chromosome-wide gene silencing and formation of an Xi. (**b**) Elements that regulate *Xist* expression within the *Xic* include the non-coding *Tsix* transcript (green). *Tsix* is transcribed in antisense orientation to and overlaps the *Xist* transcription unit, and represses *Xist* expression. Several Oct4 binding sites (red circles) have been identified within the *Xic* and mediate repression of *Xist* in a *Tsix*-dependent and a *Tsix*-independent mode. Also CTCF has been implicated in *Tsix* expression and in *Xic* pairing.

Xic–Xic pairing, it is unlikely the trigger for *Xist* upregulation but might perform a permissive function.

In mice, *Xist* expression is repressed by the antisense *Tsix* RNA (Figure 2b). The transcription unit of *Tsix* overlaps *Xist* thereby forming an antisense pair. This antisense *Xist–Tsix* pairing has provoked thoughts of potential involvement of double-stranded RNA that could be processed by Dicer to small RNAs. Indeed, such small RNAs have been detected in a recent study (Ogawa *et al*., 2008). However, a comprehensive analysis in embryonic stem cells has shown that a deletion of Dicer has little effect on the X inactivation process suggesting that Dicer has no major role in regulating X inactivation (Kanellopoulou *et al*., 2009). Deletion of Dicer appears to deregulate the processing of endogenous micro RNAs that leads to a loss of DNA methylation and thereby also affects X inactivation (Nesterova *et al*., 2008). These data are consistent with indirect effects of Dicer on X inactivation and leave the question of a role of double-stranded RNA in X inactivation open to further investigation.

Recently, the stem cell transcription factor Oct4 has been implicated in the regulation of *Xist* expression in mice (Navarro *et al*., 2008; Donohoe *et al*., 2009). Oct4 has been shown to bind DNA sequences within *Xist* intron 1 and around the promoter and enhancer sequences of *Tsix* (Figure 2b). It has been reported that Oct4 mediates repression of *Xist* in a *Tsix*-dependent and a *Tsix*-independent mode. Oct4 expression could thereby limit the upregulation of *Xist* until X inactivation is initiated in development. However, Oct4 is also expressed at high levels along with *Xist* in cell lines other than mouse embryonic stem cells including mouse epiblast-derived stem cells (Navarro and Avner, 2009). Oct4 expression is therefore unlikely the only determinant of *Xist* upregulation and an additional layer of control might await discovery.

Conservation and divergence of X inactivation across mammals

Random X inactivation is exclusively observed in placental mammals. In marsupials, the paternally inherited X chromosome is inactivated giving rise to an imprinted pattern of X inactivation. The marsupial imprinted X chromosome shares some features with the Xi of placental mammals. It lacks histone modifications associated with active chromatin consistent with its repressed state (Koina *et al*., 2009). A recent study shows that marsupial imprinted X inactivation also involves reactivation of the X chromosome after male meiosis and histone H3 lysine 27 methylation (Mahadevaiah *et al*., 2009). However, in marsupials *Xist* is not present. Interestingly, a recent report suggests that *Xist* is also not required for the initiation of imprinted X inactivation in the extraembryonic tissues in mice (Kalantry *et al*., 2009). In extraembryonic lineages *Xist* is required for maintenance of gene repression (Marahrens *et al*., 1997; Kalantry *et al*., 2009). This provides evidence for a similarity between imprinted X inactivation in marsupials and in mice. It is noteworthy that there is a clear difference between imprinted and random X inactivation, which is exclusive to placental mammals. X inactivation has recently also been investigated in human preimplantation embryos (van den Berg *et al*., 2009). Human *XIST* progressively accumulates on one of the two X chromosomes in female embryos starting at the eight-cell stage. This shows that dosage compensation in preimplantation embryos is more widely conserved among placental mammals. Among placental mammals X inactivation and *Xist* appear broadly conserved and X

inactivation has been confirmed in a tetraploid mammal (Bacquet *et al*., 2008). Yet, in certain placental mammals *Xist* may have been lost. A study reports on a mole species in which *Xist* might have been lost after complete Y chromosome degeneration and loss of male–female karyotype heteromorphy (Just *et al*., 2007). This is an interesting example of the evolutionary progression of sex determination and dosage compensation systems within placental mammals.

The *Tsix* gene, which is vital for *Xist* regulation during both random and imprinted X inactivation in mice, seems to be not functionally conserved in humans (Migeon *et al*., 2001). This could indicate that other mechanisms for *Xist* repression are used by different mammalian species. One such mechanism could be the *Tsix*-independent repression of *Xist* by Oct4 (Navarro *et al*., 2008). It is noteworthy that in mice *Xist* repression in male cells does not require *Tsix* (Ohhata *et al*., 2006). This suggests that also in mice alternative mechanisms for *Xist* repression are operating. One of these might involve Polycomb function. It has been observed that in male mouse embryonic stem cells *Xist* is activated when *Tsix* and Polycomb repressive complex 2 functions are disrupted (Shibata *et al*., 2008). This suggests that Tsix-independent repression of *Xist* requires Polycomb repressive complex 2 functions. However, indirect effects because of general chromatin changes in the absence of Polycomb repressive complex 2 cannot be ruled out. The current data suggest that a number of processes might act upstream of *Xist* to ensure faithful control of X inactivation (for review, see Wutz and Gribnau, 2007). Some of these processes might be emphasized in certain mammals and lost in others. The molecular basis of dosage compensation could therefore show a fair amount of variability among mammalian species.

Conclusions for the mechanism of X inactivation

Random X inactivation has evolved exclusively in placental mammals. This suggests that the dosage compensation system relies on processes that have mediated gene regulation more broadly in vertebrates. The notable exception is the non-coding *Xist* RNA and the regulatory sequences of the *Xic*. These have evolved specifically for the purpose of dosage compensation. There is also evidence that evolution of regulatory elements has not come to a halt and might be ongoing even in present day mammals. Regulation of X inactivation involves chromosomal pairing and antisense repression. *Tsix*, CTCF, Oct4 and Polycomb complexes have been implicated as molecular players in this process. Chromosome-wide silencing is triggered by the non-coding *Xist* RNA and appears to be a multistep process. *Xist* and SATB1 act in the initiation of gene silencing and SmcHD1 and DNA methylation have a crucial role for maintaining repression of the Xi. Also Polycomb group complexes are involved in X chromosome-wide silencing but their precise function needs to be investigated.

The targets of *Xist* on the chromosome remain unknown. A role for genomic repeats and non-genic DNA in X inactivation has been suggested by a number of studies. Genes and non-genic chromatin are spatially separated on the Xi. The observation that Xist overlaps the repeat rich center of the X chromosome territory suggests that non-genic chromatin might be the primary target of *Xist*. This brings up the idea that X inactivation might have co-opted a pre-existing mechanism for repeat element silencing. Genomic repeats comprise the remnants of mobile genetic elements that invaded the genome and subsequently were inactivated. This involves the evolution of host defense mechanisms. Could the inactive X be the result of a controlled engagement of such host defense strategies that is triggered by *Xist* expression? Long interspersed repetitive elements (LINE) elements have been suggested to perform a function in spreading X inactivation. Recently, X inactivation was compared in a mammalian species in which LINE proliferation has ceased. These data show that LINE amplification is not absolutely required for X inactivation suggesting that also other types of elements could perform a similar role in X inactivation (Cantrell *et al*., 2009).

Progress in understanding X inactivation has brought up new and more defined questions. The factors that mediate *trans*-chromosomal interactions during *Xic*–*Xic* pairing at the onset of X inactivation need to be identified. Work on the involvement of Oct4 and the developmental timing of X inactivation seems to be a promising route to identify a regulatory network that is intertwined with stem cell biology. A crucial missing piece for understanding the gene silencing pathway is the link between SATB1 and the *Xist* repeat A. Answering these questions will not only advance our understanding of mammalian dosage compensation but will also provide insights into fundamental nuclear processes for epigenetic regulation in mammals.

Conflict of interest

The authors declare no conflict of interest.

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