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X inactivation and the complexities of silencing a sex chromosome

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X chromosome inactivation represents a paradigm for monoallelic gene expression and epigenetic regulation in mammals. Since its discovery over half a century ago, the pathways involved in the establishment of X-chromosomal silencing, assembly, and maintenance of the heterochromatic state have been the subjects of intensive research. In placental mammals, it is becoming clear that X inactivation involves an interplay between noncoding transcripts such as Xist, chromatin modifiers, and factors involved in nuclear organization. Together these result in a changed chromatin structure and in the spatial reorganization of the X chromosome. Exciting new work is starting to uncover the factors involved in some of these changes. Recent studies have also revealed surprising diversity in the kinetics and extent of gene silencing across the X chromosome, as well as in the mechanisms of XCI between mammals.

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Introduction

In mammals, sex chromosome dosage compensation is achieved by silencing one of the two X chromosomes during early female development. The kinetics and mechanism of X chromosome inactivation (XCI) have mainly been studied in mice, which display an initial, imprinted form, and a later, random form of XCI. Both forms of XCI are dependent on the noncoding Xist RNA, which coats and silences the chromosome from which it is expressed. However, the mechanistic details of these two forms of XCI are still far from clear. Furthermore, up until recently it was thought that XCI could only occur during an early window of developmental time. However, recent work has revealed that Xist RNA may be apt to induce gene silencing in adult stem or progenitor cells, as well as in cancer cells — and the factors underlying this capacity are just starting to be uncovered. This opens up the possibility of understanding the epigenetic plasticity that can exist beyond early development. Xist RNA is turning out to be a multitasking molecule, regulating not only the onset of gene silencing, but also the reorganization of the X chromosome and possibly the recruitment of chromatin modifying complexes required to establish epigenetic marks. The formation of inactive X (Xi) heterochromatin and its stable propagation through cell division, are thought to be ensured by these chromatin marks, as well as by spatial and temporal segregation of the Xi. The heterochromatin of the Xi is not a homogeneous entity, however, consisting of different combinations of histone modifications and other epigenetic marks, depending on species and cell type. In this review we will focus on some of the most recent findings in the field, particularly on Xist RNA-mediated gene silencing and downstream events during X inactivation.

Chromatin architecture of the inactive X chromosome

The XCI process was discovered in 1961 by Lyon [1]. Facultative heterochromatin of the Xi had already been noted in 1949 by Barr and Bertram [2], as a dark staining body in female cat neurons, often located at the nuclear periphery or the nucleolus. Asynchronous replication timing was later discovered as another hallmark of the Xi [3]. However, the degree to which the Xi is condensed compared to other regions of the genome, and the molecular nature of its heterochromatin have remained unclear. Immunofluorescence (IF) and chromatin immunoprecipitation (ChIP) techniques are providing a more detailed picture of the chromatin status of the Xi. IF studies on mitotic chromosomes [4] or interphase nuclei [5,6] have shown that the Xi is globally enriched in several histone modifications, including H3K27me3, H3K9me2, H3K9me3, H4K20me1, H2AK119Ub as well as in the histone variant macroH2A. Conversely, the Xi is depleted for marks commonly associated with euchromatin such as H3K4me2/3 as well as H3 and H4 acetylated lysines. Such studies also revealed that Xi heterochromatin is not a homogeneous entity. In human somatic cell lines, at least two distinct heterochromatin signatures have been characterized: one defined by the presence of XIST RNA, macroH2A, H3K27me3, H4K20me1, and H2AK119Ub; and the other by features more consistent with constitutive heterochromatin including later replication timing, H3K9me3, H4K20me3, and HP1. By IF, these different heterochromatin domains appear to

remain spatially distinct during metaphase and interphase. In mouse, on the other hand, enrichment of HP1 and H3K9me3 on the Xi is much less distinctive [7,8]. Species differences are even more striking in marsupials where at metaphase, although the Xi is depleted for active marks similarly to the mouse and human Xi, no significant enrichment for inactive histone modifications (H3K9me2, H3K27me3, H3K9me3, and H4K20me3), is detected, at least by IF [9•]. Interphase Xi patterns in marsupials still require evaluation however. Interestingly, in marsupials there is no *XIST* gene [10]. This may explain the lack of histone modifications that characterize the eutherian Xi, as some of these (particularly H3K27me3) require XIST to trigger their recruitment. Whether such differences explain why gene silencing on the Xi is less stable in marsupials compared to eutherian mammals remains to be seen. The situation in monotremes is even less clear where the sex chromosome composition is extremely complex with females having five pairs of XX chromosomes and males having five XY pairs. In addition, the levels of dosage compensation are largely incomplete and variable between genes [11,12,13•].

Epigenomic analyses, such as ChIP-chip and allele-specific ChIP, are starting to provide a higher resolution view of the chromatin signatures on the Xi [14–18]. For example, ChIP-chip analysis for macroH2A1 in mouse liver cells reveals a ~1.5X enrichment in females versus males. This enrichment is distributed uniformly across the entire X chromosome, suggesting that macroH2A1 may influence global chromatin structure rather than directly inhibiting transcription at promoters of genes [19•]. DNA methylation of promoters of X-linked genes is another hallmark of XCI in eutherian somatic cells. Global microarray analyses involving methylated DNA immunoprecipitation (MeDIP) were used to assess the DNA methylation status of the Xi relative to the Xa in human primary cells. This study revealed that although CpG islands are hypermethylated on the Xi, the overall levels of methylation on the X chromosome are in fact lower in females compared to males, especially in gene-poor regions [20]. Hellman and Chess [21] analyzed the DNA methylation status of more than 1000 informative X-linked loci and found that on the active X, DNA methylation is concentrated in gene bodies, confirming previous studies (for review see [22]). The function of gene-body methylation is currently unclear, but has been observed in other species, including marsupials and plants (see [22,23]) and points to a possible role for methylation outside of promoters in gene regulation. Such studies and the advent of next generation sequencing technologies enabling genome-wide, allele-specific analyses, will hopefully provide a detailed view of the epigenomic constitution of the Xi and identify specific combinations of DNA methylation, histone modifications, and other proteins that might be predictive for XCI efficiency or stability. Such approaches may also unveil specific DNA elements that set up distinct chromatin signatures on the Xi.

Nuclear organization of the inactive X chromosome

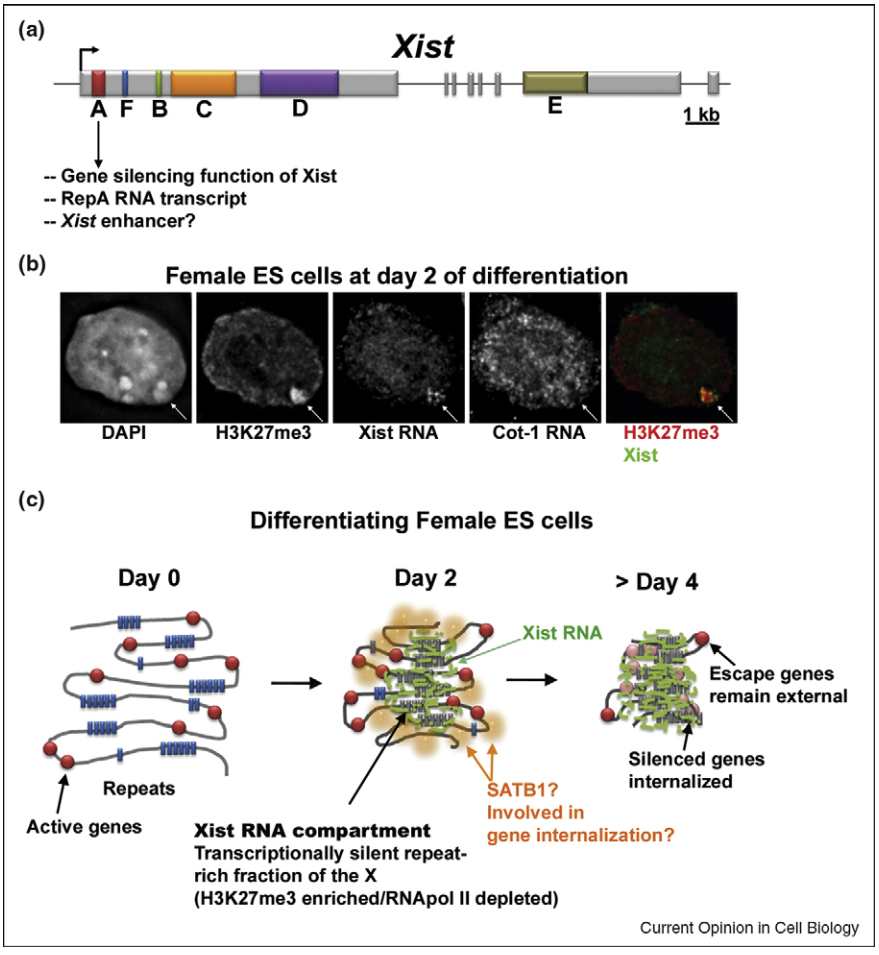
Spatial organization of the X chromosome also seems to play a role in X inactivation [24,25]. Using fluorescence *in situ* hybridization (FISH), two studies have shown that the Xi consists of a repetitive core, surrounded by an outer rim of genes and encompassed by Xist RNA [25,26]. Combined IF and RNA/DNA FISH revealed that marks such as H3K27me3 are enriched within this repetitive Xist RNA compartment (Figure 1). Only genes that escape XCI, or that lie within the pseudoautosomal region, are found to be located outside it ([25,27]; see Figure 1). Ultrastructural analysis of the Xi was recently performed in mouse and human fibroblasts [28••]. Using light and electron microscopy, the H3K27me3-labeled portion of the Xi was shown to consist of tightly packed heterochromatic fibers/domains with intervening spaces between them. Importantly, this structure is distinct from both euchromatin and constitutive heterochromatin. Given that H3K27me3 stains only part of the Xi territory at interphase, particularly in human cells [14], it will be interesting to determine whether H3K9me3-stained Xi regions show a similar organization, or whether they resemble constitutive heterochromatin.

In addition to the organization and structure of the Xi, its spatial segregation within the nucleus might also promote the inactive state, by facilitating access to factors required for heterochromatin, or limiting access to factors required for transcription. The Xi is frequently located at the nuclear periphery or at the nucleolus [28••] both of which are associated with heterochromatin (for review see [29]). Zhang *et al.* [30•] recently showed that perinucleolar association of the Xi only occurs during S phase and is Xist RNA-dependent. They proposed that nucleolar positioning might be important for faithful replication of the Xi's epigenetic state as the nucleolar periphery is enriched in factors required for replication of heterochromatic regions [30•]. Whether perinucleolar association of the Xi is the cause or consequence of its heterochromatic state remains to be determined however [7,31].

Setting up the inactive state during XCI

The early steps in imprinted and random XCI are similar, although random XCI seems to entail additional epigenetic marks, such as DNA methylation (Figure 2). The absence of DNA methylation on the imprinted paternal Xi, probably explains the ease with which the inactive state is reversed in the inner cell mass on the one hand [32], and the greater dependence of imprinted XCI on Polycomb group proteins for maintaining silencing, on the other [33]. The earliest detectable event in both the initial imprinted form of XCI and the later random form of

Figure 1

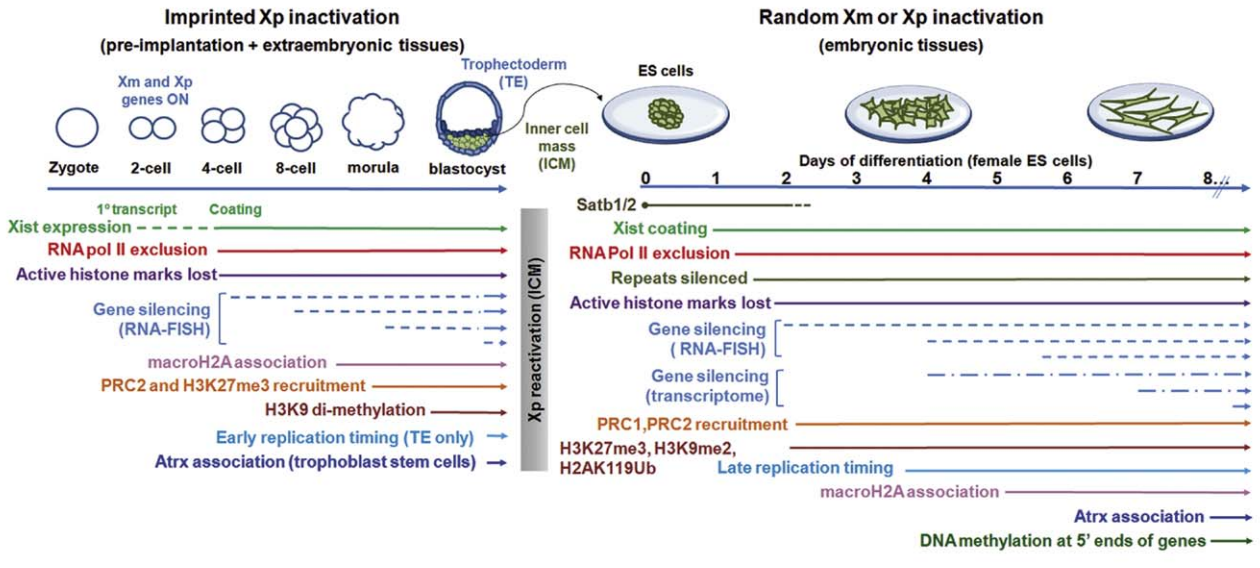


(a) Structure of the Xist gene with the conserved repeat regions labeled A–F. The A region (red) denotes the conserved A-repeat region essential for gene silencing. **(b)** Combined RNA-immunofluorescence on day 2 differentiated female ES cells, showing the Xist-coated transcriptionally silent compartment which is enriched for H3K27me3. **(c)** Model. Xist coating induces the formation of a transcriptionally silent repetitive compartment. As genes are silenced they are recruited into this compartment. A possible mediator for this internalization may be the matrix-associated protein SATB1/2.

XCI is the monoallelic upregulation of Xist RNA (Figure 2). Xist RNA coating rapidly leads to the formation of a silent, repetitive compartment that is depleted for RNA polymerase, transcription factors, and euchromatic marks. The onset of gene silencing occurs subsequently, as do PcG-induced and other chromatin changes on the Xi, although these can be functionally dissociated from gene silencing, based on analysis of Xist RNA and PcG mutants [7,25,34,35]. XCI is accompanied by spatial reorganization of genes on the X chromosome, as they are initially located outside the silent, Xist RNA compartment, but move into it as inactivation proceeds [25,27]. This relocation of genes into the heterochromatic core of the Xi may be an active process, driven by Xist RNA to facilitate silencing. Alternatively it may be a passive consequence of gene silencing, which could nevertheless participate in maintaining inactivity.

New insights into the exact timing of X-linked gene silencing during X inactivation have recently been obtained (Figure 2). XCI was thought to be a rather concerted process, occurring in the space of just a few cell divisions, during the critical time window when Xist RNA can act [36]. However, studies in mouse preimplantation embryos [37] and ES cells [38] now show that XCI is in fact a far more prolonged process. Different genes show very different XCI kinetics, with some genes being silenced well outside of the time window in which Xist is thought to act (Figure 2). Several lines of evidence point to a gradient effect, with genes closest to the Xic being silenced earliest during differentiation [37–39]. However this breaks down somewhat for regions further from the Xic. Such heterogeneity in XCI kinetics and efficiency across the X chromosome suggests highly region-specific processes; however, the underlying sequences responsible remain to be found. Nevertheless, these findings

Figure 2



Xi Feature	Factors involved	Reference
RNA polymerase II exclusion	Xist RNA coating (A-repeat independent); repetitive elements?	[25]
Loss of active histone marks (H3 and H4 acetylation; H3K4me2/3)	Xist RNA coating (A-repeat independent); Histone deacetylases? histone demethylases? histone exchange, proteolysis…?	Reviewed in [62]
PRC2 recruitment	Xist RNA coating (A-repeat independent) + ? RepA RNA?	[7,34,55]
HeK27me3	Ezh2 (PRC2)	[63]
H3K9me2	?	
PRC1 recruitment	Xist RNA (A-repeat independent) + ? PRC2/ H3K27me3	[35,64-66]
Cbx7 (mammalian Polycomb protein homolog—PRC1 component)	H3K27me3 + RNA + ?	[67]
H2AK119Ub	Ring1a/b (PRC1)	Reviewed in [63]
H4K20me1	PrSet7 (?)	[7]
Gene silencing	Xist RNA (A-repeat dependent); relocation into silent compartment?	[25,52]
Macro H2A association	Xist RNA (A-repeat independent) + downstream heterochromatin changes/factors?	[31,68-70]
Late replication timing	heterochromatin changes?	
Nucleolar localization	Xist RNA + downstream heterochromatin changes/factors?	[30]
Smchd1 localization	Xist RNA + downstream heterochromatin changes/factors?	[40]
DNA methylation at CpG islands	Smchd1? Dnmt3a/3b (de novo MTases)? Dnmt1 (maintenance MTase)	[40,71]
Satb1	?	[57]
Atrx	Xist RNA + downstream heterochromatin changes/factors?	[50]

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Kinetics of imprinted and [random inactivation]. The table details specific features of the inactive X chromosome and their possible effectors.

imply that Xist RNA may actually function outside an early differentiation time window, and/or that the changes it induces are propagated more slowly into some regions than others. They also raise the possibility that Xist-independent mechanisms of silencing may exist for some regions of the X chromosome.

Although Xist RNA seems to recruit, directly or indirectly, certain chromatin modifying complexes including PcG proteins, we still do not understand the precise nature of the changes on the Xi that lead to subsequent events during XCI, such as the shift to late replication timing, macroH2A enrichment, and DNA methylation at promoters of X-linked genes. A recent new player in X inactivation that may play a role in some of these downstream changes — the SmcHD1 protein — has recently been identified [40••]. *Smchd1* mutant female embryos display both placental and extraembryonic defects and die before stage E13.5. As the lethality observed is well after initiation of XCI, this is suggestive of a function during maintenance. Indeed, promoter DNA hypomethylation on the Xi was detected in

mutants, suggesting that Smchd1 may affect the DNA methylation deposition step [40••]. SmcHD1 is an SMC-like protein with homology to components of cohesin and condensin complexes. These proteins are implicated in dosage compensation in *C. elegans* where both X chromosomes are downregulated in female hermaphrodites [41], providing an exciting potential link between these two forms of dosage compensation.

Escape from X inactivation

Several X-linked genes can escape XCI, particularly in humans. The degree of escape can vary considerably between loci, ranging from 5% to >75% of Xa levels [42]. This heterogeneity is presumably because of the differences in the local environment of genes. LINE1 elements, which are enriched on the X chromosome, have been proposed to facilitate XCI spread and/or maintenance [43] and are depleted in the vicinity of escapees [44,45]. Insulator elements may form domains of escape by preventing the spread of heterochromatinization and/or recruitment into the silent repetitive compartment. For example, CTCF-binding sites are hypothesized to play such a role as they have been identified between inactivation and escape domains [46]. The existence of escape-promoting DNA elements was demonstrated by transgenic studies in mouse ES cells, as the escape gene *Jarid1C* was found to retain its escape capacity in several different integration sites on the Xi [47••].

What might the functional consequences of escaping XCI be? Aside from those genes that have homologs on the Y chromosome, the necessity, if any, for escape remains unclear. For some escapees, gene dosage may not need to be stringently controlled. Their level of expression is often only a fraction of that found on the Xa and may not have a large impact on overall transcript levels. A recent study comparing expression levels of X-linked genes in male versus female lymphoblast cells found that gene expression is only slightly higher in females [48]. In some cases however, where tissue-specific or lineage-specific escape is found, there may be a specific requirement for increased dosage of this gene. For example, *Atrx* has been found to escape from XCI in trophectoderm lineages, but not in the embryo. This may indicate a tissue-specific requirement for increased dosage of its product [37,49]. Interestingly, Atrx is a chromatin-remodeling factor and has been shown to associate with the Xi [50]. Furthermore, *Atrx* mutants show defects in imprinted inactivation [49] which suggests its role in maintenance is particularly important in extraembryonic tissues. Thus there may be a need for lineage-specific escape from XCI for *Atrx* in order to ensure increased levels of this protein for its role on the Xi in female extraembryonic lineages. Whether other escapees, such as *Jarid1c* and *Utx*, both of which are histone demethylases, have similar Xi-specific requirements remains to be found.

Xist RNA and its multiple roles in XCI

The developmental regulation of *Xist* and its monoallelic control have been discussed in recent reviews [51]. We will therefore focus only on the potential functions of Xist RNA. How does this long noncoding transcript encompass a chromosome in *cis* and mediate chromosome-wide silencing during a developmentally restricted time window? Deletion analyses indicate that Xist has multiple roles, likely mediated by distinct functional domains [52]. The RNA contains several conserved repeats (Figure 1a; [53]), the most conserved being the A-repeats, which when deleted abolish Xist's gene silencing function. [52,54]. An inducible Xist cDNA transgene mutated for the A-repeats is unable to silence genes but can still coat the chromosome in *cis* and recruit Polycomb complexes and H3K27me3 [52]. Interestingly, a similar deletion of the A-repeats at the endogenous *Xist* locus revealed that this region also acts as a genomic regulatory element of the gene [54]. The A-repeat region also produces an independent 1.6 kb transcript, RepA which, based on RNA immunoprecipitation experiments, associates with PRC2 Polycomb group proteins and was proposed to nucleate the recruitment of H3K27me3 to the Xi [55•]. However, inducible *Xist* transgenes deleted for A-repeats can still recruit H3K27me3 to the X chromosome, implying that there must be redundancy between Xist sequences for this function [7,34]. Furthermore, it should be noted that, whatever RepA's function may be, PRC2 does not appear to be required for the initiation of random XCI [35,56].

Increasing evidence points to Xist RNA having an architectural role that may be important for the specific 3D-organization of the Xi [25,26,28]. One of the earliest events following Xist coating is the exclusion of RNA polymerase II and the transcriptional silencing of a repeat-rich fraction of the X chromosome [25]. The formation of this transcriptionally silent compartment occurs before genes are silenced and is independent of the A-repeats. As inactivation proceeds, gene silencing is linked to their movement into this Xist-coated compartment. It is clear that the A-repeats are required for gene silencing, but whether they have a direct role in 'reeling' genes into the compartment is unclear. Exciting new work from the Wutz laboratory has revealed that the SATB1 and SATB2 proteins may participate in enabling the gene silencing function of Xist RNA [57••]. The proposed role of these proteins in the nuclear reorganization of chromosomal sequences during T lymphocyte development [58] raises the possibility that they might play a similar role in enabling gene relocation into the silent Xist repetitive compartment during X inactivation (Figure 1c). As expression of these factors is restricted to early ES cell differentiation, this could explain the developmentally restricted time window of Xist's silencing function. Importantly however, recent findings demonstrated that Xist could actually induce gene silencing

outside a developmental context, in some cancer cells [59,60] and in adult progenitor stem cells [61]. Agrelo *et al.* [57••] show that this reacquired capacity of Xist-induced silencing in adult cells comes from re-expression of the SATB1 protein. This work opens up exciting new perspectives in understanding Xist's mechanism of action and also in evaluating the epigenetic status of cancer cells.

Conclusions

The recent discovery of new factors that are involved in XCI, such as SATB1 and Smchd1, in addition to the Polycomb group proteins, provide an important step forward in the field. This brings us closer to identifying the complexes that mediate the various steps ensuring chromosome-wide silencing and maintenance of the inactive state. The unusual timing of XCI for some regions of the Xi and the diversity of chromatin patterns found on the Xi, suggest that there may be more than one way of generating inactive chromatin on the X, both between and within mammalian species. XCI is thus likely to involve complex solutions to what, at first sight, might appear as a relatively simple problem. The future promises a deeper understanding of the evolutionary diversity in X inactivation mechanisms between mammals and a more detailed view of the molecular nature of epigenetic marks on the Xi chromosome.

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