Gene silencing in X-chromosome inactivation: advances in understanding facultative heterochromatin formation

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Abstract | In female mammals, one of the two X chromosomes is silenced for dosage compensation between the sexes. X-chromosome inactivation is initiated in early embryogenesis by the *Xist* RNA that localizes to the inactive X chromosome. During development, the inactive X chromosome is further modified, a specialized form of facultative heterochromatin is formed and gene repression becomes stable and independent of *Xist* in somatic cells. The recent identification of several factors involved in this process has provided insights into the mechanism of *Xist* localization and gene silencing. The emerging picture is complex and suggests that chromosome-wide silencing can be partitioned into several steps, the molecular components of which are starting to be defined.

Dosage compensation
In mammals, the difference in chromosome complement between XY males and XX females is compensated by transcriptional silencing of genes on one of the two X chromosomes in female cells. Therefore, in both male and female cells, a single copy of each of the X-linked genes is active; this is in contrast to autosomal genes, which are expressed from two homologous chromosomes.

Facultative heterochromatin
A subtype of heterochromatin that is formed in the euchromatic environment, in which heterochromatin proteins are used to stably repress the activity of certain target genes.

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Chromatin modifications and DNA methylation define epigenetic patterns that constrain gene expression in order to stabilize cell identities. X-chromosome inactivation (XCI) in mammals is a process that exemplifies the developmentally controlled formation of silent chromatin. One of the two X chromosomes in female cells is inactivated for dosage compensation between the sexes[1], so that in both male and female mammals, a single X chromosome is transcriptionally active. The observation of a densely staining body within the nucleus of female cells marked the first description of the facultative heterochromatin of the inactive X chromosome (Xi)[2]. Pioneering genetic and cytological investigations then led the way to the discovery of the X inactivation centre (*Xic*) and the X-inactivation specific transcript (*XIST*) gene in mice and humans[3–5]. The *XIST* gene is exclusively expressed from the Xi and was suggested to act as a non-coding RNA based on the convincing argument that the majority of *XIST* RNA is localized to the nucleus and, more specifically, accumulated within the territory of the Xi[6]. Gene disruption in mice has shown that *Xist* is required for the initiation of XCI[7,8].

The process of XCI, which has been extensively studied in mouse development (FIG. 1), involves several steps that are orchestrated in a developmental manner: the counting of the number of X chromosomes; the choice of which X chromosome to inactivate; the initiation of chromosome-wide silencing; and the maintenance of stable repression of the Xi. A number of both *cis*- and *trans*-acting activators and repressors of *Xist* expression have been identified, many of which are encoded within the *Xic* (for a Review, see REF. 9). Once *Xist* expression is upregulated, it accumulates within the chromosome territory of the future Xi and establishes chromosome-wide silencing. *Xist* is required for the initiation of silencing[7,8] but not for the maintenance of gene repression in differentiated cells[10–12]. The process of XCI has therefore been logically divided into an initiation phase, in which gene silencing is established in a chromosome-wide manner, and a maintenance phase, in which the repressed state becomes stable and does not require continued *Xist* expression. The transition from initiation to maintenance of XCI involves a number of epigenetic processes, among which are DNA methylation and Polycomb group complex (PcG complex) mediated chromatin modifications. This makes the Xi an interesting model for studying epigenetic mechanisms in mammals. Recently, factors have been identified that function in the localization of *Xist* to the Xi, initiation of gene repression and stabilization of XCI. In this Review, I discuss these recent insights into the regulation of facultative heterochromatin on the Xi and the wider implications for the regulation of epigenetic states during development.
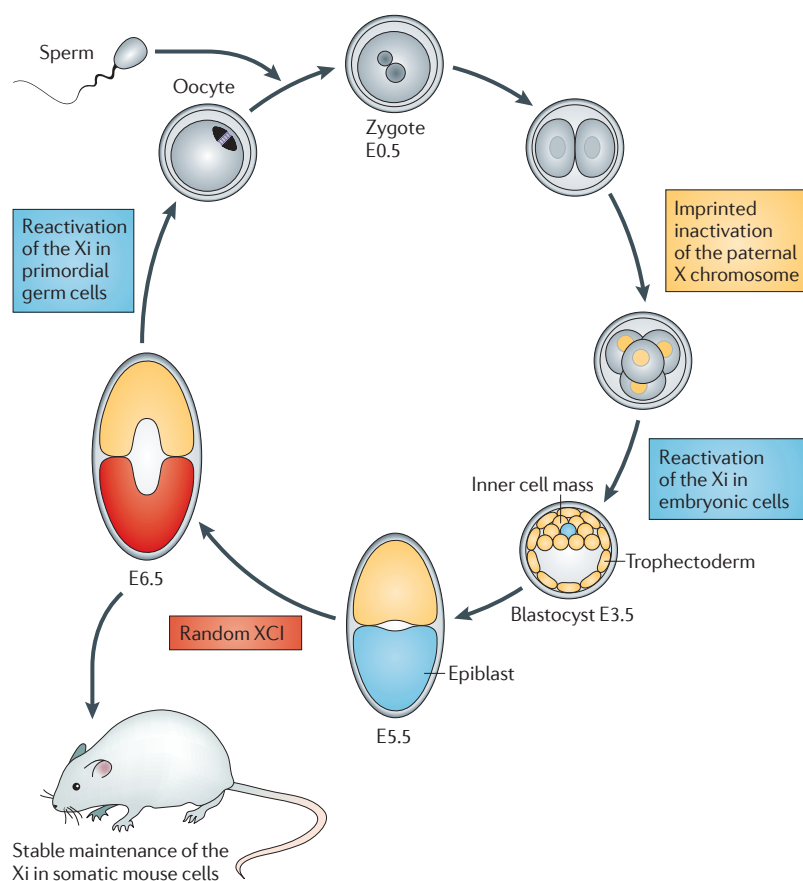
Figure 1 | **X-chromosome inactivation and reactivation during mouse development.** A schematic illustration of mouse development, showing the relevant stages for X-chromosome inactivation (XCI) and reactivation. Inactivation of the paternally inherited X chromosome is initiated in cleavage-stage embryos[33]. Imprinted inactivation (yellow shading) of the paternally inherited X chromosome is maintained in the developing extra-embryonic lineages[120]. In the inner cell mass of blastocysts, reactivation of the inactive X chromosome (Xi) occurs specifically in cells that will form the embryo (blue shading)[33,85]. Subsequently, both X chromosomes are active in the cells of the developing epiblast between embryonic day 3.5 (E3.5) and E5.5 (blue shading). Random XCI (red shading) of either the paternally or maternally inherited X chromosome is initiated around E5.5 (REF. 85). Once established, the XCI pattern is maintained in the somatic lineages of female mice. In the developing germ line, the Xi is reactivated such that both X chromosomes are active in oogenesis[83].

Initiation of chromosome-wide silencing

Localization of Xist *to the inactive X chromosome.* An active X chromosome and an Xi are simultaneously present in the cell nuclei of female mammals. Therefore, XCI requires a mechanism that can act in *cis* and sequester the X chromosome from all other chromosomes within the same nucleus — this is provided by *Xist* (FIG. 2a,b). In interphase, chromosomes appear to occupy specific volumes that are stochastically arranged within the nucleus[13] and chromosome territories have been visualized by fluorescence *in situ* hybridization (FISH). The Xi territory preferentially occupies a perinucleolar location[2,14]. Neighbouring chromosome territories appear to border each other along weakly defined regions, suggesting that distinguishing a single chromosome in interphase might be difficult.

How the *Xist* non-coding RNA is confined to a single X chromosome is an open question. A functional analysis of the mouse *Xist* RNA sequence has been carried out to investigate the sequence requirements for localization (FIG. 2c). Using an inducible system, a series of *Xist* constructs containing defined deletions was expressed from the X-linked hypoxanthine guanine phosphoribosyl transferase (*Hprt*) locus in mouse embryonic stem cells (ESCs). This study showed that several regions within *Xist* are able to mediate chromosomal localization[15]. Different regions in *Xist* seem to act additively or synergistically, but no region is absolutely required, and searches for sequences that are common among these localization regions, or exclusive to them, have so far been unsuccessful. From these observations, it has been suggested that *Xist* might attach to the chromosome via many binding sites[16]. The apparent lack of conserved motifs is a feature of many regulatory non-coding RNAs and could be the result of the difficulty of predicting functional domains in RNA sequences. Alternatively, sequence divergence could indicate a low affinity of individual binding sites. Recently, locked nucleic acid (LNA) and peptide nucleic acid (PNA) oligomers have been used to investigate *Xist* localization[17,18]. These synthetic DNA analogues base pair with RNA at a very high binding energy, thus potentially blocking other interactions. LNAs that are complementary to specific *Xist* sequences have been shown to disrupt the association of *Xist*, PcG complexes and macroH2A with the Xi[17,18]. However, although this method appears to be promising for elucidating functional sequences of *Xist*, it is presently not clear how LNAs and PNAs exert their effects in these experiments.

Although the mechanism of *Xist* localization to the Xi remains unclear, the nuclear scaffold protein SAF-A (also known as hnRNPU) appears to be a crucial component[19]. SAF-A has been identified as a protein that is enriched on the Xi in female somatic cells, and its localization to the Xi has been shown to require both the SAF-A RNA binding domain[20] and *Xist* expression[21]. Recently, SAF-A has been identified in an RNAi-based screen for factors controlling *Xist* localization[22]. Binding of *Xist* RNA to SAF-A has been observed, but it remains to be defined which sequence motifs within *Xist* mediate this binding[22]. These results indicate that *Xist* interacts with the nuclear scaffold (also known as the nuclear matrix) in order to localize in *cis* over the X chromosome from which it is transcribed. An interaction with nuclear scaffold proteins is consistent with earlier observations that *Xist* RNA can be retained in the nuclear scaffold after chromatin has been removed[6].

Microscopy studies have shown that *Xist* binding on the X chromosome overlaps with sequences that are enriched for genomic repeats[23,24]. These sequences are distinct from the gene-containing regions of the X chromosome, which are located at the periphery of the X-chromosome territory and do not overlap the majority of *Xist* binding sites. Although these studies suggest that *Xist* might primarily bind to the non-coding part of the chromosome, specific sequences on the X chromosome with which *Xist* associates remain to be identified. Potential candidates are long interspersed elements

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Chromosome territory
The volume occupied by the DNA of a single chromosome in the interphase cell nucleus.

Polycomb group complex
(PcG complex). A chromatin-modifying complex that contains proteins that were originally indentified as being required for maintenance of homeotic gene silencing in *Drosophila melanogaster*.

Nuclear scaffold
A network within the nucleus consisting of RNA and protein that is believed to organize chromatin.
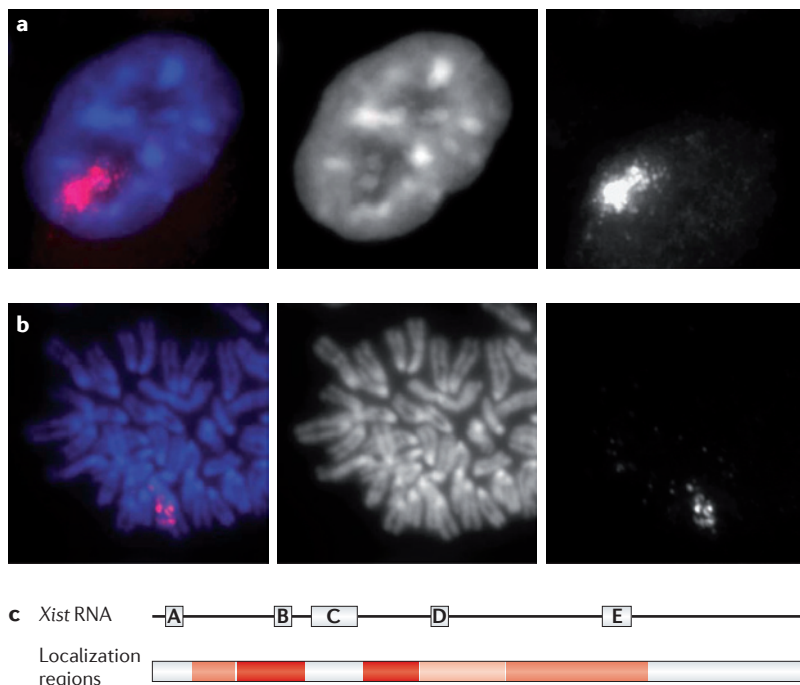
Figure 2 | **Xist localization to the X chromosomes. a** | X-inactivation specific transcript (*Xist*) detection by RNA fluorescent *in vitro* hybridization (FISH) in a female mouse-cell nucleus at interphase. *Xist* is stained red and DNA is stained blue. **b** | A mitotic spread of condensed mouse chromosomes showing residual *Xist* (red) binding. *XIST* is displaced from the inactive X chromosome (Xi) in human cells when chromosomes condense in mitosis. In mouse cells, *Xist* can be observed on condensed chromosomes but is reported to detach at anaphase[110]. A newer study reporting association of *Xist* with the Xi throughout the cell cycle[121] has, thus far, not been recapitulated by other groups. One explanation of conflicting observations of *Xist* displacement in different cell systems might be related to the activity of aurora kinase B (AURKB), which has been shown to be involved in the displacement of *XIST* in human cells[98]. **c** | A schematic representation of mouse *Xist* is shown with repeats (A to E) indicated. Below is a representation of regions in *Xist* that contribute to chromosomal attachment and localization (the shading indicates the strength of the contribution; red indicates the strongest effect, and grey indicates no contribution). The localization domains of *Xist* were determined through a deletion analysis of the *Xist* cDNA in mouse embryonic stem cells[15].

Long interspersed elements
(LINEs). Types of repetitive DNA in animal genomes that are derived from transposons.

Blastocysts
Pre-implantation embryonic stages that are characterized by the first definitive lineages. They consist of a fluid-filled cavity (blastocoel), a focal cluster of cells from which the embryo will develop (inner cell mass) and peripheral trophoblast cells, which form the placenta.

Pericentric heterochromatin
A block of heterochromatin-containing silent repeats surrounding the centromeres of the chromosomes.

(LINEs), which have been proposed to function in XCI based on their greater occurrence on the X chromosome than on autosomes[25]. A number of studies have aimed to clarify the role of LINEs in XCI. Notably, it has been shown that *Xist* can also interact with and silence autosomes when it is expressed from transgenes that are integrated into different autosomes[12,26,27]. This observation suggests that X-chromosome-specific sequences are not essential for *Xist* localization. However, autosomal chromatin is often less permissible to *Xist* localization. For example, the spreading of *Xist* is attenuated at the boundary of a translocation involving the X chromosome and chromosome 4 (REF. 28). In this case, the translocated chromosome 4 sequences had low numbers of LINEs, suggesting that a lack of LINEs could prevent spreading of *Xist* into the autosomal part of the translocation.

The correlation between silencing on autosomes with LINE density has also been examined in a comprehensive analysis of three autosomal *Xist* transgenes in mouse

ESCs[29]. Chromosomal regions that were enriched for LINE sequences were silenced more efficiently by *Xist*. However, LINE content was not sufficient to explain efficient repression in all cases, suggesting that other chromosomal features might also play a part. There is evidence that LINEs behave differently on the Xi when compared to autosomes or the active X chromosome. For example, the DNA methyltransferase gene *Dnmt3b* is specifically required for DNA methylation of LINE elements on the mouse Xi. Deficiency in human *DNMT3B* causes ICF (immunodeficiency, chromosome instability and facial abnormalities) syndrome and is associated with a lack of LINE methylation on the Xi[30]. In mice, LINEs remain transcriptionally active on the Xi at early stages of XCI[31]. At one gene locus, transcripts derived from LINEs were also found to contribute to the production of small RNAs and have been implicated in enhancing the efficiency of silencing of certain genes on the Xi[31].

Altogether, the function of LINEs in XCI remains unclear. A major caveat of the above studies is that LINEs co-occur with other genomic repeat sequences in gene-poor regions. Long terminal repeat (LTR) and inverted repeat sequences are also over-represented on the X chromosome when compared to the whole genome and could also be candidates for elements contributing to XCI[32]. Thus, the characterization of the primary targets of *Xist* on the X chromosome is an important aim of further investigations and could potentially uncover novel regulatory sequences that act in chromosomal organization.

Formation of a repressive compartment. The initiation of gene silencing by *Xist* has been studied in mouse ESCs, which are derived from female blastocysts and undergo XCI when they enter differentiation. One of the earliest events after *Xist* localization over the Xi is the depletion of transcription initiation factors, RNA polymerase II and splicing factors from the *Xist*-covered chromatin domain[23,33]. This has led to the view that the transcription machinery is excluded from the *Xist* domain, leading to a transcriptionally inactive and repressive compartment. This view is also consistent with a depletion of nascent RNAs[23,33] and the absence of histone modifications associated with active genes, such as histone acetylation or methylation of histone H3 at lysine 4 (H3K4me)[34]. It is currently unclear how the exclusion of the transcription machinery is accomplished. However, the fact that the transcription machinery is also absent from pericentric heterochromatin indicates one potential mechanism involving the rearrangement of silent chromatin within the centre of the X-chromosome territory. Importantly, at this initial stage of XCI, genes at the periphery of the Xi territory are in contact with the transcription machinery and remain active.

Subsequently, chromatin within the *Xist* domain is modified by the activity of PcG complexes (TABLE 1). Two catalytically active PcG complexes are enriched at the Xi: Polycomb repressive complex 1 (PRC1), which catalyses ubiquitylation of histone H2A at lysine 119 (H2AK119ub1)[35,36], and PRC2, which catalyses

Table 1 | **Proteins that bind to the inactive X chromosome**

Protein	Role in X-chromosome inactivation (XCI)	Is binding Xist-dependent?	Does it require Xist repeat A?	Refs
SAF-A	Xist RNA binding and stabilization of the inactive X chromosome (Xi) structure. Enrichment on the Xi is reported on the transition to maintenance of XCI	Yes	No	20–22
PRC2 • EED • EZH2 • SUZ12 • PCL2	Polycomb repressive complex 2 is enriched on the Xi from the initiation of XCI onwards and catalyses trimethylation of histone H3 at lysine 27 (H3K27me3)	Yes	No	64,65,126
PRC1 • RING1B • RING1A • MPH1 • MPH2 • MEL18	Polycomb repressive complex 1 is enriched on the Xi from the initiation of XCI onwards and catalyses monoubiquitylation of histone H2A (H2Aub1). Complex composition changes during cell differentiation	Yes	No	35,36,79
macroH2A1 macroH2A2	The histone variant macroH2A is enriched on the Xi in the maintenance phase of XCI. Two genes are known in mice. A function in stabilizing gene repression on the Xi has been postulated	Yes	No	11,21,70
ASH2L	The Trithorax group protein ASH2L is enriched on the Xi in the maintenance phase of XCI. It is thought that ASH2L is recruited as a structural component of the Xi and might interact with Polycomb group complexes	Yes	No	21
SMCHD1	SMCHD1 is enriched on the Xi in the maintenance phase of X inactivation and is required for maintaining gene repression and DNA methylation on the Xi	?	Not tested but expected	61
DNMT1	DNMT1 is required for DNA methylation of promoters on the Xi and the maintenance of gene repression	?	Not tested but expected	59
SATB1	SATB1 and other related proteins define the cellular context for the initiation of gene silencing on the Xi	?	Not tested	49

Question marks indicate that these interactions have not yet been determined. DNMT1, DNA methyltransferase 1; EED, extra-embryonic development; PCL2, also known as MTF2; SAF-A, also known as hnRNPU; SATB1, special AT-rich sequence binding protein 1; SMCHD1, structural-maintenance-of-chromosomes hinge domain containing 1; Xist, X-inactivation specific transcript.

trimethylation of H3K27 (H3K27me3)[37]. The activity of PRC1 and PRC2 leads to chromosome-wide histone modifications on the Xi. The precise function of PcG complexes in XCI is currently unclear. A disruption of either PRC1 or PRC2 function does not lead to a defect in the initiation of gene silencing[38–40]. Based on the observation that PRC1 and PRC2 are recruited by Xist independently of each other, an overlapping function has been postulated[39]. Although overlapping functions of both PRCs have been demonstrated in ESCs[41], it remains to be investigated whether they also perform redundant functions in XCI. Recruitment of PcG complexes seems to occur before gene silencing is initiated[33,37], and gene silencing can also be separated genetically from the formation of a domain of modified chromatin[23]. The initiation of gene silencing requires a conserved RNA sequence motif at the 5′ end of Xist, which is named Xist repeat A[15]. A mutation of the repeat A sequence retains the ability to localize Xist RNA to the X-chromosome territory, where it triggers several characteristic chromatin modifications of the Xi[21]. However, in this case, genes remain active[21,23]. These observations suggest that initiation of chromosome-wide silencing can be separated into two steps. First, a repressive compartment of modified chromatin is formed by Xist on genomic repeat sequences within the centre of the Xi territory. In a second step, genes become silenced and associate with the repressive compartment in a manner that requires Xist repeat A (FIG. 3). The fact that most — but not all — of the genes on the Xi are silenced is also consistent with a gene-specific inactivation mechanism (see BOX 1 for a discussion of genes that 'escape' from XCI).
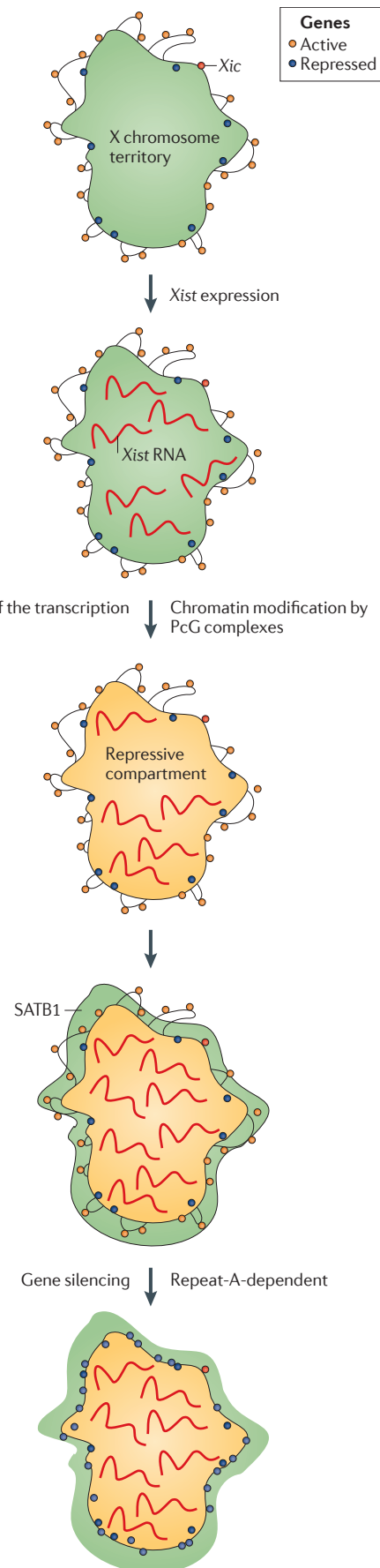
Although Xist repeat A is generally accepted to have an important role in gene silencing, it remains unclear how this sequence mediates gene repression. The identification of a small core motif of around 30 bases within the Xist repeat A region that is crucial for gene silencing has led to searches for interacting proteins. A biochemical approach that uses small RNA fragments derived from Xist repeat A has identified the splicing factor ASF (also known as SF2) as an interacting protein[42], but the essential role of ASF in splicing has thus far precluded its analysis in the initiation of gene silencing. Binding of the PRC2 proteins EZH2 and SUZ12 to repeat A RNA has also been reported[43,44], which is consistent with an earlier finding that repeat A enhances PRC2 recruitment in mouse ESCs[45]. However, Xist that lacks repeat A can recruit PRC2, and, in differentiating ESCs, PRC2 recruitment appears to be independent of repeat A. Furthermore, XCI can be initiated in the absence

of PRC2 function[37,39]. These observations suggest that PRC2 is not essential for the initiation of gene silencing on the Xi but that it might have a role in stabilizing it (see later). Taken together, these findings suggest that the mechanism of gene silencing on the Xi might involve novel pathways, and it remains an interesting area of future investigation.

The developmental context for initiation of silencing.
The ability of *Xist* to initiate gene repression is not observed in all cell types but is correlated with specific cellular contexts. Using inducible expression of *Xist* in mouse ESCs, it has been shown that *Xist* can initiate gene repression in a window in early differentiation[12], whereas induction of *Xist* expression in differentiated cells is not sufficient to induce gene repression, even though *Xist* RNA localization is correctly observed[12,46,47]. A detailed analysis of the developmental context for *Xist* function has been carried out using an inducible *Xist* expression system in mice, in which a tetracycline-regulated promoter was inserted upstream of the *Xist* transcription initiation site[48]. Consistent with earlier results in ESCs, gene repression is initiated by *Xist* expression in the cells of the early embryo. As development progresses, the number of cells in which *Xist* initiates silencing diminishes gradually. A surprising finding of this study was that the function of *Xist* in gene repression can also be observed in the haematopoietic system of adult mice. A detailed analysis of haematopoietic differentiation showed that *Xist* induction initiates gene repression in haematopoietic precursor cells but not in haematopoietic stem cells or fully differentiated blood cells[48]. These observations indicate that pathways for gene silencing by *Xist* are transiently reactivated in precursor cells of the blood system, including pre-B cells and T cell precursors[48]. The context for initiation of gene silencing is therefore confined to very specific cell types in somatic tissues. Notably, there is evidence that a silencing context is also preserved in certain tumours. *Xist* expression has been shown to cause ectopic XCI in a mouse lymphoma model[49], and *XIST* expression from transgenes has been shown to initiate gene repression in transformed human cells[50,51].

The developmental regulation of the *Xist* silencing function provides an opportunity to investigate the molecular basis of the cellular context for gene silencing. In a study of mouse T cell lymphoma, inducible *Xist* expression from the single X chromosome in tumour cells from male mice was shown to cause cell death, leading to the regression of tumours[49]. The ability to kill tumour cells was then used to isolate a tumour that became resistant to *Xist* after *in vitro* culture of tumour cells. A comparison of gene-expression patterns of parental and *Xist*-resistant tumours identified the DNA-binding protein special AT-rich sequence binding protein 1 (SATB1) as a crucial silencing factor for *Xist*-mediated gene repression in lymphoma cells[49]. In thymocytes, SATB1 forms a nuclear network that seems to overlap with the bases of chromatin loops that contain genes[52], and it has also been shown that SATB1 regulates the expression of many T cell genes[53]. The function of

Thymocytes
A subset of white blood cells (T cells) that reside in the thymus. Thymocytes perform functions in the immune response.

Genes
● Active
● Repressed

Xic

X chromosome territory

Xist expression

Xist RNA

Exclusion of the transcription machinery | Chromatin modification by PcG complexes

Repressive compartment

SATB1

Gene silencing | Repeat-A-dependent

◄ Figure 3 | **Initiation of chromosome-wide silencing on the inactive X chromosome.** Schematic representation of events during the initiation of gene silencing on the inactive X chromosome (Xi). Most chromosomal DNA is composed of genomic repeats and non-coding sequences and occupies a discrete volume within the nucleus. Genes are located at the periphery of this chromosome territory and active genes protrude into the nuclear space on chromatin loops. X-inactivation specific transcript (*Xist*) localizes to the centre of the chromosome territory and triggers the formation of a repressive compartment, a process that involves exclusion of the transcription machinery and the modification of chromatin by Polycomb group (PcG) complexes. In a subsequent step, genes are silenced — a process that is dependent on a specific cellular context in which silencing factors such as special AT-rich sequence binding protein 1 (SATB1) are present. SATB1 is thought to overlap the bases of chromosome loops where they protrude from the repressive compartment. It is envisioned that SATB1 binding to chromatin loops makes genes susceptible to repression by *Xist* in a process that involves the repeat A region of the *Xist* RNA. Silent genes then associate with the repressive compartment. *Xic*, X-inactivation centre.

SATB1 is regulated by signalling pathways, interaction with factors such as β-catenin and post-translational modifications[54,55]. Intriguingly, SATB1 can be associated with both gene activation and repression. The role of SATB1 in XCI remains to be defined, but one possibility is that it is involved in the organization of X-linked genes for silencing (FIG. 3). Binding of SATB1 to the Xi does not seem to overlap with sites of *Xist* binding but with the periphery, where genes are located[49]. It is conceivable that SATB1 binding makes chromatin susceptible to silencing by *Xist*. SATB1 is also expressed in mouse ESCs[49]. Importantly, it has been shown that viral expression of *Satb1* re-established a context for gene silencing in mouse fibroblasts, in which *Xist* does not normally initiate gene repression[49]. These observations suggest that SATB1 contributes to XCI in the embryo. However, a mutation of *Satb1* has been shown to be compatible with the development of female mice[53], which makes a requirement for SATB1 in XCI unlikely and suggests that other factors might also contribute. The homologous protein SATB2 has been suggested as one factor that might perform a similar function to SATB1 in XCI[49]. However, the precise genetic requirements for the initiation of gene silencing in the embryo remain to be defined.

Stability and maintenance

During development, the initially silenced Xi undergoes further changes that finally lead to a stably inactivated Xi in female somatic cells, which does not require *Xist* to maintain inactivation. A study in mouse ESCs has shown that a switch from *Xist*-dependent, reversible inactivation to *Xist*-independent silencing can be observed at a specific stage in differentiation[12]. The transition to stable gene silencing is also referred to as 'locking-in' of XCI, and its mechanistic basis is not fully understood. Studies in mice indicate that different pathways might contribute to the maintenance of gene repression on the Xi in embryonic and extra-embryonic lineages.

Stabilization in embryonic lineages. DNA methylation of X-linked gene promoters has been associated with repression of the Xi. In somatic cells, the Xi is hypomethylated in gene-poor regions and hypermethylated in gene-rich regions, relative to the active X chromosome[56–58]. *Dnmt1* has been shown to be required for stable maintenance of XCI in embryonic development. In mice, a mutation in *Dnmt1* leads to failure in the maintenance of gene silencing on the Xi and embryonic lethality at embryonic day 9.5 (E9.5)[59]. This finding indicates that DNA methylation is important for stabilizing XCI. However, the DNA methyltransferases DNMT3A and DNMT3B, which have a role in the establishment of methylation marks, are not essential for XCI in mice[46]. As discussed above, DNMT3B is required for DNA methylation of LINEs on the Xi, indicating that different pathways mediate DNA methylation of genes and LINEs on the Xi. This idea is also consistent with the observation that DNA methylation on the Xi is preferentially enriched on gene promoters, whereas overall sequences are less methylated on the Xi compared to the active X chromosome[57].

An insight into the pathway for maintaining gene repression on the Xi has been obtained from a genetic screen for epigenetic modifiers in mice[60]. This work has led to the discovery of the previously unstudied gene structural-maintenance-of-chromosomes hinge domain containing 1 (*Smchd1*), which is required for maintenance of gene repression and DNA methylation on the Xi in mouse development[61]. SMCHD1 protein is enriched on the Xi. Mutation of *Smchd1* in mice leads to reactivation of X-linked genes from the Xi and causes female-specific lethality[61]. By contrast, males appear to develop normally, suggesting that SMCHD1 function is highly specific for XCI. It has further been shown that initiation of XCI is unaffected by a mutation in *Smchd1*[61]; therefore, *Smchd1* is exclusively required for maintaining XCI. Taken together, these findings suggest that a specific DNA methylation pathway exists that is central to locking-in of XCI in the embryo. Notably, a protein containing an SMCHD has also been identified in plants as a factor involved in RNA-mediated DNA methylation[62]. It is therefore conceivable that SMCHD proteins might be components of an evolutionarily conserved mechanism of epigenetic gene regulation.

Stabilization in extra-embryonic lineages. Whereas *Dnmt1* and *Smchd1* are essential for the maintenance of XCI in the embryo, mutations in either gene appear to be dispensable for imprinted XCI in the extra-embryonic tissues[59,61]. These observations are consistent with reported DNA hypomethylation on the Xi in the placenta[63] and indicate that pathways other than DNA methylation contribute to locking-in of XCI in a lineage-specific manner. A mutation of the PcG group gene extra-embryonic development (*Eed*) in mice causes a female-specific phenotype in trophoblast development[64]. This phenotype might result from a failure to maintain imprinted XCI in extra-embryonic tissues, as supported by the reactivation of a reporter gene on the paternally inherited Xi in these tissues[64]. Furthermore, the EED and EZH2 proteins have been shown to associate together on the Xi in trophoblast stem cells[65]. These results indicate a role of PRC2 on the Xi chromatin in imprinted XCI. However, the situation is complex. Detailed investigation of EED-deficient

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Imprinted XCI
(Imprinted X-chromosome inactivation). Inactivation of the paternally inherited X chromosome, whereby inactivation is determined by the parental origin of the chromosome.

Trophoblast
An extra-embryonic lineage that is derived from the trophectoderm of the blastocyst, which gives rise to a cell layer of the placenta.

REVIEWS

Box 1 | **Escape from X-chromosome inactivation**

An important area of investigation is the escape of genes from X-chromosome inactivation (XCI). Certain genes on the inactive X chromosome (Xi) — including X-inactivation specific transcript (*Xist*) — are not subject to silencing. In mice, only a few such cases are known; however, a study conducted in human cells suggests that a large number of genes might escape from XCI[105]. In this study, 15% of all X-linked genes were found to be expressed from the Xi to different extents in a large panel of fibroblast samples.

The mechanism by which genes escape XCI is unclear, although the genomic environment surrounding the gene locus and the presence of boundary elements have been implicated[28,29,106]. Studies in mouse embryonic stem cells have provided evidence that promoters or other regulatory elements of the *Jarid1c* (also known as *Kdm5c*) locus might mediate escape of this gene from inactivation[107]. When bacterial artificial chromosome (BAC) transgenes containing the *Jarid1c* locus and neighbouring genes were inserted at different positions on the X chromosome, *Jarid1c* continued to escape from XCI, whereas neighbouring genes on the transgene were normally inactivated. Notably, X-linked transgenes in mice are generally subject to silencing on the Xi, even if they contain strong promoters[108,109]. These results indicate that, at least for some genes, escape from XCI might involve specific gene-regulatory elements, rather than being the result of inefficient silencing of a genomic region. In addition to these studies, a hypothesis has been put forward suggesting that chromosomal loops constrained by CCCTC-binding factor (CTCF) could shield certain regions on the X chromosome from *Xist*[106].

From an evolutionary perspective, genes that escape from XCI are thought to have been added to the X chromosome relatively recently so that they have not yet adapted to efficient XCI. These adaptations could include the gain of elements that enhance gene repression by *Xist*, such as long interspersed elements (LINEs)[31], or the loss of sequences that prohibit *Xist* spreading[28]. Some of the younger genes on the X chromosome have homologous genes on the Y chromosome owing to the addition of sequences to both sex chromosomes. Therefore, dosage compensation becomes necessary only after the Y-linked gene copy has been eroded owing to accumulation of mutations.

Histone variant
A protein that contains a histone domain and, in addition, another unrelated protein domain.

Trithorax protein
A protein that maintains the stable and heritable active state of several genes, including the homeotic genes. Trithorax group proteins were discovered in genetic screens in *Drosophila melanogaster*, in which they were found to oppose the silencing mediated by Polycomb group complexes.

trophoblast stem cells has shown that gene repression is maintained on the Xi[66]. In the absence of EED, *Xist* RNA appears to be lost from the chromosome, suggesting that EED might be required for *Xist* localization. Reactivation of genes on the Xi was observed only after differentiation of EED-deficient trophoblast stem cells was induced[66]. These observations suggest that the understanding of EED function in maintaining XCI is not complete at present and might involve indirect effects.

Xi facultative heterochromatin in somatic cells. Gene silencing on the Xi in somatic cells is remarkably stable. Thus far, chromosome-wide reactivation of genes on the Xi has not been achieved unless the cell is reprogrammed to an early developmental stage. Interference with either DNA methylation or histone deacetylation results in partial reactivation of some genes on the Xi in female mouse fibroblasts[67]. However, in this study, even a combined deletion of the *Xist* and *Dnmt1* genes, together with chemical inhibition of histone deacetylases, resulted in the reactivation of individual genes in just a small number of cells. These results demonstrate the impressive stability of gene silencing on the Xi and suggest that several epigenetic pathways might act in parallel for maintaining gene repression.

The Xi in female somatic cells has a specific chromatin composition that distinguishes it from other types of heterochromatin, such as pericentric heterochromatin[68] or nucleolar heterochromatin[69] (TABLE 2).

Immunofluorescence studies have shown that a number of proteins are specifically enriched on the Xi (TABLE 1). These include the histone variant macroH2A[70], SAF-A[20], the Trithorax protein ASH2L[21] and SMCHD1 (REF. 61). Furthermore, Polycomb proteins are recruited to the Xi and catalyse H3K27me3 and H2Aub1. In addition, the Xi lacks acetylated forms of histone H4 (REF. 71) and possesses higher levels of H4K20me than other forms of heterochromatin[45]. Recently, the distribution of macroH2A and H3K27me3 on the Xi has been investigated at high resolution using chromatin immuno-precipitation combined with chromosome-specific microarrays (ChIP–chip) or high-throughput sequencing (ChIP–seq)[72–74]. Notably, all of these studies find nearly uniform distributions of these chromatin features over the Xi in somatic cells. The absence of clear patterns of regional enrichment could indicate that the Xi chromatin composition is relatively homogenous, suggesting that, once genes are silenced, chromatin modifications spread in a manner that is indiscriminate of the underlying sequence over the Xi. By contrast, regional differences in Xi heterochromatin have been reported in human cell lines and might indicate cell-type- or species-specific differences[75].

The timing of changes in the chromatin composition of the Xi has been investigated during the differentiation of mouse ESCs[21,76–79] (FIG. 4a), and these changes seem to occur at the stage when gene silencing becomes stable. This coincidence with locking-in of XCI suggests that chromatin changes are at least markers for — if not causal factors in — the transition to stable gene repression. However, the function of chromatin components in the maintenance of XCI is not clear. Depletion of macroH2A1 by RNAi has been reported to cause the reactivation of a reporter gene on the Xi in a small percentage of fibroblast cells[80]. However, a mutation of macroH2A1 has no obvious effect in female mice, suggesting a subtle role for macroH2A in stabilizing Xi chromatin[81]. Notably, studies in mouse ESCs and mice have obtained no evidence for a role of PcG complexes in the maintenance of XCI in embryonic cells[38,39,64]. These results do not support the view that these components are crucial for the lock-in step to occur. However, future studies will be needed to clarify whether the lack of a demonstrable effect is due to compensating epigenetic pathways that act in parallel.

One important insight from studies in ESC differentiation is that most — but not all — chromatin features of the Xi require *Xist* expression. It has been shown that a loss of *Xist* expression is followed by a loss of PcG complexes and their associated histone modifications, as well as loss of macroH2A, SAF-A and ASH2L[21,37,39,45]. This surprising observation suggests that these factors are unlikely to be required for *Xist*-independent maintenance of gene repression. By contrast, hypoacetylation of histone H4 is maintained on the Xi when *Xist* is deleted by conditional mutagenesis in female mouse fibroblasts[11]. At present, DNA methylation on gene promoters and chromosome-wide hypoacetylation are two epigenetic marks that are known to be associated with stable and *Xist*-independent maintenance of XCI.

Table 2 | **The composition of different types of heterochromatin in mammals**

Heterochromatin	RNA	RNA-binding proteins	Chromatin proteins	Chromatin-modifying complexes	Chromatin modifications
Inactive X chromosome (Xi)	17–19 kb *Xist*	SAF-A	ASH2L	PRC1 (RING1B)	• H2Aub1
			macroH2A	PRC2 (EZH2)	• H3K27me3 • H4K20me1 • Loss of H4ac
Pericentric heterochromatin	34–42 nt centromeric RNA	TIP5 (?)	• HP1 • SU(VAR)3-9 • SU(VAR)4-20	NoRC (SNF2) (?)	• H3K9me3 • H4K20me3 • Loss of H4ac
Nucleolar heterochromatin	150–300 nt *pRNA* from IGS spacer	TIP5	HP1	NoRC (TIP5, SNF2)	• H3K9me3 • H4K20me3

Question marks indicate that an interaction is likely but needs to be confirmed. HP1, heterochromatin protein 1; IGS, NoRC-associated RNA; me, methylation; NoRC, nucleolar remodelling complex; PRC1, Polycomb repressive complex 1; TIP5, TTF-I-interacting protein 5; ub, ubiquitylation; *Xist*, inactive X specific transcript.

Reactivation of the inactive X chromosome

In female somatic cells, genes on the Xi are stably repressed[67]. This is in contrast to the early embryo, in which XCI is *Xist*-dependent and reversible[12]. Reversal of XCI is accomplished in certain developmental contexts and also during experimentally enforced reprogramming of somatic cells.

In female mice, reactivation of the Xi accompanies the development of the germ line[82–84]. A loss of *Xist* expression is observed at the time of primordial germ cell formation[83], and Xi-linked genes subsequently become reactivated in a gradual manner during germ cell migration. As a result, female germ cells possess two active X chromosomes during the process of oogenesis. Reversal of imprinted XCI is also observed in cells of the inner cell mass of the blastocyst[33,85]. This developmental context for Xi reactivation is maintained in mouse ESCs. Fusion of female somatic cells with mouse ESCs triggers the reactivation of the Xi from the somatic fusion partner[86,87]. This finding suggests that ESCs have pathways that can reverse all epigenetic modifications that stabilize the Xi in somatic cells. Repeated inactivation and reactivation of the Xi in development implies that opposing pathways exist for stabilization and remodelling of the Xi. However, the molecular mechanism of Xi reactivation remains to be defined.

Reactivation of the Xi also accompanies the reprogramming of female mouse somatic cells to a pluripotent state (FIG. 4b). Transgene-induced reprogramming is a stochastic process and Xi reactivation is observed as a late event in this situation[88]; however, Xi reactivation is accomplished within 2 days when somatic cells are fused to mouse ESCs. These findings suggest that faster and more efficient reprogramming could be achieved by experimental induction of a chromatin environment akin to that of mouse ESCs. The mechanism of Xi reactivation is therefore of interest for understanding the process of reprogramming. To address this aspect, the regulation of XCI in mouse ESCs has been investigated. Recent evidence suggests a link between *Xist* regulation and the transcription-factor network of pluripotent mouse ESCs. Binding sites for the transcription factor octamer-binding protein 4 (OCT4; also known as POU5F1) have been identified within *Xist* intron 1 (REF. 89), and OCT4 has also been reported to regulate *Xist* antisense gene

(*Tsix*) transcription[90], although this finding could not be reproduced in a different line of ESCs[91]. Other transcription factors, including REX1 (also known as ZFP42), Krüppel-like factor 4 (KLF4) and MYC (also known as C-MYC) have also been implicated in *Tsix* regulation in ESCs[91]. These findings suggest that XCI might be directly regulated by the transcription-factor network of pluripotent cells. However, the relevance of these results remains unclear, as a *Rex1* mutation in mice is compatible with female development[92]. A recent study has found that deletion of the OCT4 binding sites in *Xist* intron 1 does not lead to derepression of *Xist* in ESCs[93]. Furthermore, XCI is differentially regulated in epiblast-derived stem cells (EpiSCs) and ESCs in mice[94]. In EpiSCs, XCI is initiated and *Xist* is expressed despite the presence of OCT4. Therefore, the effect of ESC transcription factors on *Xist* regulation is likely to be more subtle and could be indirect. Heterogeneity in terms of XCI status is also observed in human ESCs. Recently, human ESCs have been obtained with two active X chromosomes using culture in low-oxygen conditions[95]. This study suggests that in human ESCs, XCI is initiated following culture stress but is apparently uncorrelated with pluripotency.

Taken together, these findings indicate that *Xist* is regulated within the pluripotent cell context of the mammalian embryo by many factors. Understanding the mechanism of *Xist* regulation might provide new insights into different states of pluripotency. Notably, during the production of cloned mice, aberrant initiation of XCI causes major losses of nuclear transfer embryos. It has been shown that deletion of the *Xist* gene increases the efficiency of mouse cloning considerably[96]. Regulation of XCI is therefore an important factor not only in female but also in male cells, with respect to experiments that involve changing the developmental potency of cells and embryos. Reactivation of certain genes on the silent Xi has also been considered in relation to therapeutic strategies for diseases that involve mutations in X-linked genes, such as muscular dystrophy and Rett's syndrome[97]. Rett's syndrome is a neurodegenerative disease that manifests itself in female patients and is caused by the mutation of the X-linked gene methyl-CpG-binding protein 2 (*MECP2*). Owing to random XCI, half of the cells in heterozygous individuals

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Primordial germ cells
Embryonic cells that give rise to germ cells from which the haploid gametes (oocytes in females and sperm in males) differentiate.

Inner cell mass
A small clump of cells in the blastocyst, which gives rise to the entire fetus plus some of its extra-embryonic membranes.

Pluripotent
A term used to describe a cell that has the developmental potential to differentiate into all lineages of the embryo, including the germ cells.

Epiblast
A term for the group of embryonic cells from which the embryo is structured during gastrulation. It is derived from the inner cell mass of the blastocyst.

express the mutated copy of the gene, causing a dominant phenotype. It has been shown in mice that restoring *MECP2* function in the brain can reverse the symptoms. This suggests that selective reactivation of the intact copy of *MECP2* on the silent X chromosome could be used as a cure for Rett's syndrome. Thus far, selective reactivation of genes on the Xi has not been achieved. A better understanding of the facultative heterochromatin of the Xi could contribute to the development of new strategies for therapeutic reactivation of the Xi.
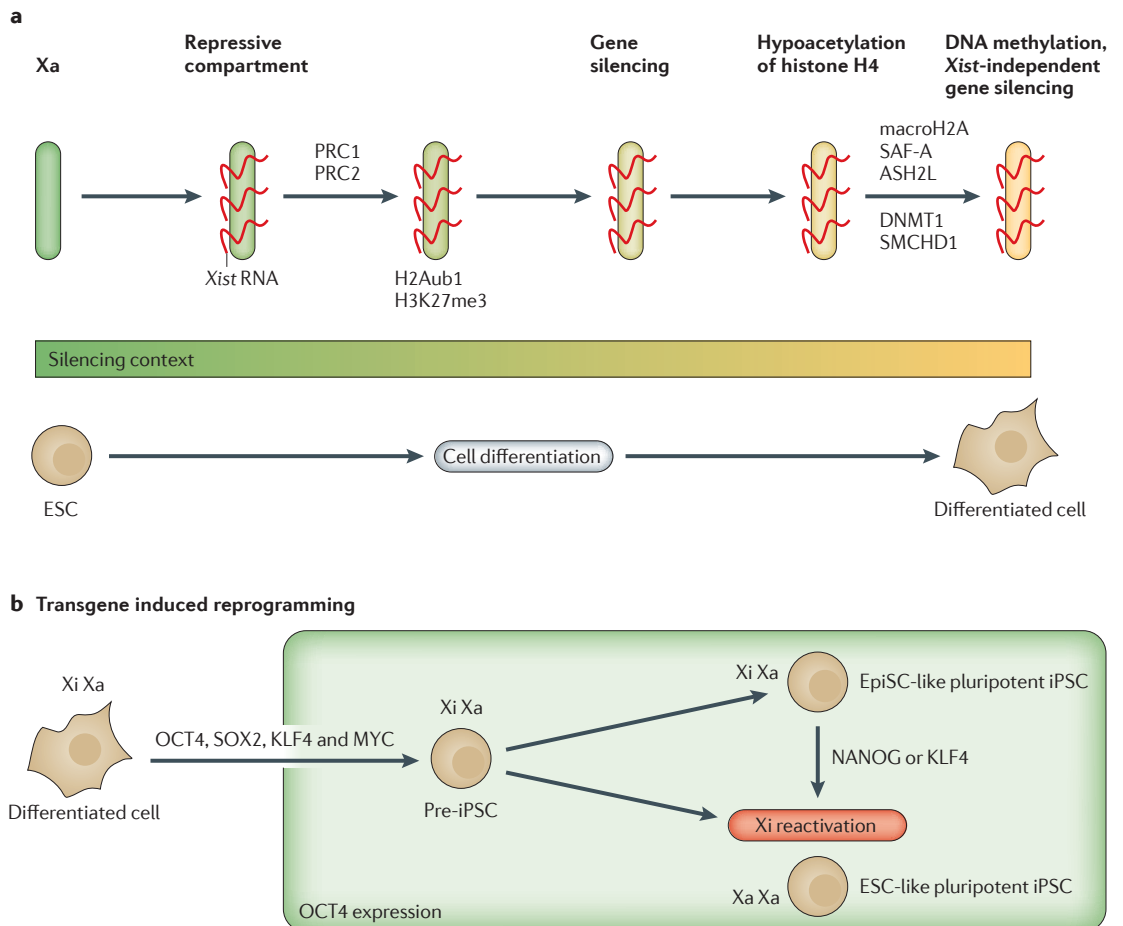

Figure 4 | **Transition to maintenance of X-chromosome inactivation. a** | X-chromosome inactivation (XCI) proceeds in a stepwise series of chromatin modifications. The relative order of events has been defined using mouse embryonic stem cells (ESCs). Female ESCs possess two active X chromosomes (denoted 'Xa' in the figure) and initiate random XCI when they enter differentiation. X-inactivation specific transcript (*Xist*) RNA accumulation in the X chromosome leads to the formation of a repressive compartment. Recruitment of Polycomb repressive complex 1 (PRC1) and PRC2 by *Xist* establishes ubiquitylation of histone H2A (H2Aub1) and trimethylation of H3 at lysine 27 (H3K27me3). Gene silencing is accomplished in a separate step. During the subsequent differentiation of the cells, chromatin on the inactive X chromosome (Xi) becomes further modified by hypoacetylation of histone H4. Subsequently, macroH2A, ASH2L and SAF-A are enriched over the inactive X chromosome (Xi). In parallel to the progressive changes of Xi chromatin, the gene-silencing function of *Xist* is lost. Gene repression is maintained from then on by other mechanisms that are independent of *Xist*. DNA methyltransferase 1 (DNMT1) and structural-maintenance-of-chromosomes hinge domain containing 1 (SMCHD1) have been implicated in maintenance of gene repression on the Xi in the embryo. DNA methylation of gene promoters on the Xi is a very late event and has been observed long after all other chromatin marks of the Xi have been established during ESC differentiation. Studies of inducible *Xist* transgenes have defined the point when XCI becomes independent of *Xist*. This 'lock-in' step appears to overlap with the recruitment of SAF-A and ASH2L and hypoacetylation of histone H4 on the Xi. This suggests that a chromatin transition marks the switch to maintenance of XCI. However, of all Xi chromatin marks, only hypoacetylation of histone H4 can be maintained without *Xist*. **b** | Xi reactivation during reprogramming of induced pluripotent stem cells. The Xi in somatic cells is stably silenced and genes are not reactivated unless the cell is reprogrammed to an earlier developmental stage. Reprogramming of somatic cells to induced pluripotent stem cells (iPSCs) can be achieved by expression of four factors: octamer-binding protein 4 (OCT4), SOX2, Krüppel-like factor 4 (KLF4) and MYC[122]. Reprogramming is a stochastic event and occurs via partially reprogrammed cell types (pre-iPSCs). Depending on the culture conditions, ESC-like[123,124] or epiblast-derived stem cell (EpiSC)-like[125] iPSCs are obtained. Both iPSCs are considered to be pluripotent. However, Xi reactivation is only associated with the formation of ESC-like cell types[88]. This indicates that reactivation of the Xi is a late event in reprogramming and requires that a specific pluripotent cell type is established.

Conclusions and future directions

Key questions for understanding X-chromosome inactivation. Recent progress has provided new insights into the mechanism of XCI, which appears to be more complex than had been initially anticipated. Identification of key factors suggests that the mechanism of chromosome-wide silencing can be partitioned into a number of steps, such as *Xist* localization, initiation of gene repression and stabilization of the inactive state. The molecular basis of each of these steps will need to be further investigated and how individual steps are connected also remains to be defined. A major challenge for future studies is the identification of chromosomal sites for *Xist* attachment and the mechanism of spreading over the Xi. A study in human cells has implicated the mitotic aurora kinase AURKB (also known as STK12) in the regulation of *Xist* attachment[98]; inhibition of this kinase blocked the release of *Xist* from mitotic chromosomes. Thus, defining the relevant substrate of AURKB will be an important step forward.

Another unresolved question regards the pathway for gene silencing. Currently, there is no known molecular link between *Xist* repeat A and factors such as SATB1 that defines the cellular context for initiation of gene repression. One potential avenue for addressing this question is an investigation of gene promoters at the onset of XCI. So far, there has been no report of early changes in the transcription units of X-linked genes after *Xist* expression. However, a large number of antisera that are specific for components of the transcription and mRNA-processing machinery have been developed, and identification of the step of gene transcription that is blocked by *Xist* could provide an entry point for understanding the silencing pathway. Of key interest, in terms of epigenetic regulation, is the stabilization of gene repression during locking-in of XCI. The identification of *Smchd1* and *Dnmt1* promises progress in defining the underlying mechanism in the near future. Potentially, it could be more challenging to define other pathways that act in maintaining XCI in the extra-embryonic lineages where *Smchd1* and *Dnmt1* are not required.

Finally, it will be important to investigate the process of XCI in other mammals. To date, most studies on the mechanism of XCI have been conducted in mice. Future studies will need to establish similarities and differences with the mechanisms that operate in other mammals, including humans (BOX 2). Such studies could also provide insights into how epigenetic systems evolve.

Broader implications from studies of X-chromosome inactivation. The Xi exemplifies pathways for compartmentalization in the mammalian cell nucleus. RNA has long been recognized as a component of the nuclear scaffold and *Xist* might provide a model for a better understanding of the function of the nuclear scaffold for organizing chromatin. SAF-A is a nuclear scaffold protein that is involved in XCI and has been implicated in a wide range of nuclear processes. Similarly, the non-coding RNA p53p21 has been shown to associate with heterogeneous nuclear ribonuclease protein K (hnRNPK)[99]. SAF-A and hnRNPK are members of abundant RNA-binding complexes, suggesting that scaffold interactions similar to *Xist* might be relevant in many different biological scenarios. Non-coding RNAs also have a role in PcG complex recruitment in homeobox (*HOX*) gene regulation[100], genomic imprinting[101–103] and developmental gene regulation[104]. This suggests that mechanisms observed in XCI might have broader implications for understanding gene regulation in mammals.

XCI also exemplifies how a stable epigenetic state can be established in development. More work is needed to determine the extent to which mechanisms in developmental gene regulation and XCI overlap. However, the fact that the *Xist* gene evolved after the lineages of marsupial and placental mammals split may suggest that XCI involves pathways that are common to vertebrate development (BOX 2). XCI therefore highlights important questions about gene regulation, and progress in understanding facultative heterochromatin formation on the Xi promises to provide broader insights into key mechanisms for epigenetic regulation in mammals.

Box 2 | X-chromosome inactivation in placental and marsupial mammals

In placental mammals, X-inactivation specific transcript (*Xist*) initiates the formation of the characteristic facultative heterochromatin of the inactive X chromosome (Xi). *Xist* is conserved throughout placental mammals, but most studies have been focused on the mouse. Differences in chromosomal localization of *XIST* RNA in mitosis and the chromatin composition of the Xi have been reported between mouse and human cells[6,75,98,110]. Recent evidence also suggests that regulation of X-chromosome inactivation (XCI) might be fundamentally different among eutherian mammals[111]. In rabbit embryos, *Xist* is initially expressed from both X chromosomes in female cells and from the single X chromosome in male cells. After partial inactivation of all X chromosomes, the pattern is corrected and one Xi is maintained in female cells. *Xist* expression is extinguished on one X chromosome in males and females[111]. Similar to the situation in the rabbit, *XIST* is reported to be initially expressed from both X chromosomes in human embryos. However, human embryos do not initiate XCI before implantation[111]. These recent results are currently hard to reconcile with an earlier report that human XCI is established by expression of *XIST* from one X chromosome at the morula stage[112], and further studies are needed to explain this discrepancy.

Marsupial mammals lack a functional *Xist* gene but possess the ancestral protein coding gene *Lnx3* that shares limited sequence homology with an exon of *Xist*[113]. In marsupials, inactivation of the paternally inherited X chromosome gives rise to an imprinted XCI pattern in the embryo[114]. In the tamar wallaby, paternal and incomplete XCI with stochastic escape has been reported[115]. Although the Xi showed an absence of histone modifications that are known to associate with active transcription, repressive histone modifications were not detected[116]. In the marsupial *Monodelphis domestica*, a heterochromatic Xi was reported that shared several histone modifications with the Xi of placental mammals[117]. However, more recent studies suggest that the chromatin of this marsupial Xi seems to more closely resemble that of mammalian pericentric repeats, with histone H3 trimethylation on lysine 9 (H3K9me3) and heterochromatin protein (HP1) binding[118]. This view is confirmed by another study showing that H3K9me3 is enriched on the marsupial Xi, whereas K27me3 was observed on both the Xi and active X chromosome[119]. Taken together, these observations suggest that differences in chromatin composition between placental and marsupial mammals may arise as a result of distinct silencing mechanisms, which involve *Xist* in placental mammals but not in marsupials.

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