

Evolutionary diversity and developmental regulation of X-chromosome inactivation

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Abstract X-chromosome inactivation (XCI) results in the transcriptional silencing of one X-chromosome in females to attain gene dosage parity between XX female and XY male mammals. Mammals appear to have developed rather diverse strategies to initiate XCI in early development. In placental mammals XCI depends on the regulatory noncoding RNA X-inactive specific transcript (*Xist*), which is absent in marsupials and monotremes. Surprisingly, even placental mammals show differences in the initiation of XCI in terms of *Xist* regulation and the timing to acquire dosage compensation. Despite this, all placental mammals achieve chromosome-wide gene silencing at some point in development, and this is maintained by epigenetic marks such as chromatin modifications and DNA methylation. In this review, we will summarise recent findings concerning the events that occur downstream of *Xist* RNA coating of the inactive X-chromosome (Xi) to ensure its heterochromatinization and the maintenance of the inactive state in the mouse and highlight similarities and differences between mammals.

Introduction

Sex chromosomes differ significantly in their gene content (Ohno 1967), which has led to the evolution of mechanisms of dosage compensation (Lyon 1961, 1962). At least three

different mechanisms have been described to adjust sex chromosome gene expression dosage between males and females. The single male X-chromosome is hypertranscribed in flies (Park and Kuroda 2001), both X-chromosomes are partially repressed in worms (Meyer 2000), while in mammals one of the two X-chromosomes is silenced in each cell (Lyon 1961). It was 50 years ago that cytological observations in mouse, rat, opossum and human (Lyon 1962; Ohno and Hauschka 1960) resulted in the Lyon hypothesis (1961), whereby dosage of X-chromosomal gene products is equalised between male and female mammals, by inactivating one of the X-chromosomes during early development in females (Lyon 1961, 1962). Fifty years on, we are still seeking the exact mechanisms that trigger the initial differential treatment of the two X-chromosomes, as well as the spread and maintenance of the inactive state. In this review, we will discuss recent studies that have contributed to our understanding of how an entire chromosome is transformed, in a step-wise fashion, from an active into an inactive and heterochromatic entity and how this state is then maintained through cell division and life span. While describing this process in the mouse, we will consider possible differences in the XCI process and the Xi chromosome itself in other mammals.

Regulation of X-chromosome inactivation during early development of eutherian mammals

Over the years the mouse has been the privileged mammal for investigation of XCI given the ease of genetics and embryology in this species. It is now apparent, however, that the XCI strategy found in the mouse may represent just one of many modes of regulating X-chromosome gene dosage in mammals (Fig. 1). In marsupials, XCI is subject

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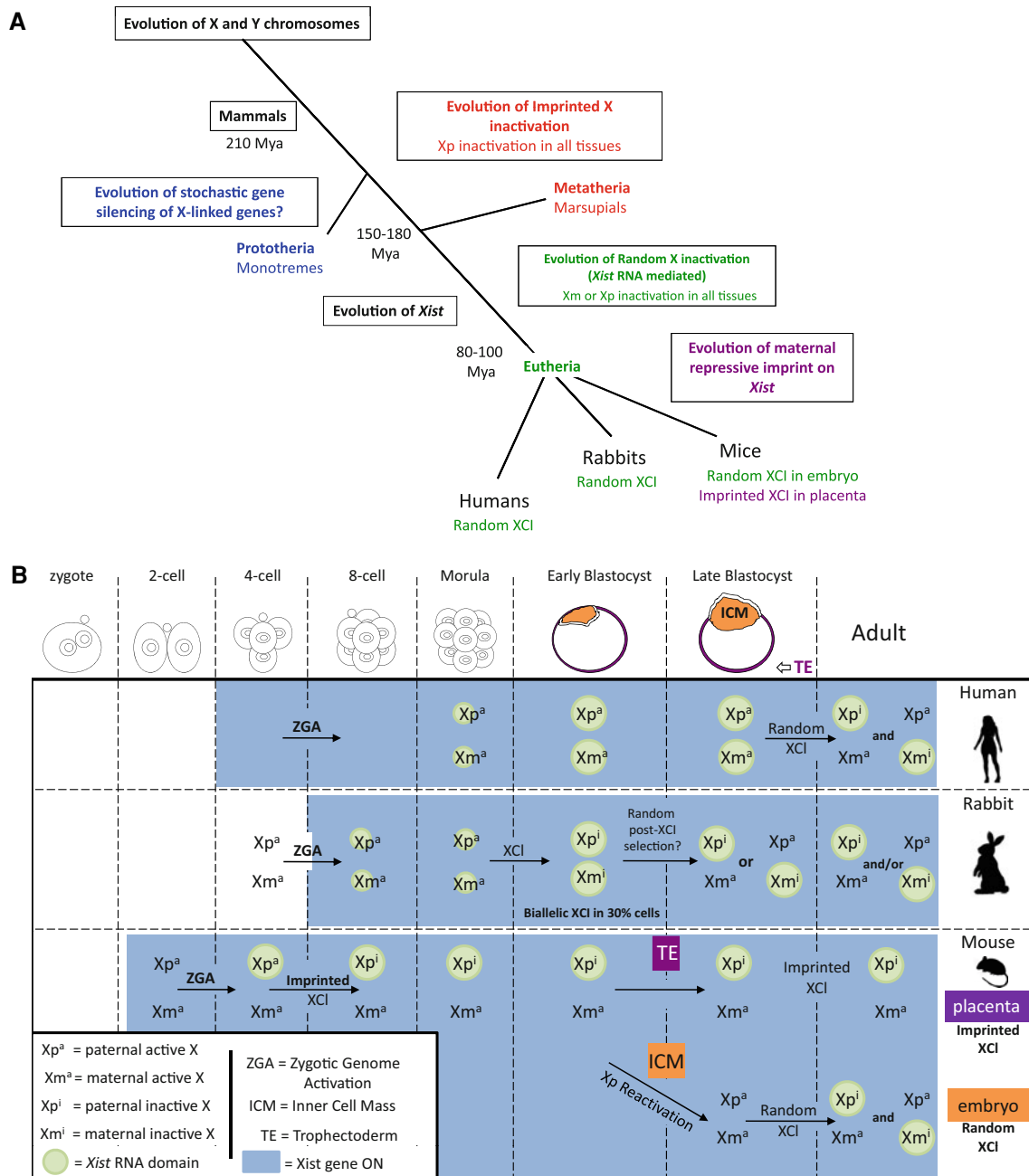


Fig. 1 Strategies and timing of XCI regulation in eutherians. **a** Hypothetical time line of events during evolution for the appearance of different XCI strategies in different mammals. **b** Developmental timing of *Xist/XIST* up-regulation and XCI in humans, rabbits and mice during pre-implantation embryogenesis. In humans, *XIST* is not imprinted and upregulation is observed on both X-chromosomes from the morula stage with partial coating of both chromosomes. Random XCI initiates at or after the blastocyst stage in both extra and embryonic lineages—the exact timing is not clear. In the rabbit, *Xist* is not imprinted and upregulation occurs on one or both of the X-chromosomes in some cells of female embryos, and eventually

leads to XCI. By the late blastocyst only one of the X-chromosomes shows *Xist* coating and X-linked gene inactivation. How XCI goes from a biallelic to monoallelic situation is unclear. In the mouse, *Xist* is imprinted to stay silent on the maternal X-chromosome. Paternal XCI is initiated by triggering *Xist* RNA coating from 4 to 8 cell stage onwards exclusively on the paternal chromosome. This imprinted form of XCI is found in the extra-embryonic tissues such as placenta. However, in the ICM of the late blastocyst, the Xp is re-activated, so that one of the two X-chromosomes then has an equal chance of being inactivated in the lineages that will give rise to the adult mouse

to imprinting, with the paternal X-chromosome (Xp) being chosen for inactivation. This form of imprinted X inactivation is very leaky being tissue- and locus-specific, the

extent to which remains to be carefully assessed (Deakin et al. 2009). In the egg-laying monotremes such as the platypus, which have five X and Y-chromosomes, dosage

compensation appears to be only partial and may be locus specific, similar to birds. Some genes on the platypus X-chromosome are not dosage compensated, while others show some form of compensation, possibly via stochastic inactivation (Deakin et al. 2008), suggesting that monotremes have retained an ancestral compensation system (Deakin et al. 2009).

In eutherians, XCI is generally thought to be random, with the paternal and maternal X-chromosomes having an equal chance of being silenced. For example, in humans, random XCI is thought to occur in both embryonic and extra-embryonic derivatives (Migeon 2002; Moreira de Mello et al. 2010). However, in the mouse, XCI is initially imprinted (Takagi and Sasaki 1975) with the silencing of the Xp from the 4–8 cell stage (Donohoe et al. 2009; Navarro et al. 2008, 2010; Okamoto et al. 2004). The Xp remains inactive in the trophectoderm (Takagi and Sasaki 1975) but is reactivated in the inner cell mass (ICM) of the blastocyst (Mak et al. 2004; Okamoto et al. 2004), followed by random XCI (McMahon and Monk 1983). This second, random wave of XCI is accompanied by a complex interplay of events including downregulation of pluripotency factors such as *Nanog*, *Oct4/Pou5f1* and *Sox2*, activation of *Xist* by dosage-sensitive X-linked competence factors such as *Rnf12* and random monoallelic regulation of *Xist* via its antisense transcription unit, *Tsix*, as will be described in the section on initiation of random X-chromosome inactivation in mice.

The status of the X-chromosome and the regulation of XCI during early female development in mammals other than mice have been unclear until recently. Although mice are clearly a powerful model for dissecting the actors involved in XCI, different strategies for regulating the onset of XCI exist in other mammals as seen in rabbits and humans (Okamoto et al. 2011) (Fig. 1). In both these species, XCI was found to begin later than in mouse, and furthermore *Xist* was found not to be subject to imprinting. This reinforces the previously proposed notion that imprinted XCI, via a *Xist* imprint may be a recent evolutionary event, occurring in mice due to their rather rapid pre-implantation development (Okamoto et al. 2005). Another major difference observed between mammals is that in rabbit and human pre-implantation embryos, *Xist* is up-regulated on both X-chromosomes in a high proportion of blastomeres, a situation rarely observed in the mouse, in vivo. This up-regulation has different consequences on gene expression. In rabbits, XCI appears to be triggered on both X-chromosomes transiently, in a subset of cells, but this situation rapidly resolved to monoallelic XCI. In humans, although *XIST* is expressed from both X-chromosomes in pre-implantation embryos, chromosome-wide XCI only begins later, after the blastocyst stage (Okamoto et al. 2011). A previous study on human pre-implantation

embryos concluded that XCI in human starts by the blastocyst stage (van den Berg et al. 2009). In contrast to Okamoto et al. (2011), this study showed *XIST* monoallelic accumulation from the 8-cell-stage and only 5% of blastomeres accumulate *XIST* on both X-chromosomes by morula and blastocyst stages (van den Berg et al. 2009). The discrepancy between this and the Okamoto et al. (2011) study could be due to technical differences, such as RNA fluorescence in situ hybridization (FISH) efficiency, which can influence the detection of biallelic versus monoallelic expression—Okamoto et al. (2011) provided high-efficiency RNA FISH data showing biallelic expression of *XIST* and of four independent X-linked genes in female blastocysts. The discrepancy may also be due to differences in embryo in vitro culture conditions.

A further species difference concerns the X-chromosome reactivation process observed in the mouse ICM, which is presumed to be due to pluripotency factor-mediated repression of *Xist* in pre-epiblast cells. In rabbits, the X-chromosome actually initiates XCI in cells of the ICM, rather than being reactivated, while in humans, *XIST* is highly expressed but does not trigger XCI in the ICM, suggesting that in both species *Xist/XIST* is rather differently controlled in the ICM (Okamoto et al. 2011). Whether this is due to differences in *Xist* regulatory sequences and/or to differences in the exact combination of pluripotency factors and other *Xist/XIST* regulators in the ICM in other mammals remains to be found. In summary, there is substantial diversity in the timing and mode of regulation of XCI initiation between mammals, even though the outcome, dosage compensation, seems to be the same. The reasons for such diversity are unclear but the most likely explanation is that the XCI process has had to adapt to the considerable variety between mammals in developmental dynamics. For example, the remarkably early zygotic gene activation found in mice compared with other mammals may have led to pressure to evolve an imprint on *Xist* in mice, in order to prevent precocious initiation of XCI on the maternal and paternal X-chromosomes, at a stage of development when gene dosage might be less easy to evaluate.

In mice, the random form of XCI follows Xp reactivation in the ICM, at around the time of implantation (day 5). A major difficulty in the study of random XCI is the small size of the embryo and the limited amount of material available at this stage. For this reason, in vitro model systems, such as embryonic stem (ES) cells (derived from the ICM of blastocysts) provide a valuable alternative. In mouse ES cells, both X-chromosomes are active (Xa), while the cells are maintained in an undifferentiated state and the XCI process can be initiated by simply inducing ES cell differentiation (Martin et al. 1978). Such cell lines have greatly facilitated the investigation of the different

steps in the process of random XCI, from the establishment through to the maintenance of the inactive state.

Initiation of random X-chromosome inactivation in mice

Studies of XCI patterns in mouse embryos and cells carrying translocated or truncated X-chromosomes revealed the existence of an X-linked region, the X-inactivation centre (Xic), that is required to trigger this process (reviewed in Augui et al. 2007). The Xic locus is thought to ensure that only a single X-chromosome will remain active per diploid autosome set; it also underlies the “choice” of which X-chromosome will be inactivated (Brown et al. 1991b) and it produces the signal that triggers *cis*-inactivation of the X-chromosome, in the form of the non-coding *Xist* transcript (Brown et al. 1991a). *Xist* is responsible for initiating silencing of the chromosome from which it is expressed (Borsani et al. 1991; Marahrens et al. 1997; Penny et al. 1996) and also seems to underlie many of the

chromatin changes that take place during XCI. Functional studies have shown that *Xist* is clearly the essential trigger in mice and given that it is conserved in eutherians, it is fair to imagine that it would play a similar role, although functional proof of its role in XCI in other mammals has not so far been demonstrated.

The regulatory network that controls *Xist* expression in mice has recently begun to be unravelled, revealing a complex interplay of *cis*-acting elements and *trans*-acting factors or RNAs, all of which are tightly integrated with the early steps of differentiation (Fig. 2). The downregulation of pluripotency and stem cell factors, such as *Oct/Pou5f14*, *Nanog*, *Rex1* and others, during ES cell differentiation coincides with *Xist* up-regulation. Genetic and developmental studies suggest that such factors play a central role in this *Xist* regulatory network, at least in mice. Although some of these factors were originally proposed to repress *Xist* directly, through binding sites in its first intron (Navarro et al. 2008), more recent studies suggest that these sites alone are not essential for the control of *Xist* and that pluripotency factors control *Xist*'s regulators, such as the

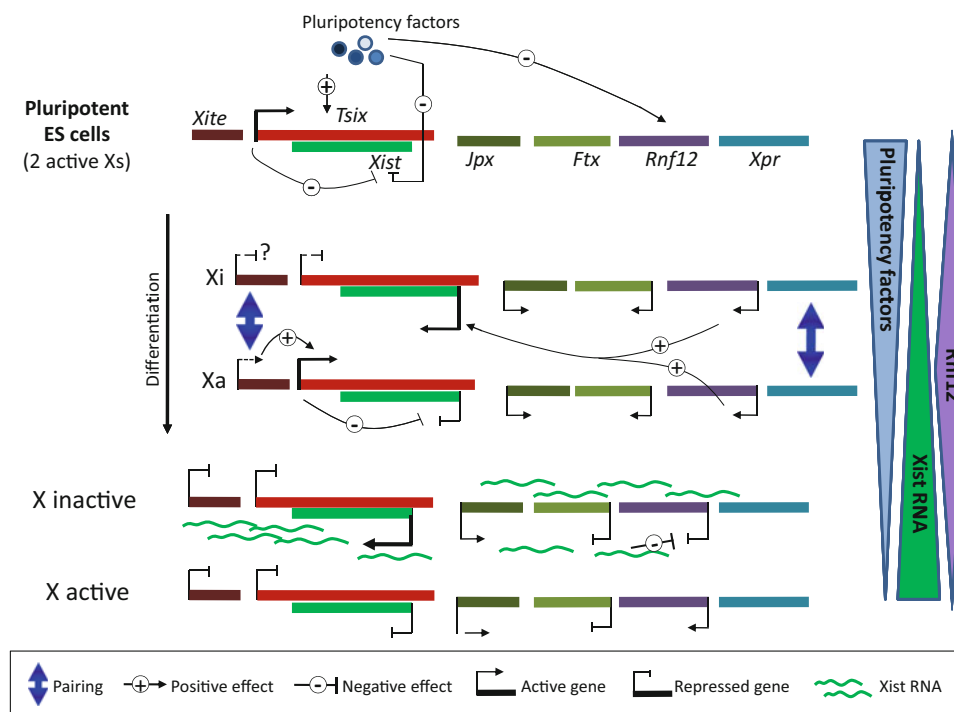


Fig. 2 Initiation of random XCI and the monoallelic regulation of *Xist* in the mice. In pluripotent ES cells, *Xist* is expressed at low levels and this repression is partly due to pluripotency factors that repress *Xist*. *Xist* regulation via pluripotency factors could be direct through binding to the intron 1 of *Xist* gene and/or indirect, via activation of *Xist*'s repressive antisense transcript, *Tsix* or repression of *Xist* activators such as the X-linked gene *Rnf12*. During differentiation the levels of pluripotency factors decrease, while the levels of *Xist* activators such as *Rnf12* increase (the only activator reported so far). Other long-range elements possibly involved in this process are the

non-coding RNAs (*Xite*, *Jpx*, *Ftx*) or *Xpr* (X-pairing region). *Xist* activation on only one allele is believed to be partly regulated by monoallelic regulation of *Tsix* and its enhancer *Xite*. The *Xpr* and *Xite/Tsix* regions have been shown to undergo transient “pairing” events that could potentially help in establishment the monoallelic expression of *Xist*. Other mechanisms could underlie monoallelic *Xist* regulation such as stochastic events and negative feedback loops involving X-linked activators such as *Rnf12*. *Xist* upregulation results in *cis*-coating of the X-chromosome and results in silencing of genes, including the activator *Rnf12*

X-linked activator of *Xist*, *Rnf12* (Barakat et al. 2011; Navarro et al. 2011), or the repressive antisense transcript to *Xist*, *Tsix* (Navarro et al. 2010). Although downregulation of pluripotency factors can partly explain *Xist* activation during differentiation, it does not explain why *Xist* is upregulated only in female, not male cells. Recently, several X-linked loci have been implicated in XX-specific activation (sensing, competence) of *Xist*. These include the *Rnf12* gene, which encodes an ubiquitin ligase protein that can directly act on the *Xist* promoter (Barakat et al. 2011; Jonkers et al. 2009). In the context of increased dose of the RNF12 protein, *Xist* expression can be triggered even in male cells. However, RNF12 is probably not the only dosage-sensitive activator as its deletion on one allele does not abolish *Xist* activation (Jonkers et al. 2009). Other loci that have been proposed to be implicated in XX-specific *Xist* expression include the X-pairing region (*Xpr*) (Augui et al. 2007) and the *Jpx* non-coding RNA locus (Tian et al. 2010).

XX-specific activation of *Xist* via sensing or competence mechanisms does not explain how only one of the two X-chromosomes up-regulates *Xist* during random XCI, a process known as “choice”. As mentioned earlier, mono-allelic regulation of *Xist* seems to be less well coordinated in rabbits and humans, compared with the mouse. How is such tight monoallelic regulation of *Xist* expression achieved in the mouse? The *Xist* antisense transcription unit, *Tsix*, seems to play a critical role in choice of *Xist* allele to be upregulated. *Tsix* transcription across the *Xist* promoter is accompanied by repressive chromatin modifications (Navarro et al. 2005; Sado et al. 2005; Sun et al. 2006), although it is not clear whether this is simply a result of antisense transcription across the *Xist* locus or due to a role for the *Tsix* RNA, for example in recruiting the chromatin modifying machinery to silence the *Xist* gene *in cis*. Deletion of *Tsix* in the mouse leads to preferential up-regulation of *Xist* on the deleted allele (Lee and Lu 1999). Furthermore, deletion of both *Tsix* alleles in female ES cells leads to chaotic *Xist* up-regulation (Lee 2005), implying a critical role for *Tsix* in the coordination of monoallelic *Xist* expression. However, it is important to note that *Tsix* is poorly conserved in other eutherians and antisense transcription across *XIST* has not been found in humans for example (Chang and Brown 2010; Chureau et al. 2002; Migeon 2002; Migeon et al. 2001). This apparent lack of *Tsix* in other eutherians might explain the species differences in *Xist*'s monoallelic regulation in rabbits and humans (Okamoto et al. 2011). In the mouse, the precise manner in which *Tsix* controls monoallelic expression of *Xist* is still unclear, but one hypothesis, recently supported by live-cell imaging data, is that transient pairing events at the level of *Tsix* result in asymmetric *Tsix* expression, and this in turn facilitates asymmetric *Xist* up-regulation (Bacher et al. 2006; Masui et al. 2011; Xu et al. 2006).

***Xist* RNA-mediated silencing and heterochromatinization of the inactive X-chromosome**

In the mouse, the upregulation of *Xist* is critical for downstream events in XCI leading to gene silencing and heterochromatinization. Deletions of *Xist* have demonstrated its essential role in inducing chromosome-wide inactivation *in cis* (Marahrens et al. 1997; Penny et al. 1996). This long, non-coding transcript has fascinated researchers since its discovery two decades ago (Brown et al. 1991a), yet very little is known about its unusual behaviour and mechanism of action. *Xist/XIST* is a polyadenylated, capped, spliced RNA, devoid of any significant open reading frame. It is retained in the nucleus as a 15- to 17-kb-long RNA and coats the chromosome from which it is expressed (Brockdorff et al. 1992; Brown et al. 1992; Clemson et al. 1996). To date, *Xist* is the only gene expressed exclusively from the Xi in somatic cells. Although the structure and organisation of the *Xist* gene does not differ greatly between mammals, its overall sequence conservation is poor (Brockdorff et al. 1992; Brown et al. 1992). The exception to this concerns a series of conserved repeats motifs in *Xist*, termed A to F (Fig. 3a), although their sequence and size can also be quite variable between species (Yen et al. 2007). The A repeat, lying at the 5' end of *Xist* exon 1, is the most conserved and has been shown to be essential for the silencing function of *Xist* (Wutz et al. 2002) (discussed in “*Xist* RNA coating”).

Xist RNA coating

The precise manner in which *Xist* RNA coats the chromosome from which it is expressed *in cis* remains mysterious. This capacity does not appear to be restricted to the X-chromosome as *Xist* transgenes located on autosomes and X:autosome translocations display *Xist* RNA coating of autosomal regions *in cis* (Heard et al. 1999; Herzing et al. 1997; Lee and Jaenisch 1997; Lyon 1998a; Tang et al. 2010), although the degree of *Xist* coating and gene silencing can be less efficient on autosomes compared with the X-chromosome (Duthie et al. 1999; Popova et al. 2006). This implies that intrinsic characteristics of the sequence or chromatin environment of the X-chromosome might facilitate *Xist* RNA coating and/or spreading of the inactive state and possibly also its maintenance. Repeats such as LINEs are clearly enriched on the X-chromosome compared with autosomes (Bailey et al. 2000; Lyon 1998b). However, these elements do not seem to provide a scaffold for *Xist* RNA coating, but rather they help in the formation of heterochromatin, as will be discussed in the section on the formation of a silent nuclear compartment by

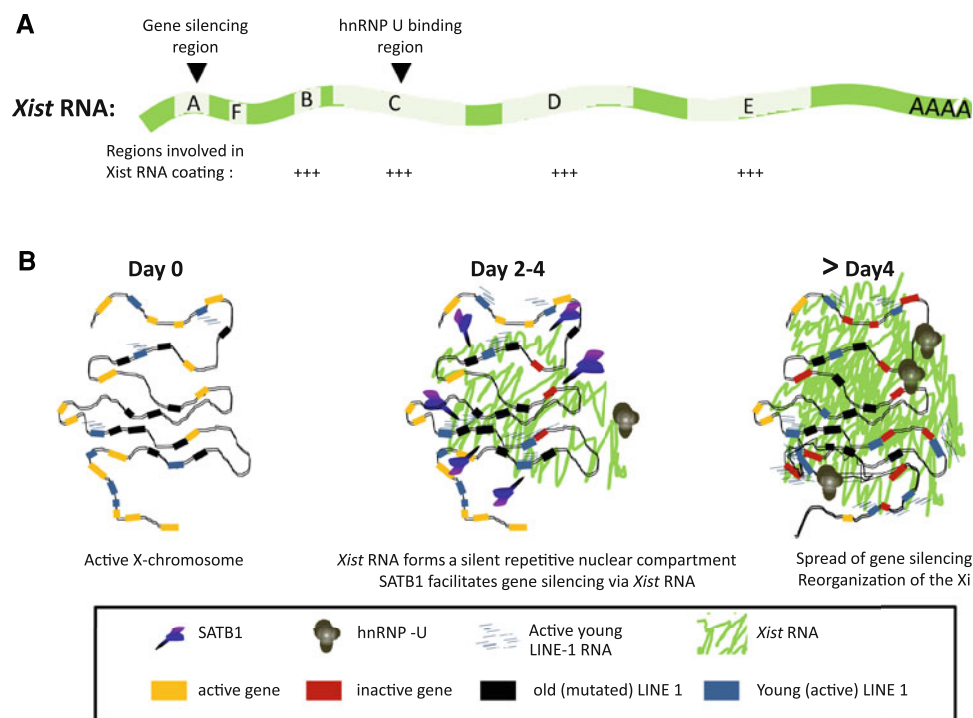


Fig. 3 *Xist* and the formation of the inactive compartment. **a** Structure of the *Xist* gene showing the conserved repeat regions labelled A to F. Specific functions and characteristics of the different regions of *Xist* are highlighted by arrowheads. **b** Model for the formation of the silent nuclear compartment by *Xist* RNA: *Xist* RNA coating occurs immediately upon differentiation and forms a nuclear silent compartment where X-linked genes are recruited as they become silent in a locus-specific manner. The formation of this silent and heterochromatic compartment is probably helped by the silent

repetitive elements, especially LINE elements that are abundant on the X-chromosome; this occurs when the matrix attachment protein SATB1 is present and might facilitate gene relocation into the Xi. At this stage of differentiation (day 4), young LINE-1 elements are highly expressed and they might play a role in the spread of silencing into regions prone to escape, but which will eventually become silenced later (not shown). The hnRNP U matrix attachment protein becomes enriched on the Xi around days 4–5 of differentiation and participates in the maintenance of *Xist* RNA coating

Xist RNA (Chow et al. 2010). The precise nature of the DNA sequences to which *Xist* RNA might bind still remains a mystery. In fact, as DNase treatment does not disrupt the *Xist* RNA nuclear signal in any way (Clemson et al. 2006), suggesting that *Xist* RNA may interact only indirectly with chromosomal components.

In order to define the regions of the *Xist* transcript that are involved in chromosome coating, gene silencing and chromatin modifications, various deletion derivatives of an inducible *Xist* cDNA were stably integrated into the X-linked locus (*Hprt*) in male ES cells. In this way, it was shown that several distinct regions of *Xist* can act cooperatively and redundantly to ensure the coating of the chromosome (Wutz et al. 2002) (Fig. 3a). These regions are also important for inducing some of the chromatin modifications associated with the Xi. Failure of *Xist* RNA to coat the Xi results in failure to inactivate the chromosome (Wutz et al. 2002). Importantly, *Xist* RNA lacking the highly conserved A-repeat region was capable of coating the X-chromosome but did not lead to chromosome-wide gene silencing (Chow et al. 2010; Wutz et al. 2002). The *Xist* A-repeat mutant could also induce histone

modifications and other chromatin changes on the X-chromosome. This uncouples the properties of coating and silencing by *Xist* RNA and also demonstrates that *Xist* RNA is likely to have multiple, possibly independent, roles in gene silencing and heterochromatin formation of the Xi. The above studies were performed in differentiating ES cells, using a Tet-inducible *Xist* transgene. Another report used locked nucleic acid (LNA) interference probes to interfere with different parts of the endogenous *Xist* RNA in differentiated female ES cells and transformed mouse embryonic fibroblasts (MEFs). This revealed that targeting of the *Xist* repeat C region causes *Xist* RNA to be displaced from the Xi (Sarma et al. 2010). Interestingly, this region has been shown to interact with RGG domain of the hnRNP U/SP120/SAF-A, a matrix attachment protein whose localisation to the Xi depends on this RGG domain (Hasegawa et al. 2010; Helbig and Fackelmayer 2003). Depletion of the hnRNP U results in the dissociation of *Xist* RNA from the Xi and its dispersion into the nucleoplasm in differentiated female cells (Hasegawa et al. 2010). Furthermore, differentiating ES cells depleted for hnRNP U initiate XCI in a smaller percentage of cells due to failure

of *Xist* to coat the X-chromosome properly (Hasegawa et al. 2010). This is the only factor so far shown to be crucial for *Xist* localization to the Xi. It remains to be clarified whether hnRNP U is actually required to initiate the *Xist* RNA coating process or instead to maintain it. The latter currently would be the likely scenario given that hnRNP U is only recruited to the Xi several cell cycles after *Xist* coating during ES cell differentiation (Pullirsch et al. 2010) (Figs. 3b, 4b).

Developmental time window for *Xist* RNA induction of XCI

Xist RNA upregulation during early development leads to XCI. On the other hand, aberrant *Xist* expression from the active X-chromosome in somatic cells following treatment with DNA demethylating agents does not cause gene silencing (Tinker and Brown 1998). Such studies suggested that *Xist* might only be capable of triggering XCI during an early developmental time window. The use of inducible *Xist* cDNA transgenes, whereby *Xist* can be expressed or repressed at different time points during ES cell differentiation, demonstrated that *Xist* is only competent to initiate XCI when expressed in undifferentiated and early differentiating ES cells (first 72 h of differentiation). Induction of *Xist* after this time, or in fully differentiated MEFs, no longer resulted in XCI (Wutz and Jaenisch 2000). Recently, it was found that ectopic *Xist* induction might also be able to induce XCI in certain types of adult cells, such as pro-B cells (Savarese et al. 2006), or in some lymphomas (Agrelo et al. 2009). The latter study in fact enabled the identification of a factor, SATB1, that might participate in *Xist*'s silencing capacity (Agrelo et al. 2009). SATB1 is a nuclear protein that is highly expressed in thymocytes and has previously been implicated in regulating chromatin structure (Cai et al. 2003). It has also been shown to interact physically with chromatin remodelling factors (Yasui et al. 2002). Although previously considered to be rather tissue specific, SATB1 and the closely related SATB2 protein are in fact expressed transiently during an early window of ES cell differentiation that exactly matches the period during which *Xist* is competent to trigger XCI. Indeed, knock-down of *Satb1* and/or *Satb2* in mouse ES cells prevented *Xist*-mediated silencing and ectopic expression of these factors in MEFs, where *Xist* RNA is artificially induced, enables *Xist* to repress transcription to some extent (Agrelo et al. 2009). The exact role of *Satb1/2* for XCI in vivo remains to be defined, however. Furthermore, the development time window and factors involved in *Xist* RNA functions in other mammals have not yet been evaluated. However, findings in pre-implantation human embryos, where *XIST* RNA is expressed and yet chromosome-wide XCI is not triggered (Okamoto et al.

2011), suggest that the *XIST* RNA competence time window can be dissociated from the onset of *XIST* up-regulation found in mice.

Cell cycle time window for *Xist* RNA coating

In addition to the developmental time window during which *Xist* RNA can induce silencing, an important question concerns whether *Xist* RNA can coat the chromosome throughout the cell cycle or whether events downstream of *Xist* coating are sufficient to maintain the inheritance of the inactive state during early development. Following *Xist* RNA coating in *cis*, downstream events are triggered in a step-wise manner over a period of several cell cycles during differentiation and many of these seem to be implicated in helping to lock in the inactive state and provide cellular memory (Kohlmaier et al. 2004). Once established, the Xi no longer needs *Xist* RNA to maintain global inactivity (Csankovszki et al. 1999). However, during early development, gene silencing is initially *Xist* dependent and is totally reversible if *Xist* RNA coating is removed. Studies investigating the cell cycle kinetics of *Xist* RNA association with the Xi have reported that it remains associated throughout interphase but is displaced during mitosis in human and mouse cells (Clemson et al. 1996; Duthie et al. 1999; Hall and Lawrence 2003; Smith et al. 2004). Initial reports suggested that whereas human *XIST* RNA is rapidly lost from the chromosome by metaphase, murine *Xist* RNA remains associated up to telophase, but then dissociates. However, recent re-evaluation of *Xist* RNA coating during the cell cycle in differentiating female mouse ES cells (Jonkers et al. 2008) revealed that a significant number of female differentiated ES cells and MEFs exhibit *Xist* RNA coating even at telophase. Furthermore, experiments using MS2-tagged *Xist* RNA suggest that it is retained on the Xi throughout the cell cycle and does not diffuse into the nucleoplasm (Jonkers et al. 2008). Thus, *Xist* RNA could indeed provide a “memory” of the inactive state at least during an early developmental time window, which is when *Xist* expression is essential for XCI.

Formation of a silent nuclear compartment by *Xist* RNA

Soon after *Xist* is upregulated and accumulates on the X-chromosome *in cis*, the formation of a transcriptionally inert compartment within the *Xist* RNA domain can be observed within a relatively short period of time. Notably, the *Xist* RNA-coated chromosome shows rapid loss of the basic transcriptional machinery such as RNA POL II, TAF10 and TBP proteins, as well as a depletion of *Cot1* repetitive RNA and a loss of euchromatic histone modifications (Chaumeil et al. 2002, 2006; Okamoto et al. 2004). Importantly, the X-chromosome sequences lying within the

newly formed *Xist* domain are believed to consist mainly of silent repeats (Chaumeil et al. 2006; Chow et al. 2010). X-linked genes on the other hand are initially located outside or on the edge of this *Xist* RNA compartment but are moved into it as they become inactivated (Chaumeil et al. 2006; Chow et al. 2010) (Fig. 3b). Whether this relocation of genes into the *Xist* RNA compartment is a cause or a consequence of *Xist* RNA-mediated silencing, is still an open question. Interestingly, the appearance of this compartment does not depend on the silencing activity of *Xist*, since it is still formed in A-repeats *Xist* mutant. However, relocation of the genes into the compartment as they get silenced is dependent on the A-repeats (Chaumeil et al. 2006). Once XCI has been accomplished, the Xi is organised as a repetitive core surrounded by a rim of silent genes both in humans and mice (Chaumeil et al. 2006; Clemson et al. 2006). Only genes that escape XCI (including *Xist* itself) are located at the edge or outside of the *Xist* RNA domain in somatic cells (Chaumeil et al. 2006). Indeed, escape genes may be flanked by sequences permissive for their externalisation or looping out from the *Xist* compartment to be in contact with the transcription factor in the nucleoplasm (reviewed in Heard and Bickmore 2007).

The silent *Xist* RNA compartment initially encompasses X-chromosome repeats. Repetitive elements have long been implicated in silencing and heterochromatin formation (Eymery et al. 2009). In the context of XCI, LINEs and possibly other repeats within *Xist* RNA repressive compartment might have an important role by helping to nucleate heterochromatin of the chromosome. Consistent with this, genes in LINE-rich regions show much more efficient silencing and relocalization into the inactive *Xist* RNA compartment than genes in LINE-poor regions based on studies on ectopic *Xist* expression on autosomes (Chow et al. 2010; Tang et al. 2010). Genes escaping silencing in LINE-poor regions remain at distances far from the repressive compartment (Chow et al. 2010). Therefore, the high density of repeats, in particular, LINEs on the X-chromosome might provide a helpful environment for heterochromatin formation and the spread of inactivation. Interestingly, in regions of the X-chromosome that are prone to escape silencing (for example, around the *Huwe1/Jarid1c* locus), expression of young, active LINE-1 elements has been observed on the X-chromosome undergoing inactivation during ES cell differentiation (Chow et al. 2010). It has been proposed that these transcribed LINEs might facilitate the local propagation of XCI, especially into regions of the X-chromosome that are otherwise prone to escape.

Chromatin changes during X-chromosome inactivation

The chromatin of the Xi is dramatically altered following *Xist* RNA coating. The alterations appear to occur in a

step-wise fashion and two major waves of events have been described which may or may not be inter-dependent. The initial wave of chromatin changes is tightly coupled to *Xist* coating, occurring within the first 2–3 days of ES cell differentiation. The second wave (4–8 day of differentiation) corresponds to the shift from reversible to irreversible XCI and may depend on the changes induced during the first wave (Wutz and Jaenisch 2000) and is accompanied by the acquisition of a chromosomal memory of unknown nature (Kohlmaier et al. 2004). Extinction of *Xist* expression after this time window (first 3 days of differentiation) does not cause major reactivation of genes, even if some of the characteristics of the Xi chromatin are disrupted (Csankovszki et al. 2001; Kohlmaier et al. 2004; Pullirsch et al. 2010; Wutz and Jaenisch 2000; Zhang et al. 2007).

It remains largely unknown how *Xist* mediates these chromatin events and whether they are direct or indirect consequences of *Xist* RNA coating. Nevertheless, it is important to consider the types of processes that could be implicated in the generation of facultative heterochromatin as a consequence of *Xist* coating. One mechanism is the recruitment of histone modifying complexes that would lay down post-translational modifications (PTM) on histones, mainly on their N-terminal tails that protrude out from the nucleosome, the fundamental unit of chromatin. Several PTMs have been described such as methylation, acetylation and ubiquitylation among others that have a direct impact on transcription, repair, replication or condensation (reviewed in Kouzarides 2007). PTMs could either disrupt the contacts between adjacent nucleosomes or recruit nonhistone proteins, their “readers” (Bannister and Kouzarides 2011). Indeed, the notion of a histone code (Strahl and Allis 2000; Turner 2000) whereby combinations of histone modifications could be targets for specific proteins is attractive, particularly in the case of the Xi to provide a specific signature of facultative heterochromatin that would distinguish it from constitutive heterochromatin and from euchromatin. A second mechanism that could be implicated in the heterochromatinization of the Xi is the replacement of histones by new synthesised histones including new histone variants. Histone variants have sequence variations providing a means to generate diversity in the chromatin structure (Banaszynski et al. 2010). A third mechanism could be the recruitment of chromatin remodelling machinery that changes accessibility to the DNA sequence by remodelling nucleosomes in an ATP-dependent manner. Finally, the recruitment of structural chromosome proteins, such as condensins and cohesions, able to affect higher order chromatin conformation and the compaction state of the chromosomes (Hirano 2006), as has been found for X-chromosome dosage compensation in *C. elegans* (Meyer 2010) might confer a fourth mechanism

through which *Xist* coating might cause the heterochromatinization of the Xi.

Early chromatin changes during X-chromosome inactivation

Soon after *Xist* RNA accumulates a depletion in the histone modifications generally associated with gene activity, such as H3K4me2/3, H3K36 methylation and H3 and H4

acetylation is observed for the part of the X-chromosome within the *Xist* RNA domain, based on immunofluorescence (IF) detection (Chaumeil et al. 2002, 2006; Heard et al. 2001; Keohane et al. 1996; O'Neill et al. 2008). Shortly after this loss of active marks, an enrichment of repressive marks is observed—including H3K27me3, H3K9me2, H4K20me1, H2AK119ub and others (Fig. 4b). This first wave of alterations in the chromatin landscape of the X-chromosome chosen for inactivation happens within

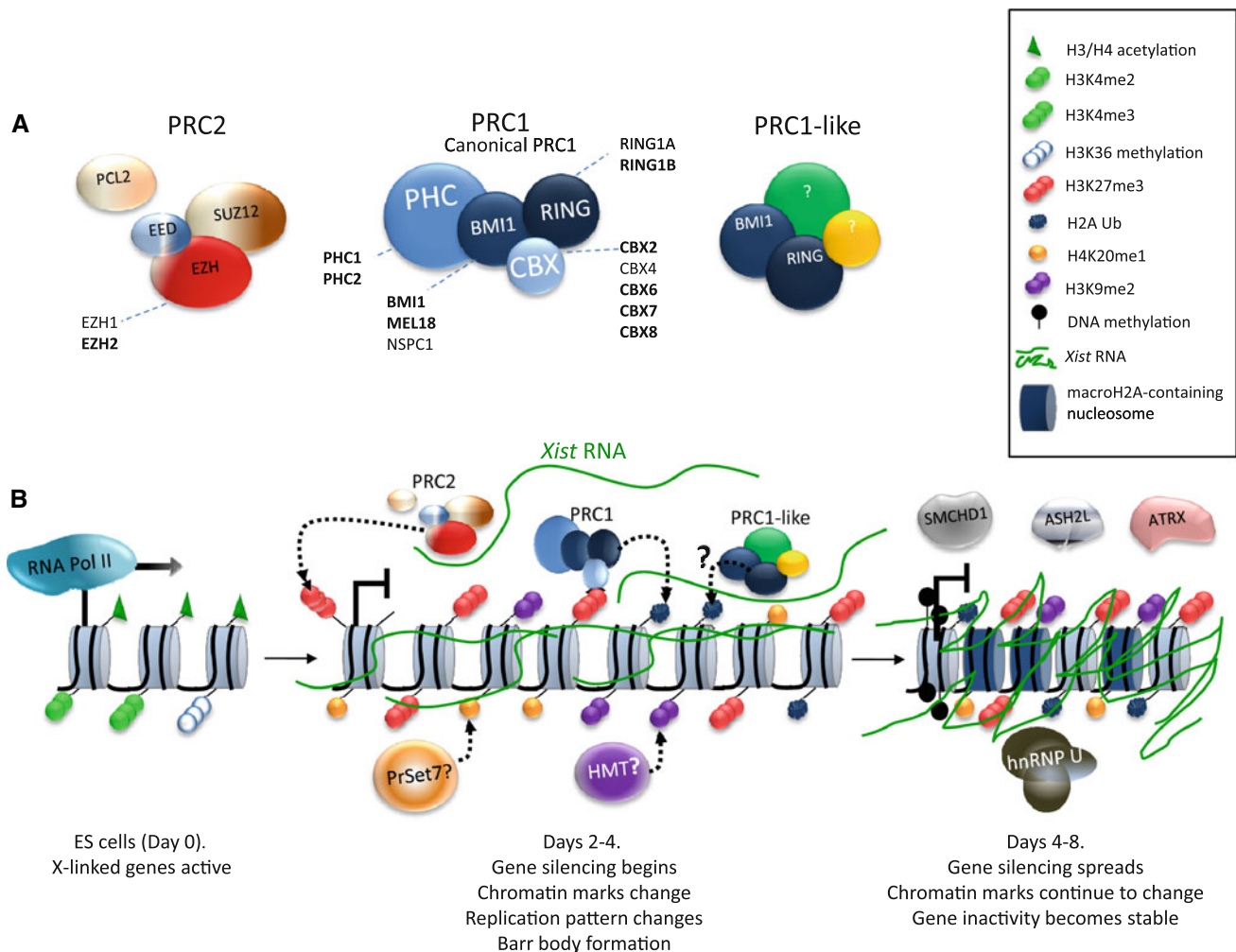


Fig. 4 Chromatin dynamics at the initiation of the XCI. **a** Composition of the polycomb group 2 and the polycomb group 1 complexes. PRC2 is composed of three core elements SUZ12, EED and EZH and several co-factors including PCL2. The PRC1 complex has four core components: CBX, BMI1, RING and PHC. RING and the BMI1 component can also be found in other kind of complexes that we mention as PRC1-like complexes. All the alternative components are shown and in *bold* highlights the ones that have been documented as enriched in the Xi. **b** Schematic representation of the epigenetic events during XCI. In undifferentiated ES cells, the chromatin around the promoter of an X-linked gene is shown transcribed, with the presence of euchromatic marks such as H3K4me2/3, H3K36 methylation and H3 and H4 acetylation. Days 2–4: when differentiation is induced, *Xist* is upregulated on one X-chromosome and coats the

X-chromosome from which it is expressed in *cis*. Gene silencing is initiated and *Xist*-mediated chromatin modifying complexes such as PRC1 and PRC2 and others are recruited to the Xi, resulting in the accumulation of PTMs typical of facultative heterochromatin such as H3K27me3, H2AK199ub, H3K9me2 and H4K20me1. The replication pattern of the Xi changes and the condensation of the Xi become apparent. Days 4–8: during this period, gene silencing is spread and maintained thereafter. Further epigenetic events take place such as the acquisition of DNA methylation at the CpGs of the 5' end of genes and the incorporation of macroH2A histone variant; chromatin modifications acquired previously are maintained; there is also the recruitment of different proteins such as SMCHD1, ASH2L, ATRX and hnRNP U that will lock in the inactive state

a relatively small time window lasting no more than 1–2 cell cycles. Indeed, whether cell division is actually required for some of these chromatin changes to take place remains an open question. It is also during this time window that gene silencing initiates on the X-chromosome, with genes shifting from outside to inside the *Xist* RNA compartment, which is depleted for actively marked chromatin and enriched for inactive chromatin, as they become silenced. The dynamics of gene silencing (and relocation) differs greatly among X-linked genes during ES cell differentiation (Chow et al. 2010; Lin et al. 2007; Patrat et al. 2009). The Xi also shifts its replication timing (Casas-Delucchi et al. 2011) and assumes a more compact conformation during this time window. Thus, the early wave of chromatin changes affecting the *Xist* RNA-coated X-chromosome in the mouse is preceded by a change in nuclear organisation of the X-chromosome and followed by a temporal change in replication. The interdependence between these events still remains to be defined (Chadwick and Willard 2002; Okamoto and Heard 2009; Okamoto et al. 2004) (Figs. 3b, 4b).

Potential mechanisms involved in early chromatin changes on the inactive X-chromosome

The role of chromatin modifications in XCI and the mechanisms by which these changes are induced are still largely mysterious. First we consider the mechanisms by which euchromatic marks are lost from the Xi. H3K4 methylation is a mark normally associated with active transcription, with H3K4me3 and H3K4me2 being strongly enriched at active promoters, whereas H3K4me1 has been more associated with enhancers (Heintzman et al. 2007). Rapid global loss of H3K4 methylation marks, based on immunofluorescence detection, is one of the earliest chromatin events observed during XCI (Chaumeil et al. 2002; Heard et al. 2001; O'Neill et al. 2008). Curiously, the three different forms behave differently during XCI, with the H3K4me3 mark being lost before H3K4me2, while H3K4me1 is in fact depleted on both the Xa and Xi compared with autosomes, during ES cell differentiation (O'Neill et al. 2008; da Rocha unpublished observations). Loss of these marks could either be passive through DNA replication and the exclusion in some way of the H3K4 methyltransferase complexes following *Xist* coating. The rapidity of this H3K4 methylation loss—maybe even within a single cell cycle—might imply active mechanisms, however. For example, histone exchange might affect the *Xist* RNA-coated X-chromosome; an alternative, not mutually exclusive mechanism, could involve histone demethylases (Christensen et al. 2007; Shi et al. 2004). The latter would be consistent with the more rapid loss of

H3K4me3 compared with H3K4me2. Chromatin-modifying factors recruited to the X-chromosome in a *Xist*-dependent manner could play a part in such active mechanisms of histone methylation loss.

The loss of histone acetylation on the Xi is another characteristic change during early XCI. Acetylation of histone lysine residues results in the neutralisation of the basic charge of this amino acid and is believed to open up chromatin present at promoters of active genes (reviewed in Kouzarides 2007). Histone hypoacetylation on the Xi is first observed for histone H3K9 and subsequently for histone H4 (H4K5ac, H3K8ac, H3K12ac) (Chaumeil et al. 2002) (Fig. 4b). This difference in timing could imply different mechanisms that may be involved. Studies using general deacetylase inhibitors such as Trichostatin A (TSA) suggest the implication of histone deacetylases of the HDAC family in XCI (Casas-Delucchi et al. 2011; Csankovszki et al. 2001; Do et al. 2008; O'Neill et al. 1999) in establishing the depletion, or in maintaining the deacetylated state, a hallmark of the Xi. To date, however, no specific implication of any of the 11 HDACs has been found during XCI, and indeed, some degree of redundancy might be anticipated among these enzymes.

Several histone PTMs become enriched on the *Xist* RNA-coated X-chromosome during XCI. The mechanisms underlying the recruitment of heterochromatic marks on the Xi are better understood. The enrichment of H3K9me2, H3K27me3, H4K20me1 and H2AK119ub are the best documented Xi marks in the mouse (de Napoles et al. 2004; Fang et al. 2004; Heard et al. 2001; Kohlmaier et al. 2004; Peters et al. 2002; Plath et al. 2003). Certain candidate modifying complexes responsible for these modifications are also observed to be recruited transiently at early stages of XCI (Kohlmaier et al. 2004; Schoeftner et al. 2006): in particular, the Polycomb repressive complex 2 (PRC2) that catalyses the tri-methylation of H3K27 (Plath et al. 2003; Silva et al. 2003) and the PRC1 complex that mono-ubiquitinates the K119 at H2A (de Napoles et al. 2004; Plath et al. 2004). These complexes and their recruitment to the Xi will be discussed below.

The H3K27me3 mark is strongly enriched on the Xi both at interphase and during mitosis (Plath et al. 2003; Silva et al. 2003). Recent ChIP-seq data in differentiating mouse female ES cells has shown that this mark is enriched fairly uniformly across the Xi, but preferentially in gene-rich regions. H3K27me3 is present at promoters, but not restricted to them and does not seem to spread from the *Xist* locus (Marks et al. 2009). Far less is known about the mechanisms of Xi enrichment of H3K9me2 and H4K20me1. Several H3K9 MTases are known to exist in the mouse and one or more of these might be implicated. H4K20me1 seems to be cell-cycle regulated, only appearing

on the Xi during late S phase and mitosis (Oda et al. 2009). This closely matches the expression pattern of the only H4K20 monomethyltransferase known, Pr-SET7 (Oda et al. 2010), suggesting that this could indeed be responsible for this mark. The precise role of these different histone modifiers and modifications to Xi heterochromatin formation remains largely to be determined. Functional studies involving knock outs are confounded by redundancy on the one hand and lethality due to pleiotropic effects on the other (Oda et al. 2009).

Considerable progress has been made in recent years on our understanding of the Polycomb complexes in XCI. Genetic studies point to a critical role for PRC2 in the maintenance of the inactive state (Wang et al. 2001) at least in extra-embryonic tissues of the mouse. The PRC2 complex is composed of three major components, the histone methyltransferase EZH2 (or EZH1), the zinc-finger-containing SUZ12 and the WD40 repeat protein EED that are essential both for the stabilisation and for the methyltransferase activity of EZHs proteins (reviewed in Margueron and Reinberg 2011). Several other PRC2 co-factors have recently been reported in ES cells and other proliferative cells such as the Polycomb-like 2 protein—PCL2/MTF2 (Fig. 4a). PRC2 components are very quickly recruited to the X-chromosome, after *Xist* RNA coating and at the same time as H3K27me3 enrichment is observed (Fig. 4) (Plath et al. 2003; Silva et al. 2003). Interestingly, the enrichment of PRC2 complex is transient—only being observed in the first 2–5 days of differentiation, even though H3K27me3 persists thereafter (Plath et al. 2003; Silva et al. 2003). The Polycomb complex 1 is recruited at very similar stages to PRC2 (de Napoles et al. 2004; Fang et al. 2004; Plath et al. 2004). PRC1 is composed of four subunits, containing an E3 ubiquitin ligase (either RING1A/RING1 or RING1B/RNF2) responsible for the ubiquitylation of K119 at histone H2A. In contrast to PRC2, there are several PRC1 complexes due to the great number of paralogs of its subunits (Fig. 4a) (reviewed in Simon and Kingston 2009). Given the numerous putative combinations of PRC1 complexes, it is not surprising that the individual proteins may have different kinetics, or degree of recruitment to the Xi. For example, among the five CBX subunits, CBX7 seems to be preferentially associated with the Xi (Bernstein et al. 2006). Unlike PRC2 complex, PRC1 proteins do not seem to be enriched on all cells with a Xi (de Napoles et al. 2004; Plath et al. 2004; Schoeftner et al. 2006). Intriguingly, the ubiquitin ligase subunit RING1B has been found enriched in 50% of *Xist* RNA domains during interphase and on mitotic chromosomes (Fang et al. 2004; Schoeftner et al. 2006), but the mark that this complex lays down, H2AK119ub, is present on the Xi in the majority of *Xist* RNA domains throughout the cell cycle (de Napoles et al. 2004; Plath et al. 2004; Schoeftner et al. 2006).

PRC1 recruitment to chromatin has been classically defined to rely on PRC2-dependent deposition of H3K27me3. The tri-methylation of H3K27 is then thought to be recognised by one of the Polycomb homolog, CBX proteins, via its chromodomain, which will bring with it the PRC1 complex, leading to both RING1B/RING1A-mediated H2AK119ub, the establishment of chromatin condensation and the maintenance of stable gene silencing (Fig. 4b) (reviewed in Simon and Kingston 2009). This series of events may hold true for the Xi in extra-embryonic tissues, as embryos mutated for the PRC2 component EED fail to recruit PRC1 and H2AK119ub to their Xi (Kalantry et al. 2006). Interestingly, the same does not hold true in the ES cell system. Loss of EED in a male cell line, in which ectopic *Xist* expression can be induced from an autosome, results in failure to recruit some PRC1 subunits, but does not affect RING1B recruitment or enrichment of H2AK119ub (Schoeftner et al. 2006). Thus, RING1B can be recruited to the Xi in the absence of H3K27me3—perhaps in a *Xist* RNA-mediated manner by some other protein complex. In this context it is noteworthy that RING1B is part of other PRC1-like complexes (Gearhart et al. 2006; Sanchez et al. 2007) (Fig. 4a). It remains to be seen if PRC2-independent recruitment of PRC1 components also occurs in a normal female context where *Xist* is not ectopically induced at high levels.

The possible mechanism of PRC2 recruitment to the Xi has been subject of high interest. EZH2 has been extensively studied as the putative binder to long non-coding RNAs that might be important for PRC2 recruitment to target genomic sites (Kaneko et al. 2011; Zhao et al. 2010) and in the case of XCI, both EZH2 and SUZ12 have been shown to bind to the A-repeat region of the *Xist* transcript in undifferentiated and differentiating ES cells (Kaneko et al. 2011; Kanhere et al. 2010; Maenner et al. 2010; Zhao et al. 2008) (Fig. 4). The A-repeat element is unlikely to be the only means by which PRC2 is recruited to the Xi, however, since an inducible *Xist* gene deleted for the A-repeat region still results in global H3K27me3 accumulation, in early differentiating ESCs, although with a slower kinetics (Kohlmaier et al. 2004; Plath et al. 2003). A recent study has shown that the Polycomb-like 2 protein (PCL2/MTF2), a co-factor of PRC2 in ES cells and proliferative cells (Li et al. 2010; Walker et al. 2010) affects, to some extent, PRC2 recruitment or maintenance and the deposition of H3K27me3 to the Xi (Casanova et al. 2011) (Fig. 4). The relative contributions of the *Xist* A-repeat PRC2 association, versus a PCL2-mediated mechanism for PRC2 accumulation on the Xi remain unclear. One hypothesis could be that PRC2 is recruited directly by *Xist* RNA via its A-repeat region (and perhaps helped by other parts of the RNA) and the association with the Xi is then stabilised by the recognition of specific histone modifications, via the two PHD

domains or the TUDOR domain of PCL2 (Fig. 4). However, it is still not clear whether EZH2 binding to the *Xist* RNA is a direct association, nor whether the role of PCL2, or other PRC2 co-factors, is to recruit or to maintain PRC2 deposition on the Xi.

Chromatin landscape of the inactive X-chromosome in other mammals

Although most studies on chromatin changes during the onset of XCI have been conducted in the mouse, there are several reports on the characteristics of the Xi in somatic cells of other mammals, even though this was not during the onset of XCI (Table 1). In general, depletion of euchromatic marks is consistently observed on the Xi in all mammals (Chadwick and Willard 2003; Chaumeil et al. 2002; Koina et al. 2009; Rens et al. 2010). On the other hand, some of the repressive marks associated with the Xi in humans and marsupials are different (Table 1). For example, in humans it has been suggested that the Xi heterochromatin can be subdivided into two sub-types. One of these closely resembles the mouse Xi, characterised by *Xist* RNA coating, H3K27me3 and macroH2A; the second resembles constitutive heterochromatin characterised by H3K9me3, H4K20me3 and binding of heterochromatin protein 1 (HP1) (Chadwick and Willard 2004), which are not observed to be particularly enriched on the mouse Xi. In the case of the marsupial Xi, H3K9me2 and H3K27me3 are present but its enrichment seems to be less striking compared with mouse and human, being restricted to specific windows of the cell cycle. Constitutive heterochromatin marks such as H3K9me3, H4K20me3 and HP1 are also present on the marsupial Xi (Chaumeil et al. 2011; Koina et al. 2009; Mahadevaiah et al. 2009; Rens et al. 2010; Zakharova et al. 2011). All in all, these differences between mammals suggest that the Xi can be constituted of several types of heterochromatin. The exact constitution of these chromatin signatures may be dependent on differences in X-chromosome sequence content between species on the one hand and on differences in the nature and recruitment of chromatin-modifying enzymes on the other.

Replication timing of the inactive X-chromosome

One of the most conserved and well-documented features of the Xi concerns its replication timing. The Xi has been shown to replicate asynchronously with respect to its active homologue in the human, mouse, rabbit and marsupial (Gartler and Riggs 1983; Kinsey 1967; Robinson et al. 1994; Sharman 1971). Asynchronous replication of the Xi compared with its active counterpart could provide temporal segregation of the two X-chromosomes and minimise the exposure of the Xi to transcription factors, thus

facilitating the maintenance of transcriptional silencing. Indeed, asynchronous replication is the most evolutionarily conserved feature of the Xi (reviewed in Heard and Bickmore 2007). In mouse, in adult somatic cells and in most tissues of the embryo, the Xi is thought to replicate during late S phase, except in extra-embryonic tissues, where early replication has been found (Takagi and Sasaki 1975). In a recent publication, evidence was found that the Xi can replicate during early to mid S phase in mouse myoblast C2C12 cells and MEFs (Casas-Delucchi et al. 2011). The general conclusion seems to be that the bulk of the Xi tends to replicate as a single block, which is often, but not always, asynchronous in its replication timing when compared with the Xa and the rest of the genome.

The time of onset of asynchronous replication during the initiation of XCI corresponds to the first 2–3 days of differentiation which is the time window in which *Xist*-mediated gene silencing and chromatin changes occur (Chaumeil et al. 2002; Keohane et al. 1996) (Fig. 4). A recent study suggested that histone hypoacetylation could be causal in the change in replication timing of the Xi although this remains to be further investigated as the study was based on TSA treatment with arguably pleiotropic effects (Casas-Delucchi et al. 2011). What exactly the replication profile of the Xi represents in terms of origin firing and usage and whether it plays a role in the initiation or maintenance of XCI still remain open questions. At the single gene level in humans, late replication timing is highly correlated with the transcriptional activity of specific genes, rather than being a global chromosome-wide characteristic of the Xi, as late replicating X-linked genes were found to be transcriptionally inactive, whereas genes that escape XCI are synchronously replicated on the Xa and the Xi (Boggs and Chinault 1994).

Nuclear compartmentalization of the inactive X-chromosome

In addition to chromatin marks and asynchronous replication, the spatial segregation of the two X-chromosomes into different nuclear compartments might also help in the differential treatment required to adopt and maintain opposite epigenetic and transcriptional states (Cremer et al. 2006). Indeed, as discussed above, the Xi in both mouse and human is organised as a repetitive inner core surrounded by genes and *Xist* RNA localised often in contact with the nuclear periphery or with the nucleolus (Chaumeil et al. 2006; Clemson et al. 2006; Rego et al. 2008; Zhang et al. 2007). It has also been proposed that the Xi migrates to a perinucleolar compartment that is specialised in replicating condensed chromatin (SNF2H-enriched) during mid-to-late S phase, in order to faithfully duplicate its epigenetic marks and stably repress resident genes (Zhang

Table 1 Comparison of the epigenetic marks that characterised the facultative heterochromatin of the Xi in the mouse, humans and marsupials

Epigenetic marks/factors	Mouse	Human	Marsupials
Xist coating	+	+	–
	(Interphase and mitosis)	(Interphase)	
H3K4 hypomethylation	+	+	+
	(Interphase and mitosis)	(Interphase and mitosis)	(Interphase and mitosis)
H3/H4 hypoacetylation	+	+	+
	(Interphase and mitosis)	(Interphase and mitosis)	(Interphase and mitosis)
H3K36 hypomethylation	+	+	nd
H3R17 hypomethylation	+	+	+
		(Interphase only)	
H3K9me2	+	+	+
	(Interphase and mitosis)	(Interphase and mitosis)	(Late S phase and G2)
			Enrichment less striking compared to mouse and humans
H3K9me3	–	+	+
		(Interphase and mitosis)	(Interphase and mitosis)
		Banded pattern	
H3K27me3	+	+	+
	(Interphase and mitosis)	(Interphase and mitosis)	(Late S phase, G2 and mitosis)
		Banded pattern	Enrichment less striking compared to mouse and humans
H4K20me1	+	nd	–
	(Interphase and mitosis)		
H4K20me3	–	+	+
		(Interphase and mitosis)	(Interphase and mitosis)
		Banded pattern	
H2AK119ub	+	nd	nd
	(Interphase and mitosis)		
macroH2A	+	+	nd
	(Interphase and mitosis)	(Interphase and mitosis)	
		Banded pattern	
HP1	–	+	+
		(Interphase)	(Interphase)
DNA methylation at 5' end of genes	+	+	–
		(Less in the placenta)	

+: presence; –: absence; nd: not determined; in brackets is the presence or absence of the mark during cell cycle when determined

et al. 2007). Whether this location is a cause or consequence of the inactive state is still not clear. Nevertheless, we can conclude that the Xi is not only epigenetically marked at the level of its chromatin, but also in terms of its temporal and spatial segregation.

Inactive X-chromosome compaction and higher order chromatin conformation

The Xi is detected cytologically as a condensed DNA mass using common nucleic acid dyes. Barr and Bertram (1949) first observed that this sub-nuclear structure is kept in many female cells in different organisms, and hence it is

commonly called the Barr body. Analysis at the ultra-structural level by electron microscopy has revealed that the Xi does not appear as a solid mass similar to pericentric heterochromatin, but rather it appears as a series of separate, but tightly packed fibres (Rego et al. 2008). This difference in structure may reflect the different chromatin composition of facultative heterochromatin of the Xi when compared with constitutive heterochromatin. In terms of volume, the Xi may be slightly smaller than the Xa (Naughton et al. 2010), consistent with Xi being more condensed, although other studies have reported that the volume is similar but the overall texture is different (Chaumeil et al. 2006; Clemson et al. 2006). Compaction

of the Xi is an early event as the DAPI-dense Barr body is detected early in differentiation following *Xist* coating (Chaumeil et al. 2006). It may undergo a further degree of compaction and/or higher order organisation at later stages based on FISH studies (Chow et al. 2010). Biochemical approaches, involving chromatin fractionation after micrococcal nuclease digestion, have shown that both the Xa and Xi have similar bulk 30 nm chromatin fibre structures in human somatic cells, its differences only being detected locally at the promoters of genes that are expressed on the Xa and silent on the Xi. Analysis of large-scale chromatin compaction using 3D RNA/DNA FISH with probes 2 Mb apart revealed that only in gene-rich regions, the Xi indeed shows more compaction than the Xa (Naughton et al. 2010).

The molecular basis of the more compact state of the Xi remains to be elucidated but is likely to depend on multiple aspects of its chromatin content and associated proteins and nucleic acids. One candidate could be the PRC1 component, RING1B, which has recently been shown to compact chromatin in vivo in the *Hox* cluster independently of its ubiquitination activity (Eskeland et al. 2010). So far no such role has been found on the Xi (de Napoles et al. 2004), but this could also be due to redundant roles of different chromatin factors.

Late epigenetic changes and chromosome structure of the Xi

Although major alterations in gene activity, chromatin status, nuclear compartmentalisation and replication timing of the Xi are observed in the first few days of differentiation, XCI is only fully accomplished several days later (Chow et al. 2010).

In this second wave (day 4 of differentiation onwards), many of the changes initiated during the first wave are maintained or extended to other regions (Marks et al. 2009). Interestingly, marks such as H3K27me3 and H2AK119ub are maintained even though the enrichment of PRC2 and PRC1 complexes are no longer detectable by IF (de Napoles et al. 2004; Silva et al. 2003). Instead, new players such as the Trithorax protein ASH2L and the chromatin remodelling factor ATRX (Baumann and De La Fuente 2009; Pullirsch et al. 2010) appear on the Xi (Fig. 4b). The roles of these proteins in XCI remain to be assessed. At first sight, the recruitment of ASH2L is counter-intuitive since it belongs to a family of H3K4 methyltransferases—which lead to histone modifications that are actually depleted on the Xi. However, it cannot be excluded that transient H3K4 methylation may be followed by rapid demethylation on the Xi during the cell cycle. Alternatively, ASH2L on the Xi could reflect another function of this protein. Another protein that is enriched

during this period is the matrix attachment protein hnRNP U, important for RNA coating (Pullirsch et al. 2010). During this later phase, DNA methylation at promoters of X-linked genes, a step that seems to be very important for the maintenance of the inactive state, is also believed to occur. DNA methylation is somehow dependent on the recently discovered structural chromosome protein SMCHD1, which results in loss of DNA methylation and some degree of X-linked gene reactivation in mice mutated for this gene (Blewitt et al. 2008). Another chromatin change during this second phase is the recruitment of the histone variant macroH2A (Costanzi and Pehrson 1998). Finally, the organisation of the Xi also seems to become altered between days 4 and 8 of differentiation (Chow et al. 2010) (Figs. 3b, 4b). The changes during this second phase of the XCI process are likely to be involved in maintenance and stable locking in of the Xi.

DNA methylation and XCI

DNA methylation has long been considered to be an important feature of the Xi and the maintenance of the inactive state (Riggs and Xiong 2004). Methylation patterns can be inherited from cell to cell, thanks to maintenance DNA methyltransferase activity that recognises and methylates hemimethylated CpG sites during DNA replication (reviewed in Altun et al. 2010). Although promoters and CpG islands of X-linked genes tend to be methylated on the Xi (Norris et al. 1991; Tribioli et al. 1992), the overall methylation levels have been shown to be less than those on the Xa by cytogenetic studies in both humans and marsupials (Loebel and Johnston 1993; Viegas-Pequignot et al. 1988). This has been confirmed by recent studies using global microarray analyses involving methylated DNA immunoprecipitation (MeDIP) to assess the DNA methylation status of the Xi relative to the Xa in human primary cells (Weber et al. 2007). Intriguingly, the Xa shows increased methylation levels in gene bodies compared with the Xi, although the significance of such gene-body methylation still remains to be elucidated (Hellman and Chess 2007). The precise kinetics of DNA hypermethylation at promoters during XCI has never been systematically investigated, but should now be feasible thanks to next generation sequencing technologies that enable allele-specific assessment of epigenomic states.

Although the mechanism by which DNA methylation becomes recruited to different genes on the Xi is still not clear, this mark is an important player in the maintenance of the inactive state in somatic cells of eutherian mammals, as shown by studies involving 5-aza-C treatment or deletion of the maintenance DNA methyltransferase, *Dnmt1* (Csankovszki et al. 2001; Graves and Young 1982; Mohandas et al. 1981). Furthermore, instability of silencing

of the Xi has been reported in patients suffering immunodeficiency centromeric instability facial anomalies (ICF), a syndrome caused by a defect in the de novo DNA methyltransferase *DNMT3B* (Hansen et al. 2000). In both cases, the effects observed in derepression were minimal and gene-specific, arguing that other repressive mechanisms are sufficient to maintain silencing of most loci. Indeed, in extra-embryonic tissues the promoters of X-linked genes do not appear to be methylated on the Xi even though it is globally inactive. This may account for the greater dependence on Polycomb-mediated maintenance of Xi inactivity in these tissues (Wang et al. 2001). Furthermore, although DNA methylation on the Xi is found in all eutherians examined to date, it is not a feature of promoters of X-linked genes on the Xi in marsupials (Hornecker et al. 2007; Kaslow and Migeon 1987; Loebel and Johnston 1996). However, the precise extent to which this mark is present on the Xi in other mammals needs to be systematically elucidated.

XCI and the histone variant macroH2A

The only histone variant to date that has been found to be enriched on the Xi is macroH2A. This is an unusual histone variant consisting of a domain similar to that of the conventional H2A (H2A-like domain) fused to a large non-histone region (Pehrson and Fried 1992). The enrichment of macroH2A during XCI suggests that it might play a role in this process. The presence of this histone variant in chromatin is correlated with transcriptional repression. The chromatin-remodelling complex SWI/SNF is unable to remodel in the presence of macroH2A. MacroH2A also represses RNA polymerase II transcription (Doyen et al. 2006). In agreement with this, macroH2A is depleted from active genes (Changolkar and Pehrson 2006). Three macroH2A variants were described (Changolkar and Pehrson 2006); two of them have been shown to be enriched on the Xi (Chadwick et al. 2001; Costanzi and Pehrson 1998) from around day 4–5.

If studies in human and mouse indicate that macroH2A is enriched on the Xi (Chadwick et al. 2001; Costanzi et al. 2000). MacroH2A was found to be uniformly distributed across the entire Xi chromosome by ChiP-on-chip analysis in human liver cells. This result suggests a potential role for macroH2A in large-scale chromosome structure, rather than directly inhibiting transcription and in genome stability (Mietton et al. 2009). Cell cycle analysis in human and mouse show high local concentration of macroH2A during S phase. This may be one of many redundant mechanisms to ensure the replication of the inactive state (Chadwick and Willard 2002). Interestingly, macroH2A like PTMs such as H2AK199ub and H3K27me3, remains

associated with both the human and mouse Xi during mitosis. MacroH2A is detected as distinct bands that appear to mimic the bands seen with *Xist* RNA on the human Xi during mitosis (Chadwick and Willard 2004).

Deletion of *Xist* RNA leads to loss of macroH2A on the Xi (Csankovszki et al. 1999). *Xist*-dependent recruitment is supported by a *Xist*-inducible systems in XY cells (Wutz et al. 2002) in which cell differentiation and *Xist* induction were sufficient to recruit macroH2A (Pullirsch et al. 2010). However, macroH2A recruitment to the Xi does not depend on *Xist* transcript's silencing function, since is still recruited in the A-repeats *Xist* mutant (Pullirsch et al. 2010). The rather late recruitment of macroH2A to the Xi (>day4) suggests that it has a role in the maintenance of the inactive state. However, just as other chromatin marks of the Xi, it is not essential since its deletion does not result in detectable re-activation of the genes on the Xi or impact in any measurable way on the XCI process (Changolkar et al. 2007). Nevertheless, conditional mutations of this variant might reveal a role at different stages of development.

Perspectives on epigenetic mechanisms and x-linked gene silencing during X-chromosome inactivation

Changes in chromatin composition and DNA methylation accompany the progressive gene silencing observed in the Xi, resulting in a remarkable locked state very refractory to reactivation and inherited through cell division. As we have seen, although some of these chromatin changes may be involved in the gene-silencing events during XCI, most of the evidence points to such changes representing epigenetic marks, that provide the cellular memory of the inactive state, at least, in certain developmental contexts.

The loss of euchromatic marks, such as H3K4 methylation and H3 acetylation are the first described chromatin modifications in the Xi. Therefore, we could speculate that they might be the initial triggering of silencing, resulting in loss of the transcriptional machinery that is then maintained by the recruitment of heterochromatic marks. This remains to be assessed. However, it could well be that loss of euchromatic marks is just a consequence of the removal of the transcriptional machinery, as genes become recruited into the silent *Xist* RNA compartment. On the other hand, removal of euchromatic marks on the Xi could be a crucial step for the subsequent recruitment of certain chromatin-modifying complexes. The coordinated regulation of gene repression by the H3K4 demethylase JARID1A together with PRC2 has been suggested to occur for a fraction of PRC2 target genes during ES cell differentiation (Pasini et al. 2008). It will be interesting to know whether, for example, the loss of H3K4 methylation has a similar

impact on the efficiency of PRC2 recruitment in the context of XCI.

H3K27me3 is the histone mark that has been mostly extensively studied in the context of XCI. However, this modification clearly does not provide the initial trigger for gene silencing along the Xi. In the *Eed*^{-/-} mouse, devoid of H3K27me3, the Xi remains silenced at the blastocyst stage in both ICM and trophoectoderm (Kalantry and Magnuson 2006; Kalantry et al. 2006; Wang et al. 2001). *Xist*-mediated silencing is also not significantly perturbed in ES cells lacking *Eed* (Schoeftner et al. 2006) or knockdown for the three core PRC2 proteins (Zhao et al. 2008). Furthermore, H3K27me3 is well recruited to the Xi by the *Xist* A-repeat mutant described as being incompetent for gene silencing (Kohlmaier et al. 2004). Therefore, global H3K27me3 enrichment is not sufficient to cause gene silencing. Importantly, however, a role for this mark in maintenance of X-linked gene silencing of the imprinted XCI in the murine extra-embryonic differentiated trophoblast has been established. Mice deleted for *Eed* can initiate imprinted XCI, but reactivation of X-linked genes and a GFP reporter transgene are observed in the extra-embryonic differentiated trophoblast (Kalantry et al. 2006; Wang et al. 2001). Curiously, general X-linked inactivation is still observed in undifferentiated trophoblast in these mutants, a stage when EED levels are normally highly enriched in the Xi. At this stage, *Eed* mutants begin to lose *Xist* RNA coating and heterochromatic marks (H3K27me3, H4K20me1, H2Aub and macroH2A), but reactivation of X-linked genes occurs only in differentiated trophoblast cells, coinciding with the time when H3K4me2 and H3 and H4 acetylation is regained in the Xi (Kalantry et al. 2006). The mechanism by which H3K27me3 loss affects maintenance of XCI in such cells remains unclear. Interestingly, reactivation of X-linked expression is not observed in other extra-embryonic lineages such as visceral endoderm and extra-embryonic ectoderm (Kalantry et al. 2006) and random XCI is not affected in the embryonic tissues (Kalantry and Magnuson 2006).

As discussed above, DNA methylation of X-linked promoters is considered a late epigenetic event in the process of XCI, acquired after initial transcriptional repression. Failure to maintain silencing through DNA methylation loss during development has been described in mice mutated for the *Smchd1* gene, resulting in female-specific death at around E11.5 (Blewitt et al. 2008). The SMCHD1 protein has a SMC hinge domain, also present in proteins of the cohesion/condensin family. Female mutants for this protein display normal *Xist* RNA coating and H3K27me3 enrichment on the Xi, but show little sign of DNA methylation at 5' end of X-linked genes when analysed at around E9.5–E10.5 (Blewitt et al. 2008). It remains to be understood whether the effect in DNA methylation is

direct or indirect and whether this is happening at the level of recruitment of the DNA methyltransferase machinery or at the maintenance of the methylated state once established. Furthermore, we cannot rule out that SMCHD1 has effects other than affecting DNA methylation that could also lead to X-linked gene reactivation. Whatever is the reason for failure to keep XCI, SMCHD1 remains the only chromatin/chromosome structure factor so far described to cause failure of maintenance of the inactive state of Xi in the embryo proper.

Maintenance of the inactive state of the Xi in females clearly involves different layers of epigenetic regulation, including delayed replication kinetics, nuclear organisation and chromatin changes such as histone hypoacetylation, as well as heterochromatic marks, macroH2A and DNA methylation. With the exception of *Smchd1*, loss of any one of these marks is not sufficient to unlock the inactive state of the Xi in somatic, embryonic cells (Csankovszki et al. 2001; Hernandez-Munoz et al. 2005; Nusinow et al. 2007). Indeed, synergy between epigenetic mechanisms appears to preserve and reinforce the inactive state. Dissecting out the exact nature of these synergies and the molecular links between epigenetic marks remains an exciting, though challenging prospect as it will require sophisticated molecular genetics tools (conditional knock outs and/or transgenes) combined with biochemistry and single cell analyses. Such studies are clearly relevant from a medical point of view, as the X-chromosome harbours many genes that affect human health, particularly mental retardation disorders. For example, in the case of X-linked disorders such as Rett syndrome, which causes severe neurological effects in girls who are heterozygote for mutations in the X-linked *MECP2* gene, understanding how the wild type, but silent allele of this gene on the Xi might be specifically induced to reactivate in affected cells, could provide a major step forward in terms of therapeutic strategies.

In conclusion, in the 50 years since Mary Lyon first proposed the process of XCI, we have a much deeper understanding of this phenomenon, but there is still a long road to its full understanding ahead of us. In particular, the major differences that are emerging in the X-inactivation strategies in different mammals suggest that this process will need to be explored not just in the mouse, but also in other species.

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