# Chromatin Structure and Nuclear Organization Dynamics during X-Chromosome Inactivation

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Early development of female mammals is accompanied by transcriptional inactivation of one of their two X chromosomes. This leads to monoallelic expression of most of the X chromosome and ensures dosage compensation with respect to males (XY). One of the most surprising aspects of this phenomenon is that the two X homologs are treated differently even though they are present within the same nucleus. In eutherian mammals, such as humans and mice, either the maternal or the paternal X is inactivated during early embryogenesis. Once set up, the silent state is epigenetically transmitted as cells divide, so that adult females are mosaics of clonal cell populations, which express either of their two X chromosomes. The past years have been marked by the discovery of several molecular events that accompany chromosome-wide silencing.

A central player in the initiation of X-chromosome inactivation (XCI) is the long, noncoding Xist RNA (X inactive–specific transcript), which is produced only from the inactive X chromosome. Xist expression during early embryogenesis is both necessary (Marahrens et al. 1997, 1998) and sufficient (Wutz and Jaenisch 2000) for silencing to take place. A complex regulatory network ensures that Xist is up-regulated at a very precise stage of early development, exclusively in female cells, and only from one of the two X chromosomes (for review, see Nora and Heard 2009). Its ability to repress transcription strictly in *cis* has been linked to its unusual capacity to coat the chromosome from which it is produced (Fig. 1). Once Xist has been up-regulated during early development or during differentiation of mouse embryonic stem cells (mESCs), it continues to be expressed from the inactive X even in fully differentiated somatic cells. Nevertheless, it is actually dispensable for the maintenance of transcriptional repression (Wutz and Jaenisch 2000; Csankovszki et al. 2001), probably because Xist expression initiates an ordered series of changes in chromatin structure and chromosomal higher-order organization (Figs. 2 and 3), which together act in synergy to ensure epigenetic transmission of the inactive state. The mechanism by which transcriptional silencing is established by Xist RNA remains an important open question, however. The goal of this chapter is to review what is known regarding the changes in nuclear organization and chromatin structure mediated by Xist RNA and to discuss their links with the initiation or maintenance of transcriptional repression.

## XIST-MEDIATED REGULATION OF TRANSCRIPTION AND CHROMATIN STRUCTURE

Assessment of the kinetics with which XCI is established has demonstrated that the numerous features that ultimately distinguish the two X chromosomes in females do not all appear at the same time on the chromosome undergoing inactivation. Rather, changes happen in a stepwise fashion, both temporally during cellular differentiation and spatially across the 150 Mb of the X chromosome. This is also the case for transcriptional repression, with silencing kinetics being very variable along the X chromosome (Lin et al. 2007; Patrat et al. 2009; Chow et al. 2010). Immuno-FISH experiments, which enable both chromatin features and transcriptional activity to be analyzed simultaneously at the single-cell level (Chaumeil et al. 2008), have revealed that one of the earliest events following Xist coating of the X chromosome is the formation of a silent nuclear compartment corresponding to the repeat-rich core of the chromosome (Chaumeil et al. 2006). This fraction of the nuclear space is characterized by the absence of transcriptional activity, evident by the depletion of general transcription factors (such as the RNA polymerase II or TBP) and the absence of heterogeneous nuclear RNAs transcripts (hnRNAs, recognized by probes against the highly repetitive $C_0t-1$ fraction as in Fig. 2) (Hall et al. 2002). At this stage, loci are still transcribed but are found outside of the Xist RNA domain and remain in contact with the transcription machinery. Shortly after, selective enrichment or exclusion for numerous posttranslationally modified (PTM) histones occurs on the bulk of the X-chromosome territory that is coated by Xist. Silencing of genes is then established as differentiation proceeds, and is accompanied by their relocation into the Xist RNA-coated nuclear compartment (Chaumeil et al. 2006). It is still unknown whether this change in nuclear organization actively participates in transcriptional repression or whether it is simply a reflection of transcriptional status. Strikingly, loci that escape X inactivation remain outside of the Xist-coated compartment (Fig. 2). It is not clear whether silencing and the chromatin changes induced on the X chromosome by Xist RNA require cell division or DNA replication. Although a global

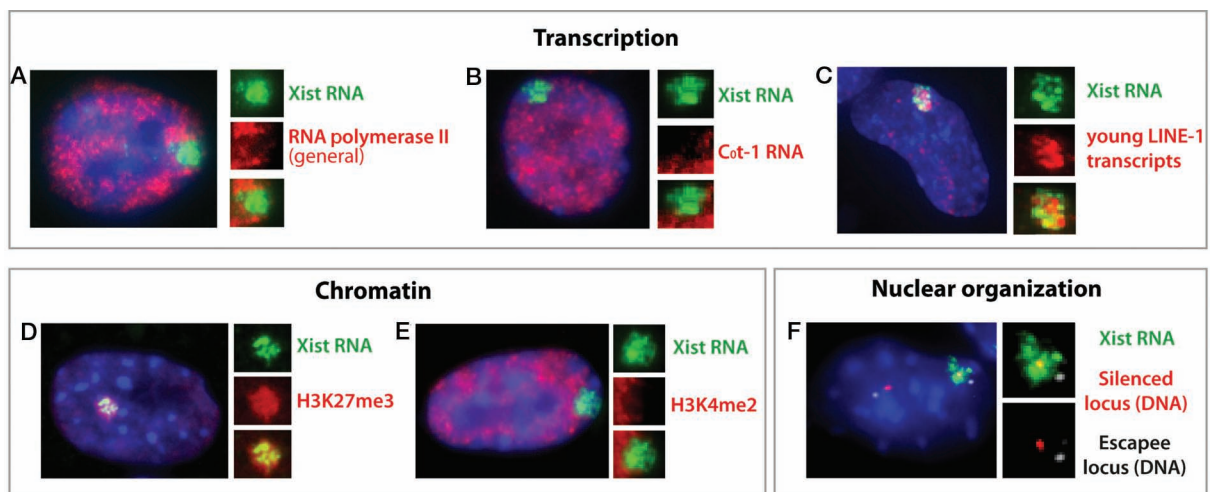## A  Xist RNA structure
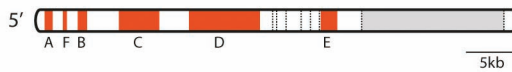


## B  Xist RNA expression pattern
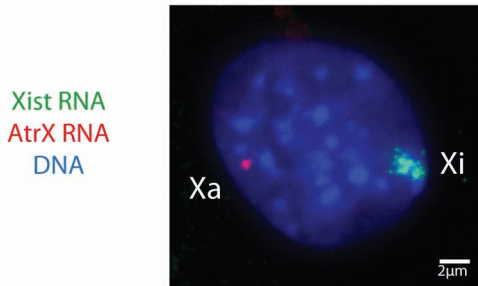


**Figure 1.** Xist RNA structure and expression pattern. (*A*) Dashed lines indicate the position of exon–exon junctions. (Gray box) An alternative exon identified by Ma and Strauss (2005). (Red boxes) The positions of tandem repeat elements, as identified in Brockdorff et al. (1992). (*B*) In female somatic cells, Xist RNA (green) paints the inactive X chromosome (Xi); nascent X-linked transcripts, here from the *Atrx* locus (red), can only be detected from the active X (Xa). DNA is counterstained by DAPI (blue).

shift to late replication timing is observed on the X chromosome undergoing inactivation, this only becomes evident later on in differentiation, suggesting that it is not necessary for the chromosome-wide changes observed early on (Chaumeil et al. 2002).

## PUTATIVE COFACTORS OF XIST RNA FOR THE ESTABLISHMENT OF THE INACTIVE STATE
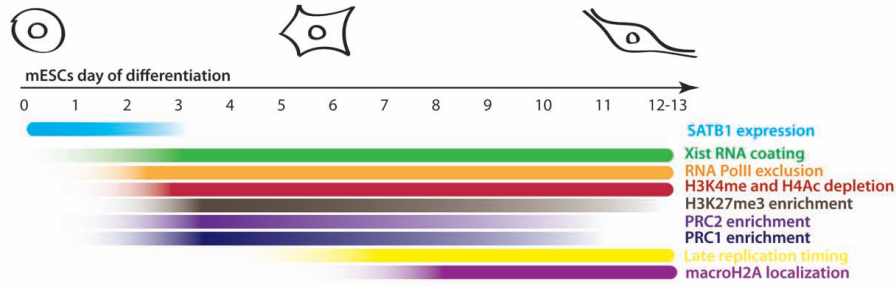
Xist-RNA-mediated chromatin modifications and silencing must rely on cofactors that are developmentally regulated. This has been demonstrated by the use of inducible Xist cDNA transgenes, which, when expressed in undifferentiated or early differentiating mouse embryonic stem cells, can trigger chromosome-wide chromatin changes and transcriptional repression (Wutz and Jaenisch 2000). In contrast, Xist expression cannot normally trigger X inactivation in differentiated cells (Fig. 3A–I). Recent studies suggest that this could be due to the developmentally restricted expression pattern of the matrix attachment protein SATB1, which is expressed in undifferentiated and early differentiating mESCs but not in most differentiated cell types (Fig. 3A) (Agrelo et al. 2009). One exception is adult hematopoietic precursors, which transiently reexpress SATB1, as well as lymphoma cells, and Xist induction leads to *cis*- inactivation in both of these cell types (Savarese et al. 2006; Agrelo et al. 2009). Interfering with SATB1 expression diminishes the efficiency at which Xist induction is able to trigger silencing (Agrelo et al. 2009).



**Figure 2.** Xist RNA coating defines a unique nuclear compartment and chromosomal structure. (*A*) (Immuno)-RNA FISH in differentiating female mESCs shows that the Xist RNA territory corresponds to a nuclear compartment devoid of RNA polymerase II, and (*B*) primary transcripts of the repetitive $C_0t – 1$ fraction. (*C*) At late differentiation stages, expression of young LINE-1 elements can, however, be detected from the inactive X. Chromatin of the inactive X chromosome is enriched in posttranslationally modified histones typically associated with transcriptional repression (*D*) and reciprocally depleted in marks generally found in transcribed regions (*E*). (*F*) Chromosome topology within the Xist domain: Loci that are silenced are usually within the Xist RNA domain, whereas escapee loci are usually located outside, as detected by combined RNA–DNA FISH.

**Figure 3.** Developmental time-line of X-inactivation and Xist RNA functions. (*A*) Simplified kinetics of XCI during differentiation of female mESCs. (*B*) Inducible Xist cDNA constructs have been used to define the time windows during which Xist can trigger chromosome-wide H3K27me3 enrichment or transcriptional silencing (Wutz and Jaenisch 2000). This has revealed that Xist expression can only efficiently induce H3K27me3 if expressed within the first 24 h, and silencing within 48 h (competence window for Xist action). H3K27me3 enrichment depends on continued Xist expression, whereas silencing can be maintained in the absence of Xist, provided initial repression was initially achieved between 48 and 72 h (switch to irreversible Xist-independent silencing). H3K27me3 and gene silencing can be reestablished in differentiated cells to some extent if Xist has been transiently expressed during the first 72 h (chromosomal memory). Of the developmentally regulated factors revealed by such experiments, SATB1 has been linked with Xist RNA's ability to achieve transcriptional repression, but not H3K27me3 enrichment (Agrelo et al. 2009). NB "Yes/No" pictograms are used here to represent trends, and readers are referred to Wutz and Jaenisch (2000), Kohlmaier et al. (2004), and Pullirsch et al. (2010) for original quantitative data.

Interestingly, ectopically expressing SATB1 in cells that normally do not express it, such as mouse embryonic fibroblasts, renders them competent for Xist-mediated silencing to some extent. SATB1 has also been previously implicated in the control of chromatin folding (Cai et al. 2006) and has been reported to physically interact with several chromatin-remodeling factors (Yasui et al. 2002), suggesting that this protein might have the ability to couple changes in chromatin composition and organization—events that are known to accompany Xist-mediated silencing. How SATB1 may render Xist RNA capable of triggering transcriptional repression remains unknown, however, and the fact that it is not enriched on the inactive X suggests that intermediate factors must exist. In addition, SATB1$^{-/-}$ female individuals can survive to birth, suggesting that other factors can supplement its function in X inactivation (Alvarez et al. 2000). Intriguingly, several human cell lines derived from tumors have been shown to be competent for XIST-mediated silencing (Hall et al. 2002; Plath et al. 2003; Chow et al. 2007; for review, see Agrelo and Wutz 2009); whether this is linked to ectopic SATB1 expression remains an open question.

Although the establishment of chromosome-wide silencing depends on Xist expression, the inactive state can be globally maintained independently of Xist RNA (Csankovszki et al. 1999; Wutz and Jaenisch 2000; Zhang et al.

2007). The factors implicated in maintenance of silencing are also developmentally regulated. Indeed, a transition from Xist-RNA-dependent silencing to Xist-independent maintenance of inactivity occurs during differentiation. Transient ectopic expression of Xist during the first 3 days of mESC differentiation is sufficient to establish stable silencing that can then be maintained without Xist for more than 10 cell cycles (see Fig. 3B) (Wutz and Jaenisch 2000). On the other hand, if Xist is induced in undifferentiated mESCs, X inactivation occurs but is fully reversible (if cells are kept undifferentiated) as reactivation is observed following Xist down-regulation. What renders early-differentiating cells capable of maintaining the repressed state—or what prevents this in undifferentiated mESCs—is still not clear. The numerous factors that are dynamically regulated during differentiation and the plastic epigenomic landscape they control are obvious candidates.

## HETEROGENEITY IN THE ESTABLISHMENT OF XIST-RNA-MEDIATED SILENCING

Contrary to what might be expected given that Xist RNA is essential for chromosome-wide silencing and can only act during the first 2–3 days of differentiation, X inactivation does not seem to be a concerted process along the X chromosome. Indeed, complete silencing is only fully achieved after as late as 8 days of differentiation for some loci (Lin et al. 2007), such as *Huwe1* (Patrat et al. 2009; Chow et al. 2010). Furthermore, the efficiency of silencing appears to be highly variable with several genes escaping from X inactivation especially in humans (Carrel and Willard 2005). This heterogeneity in both the speed and efficiency of X inactivation highlights the fact that multiple mechanisms are likely to be at play in achieving transcriptional repression. Local genomic content appears to be critical in defining the capacity of a locus to respond to Xist-induced silencing, and LINE-1 repeats have emerged as strong candidates for facilitators of transcriptional repression (Carrel et al. 2006). Indeed, the X chromosome is almost twofold enriched in LINE-1 elements compared to autosomes (Boyle et al. 1990; Abrusán et al. 2008), and regions of the X that are rich in LINE-1s correspond to the parts of the chromosome that are silenced most efficiently, particularly on the human X (Wang et al. 2006; Chow et al. 2010; Tang et al. 2010). This also holds true in X:autosome translocations, where Xist RNA can spread into and silence autosomal sequences, with regions that are more LINE-1-rich being inactivated more efficiently than those that are LINE-1-poor. Such observations led to the proposal of the "LINE hypothesis" by Lyon in 1998.

How exactly LINE-1s might play a role in favoring Xist-mediated repression is not known, but several observations reveal links with some key steps of X inactivation. First, at the very early steps of silencing, the fraction of the X chromosome that is initially coated by Xist RNA corresponds to repetitive elements, including LINE-1s (Chaumeil et al. 2006; Chow et al. 2010). These repeats have been proposed to facilitate initial repression, for example, by the sequestration of neighboring sequences into the Xist-coated silent compartment (Chow et al. 2010; Namekawa et al. 2010).

Another not mutually exclusive role could be that they focally introduce chromatin features favoring silencing, as proposed in fission yeast (Zaratiegui et al. 2011). Intriguingly, specific subclasses of LINE-1s ($G_f$ and $T_f$) corresponding to young transposable elements were recently found to show persistent transcription from the X chromosome undergoing inactivation at late stages of differentiation in mESCs (Fig. 2) (Chow et al. 2010). Furthermore, expression of Xist transgenes from autosomes also results in prolonged expression from the Xist-RNA-coated chromosome. Thus, facultative heterochromatin induced by Xist RNA appears to exhibit prolonged young LINE expression during differentiation. In addition, although expressed LINE-1 repeats are found to cluster initially outside of the Xist RNA territory, they are found intermingled with it at later stages (Fig. 2), which may reflect changes in the spatial organization of the X chromosome during X inactivation. Paradoxically, inspection of the genomic distribution of these young LINE-1s on the X chromosome revealed that members of the active $T_f$ subfamily are significantly enriched in 1-Mb intervals surrounding loci that escape from inactivation—but not in shorter (10 or 100 kb) intervals (Chow et al. 2010). Close inspection of one such escapee, *Jarid1c* (*Kdm5c*), revealed that full-length LINE-1s are not closely associated with this locus, but rather with neighboring transcription units, such as *Huwe1*, which are subject to X inactivation. *Huwe1* displays unusually slow silencing kinetics, and its transcriptional repression coincides with the time at which young LINE-1s are transcribed from the inactive X chromosome. Interestingly, the full-length $T_f$-type LINE-1 element downstream from *Huwe1* is able to drive antisense transcription, and small RNAs (19–22 nucleotides) matching the 3′ end of *Huwe1* have been detected in differentiating female cells—although their exact origin and role remain to be determined. Thus, the emerging picture is that LINE-1s may be involved at several levels during the early steps of X inactivation. Both young and old LINE-1s seem to participate in the formation of the silent nuclear compartment triggered by Xist RNA early on during X inactivation. At later stages, expression of young LINE-1 elements from the X chromosome undergoing inactivation coincides with repression of regions that might be refractory to silencing, such as those located close to escapee genes (for review, see Chow and Heard 2010). The exact molecular mechanisms by which active LINE-1s might favor Xist-mediated transcriptional repression in such regions remain to be explored further.

The local density of repetitive elements is unlikely to be the sole parameter influencing the efficiency of Xist-mediated silencing, as dramatic differences can sometimes exist between neighboring loci—particularly on the human X (Carrel and Willard 2005). The precise genomic signatures associated with escape from XCI versus efficient silencing are likely to be complex and diverse (Wang et al. 2006). Establishment of XCI is thus somehow reminiscent of position effect variegation in *Drosophila*, in that both domain-wide influences and local intrinsic features likely control the silencing propensity of a given locus (Vogel et al. 2009). Chromatin structure represents

an obvious candidate for mediating the spread of regulatory instructions over distant sites.

## CHROMATIN STRUCTURE AND X INACTIVATION

It was actually the unusual heteropycnotic structure of the inactive X (dark staining with DNA dyes), rather than its transcriptional silence, that led to its initial description by Barr and Bertram (1949). It was also noted that this structure often resided close to the nucleolus, which they originally referred to as the "nucleolar satellite," although the heterochromatic mass of the inactive X is nowadays called the Barr body. The following section focuses on the description of the chromatin composition and higher-order structure that comprise the inactive X chromosome.

### Chromatin Composition of the Inactive X Chromosome

The molecular determinants accounting for epigenetic transmission of the inactive state of the X chromosome through cell division have largely remained a mystery. Chromatin structure has emerged as a prime candidate, both because of its intimate link with genome metabolism and because of the existence of mechanisms allowing for the propagation of chromatin states through replication (for review, see Bonasio et al. 2010). Indeed, the inactive X chromosome has many chromatin and structural features that distinguish it from its active homolog. The best known is DNA methylation of X-linked promoters on the inactive X. Immunofluorescence studies on mouse and human somatic cells have revealed that the inactive X-chromosome territory is globally depleted of transcription factors and chromatin features linked to active transcription. These include a global absence of RNA polymerase II and several general transcription factors such as Myc or Sp3 (Chadwick and Willard 2003; Chaumeil et al. 2006), as well as decreased levels of a subset of PTM histones including H3K4me2, H3K4me3, H3acK9, H3R17me, H3K36me3, and H3/H4 (but not H2A) acetylation (Fig. 2) (Jeppesen and Turner 1993; Heard et al. 2001; Chaumeil et al. 2002, 2006; O'Neill et al. 2008). In addition, the histone variants H2A-Bbd, H2A.Z (Chadwick and Willard 2001), and the phosphorylated form of macroH2A, mH2AS137ph (Bernstein et al. 2006), also seem to be excluded from the inactive X-chromosome territory.

The general absence on the inactive X chromosome of components associated with transcriptional activity, on the one hand, is mirrored by an enrichment for numerous proteins, compared to the rest of the genome, on the other. Many of these proteins are integral to chromatin, binding DNA directly or indirectly, and have been proposed to participate together in establishing or maintaining transcriptional repression. The histone variant macroH2A and the histone linker H1 appear to be enriched on the inactive X, as are numerous histone modifications including H3K9me2, H3K9me3, H3K27me3, H4K20me1, and H2AK199Ub (Fig. 2) (Mermoud et al. 1999, 2002; Costanzi et al. 2000; Heard et al. 2001; Boggs et al. 2002; Peters et al. 2002;

Chadwick and Willard 2003; Plath et al. 2003; Silva et al. 2003; de Napoles et al. 2004; Fang et al. 2004; Kohlmaier et al. 2004). Some of the protein complexes responsible for catalyzing the above histone modifications are also enriched on the inactive X, but not necessarily at all stages of X inactivation (at least when assessed by immuno-fluorescence). Among the best studied of these are the Polycomb group (PcG) protein complexes PRC2, which includes Eed, Ezh2, and Suz12 proteins and mediates H3K27 methylation, and PRC1, which includes Ring1a/, Ring1b/Rnf2, Bmi-1, Cbx proteins, Phc1/Mph1 and Phc2/Mph2, which binds H3K27me3 and mediates H2AK119 ubiquitination (de Napoles et al. 2004; Plath et al. 2004; for review on PRC1 and PRC2 composition, see Simon and Kingston 2009). Enrichment of PRC2 and PRC1 complexes on the inactive X is only observed transiently during early differentiation. The possible mechanisms underlying Polycomb group complex recruitment to the X chromosome will be discussed below. In addition to Polycomb repressive complexes and PTM histones and variants, a number of other factors have also recently been uncovered as partners of the inactive X chromosome in mice, although their exact role(s) still remain to be deciphered. These include the Trithorax protein Ash2l, the chromatin remodelling (X-linked) factor AtrX, and the SMC-like protein SmcHD1, as well as the matrix attachment protein hnRNP-U/Saf-A (Blewitt et al. 2008; Baumann and De La Fuente 2009; Hasegawa et al. 2010; Pullirsch et al. 2010). The human inactive X is also enriched in hnRNP-U/Saf-A, as well as other factors such as BaHD1, HMG-I/Y, and PARP-1, as well as HP1α, HP1β, HP1γ (Chadwick and Willard 2003; Helbig and Fackelmayer 2003; Nusinow et al. 2007; Bierne et al. 2009). It is intriguing that some of these factors appear to show species differences in their enrichment of the inactive X chromosome. For example, HP1 proteins are clearly enriched in some regions of the human inactive X (Chadwick and Willard 2003) but do not seem to show similar patterns in the mouse (Heard et al. 2001).

### Heterogeneity of the Inactive X Chromosome's Heterochromatin

Very few of the above chromatin features and factors can actually be detected on the inactive X chromosome in all interphase cells within any given population. This suggests either that epitope accessibility varies—in cases in which antibodies have been used (Duan et al. 2008)—or that the presence of these features might be dynamically regulated, for example, during the cell cycle, as has been found for other heterochromatic regions of the genome (Chadwick and Willard 2002; Fischle et al. 2005; Hernández-Muñoz et al. 2005; Hirota et al. 2005). Importantly, much of the chromatin signature of the inactive X chromosome persists during metaphase (Jeppesen and Turner 1993; Chaumeil et al. 2002; Mak et al. 2002), implying that these features could carry information through mitosis and participate in epigenetic transmission of the inactive state. Studies on the human inactive X at metaphase have revealed that its chromatin composition is not homogeneous, being organized into at least two distinct types that differ in their protein

composition. One fraction appears to be associated with H3K9me3, H3K20me3, and HP1, whereas the other fraction seems to be associated with macroH2A, H3K27me3 (Chadwick and Willard 2004). Intriguingly, the mouse inactive X seems to consist mainly of the latter category, although chromatin features are not homogeneously distributed in this species either (Mak et al. 2002; Smith et al. 2004). In human cells, the spatial partitioning of these two subtypes of heterochromatin can also be seen during interphase to some extent, with macroH2A, H3K27me3 being XIST-RNA-associated, but not H3K9me3, H3K20me3, and HP1. Thus, the manner in which heterochromatin folds in the nucleus may be linked to its local biochemical composition (Chadwick and Willard 2004).

Chromatin immunoprecipitation (ChIP) experiments on interphase cells have provided a more precise assessment of the subregions of the inactive X that are actually bound by several of these chromatin features, and revealed that the partitioning visualized by immuno-fluorescence reflects unequal distribution of different chromatin features (Rougeulle et al. 2004). For example, ChIP mapping on the X chromosome undergoing inactivation has revealed that H3K27me3 becomes preferentially abundant in gene-rich regions (Marks et al. 2009), whereas macroH2A is found to be rather homogeneously enriched along the inactive X (Mietton et al. 2009; Gamble et al. 2010). Such differences likely reflect the diversity in the pathways through which these various histone subunits, variants, and PTMs can be incorporated into chromatin (for review, see De Koning et al. 2007), and more specifically suggests that not all kinds of genomic elements are treated equally during X inactivation. Noticeably, enrichment in H3K27me3, which is mostly found at repressed CpG-rich promoters in undifferentiated mESCs (Mikkelsen et al. 2007), is actually not restricted to promoters on the inactive X, but is dispersed throughout several regions of the chromosome—with no clear evidence for progressive spreading from the Xist locus (Marks et al. 2009). Given its heterochromatic nature, one might expect the inactive X to be rather nucleosome-rich. It is still not clear whether higher nucleosome density contributes at least partly to the relative enrichment for chromatin proteins, as detected by ChIP and microscopy, on the inactive X compared to the rest of the genome (Perche et al. 2000).

### DNA Methylation

Epigenetic transmission of the inactive state of the X chromosome has been proposed to involve DNA methylation, the impairment of which leads to increased rates of sporadic reactivation on the inactive X (Mohandas et al. 1981; Csankovszki et al. 2001). The location of methylated CpGs differs between the two X chromosomes, with the inactive X being hypermethylated on promoter-associated CpG islands and the active X being hypermethylated throughout transcribed sequences (Hellman and Chess 2007). However, the extent of promoter hypermethylation on the inactive X is quite heterogeneous, and some loci can be repressed without necessarily having a methylated promoter or CpG island (Yasukochi et al.

2010). DNA methylation has been proposed to be a feature that is acquired at a relatively late stage of X inactivation (Lock et al. 1987), well after initial transcriptional repression. However, the kinetics of DNA methylation across the X chromosome during differentiation still remains to be investigated. Interestingly, maintenance of DNA methylation appears to require the SmcHD1 protein, which is found to be enriched on the inactive X chromosome in differentiated cells, and when mutated leads to partial reactivation of the inactive X at rather late stages of development (Blewitt et al. 2008). Importantly, SmcHD1 is the only known factor the impairment of which disrupts maintenance of the silent state of the inactive X in the embryo proper. How SmcHD1 acts on the inactive X chromosome remains an open question, but its SMC hinge domain—shared with proteins of the cohesin family—opens interesting perspectives linking the structure of the inactive X chromosome and epigenetic maintenance of its transcriptional repression. It remains, however, to be understood how the loss of SmcHD1 impairs proper maintenance of silencing, and whether reactivation is a direct cause of the loss of promoter methylation.

### Nuclear Organization and High-Order Chromatin Conformation of Inactive X Chromosome

The active and inactive X chromosomes differ not only in their biochemical composition, but also in their conformation and organization within the nucleus. The inactive X chromosome is frequently found in contact with the nuclear periphery and/or the perinucleolar region (in a cell cycle–dependent fashion), both of which are known as nuclear landmarks of heterochromatin (Zhang et al. 2007; Rego et al. 2008; for review, see Zhao et al. 2008). Whether this location is essential to the inactive status (during initiation or maintenance) is not clear. At the cytological level, the bulk of the inactive X-chromosome territory appears to be composed of repeat elements, which form a repetitive core around which gene-rich regions tend to be organized (Chaumeil et al. 2006; Clemson et al. 2006; Chow et al. 2010). At the ultrastructural level, the inactive X does not appear as a solid mass, but is instead composed of regularly spaced 30–400-nm electron-dense substructures, which correspond to separated tightly packed chromatin globules (Rego et al. 2008). Mapping of MNase-sensitive sites within in vitro 30-nm folded chromatin suggested that at this level, the two X chromosomes have similar structures overall, with an obvious looser compaction of the active X only visible at promoter regions of transcribed loci (Naughton et al. 2010). However, a higher general compaction of the inactive X can be seen by three-dimensional (3D) DNA FISH at scales superior to 2 Mb. Surprisingly, this seems only to hold true for gene-rich but not for gene-poor regions, suggesting again that chromatin organization along the X chromosome is highly modular and dependent on the activity of underlying DNA sequence. Furthermore, inhibition of transcription has been reported to lead to compaction of gene-rich regions of the active X, producing a similar de-

gree of compaction to that seen on the inactive X, thus further supporting the idea that transcription keeps chromatin in a rather decondensed state. However, the overall volumes of the active and inactive X-chromosome territories, as assessed by chromosome painting using DNA FISH, are seen to differ only moderately, if at all (Eils et al. 1996; Naughton et al. 2010). However, their shape and texture do appear to differ, with the active X-chromosome territory having a more irregular surface than the inactive X (Eils et al. 1996; Clemson et al. 2006), which probably reflects their different internal organization. It should be noted, however, that chromosome paint probes tend to exclude repetitive elements, which actually comprise a substantial fraction of the X chromosome (Chow et al. 2010). The differences in the detailed architecture of the active and inactive X chromosomes at interphase thus still remain very much an open question.

## ROLE OF XIST RNA IN ALTERING CHROMATIN STATES

Xist expression seems to be the initial trigger for the acquisition of the features that distinguish the inactive X from its active homolog. However, the molecular basis of Xist RNA action still remains to be elucidated. Xist RNA can coat the chromosome territory that it is transcribed from in *cis* (Fig. 1), but how it does so and what structural and functional interactions it makes with chromatin remain to be understood. The use of inducible Xist cDNA transgenes, deleted for different sequences, have shown that chromosome coating relies on several elements within the 17-kb RNA that seem to act in a cooperative, but redundant, fashion (Wutz et al. 2002). It should be noted, however, that proper Xist RNA chromosome coating is also dependent on its expression level (Sun et al. 2006). Furthermore, the ability of Xist RNA to induce chromosome-wide chromatin modifications is affected by the chromosomal location of Xist (Kohlmaier et al. 2004). Thus, although ectopic Xist induction can certainly help to define some of the key sequences in the RNA, the overexpression in such induction systems may bypass some of the RNA's intrinsic properties or requirements to a certain extent. For example, although Xist cDNA isoforms lacking the repeat C region (Fig. 1) can still coat the chromosome in *cis* when ectopically induced (Wutz et al. 2002), the use of interfering oligonucleotides targeting the C region of the endogenous Xist transcript is sufficient to abrogate Xist RNA coating in female cells (Beletskii et al. 2001; Sarma et al. 2010). Interestingly, the repeat C region has recently been found to interact with the nuclear matrix attachment protein hnRNP-U/SAF-A (Hasegawa et al. 2010). Furthermore, this interaction requires the hnRNP-U/SAF-A RGG domain, which is also necessary for enrichment of this protein over the inactive X-chromosome territory (Helbig and Fackelmayer 2003). Importantly, hnRNP-U/SAF-A is essential for proper Xist RNA coating of the inactive X, in both differentiating embryonic stem (ES) cells and somatic cells (Hasegawa et al. 2010). Interestingly, and contrary to the situation in the mouse, inducible human XIST RNA seems to require the repeat A region to accumulate in *cis*,

suggesting species differences in the role of these conserved elements (Chow et al. 2007).

Xist RNA mutations that prevent chromosome coating at the onset of XCI initiation also prevent silencing and the recruitment of chromatin modifications. However, abrogating Xist coating after XCI has been established (using an inducible Xist knockout, or repression of an inducible cDNA, e.g.) does not affect global H4 hypoacetylation or late replication, and major transcriptional reactivation is not seen (Csankovszki et al. 1999; Zhang et al. 2007; Pullirsch et al. 2010). In contrast to this, macroH2A, H3K27me3, Ash2l, and hnRNP-U/SAF-A enrichment are all lost from the inactive X chromosome when Xist RNA is removed, whatever the differentiation stage looked at. This implies that these chromatin features rely on continuous Xist expression, and also that they are not essential for epigenetic maintenance of the inactive state (Fig. 3B) (Csankovszki et al. 1999; Kohlmaier et al. 2004; Pullirsch et al. 2010). The transient displacement of Xist RNA from the X using LNA oligonucleotides against the Xist repeat C region revealed that PRC2 localization is rapidly disrupted (within 60 min), with recovery occurring slowly (during a day) and in a rather uniform fashion across the inactive X (Sarma et al. 2010). Interestingly, H3K27me3 enrichment on the inactive X seems to remain stable within that time period, suggesting that short-term maintenance of this mark does not require permanent association with PRC2.

The exact mechanisms underlying recruitment of Polycomb group proteins to a Xist-RNA-coated chromosome are still not known. Protein subunits of the PRC2 complex have been reported to bind the Xist RNA repeat A element (Fig. 1) (Zhao et al. 2008; Kaneko et al. 2010; Kanhere et al. 2010; Maenner et al. 2010). Interestingly, the endogenous repeat A region can also produce a short transcript, RepA, independent of Xist, and the expression of which can ectopically recruit PRC2 through RNA–protein interaction (Zhao et al. 2008). What part this plays in X inactivation is still unclear. Indeed, the repeat A region, or RepA RNA, is unlikely to be the only region through which Xist can trigger the recruitment of Polycomb proteins, as the induction of an Xist cDNA transgene lacking this region still results in chromosome-wide PRC2 enrichment in early differentiating mESCs (Kohlmaier et al. 2004; Pullirsch et al. 2010). Investigation of the role of the repeat A region at the endogenous Xist locus is hampered by the fact that this element is necessary for appropriate Xist expression (Hoki et al. 2009; Royce-Tolland et al. 2010).

Importantly, the capacity of Xist RNA to induce chromatin modifications appears to be developmentally regulated, just like its capacity to induce silencing, as discussed above. Indeed, although inducible Xist expression readily triggers chromosome-wide enrichment of PRC2 and H3K27me3 in undifferentiated mESCs, this is not the case in cells that are at advanced stages of differentiation (Fig. 3B) (Kohlmaier et al. 2004). Similarly, Xist induction on the X chromosome in male embryos can only lead to efficient H3K27me3 enrichment during early stages of development (before embryonic day 12.5, E12.5). Furthermore, although induction of Xist RNA deleted for the repeat A

region is able to lead to chromosome-wide enrichment of H3K27me3 during early differentiation, this is not the case when the mutant Xist is ectopically expressed in undifferentiated mESC. The factors responsible for restricting Xist RNA's ability to trigger chromatin modifications to the cellular context of early development remain to be identified (Fig. 3A).

One important question concerns the nature of the chromosomal memory that is acquired after Xist RNA has triggered X inactivation. Intriguingly, Xist down-regulation (using an inducible cDNA transgene) in undifferentiated or differentiating mESCs does not lead to instantaneous loss of H3K27me3 enrichment, and complete erasure is not seen even after 48 h of culture (Kohlmaier et al. 2004). This suggests that H3K27me3 can be maintained even in the absence of Xist RNA coating (and PRC2 binding) (Sarma et al. 2010) for a short time period, lasting for more than one cell division. Surprisingly, if Xist is induced late during differentiation, H3K27me3 is much more efficiently recruited if the cells previously experienced a transient pulse of Xist expression at an earlier time point (Fig. 3C). This sensitization to Xist-mediated chromatin modification, or "chromosomal memory," seems to be set up within a short developmental time window, between 48 and 72 h of mESCs differentiation, and once more highlights the importance of unknown developmentally regulated factors in the regulation chromatin structure by Xist RNA (Fig. 3B). It is important to note, however, that different factors may control permissiveness to Xist-mediated chromatin changes and silencing, the shift to Xist-independent maintenance of silencing, and the "chromosomal memory" discussed here (Fig. 3B). For example, SATB1 expression, which may participate in the developmentally restricted ability of Xist to induce transcriptional repression, does not seem to be required for Xist-mediated H3K27me3 enrichment (Agrelo et al. 2009). The precise manner in which Xist's multiple functions are controlled by stage-specific factors during early development remain to be explored.

## IS THERE A HIERARCHY IN THE INDUCTION OF CHROMATIN CHANGES DURING X INACTIVATION?

The ever-growing list of known chromatin modifications associated with the inactive X stands in contrast with our limited understanding concerning how they are recruited following Xist expression. Their sequential appearance as XCI is established (Chaumeil et al. 2002, 2006) suggests that the different layers of chromatin rearrangements might relay each other to some extent. For example, PRC2 binding has been proposed to be a prerequisite for PRC1 recruitment to autosomal targets of Polycomb complexes such as the Hox loci (Eskeland et al. 2010). In turn, PRC1 affects higher levels of chromatin organization by mediating compaction of the Hox clusters—in a manner that does not depend on its histone ubiquitin transferase activity. However, Xist expression can trigger the recruitment of some PRC1 subunits (such as Ring1b, but interestingly not mPH1 and mPH2) in cells lacking PRC2

activity (owing to the absence of Eed) (Schoeftner et al. 2006). This indicates that Xist can recruit Polycomb group proteins in a manner that is not necessarily reminiscent of what happens at autosomal loci. Furthermore, this also indicates that Xist is able to recruit at least some of these proteins independent of each other, and raises the question as to whether independent subunits—as opposed to whole Polcomb repressive complexes—may be targeted to chromatin by Xist RNA.

Intriguingly, enrichment of Polycomb repressive complexes is developmentally regulated, and the histone modification they catalyze can persist on the inactive X even when these proteins are not specifically localized to it. Indeed, PRC2 recruitment is stage-specific, as judged by immuno-fluorescence, with extraembryonic cells displaying stable Eed and Ezh2 enrichment over the inactive X, and embryonic stem cells apparently exhibiting only transient localization during early differentiation (Silva et al. 2003; Plath et al. 2003, 2004). Surprisingly, H3K27me3 enrichment remains evident even in differentiated cells. It is not clear how H3K27me3 is maintained without apparent localization of PRC2 across the inactive X, but low PRC2 levels or transient binding might be sufficient. Similarly, PRC1 subunits also mark the inactive X in undifferentiated extraembryonic cells, as well as the X undergoing inactivation in differentiating embryonic stem cells. However, different PRC1 members show different kinetics and degrees of enrichment, suggesting that they might be recruited individually—or as part of other complexes than the canonical PRC1 (Plath et al. 2004). The Ring1b subunit is necessary for the stability of Pch1/Mph1 and Phc2/Mph2, which are thus not recruited by Xist in $Ring1b^{-/-}$ cells (Leeb and Wutz 2007). Importantly, H3K27me3 and H3K20me1 deposition remains unaffected by the absence of Ring1b, suggesting that Xist RNA independently recruits the complexes that deposit these marks.

The interplay of PRC2 and other chromatin features is not only locus-specific, but also cell-type-specific. Indeed, in the extraembryonic trophoblast tissue of mouse, loss of Eed results in loss of Xist coating as well as H4K20me1, macroH2A, PRC1 proteins, and H2AK119Ub enrichment over the inactive X (Kalantry et al. 2006). Despite this, the inactive X remains silent, and overall H3K4me2 levels remain low from its territory. However, reactivation of some X-linked loci can be observed during differentiation of these Eed mutant trophoblast cells, and the former inactive X regains H3K4me2 as well as H3 and H4 acetylation. Dependence on PRC2 function is nevertheless restricted to the trophoblast, as other extraembryonic lineages such as the visceral endoderm or the extraembryonic ectoderm do not reactivate the paternal X chromosome in $Eed^{-/-}$ embryos (Wang et al. 2001; Kalantry et al. 2006). It is surprising, however, that the loss of Eed does not lead to reactivation at the stage where it is normally enriched on the inactive X of wild-type embryos (undifferentiated trophoblast), but instead leads to reactivation at subsequent stages, when it is no longer obviously enriched on the inactive X (differentiated trophoblast). This indicates that instead of directly controlling transcriptional silencing, PRC2 is likely involved in setting up an epigenetic signa-

ture that is only read subsequently by unknown silencing factors. This is reminiscent of the chromosomal memory that is set up on the inactive X during mESC differentiation, although the fact that Eed is not required here indicates that the molecular players must differ (Schoeftner et al. 2006). One hypothesis that has been proposed to explain the sensitivity of the inactive X in differentiated trophoblast cells to lack of PRC2 activity when compared to the embryonic lineage is that in extraembryonic tissues, X-linked promoters show relatively low DNA methylation levels (Cotton et al. 2009). This hypothesis, however, remains to be tested in the context of embryonic cells lacking both PRC2 and de novo DNA methylation activity.

## WHAT IS THE IMPORTANCE OF CHROMATIN MODIFICATIONS IN THE ESTABLISHMENT OR MAINTENANCE OF X INACTIVATION?

Changes in chromatin composition accompany the progressive silencing of the X chromosome. Although it is tempting to speculate that chromatin modification could directly drive transcriptional repression during X-chromosome inactivation, clear evidence for this is still lacking, and it is not known how initial silencing is triggered. As mentioned in the previous section, the inactive X remains silent in undifferentiated mouse trophoblast cells lacking Eed, although Xist coating and other chromatin hallmarks of the inactive X are lost. Similarly, Xist-mediated silencing is unperturbed in cells of the embryo proper or mESCs that lack Eed (Kalantry and Magnuson 2006; Schoeftner et al. 2006). Furthermore, deletion of the repeat A region of Xist does not prevent its ability to recruit enrichment of H3K27me3 over the X-chromosome territory during mESC differentiation (nor global H4 hypoacetylation), although transcriptional repression and 3D reorganization of the X is not fully achieved (Wutz and Jaenisch 2000; Kohlmaier et al. 2004; Chaumeil et al. 2006; Pullirsch et al. 2010). Interestingly, in this situation the repeat-rich $C_0t$ – 1 fraction of the X seems to be properly silenced and to correctly coalesce in the core of the Xist-expressing chromosome territory (Chaumeil et al. 2006), suggesting that genomic repeats and unique (gene) sequences may be silenced by Xist through different mechanisms. Nevertheless, this indicates that global H3K27me3 enrichment is neither necessary nor sufficient for X inactivation to occur. This is further supported by the observation that loss of SmcHD1 does not lead to loss of H3K27me3 enrichment even though some loci are reactivated from the inactive X (Blewitt et al. 2008). In the same vein, the PRC1 component, Ring1b, is dispensable for Xist-mediated silencing in mESCs (Leeb and Wutz 2007), and mice in which *H2afy* (encoding MacroH2A1) is impaired show no defect in terms of X inactivation (Changolkar et al. 2007). However, multiple pathways are likely to act in parallel, rendering genetic analysis difficult. Clearly, the understanding of the different means by which Xist RNA mediates transcriptional repression and the precise role of chromatin structure and organization in the process of X-chromosome inactivation remain exciting challenges for the coming years.

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