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Review

Forming facultative heterochromatin: silencing of an X chromosome in mammalian females

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Abstract. Compensating for the dosage difference in X-linked genes between male and female mammals involves the formation of an extremely stable heterochromatin structure on one of the two X chromosomes in females. The inactive X acquires numerous features of silent chromatin, including the expression of a noncoding RNA, a switch to late replication, histone modifications, recruitment of the histone variant macroH2A and DNA hypermethylation. Although the induction of inactivation in differentiating mouse embryonic stem cells suggests that the onset of each of these features appears to occur in a sequential manner, it is likely that there is a much more complex interplay between the different features which leads to the extremely stable silencing observed in female somatic cells. Expression of the untranslated RNA, *XIST*, is required in *cis* for the establishment of the heterochromatic state. Recent results have started to elucidate how expression of *Xist* is controlled, including the role of the antisense transcript *Tsix*.

Key words. X chromosome inactivation; dosage compensation; facultative heterochromatin; *XIST* RNA.

Introduction

In mammals, one of the pair of X chromosomes in females is silenced, thereby achieving dosage equivalency with males who have a single X chromosome and the sex-determining Y chromosome. Diverse mechanisms to compensate for this dosage problem have arisen independently in different organisms. In contrast to the chromosome-wide inactivation that occurs in female mammals, males in the fruit fly *Drosophila melanogaster* hypertranscribe genes on their single X approximately twofold [1]. *Caenorhabditis elegans* has come up with yet a different way to deal with dosage differences by partially down-regulating transcription from both X chromosomes in the XX hermaphrodite [2]. Mammalian X inactivation is unique in that the two X chromosomes within a single female cell are differentially regulated. The initial choice of chromosome to inactivate is random in humans, although in marsupials it is always the paternal X which is inactivated (reviewed in [3]), as is also the case in rodent and bovine extraembryonic tissues [4, 5]. Once an X chromosome is inactivated, the same X is silenced in all descendent cells, resulting in females being mosaics for their X inactivation pattern. Thus X inactivation is a model of epigenetic inheritance in which stable changes in gene function occur without changes in the primary DNA sequence.

The epigenetic silencing of the mammalian X chromosome includes acquisition of many of the features associated with heterochromatin, and we will review these in approximately the order in which they are observed to be acquired by the inactive X chromosome (see fig. 1). This review will concentrate on human X-chromosome inactivation; however, the availability of murine embryonal carcinoma (EC) and embryonic stem (ES) cells that undergo inactivation upon induction of differentiation [6]

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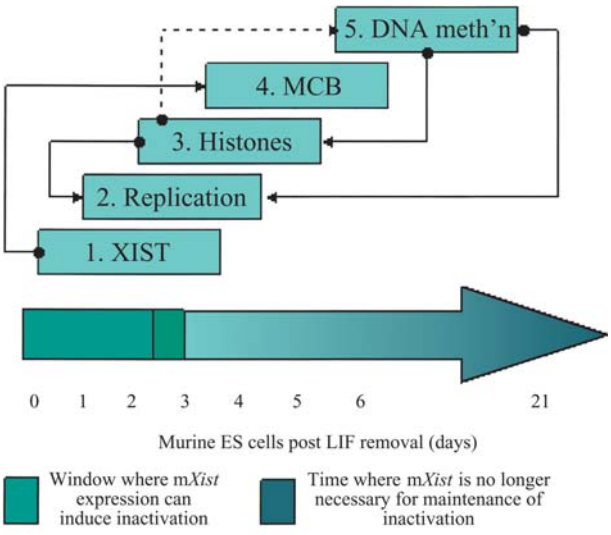

Figure 1. Schematic representation of the steps involved in the formation of a heterochromatic inactive X. Interactions between features are shown by connectors. Approximate timing of the events following induction of differentiation in mouse ES cells is shown below the arrow [7]. In mouse, induction of *Xist* first results in reversible inactivation which is later stabilized, while later induction of *Xist* is unable to induce silencing [23].

means that in many cases more detail is available for mouse, so this will be presented. The timing of the events of inactivation as outlined in figure 1 was determined in female mouse ES cells induced to undergo differentiation [7]. In general, we discuss the process of inactivation in embryonic lineages or somatic cells, and will note differences from the situation in extraembryonic tissues, or spermatogenesis where transient X chromosome silencing occurs during the pachytene stage when the inactivated X and the Y pair at their pseudoautosomal regions, forming the sex chromatin body or X-Y body. The timing of events has been suggested to be slightly different in extraembryonic tissues, notably with the potentially earlier involvement of macrohistone H2A [8].

XIST expression

One of the first events in the inactivation cascade is the expression of a transcript exclusively from the inactive X, called the X(inactive)-specific transcript, or *XIST*. *XIST* was the first gene to be mapped within the master control region on the X called the X-inactivation centre (XIC), which is required in *cis* for inactivation to occur. *XIST* encodes an untranslated, functional RNA molecule that remains in the nucleus and associates with the inactive X. For human *XIST*, this association is retained only during interphase; however, in mouse, *Xist* continues to associate with the X until early metaphase [9–11]. Although the *XIST* transcript remains nuclear, it is still spliced and polyadenylated and is highly heterogeneous with many different isoforms arising from alternate initiation sites, termination sites and splicing variants.

XIST has been identified and sequenced in mouse, voles, cow and humans [9, 11–13]. Comparative analyses between species show that there is a fairly low degree of sequence constraint within the *XIST* primary sequence, with exonic sequences having 66% identity between mouse and human, and 62% identity between mouse and cow [12]. The basic intron-exon structure of the gene, however, has been largely conserved, prompting suggestions that structural features of the transcript rather than the primary sequence are important for its function. One of the unique conserved features of the *XIST* transcript is the presence of tandem repeats, which make up over one-third of the *XIST* sequence in rodents, humans and cows [12, 13]. Six of these repeats (A–F) have been identified across the *XIST* gene and repeat A, at the 5′ end of exon 1, shows the highest conservation in all species examined [9, 11, 13] (see fig. 2). The presence and conservation of these repeats are highly suggestive of a functional role, possibly in the binding of specific proteins or in the formation of particular secondary structures.

Xist expression patterns during the initiation of inactivation have been studied in mouse preimplantation embryos and in ES cells. Prior to inactivation, *Xist* is expressed from both X chromosomes in females and the single X in males as a low-level unstable transcript that can be seen as small punctuate signals by RNA-fluorescence in situ hybridization (FISH). Upon differentiation, *Xist* is upregulated on one of the two X chromosomes, forming a large stable signal that coats the X which is subsequently inactivated, whereas on the future active X, *Xist* expression is silenced [14, 15]. Because accumulation of stable *Xist* RNA is one of the first events in the inactivation cascade, starting at about day 1 of differentiation, it is thought to recruit factors required for the silencing of the chromosome [16]. *Xist* is also expressed in mouse extraembryonic tissues where X inactivation is imprinted and the paternally derived X is preferentially inactivated. *Xist* is expressed exclusively from the paternal X and, as in the cells of the embryo proper, expression precedes inactivation. Therefore, based on these dynamic changes in expression, *Xist* appears to be involved in the initiation of both random and imprinted inactivation [17]. *XIST* expression prior to inactivation is also observed in humans [18, 19] and cows [20].

Mouse knockouts have demonstrated that *Xist* is necessary for inactivation, as chromosomes that contain deletions removing the *Xist* promoter or interstitial regions of the gene are unable to be inactivated [21, 22]. In addition, *Xist* alone is sufficient for inactivation since an inducible *Xist* complementary DNA (cDNA) construct introduced into ES cells can induce long-range transcriptional silencing as well as late-replication and histone H4 hypoacetylation of surrounding chromatin [23]. However,

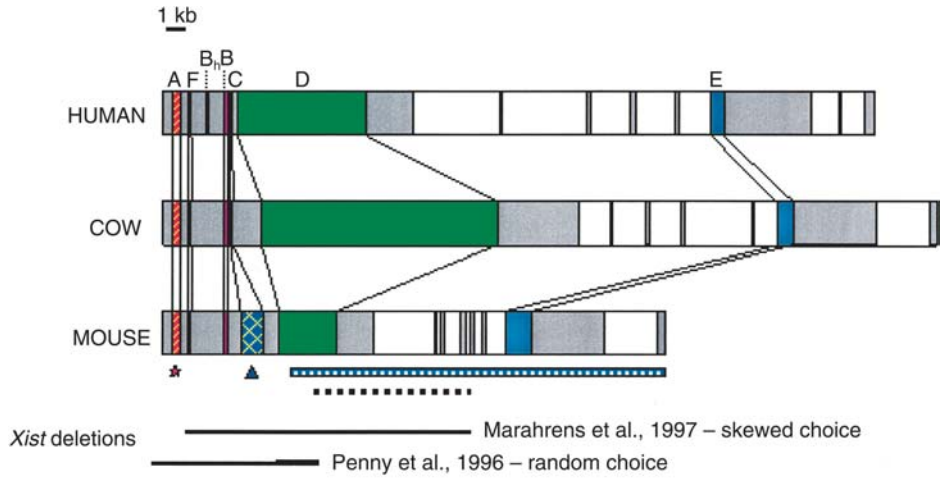

Figure 2. Conserved repetitive elements in human, cow and mouse *XIST*. The grey blocks indicate the exonic regions. The conserved A repeats, marked with the red star, are required for silencing in mouse and also contribute to *XIST* transcript localization [25]. PNA probes directed against the C repeats (triangle) in differentiating female mouse ES cells also disrupt transcript localization [26]. The rectangle contains regions(s) required for mH2A localization [25]. The solid black lines represent *Xist* deletions that disrupt *Xist* function and lead to nonrandom X-chromosome inactivation. Only the deletion spanning exons 1–5 leads to primary nonrandom inactivation, implicating the region represented by the dotted line in X-chromosome choice. For a complete description of the targeted mutations in the *Xist/Tsix* region see [207].

the stability and reversibility of *Xist*-induced silencing is dependent on developmentally regulated factors, since its ability to silence depends on the time at which it is expressed during differentiation (see fig. 1). In undifferentiated ES cells, the *Xist* transgene can induce silencing, but this 'primary' silencing is reversible and dependent on continued *Xist* expression. It is also not associated with other features of inactivation such as histone hypoacetylation and late replication timing. After 72 h of differentiation, silencing becomes irreversible, and *Xist*-independent and other features of the inactive X are acquired. There appears to be a critical window in the first 48 h of differentiation when *Xist* can initiate silencing; after this time the cell becomes refractive to *Xist* expression. However, there has been a report of *XIST*-mediated silencing in human somatic cells, suggesting that this developmental requirement may not be so strict in some cell types [24]. Alternatively, this difference may point to species or experimental differences.

Functional domains have been identified in mouse knockouts using a series of deletions introduced into a *Xist*-inducible cDNA construct integrated into the *Hprt* locus on the X chromosome and expressed in ES cells [25]. The conserved A repeats at the 5′ end of *Xist* are required for silencing and also contribute to localization of the transcripts to the chromosome, but are not absolutely required for the latter function. Transcripts deleted for the A repeats were able to localize to the chromosome, but could not silence the surrounding chromatin, demonstrating that localization of the transcript is not sufficient for silencing. Computational analysis of the A repeats predicts that the RNA folds into two stem loops which are repeated 7.5 times in mouse. Specific mutations in these repeats show that the RNA secondary structure, not the specific sequence, is important since mutations that retain the stem loops retain silencing activity. The activity of these repeats is also copy number dependent, requiring at least 5.5 units for silencing, but position independent since moving the repeats to the 3′ end of the transcript had no effect on function [25]. These conserved stem loops may provide binding sites for specific protein factors that mediate chromatin conformational changes that lead to inactivation. Not only is *Xist* required for silencing, but sequences within *Xist* also appear to be involved in the choice of which chromosome will be inactivated. Although some *Xist* deletions lead to nonrandom inactivation patterns due to secondary selection [21], others appear to result in primary nonrandom inactivation and implicate specific regions within *Xist* in the initial choice of which chromosome will be inactivated [22] (see fig. 2).

The ability of the *XIST* transcript to associate in *cis* with the chromosome from which it is transcribed is essential for initiation of silencing. *Xist* deletion constructs created by Wutz et al. [25] have identified regions within *Xist* that are involved in the association of the transcript to the chromosome and found that localization requires multiple domains throughout the *Xist* transcript that work cooperatively and in a functionally redundant manner (see fig. 2). From these various constructs it was concluded that although localization does not necessarily lead to silencing, silencing does require a localized transcript. A similar result was obtained using antisense probes constructed with peptide nucleic acids (PNA) targeted to the conserved C repeats, which disrupted localization of *Xist*

and subsequent Barr body formation in differentiating female ES cells [26].

Xist action and localization likely requires association with proteins. *Xist* is necessary for localization of the histone variant macroH2A to the inactive X. MacroH2A will be discussed further below, but association with the inactive X apparently involves the 3′ region of *Xist* (fig. 2) [25]. A few additional proteins interacting directly or indirectly with *XIST* have been described, but so far the role of these proteins in X-chromosome inactivation has not been determined. BRCA1 has recently been shown to colocalize with the inactive X [27]. BRCA1 has been implicated in many cellular functions, and through the analysis of *BRCA1* –/– cancer cells and reconstitution of BRCA1-deficient cells with wild-type BRCA1, it appears also to support localization of *XIST* and formation and/or stabilization of the silenced state of an inactive X. A general RNA-binding protein, hnRNPC1/C2, has been shown to interact with the region of the conserved repeats in the 5′ region of *XIST* in vitro [28]. Although this interaction does not appear to be specific for the *XIST* transcript, specialized heteronuclear protein isoforms may be important for *XIST*'s unique transcript processing and localization requirements [16]. In addition, antibodies in the serum of an autoimmune patient were found to recognize an as yet unidentified protein enriched on the inactive X [29], and there is a transient association of Eed/Ezh2 with the inactivating X chromosome, which requires *Xist* RNA [30].

The importance of *XIST* expression in the initiation stage of inactivation has clearly been established, and its continual synthesis in somatic cells argues that it may also have a role in maintenance. Its role in these later stages, however, may be more redundant, because although *XIST* expression appears to contribute to the stability of silencing [31], loss of *XIST* does not lead to reactivation of the chromosome in somatic cells [32–34]. An understanding of the initiation of inactivation will require understanding how *XIST* expression is regulated, and we will briefly review what is known about the transcriptional regulation of *XIST*, the role of an antisense transcript and chromatin changes in regulation, and *trans*-acting factors potentially involved in *XIST* regulation.

XIST regulation

Transcriptional regulation

Because changes in *Xist* steady-state levels have important consequences in the inactivation process, identifying elements that regulate its expression will be key to understanding the mechanism of X-chromosome inactivation. An increase in mouse *Xist* RNA steady-state levels is important in the initiation of inactivation and appears to involve a change in transcript stability, which goes from a half-life of ~30 min prior to inactivation to ~5 h after differentiation [14, 15]. Although transcriptional run-on experiments have determined that an increase in transcriptional activity does not appear to be involved in *Xist* upregulation at this stage, increases in transcriptional levels do appear to affect *Xist*'s stability and its ability to coat and inactivate surrounding chromosomal material. High levels of *Xist* expression from an inducible promoter are able to initiate inactivation in undifferentiated ES cells, which suggests that stabilization may not require developmentally specific stabilization factors [23, 25].

To identify developmentally specific transcription factors that modulate *Xist* transcriptional activity during inactivation, elements within the *Xist* promoter region have been characterized. Mutational analyses in promoter-reporter gene constructs have defined the minimal promoter to be about –81 to +1 in mouse and –93 to +31 in humans relative to the transcriptional start sites [35, 36]. The promoter regions in both species contain binding sites for ubiquitous transcription factors and are constitutively active with no sex-specific activity. Thus elements responsible for the developmentally regulated expression patterns of the *XIST* transcript must lie outside this minimal region and act to silence expression on the X chosen to remain active and upregulate expression on the X to be inactivated. Intriguingly a base-pair change in the promoter region of the human *XIST* gene has been found in individuals with nonrandom inactivation [37, 38], although the change is also found in family members who do not have extremely skewed inactivation, complicating assessment of the importance of this site.

Developmentally regulated *Xist* expression is dependent on differential methylation at CpG sites in the promoter region and at the 5′ end of the first exon of *Xist*. Methylation at these sites correlates with the activity of the gene and precedes the onset of *Xist* expression, which suggests that it has an active role in controlling expression [39–41]. In addition, targeted disruption of DNA methyltransferase 1 (*Dnmt1*) results in inappropriate expression of *Xist* from the single X in males and from both X's in females upon differentiation. However, although methylation seems to be important for *Xist* regulation after differentiation, low-level *Xist* expression prior to inactivation is unaffected by the *Dnmt1* mutation [42, 43]. Human *XIST* also shows differential promoter methylation [44], and in somatic cells demethylation seems to be sufficient to induce expression from the active X chromosome [45, 46].

Tsix – antisense regulation?

An antisense transcript, *Tsix*, has been identified in the *Xist* region and has been shown to be an important regulator of *Xist* expression during the initiation stages of inactivation. *Tsix* encodes a heterogeneous transcript, and its main initiation site lies 12 kb downstream of *Xist* and transcription spans over 40 kb, overlapping the entire *Xist*

gene. *Tsix* is also spliced and, like *Xist*, has many different transcript isoforms with an alternate minor start ~28 kb downstream of *Xist* and several different splicing variants [47, 48]. Interestingly, the spliced forms eliminate all overlap with *Xist* except for a 2-kb region at the 5′ end of *Xist* which includes the conserved repeats important for silencing.

Tsix expression, like *Xist,* goes through dynamic changes early in development, and its expression pattern suggests a role as a negative regulator [47, 49, 50]. Prior to inactivation both *Xist* and *Tsix* are coexpressed on both X's in females and the single X in males, appearing as pinpoint signals by RNA-FISH. Upon differentiation, *Tsix* expression is retained on the future active X, repressing *Xist* upregulation, whereas on the future inactive X, *Tsix* is silenced and *Xist* accumulation then leads to silencing. However, *Xist* regulation may be more complex since extinction of the *Tsix* signal does not always precede *Xist* upregulation, with some nuclei expressing both an accumulating *Xist* signal as well as a pinpoint *Tsix* signal [51]. Targeted deletions and insertions into the *Tsix* gene have further substantiated its role as a negative regulator of *Xist* and provide clues to the mechanism of its action. Targeted deletions of the *Tsix* main promoter disrupt random choice and lead to upregulation of *Xist* and inactivation on the deleted chromosome [47, 50, 51]. In addition, ectopic expression of *Tsix* through the targeted insertion of an inducible or constitutively active promoter also leads to suppression of *Xist* upregulation and inactivation [52, 53]. Active transcription in the region appears to be important for its function, since insertion of transcriptional stop signals also leads to preferential inactivation of the mutated chromosome [52].

Based on *Xist* and *Tsix* expression patterns and the results of *Tsix* knockout and ectopic promoter knockin experiments, several mechanisms of repression have been proposed [48, 49, 54]. It is possible that the antisense transcript itself is not important, and *Tsix* may regulate *Xist* through an enhancer competition-type model, where activity at a downstream promoter outcompetes *Xist* for a shared enhancer (analogous to early models for the proposed action of the oppositely imprinted genes *H19/IGF2* [55]). However, insertion of a transcriptional stop signal that truncates the *Tsix* transcript leads to nonrandom inactivation, suggesting that simply activating the promoter is not sufficient to suppress *Xist* function and that either the antisense transcript itself or the act of transcription through the *Xist* locus is important [52]. In these transcription-dependent mechanisms, *Tsix* inhibition of *Xist* may or may not require the RNA transcript. A mature, processed antisense transcript may not be important, and the simple act of transcription may function to either modify surrounding chromatin, or inhibit the movement of RNA polymerase in the sense direction. If the transcript is important, *Xist/Tsix* RNA duplex formation could lead to the degradation of the *Xist* transcript or mask important domains on the *Xist* transcript, preventing protein interactions and proper function. However, splicing of the *Tsix* transcript eliminates almost all overlap with *Xist* except in the 5′ region of exon 1, where the conserved repeats required for silencing reside [47, 48]. Quantitative analyses of *Xist* and *Tsix* transcript levels in undifferentiated mouse ES cells revealed that the *Tsix* RNA is present at much higher levels than *Xist* (>10–100-fold molar excess), supporting transcript-dependent models where sufficient levels of the antisense transcript are required to bind and block the activity of all the *Xist* transcripts [48].

Antisense transcription has also been detected at the human *XIC,* and conserved features may provide clues about the mechanism of *Tsix* regulation [56, 57, Chow et al., Genomics **82:** 309–322]. The human *TSIX* transcript only partially overlaps *XIST* and does not extend into the 5′ region; therefore, unlike mouse, there is no overlap with the conserved 5′ repeats. In addition, the presence of the antisense transcription in humans does not preclude stable, localized *XIST* expression, which may mean that the human homolog has lost its ability to negatively regulate *XIST* expression. However, 30–60% of *Tsix* transcripts in mouse remain unspliced, and transcript levels are highest at the 5′ end of *Tsix,* which may be suggestive of a functional role for transcription in this region where human antisense is also expressed [48].

Chromatin structure flanking *Xist*

Changes in chromatin modifications 5′ of *Xist* coincide with initiation of inactivation and thus implicate flanking chromatin in the regulation of *Xist* expression. In particular, the existence of regulatory elements upstream of *Xist* has been suggested by several studies. Johnston et al. [58] originally proposed that stabilization of the *Xist* transcript during the initiation of inactivation may involve a switch from an upstream promoter (Po); however, the presence of several ribosomal protein pseudogenes in the Po region [59], and the *Tsix* antisense transcript, which extends upstream of *Xist*, may have confounded the original observations of Po expression. Still, the region upstream of *Xist* does appear to contain important regulatory sequences since transgenes deleted for the region, although they express unstable transcripts in undifferentiated ES cells, are unable to express stable *Xist* when differentiated [60]. Several histone modifications also point to important elements in the region. A unique hotspot of histone H3 lysine methylation has been identified 40–100 kb upstream of *Xist* and has been proposed to act as a nucleation site for heterochromatin spread [61]. In addition, a region of histone H4 hyperacetylation specific to undifferentiated female ES cells has been identified up to 120 kb upstream of *Xist*. Upon differentiation, the region becomes hypoacetylated, and this

change has been hypothesized to be important for *Xist* stabilization and progression of inactivation [62].

In mouse, a regulatory element called the X-controlling element (Xce) also appears to reside downstream of the *Xist/Tsix* region and affects the choice of which chromosome will be inactivated. Four different *Xce* alleles have been identified and vary in 'strength', with the weakest, Xce^a, most likely to be inactivated and Xce^d, the strongest, least likely to be inactivated when present in a heterozygote. Developmentally regulated chromatin alterations as well as extensive low-level intergenic transcription have been observed in this region and have been termed *Xite*, for X-inactivation intergenic transcription elements [63]. *Xite* has been proposed to be a candidate locus for *Xce* since deletions result in skewed choice, and although the mechanism is unclear, *Xite* appears to modify choice by enhancing the likelihood of *Tsix* persistence on one of the X chromosomes, which will then remain active.

Xist regulation through *trans*-acting factors

Trans-acting factors have been hypothesized to be involved in the X-inactivation pathway and the choice of which X to inactivate. Diploid mammalian cells always retain one X chromosome active, which has led to models suggesting that an autosomal factor binds a counting element in the XIC (reviewed in [17]). A 65-kb deletion 3′ of *Xist* abolishes retention of an active X (even in male cells), and thus may define the location of binding of such a counting element [64]. One way that these hypothetical factors could carry out these functions is to regulate *XIST* expression. Binding sites for the ubiquitous chromatin insulator and transcriptional regulator CTCF have been found in the *Tsix* promoter region and implicate it as a candidate *trans*-acting factor for X-chromosome choice. It has been proposed that CTCF could mark the active X by *Xist* suppression through *Tsix* activation or by blocking access to putative enhancers [65].

Through mutagenesis screens two, as yet unidentified, autosomal loci have been implicated in X chromosome choice and may thus also be involved in *Xist* regulation [66]. In a mouse phenotypic screen, females were identified in the progeny of mutagenized males and screened for changes in the expected X-inactivation ratios. Two females with altered X-inactivation patterns were identified, and the mutations were found to segregate in an autosomal dominant manner. Because the alterations in X inactivation ratios are observed early in development, they are thought to represent mutations affecting primary choice rather than secondary cell selection.

Mechanism of *Xist*-mediated silencing

While there is substantial evidence supporting a role for *XIST* in establishing X chromosome silencing, there is growing evidence that an RNA-mediated silencing function may not be unique to *XIST* and X-chromosome inactivation. Naturally occurring antisense transcripts are found both in *cis* and *trans* throughout the human genome, with a survey showing that ~2% of messenger RNAs (mRNAs) have associated antisense expression [67], supporting a possible widespread role for double-stranded RNAs in various aspects of chromatin configuration. In particular, antisense transcripts seem to be enriched in imprinted domains, which also show epigenetic silencing of one allele, although based upon parental origin. The direct involvement of antisense transcripts in imprinted expression has been demonstrated at the *Igf2r* imprinted gene cluster, where premature truncation of the antisense transcript, *Air*, leads to derepression not only of the overlapping *Igf2r* gene, but also two paternally repressed genes located further upstream, *Slc22a2* and *Slc22a3*, which do not overlap with the antisense [68]. However, while *Tsix* apparently regulates *Xist* expression, antisense expression is not required for the establishment of inactivation, as a cDNA construct is able to establish silencing [23].

Two untranslated RNA transcripts, roX1 and roX2 (RNA on the X), are involved in sex-specific hypertranscription in male *Drosophila melanogaster,* functioning in conjunction with the male-specific lethal complex, which consists of five protein subunits: male-specific lethal proteins (msl-1, msl-2 and msl-3), maleless (mle) and males absent on the first (mof) [1]. It is not clear how the complex recognizes sequences on the X chromosome, but it appears to involve about 35 chromatin entry sites along the X chromosome, two of which are the roX1 and roX2 genes. These sites are hypothesized to act as nucleation centres for the spread of MSL-mediated hypertranscription [69]. Binding specificity of the complex is dependent upon MSL1, a novel acidic protein, and MSL2, a RING-finger protein [70, 71]. MOF, a histone acetyltransferase, may have a central role in the conformational change mediated by the complex, since the male X is highly enriched for an isoform of histone H4 that is acetylated on lysine 16 [72]. Interestingly, MOF has been shown to interact with roX2 RNA through its chromodomain in vivo, and its specific localization to the X chromosome is RNase sensitive, which suggests that the RNA may be involved in targeting the acetyltransferase to the chromosome [73].

In addition to their roles in dosage compensation, noncoding RNA transcripts are emerging as crucial components in directing chromatin modifications at other heterochromatic regions. The first link between RNA and chromatin modifications was observed in plants, where short double-stranded RNAs could direct the methylation and silencing of homologous DNA sequences [74]. Recent findings in the fission yeast *Schizosaccharomyces pombe* have implicated the RNA interference (RNAi) pathway, and RNA transcripts derived from repetitive se-

quences at the centromere and the mating-type region, in the assembly of heterochromatic chromatin [75, 76]. A role for an RNA component in the higher-order structure in pericentromeric heterochromatin also appears to be conserved in mammalian cells since RNase treatment of permeabilized mouse cells results in loss of the histone H3 lysine (K) 9 methylation and HP1 binding normally present at the centromere [77]. The precise role of RNA transcripts in directing chromatin changes remains unclear; however, models have hypothesized that double-stranded RNA transcripts processed through the RNAi pathway recruit chromatin-modifying proteins such as histone methyltransferases or deacetylases through their chromodomains to nucleate heterochromatin formation [75, 76]. The chromodomain has been reported to interact directly with RNA [73]. At the *Xist* locus, there has so far been no evidence that the transcripts are processed by the RNAi machinery; however, *Xist* expression is required for the establishment of inactive heterochromatin, which involves recruitment of the Polycomb group proteins Eed and Ezh2. The Eed/Ezh2 complex has recently been shown to localize to the inactive X during both random and imprinted X-chromosome inactivation, and is essential for histone methylation at K9/27 [30, 78]. Interestingly, Eed is also required for epigenetic regulation of a subset of autosomal imprinted loci [79].

While knockout and transgenic experiments in mice have shown that *Xist* is sufficient to initiate silencing, the mechanism by which *Xist* expression recruits the ensuing features of heterochromatin requires further elucidation. As the initiating event in inactivation, it seems likely that *Xist* would be a constant feature of mammalian dosage compensation; however the marsupial homolog has not yet been identified, perhaps due to a low level of sequence conservation. *XIST/Xist* is also expressed during spermatogenesis [80–82], while *Tsix*, if expressed, is expressed at substantially lower relative levels than seen in undifferentiated ES cells [83, 84]. Surprisingly, however, male mice carrying deletions of *Xist* that prevent somatic X-chromosome inactivation are still fertile [22], and apparently able to undergo meiotic sex-chromosome inactivation [83], confusing the role of *Xist* in spermatogenesis.

Replication timing

That heterochromatin is late replicating has been known for decades [85], and there is now evidence to suggest that this late replication actually contributes to the silencing of DNA, rather than simply being the result of inactive chromatin. Asynchronous replication of the immunoglobulin and T cell receptor genes is established at the time of implantation, and it is then the early-replicating allele that tends to be the one to undergo rearrangement and subsequent expression (reviewed in [86]). Furthermore, a recent study demonstrated that exogenous DNA injected into cells early in S phase is more likely to be transcriptionally active than DNA injected in late S phase, supporting the conjecture that DNA synthesized in late S phase is incorporated into a repressive chromatin state [87].

In order to replicate the entire mammalian genome within S phase, there are thousands of origins replicating individual replicons on the order of ~100 kb in size. Replication patterns generally correspond to banding patterns, demonstrating that the timing of replication of several replication origins is coordinately regulated to yield a like-replicating domain, whose boundaries coincide with R/G banding boundaries [86]. The identification of individual metazoan origins has been difficult, and no consensus sequence has been identified; however, origins in mammalian chromosomes have been suggested to be enriched in CpG islands [88]. It has also been suggested that epigenetic chromatin structure may contribute to the definition of mammalian origins, an argument supported by the association of the Origin Recognition Complex (ORC) with HP1 and histone acetyltransferase in humans [89]. While several studies have identified origins on the X chromosome [90, 91], only recently has it been shown for origins in the *G6PD* and *HPRT* genes that the same origin is used on both the active and inactive X chromosomes [92].

Evidence for late replication of the X chromosomes was first obtained by observation of the whole chromosome (e.g. [93, 94]), and later extended by the study of individual genes (see table 1). The introduction of a combination of bromodeoxy-uridine (BrdU) incorporation and Giemsa staining allowed the more precise definition of replication patterns on a chromosome [95]. While variability is observed between cells of the same type, as well as between cell types, bands Xp22, Xp11, Xq13 and Xq26 are often earlier replicating than the rest of the inactive, late-replicating X chromosome [96–99]. It was suggested that at least some of these early replicating segments on the inactive X chromosome may contain genes escaping inactivation [100]. In some situations the inactive X can be early replicating, as was shown for a mouse thymic lymphoma cell line. Interestingly, the inactive X in this line could be induced to become late replicating by fusion with EC cells, suggesting that replication timing was under the control of *trans*-acting factors [101]. Additionally, during mouse development the first evidence of a differential replication pattern for one X (allocyclic replication) is a shift to earlier replication [102].

Studies of X chromosome replication timing for specific regions or loci have used a variety of approaches (see table 1), with the general conclusion that silent genes/regions tend to replicate late. Genes silenced in a tissue-specific manner (e.g. F8, F9) are generally late replicating, although they show somewhat earlier replication on the ac-

Table 1. Summary of replication patterns at X-linked loci.

Locus/Region	Result	Technique	Reference
HPRT1 F8	inactive X late limited asynchrony	Southern analysis following BrdU incorporation	[103]
HPRT1 FRAXA[#] RPS4X ZFX[#] XIST	asynchronous asynchronous synchronous synchronous inactive X early	FISH	[104]
F8 FMR1 XIST	both X's late later when mutant active X late	FISH	[105]
XIST HPRT1 FMR1 F9 SLC16A2 PGK1 IDS G6PD	inactive X late inactive X late inactive X very late inactive X later inactive X late inactive X late inactive X late inactive X late	flow cytometry of BrdU labeled DNA	[109, 106, 110]
PGK1 XIST	active X early active X early	quantitative allele specific PCR after flow cytometry	[107]

[#] The RPS4X and ZFX genes are expressed from the active and inactive X chromosomes.

tive X chromosome [103]. A common approach to analyzing replication timing of individual loci has been to use FISH, under the assumption that probes showing two signals (double dot) have replicated, while ones showing a single dot have not yet replicated, so that replication asynchrony is evidenced by the proportion of cells showing single/double signals. Using this technique, two early studies suggested the silent *XIST* on the active X chromosome was late replicating [104, 105]; however, a further study using an approach based on the isolation of newly replicated DNA that had incorporated BrdU showed the *XIST* locus on the inactive X to be late replicating [106], and these authors suggested that the FISH double dot would reflect not only replication but also chromatin conformation allowing separation of the replicated chromatids. The early replication of the silent *XIST* allele on the active X chromosome has been supported by a direct quantitative measurement using allele-specific polymerase chain reaction (PCR) following flow cytometry [107]. Interestingly, the *FMR1* gene is a late-replicating domain that replicates even later when on the inactive X, or silenced by expansion of the 5′ CCG repeats in fragile X syndrome. The distal boundary of this domain is 350–600 kb 3′ of *FMR1* in proximal q27, supporting the theory of coordinate activation of adjacent replicons. Apparently at least three replicons can be delayed in their replication after expansion of the FRAXA trinucleotide repeat [108]. Late replication is a consistent feature of silent heterochromatin, and appears to be established early in the silencing of the X chromosome. Replication asynchrony can be disrupted by factors influencing DNA methylation or chromatin modifications (see below); however, a late-replicating X chromosome is observed in marsupials [111], suggesting that it is a conserved event in the inactivation process.

Histone modifications

Over the past few years there has been a rapid increase in information describing the role of histone modifications in controlling gene expression, including the suggestion of a histone regulatory 'code' superimposed upon the genetic code (reviewed in [112]). The histones, which form the protein substructure of chromatin, are small highly basic proteins, and their amino-terminal 'tails' can be modified by acetylation, phosphorylation, methylation, ubiquitination and ADP ribosylation (reviewed in [113]). In addition to this plethora of modifications, there are interactions between modifications at different amino acid sites of the different histones (reviewed in [114]). Studies of histone modifications on the inactive X chromosome have focused upon acetylation and methylation. As most analyses of histone modifications are based on reactivity to modification-specific antibodies, caution must be used in interpreting results because individual antibodies may not recognize all levels of modification (for example, mono-, di- and trimethylation), or may be specific to combinations of modification at adjacent residues (reviewed in [114]).

Histone methylation

The inactive X has been reported to be methylated at K9 of histone H3 [61, 115] and hypomethylated at K4 of hi-

stone H3 [116], similar to modifications seen in pericentromeric heterochromatin. However, recent reports suggest that inactive X may show a more distinctive K27 trimethylation accompanied by Eed/Ezh2 binding as an early, transient event in the establishment of inactivation [30, 78]. The *C. elegans* homologs of these genes (MES-2 and MES-6) are needed for germline silencing [117].

In somatic cells, histone methylation is most dramatic at the promoter, but can be observed in the body of silenced genes [61], and differential methylation is also maintained in rodent/human somatic cell hybrids [116]. There are regions of the inactive X that seem to stain positively for antibodies to methylated H3 (K4). These include the Xp pseudoautosomal region, Xp11 and Xq25-26 [116] and may reflect regions with genes that escape X-chromosome inactivation [118].

Many histone methyltransferases have been identified recently, and the SET domain, which is the canonical sequence found in most histone methyltransferases, is found in over 70 genes [113]. The Polycomb group protein Ezh2 contains a SET domain, which presumably accounts for the initial methylation of the inactive X; however, as the association with Eed/Ezh2 is transient, other methyltransferases are likely involved in the maintenance of methylated chromatin. The first histone lysine methyltransferase identified was from the SUV39H family that methylates histone H3 at K9. This methylation allows binding of the chromo domain of heterochromatic HP1 proteins, which form complexes to recruit the SUV39H methyltransferases, both of which are enriched at pericentromeric heterochromatin, providing a mechanism for propagation of heterochromatin [119]. However, the histone H3-K9 methylation of an inactive X occurs in the absence of SUV39h [113]. There is also no enrichment of HP1 alpha, beta or gamma on the inactive X in somatic cells [115]. However, in the X/Y body seen in spermatogenesis, Suv39h2, a testis-specific isoform of the histone methyltransferase, and the HP1 homologue, M31, are recruited to the silenced chromatin [120].

Histone acetylation

In general, histone H4 acetylation is associated with transcriptionally active chromatin, and immunostaining of chromosomes with antiacetylated histone H4 mimics an R banding pattern and is maintained through the cell cycle. The inactive X stains very poorly with such antibodies, although the mouse inactive X chromosome shows a banding pattern with three bands visible [121]. Similar regions are visible on the human inactive X after treatment with the histone deacetylase inhibitor butyrate, and these include the Xp pseudoautosomal region, the proximal short arm (Xp11.2-Xp11.3) and Xq22 [121]. Antibodies to acetylated histone H3 also showed the inactive X to be underacetylated relative to the active X and autosomes. Again, however, bands were observed at the Xp pseudoautosomal region and Xq22 [122]. Hypoacetylation of histone H2A is also seen on the inactive X [122].

Chromatin immunoprecipitation (ChIP) is a procedure involving cross-linking of DNA with chromatin proteins before immunoprecipitation, thereby coprecipitating DNA with antibodies to chromatin-associated proteins. Using ChIP with antibodies to acetylated histone H4, Gilbert et al. demonstrated that H4 acetylation at promoter regions correlated with expression in hamster hybrids retaining the human active or inactive X chromosome. Furthermore, the highest levels of acetylation were found at promoters relative to even a few kilobase pairs away [123]. In contrast, Morrison and Jeppesen showed underacetylation of a polymorphic marker on the inactive X in a lymphoblast line with nonrandom inactivation over 20 kb from the promoter [124]. While this could reflect differences acquired in hybrids, the existence of promoter-specific effects is supported by the association of histone acetyltransferases (HATs) and histone deacetylases (HDACs) with the transcriptional machinery. In contrast, cytological observations show that even in metaphase chromosomes, histone acetylation reflects the R bands where the majority of constitutively expressed genes are located and shows a global restriction from the inactive X, supporting that differences beyond promoter regions also exist [124]. Thus it seems likely that the strongest difference is found at promoter regions, but that there are also more globally distributed differences that give rise to cytologically observed differences in acetylation levels.

While histone acetylation is maintained as a balance between the activity of HATs and HDACS, there are no known histone demethylases, so histone methylation may be a more stable epigenetic mark that is maintained, once established, until replication or replacement of the histone. The balance between various modifications is demonstrated by the negative relationship between methylation of histone H3 K4 (which correlates with transcriptional activity) and methylation at K9 (which correlates with silencing), as well as the fact that K9 of histone H3 can only be acetylated or methylated [114]. Hypoacetylation of histone H4 is also observed for the inactive X chromosome of marsupials, suggesting that it is a primordial feature of mammalian X inactivation [111]. Interestingly, histone hypoacetylation observed on the somatic inactive X chromosome is not detected on the X/Y body [125], while histone methylation is, as discussed above.

Macrohistone H2 and the macrochromatin body

In addition to modifications of the core histones, there is also preferential incorporation of variant histones into the

chromatin of the inactive X chromosome. The macrohistone H2A1 (mH2A1) histone H2A variant is three times the size of conventional H2A. The N-terminal third of the gene shows 65% amino acid identity to histone H2A, while the novel C-terminal portion shows homology to several genes, including several proteins encoded by RNA viruses, *BAL*, a gene overexpressed in aggressive lymphoma [126], as well as another histone H2A variant, macrohistone H2A2 (mH2A2) that shows ~70% amino acid identity to mH2A1. The mH2A1 gene (*H2AFY*) is an intron-containing gene that maps to human chromosome 5q31-32, and encodes two alternative splice variants, macroH2A1.1 and macroH2A1.2, which differ by the utilization of an alternate pair of sixth exons in the non-histone, leucine-zipper region. MH2A1 is present in both male and female cells; however, in female somatic cells the histone variant is condensed into a 'macrochromatin body' or MCB that colocalizes with the inactive X chromosome [127]. The mH2A2 gene (H2AFY2) maps to human chromosome 10q22.3 and shares a very similar genomic organization to mH2A1, suggesting a relatively recent evolutionary origin by gene duplication. This variant is also concentrated upon the inactive X chromosome in female somatic cells [128], although other histone variants such as H2A.X, H2A.Z or H2A-Bbd (Barr body-deficient) are not enriched, or are excluded from the inactive X [129].

The N-terminal histone homologous domain is sufficient to direct MCB formation; however the non-histone domain could also direct MCB formation when fused to H2A or H2B domains [130]. The function of mH2A in X-chromosome inactivation is unknown; however, the non-histone region of mH2A1 can repress transcription when recruited to a site by fusion to GAL4 binding sites [131], and the leucine zipper region of the non-histone domain of mH2A1.2 has been reported to bind the MATH domain of the Spop protein [132]. Furthermore, the non-histone domain has been shown to disrupt transcription factor binding; while the H2A-like domain apparently blocks SWI/SNF nucleosome remodeling [133].

In differentiating ES cells, mH2A1 relocalizes from the centrosome to the inactive X chromosome after initiation and propogation of X inactivation [134, 135]. In mouse embryos, association with the inactive X begins between the 8- and 16-cell stage, and therefore MCB formation may be an earlier event in the extraembryonic tissues [8]. Association of mH2A1 with the centrosome is seen in all cell types, but the detection can be influenced by fixation techniques [136]. Centrosomic association is cell-cycle dependent, increasing as the association with the inactive X dissipates in late S phase, perhaps reflecting a degradation pathway, possibly shared by several other chromatin proteins [137].

Conditional deletion of *Xist* results in loss of the MCB, despite ongoing maintenance of silencing, and *Xist* transgenes can recruit mH2A1 localization to autosomal sites [32]. Induction of *Xist* in undifferentiated ES cells did not yield an MCB; however, upon induction of *Xist* expression in differentiated MEF lines, where inactivation does not occur, MCB formation can be detected [138], suggesting that *Xist* may recruit mH2A in a developmentally regulated manner independent of inactivation. Although a direct interaction has yet to be demonstrated, continued *Xist* expression is required for mH2A localization, and a specific domain at the 3′ end of *Xist* has been identified that is required for MCB formation [25]. In metaphase inactive X chromosomes, mH2A1 can be seen to preferentially associate with several regions, including Xp22, Xp11, Xq13 (indistinguishable from *XIST* location) and Xq22-24, at the DXZ4 repeat and overlapping the site of histone H3 lysine-4 methylation [137].

The mH2A1 gene is conserved beyond mammals – it shows greater than 90% amino acid identity between human and chicken, supporting a role in addition to its involvement in X-chromosome inactivation. Other histone variants show associations with heterochromatin, notably a histone H3 variant is associated with centromeric heterochromatin [139], and the H2A.Z variant has recently been shown to be found at transitions between euchromatin and heterochromatin [140]. mH2A1.2 is also present in the XY body during spermatogenesis [141, 142], as is the histone H2A variant H2AX, which is required for sex body formation and meiotic sex chromosome inactivation [143].

DNA methylation

DNA methylation is an epigenetic modification that involves the transfer of a methyl group to the 5 position of a cytosine residue in CpG dinucleotides in mammals. DNA cytosine methylation is developmentally regulated and itself plays an important role in the regulation of gene expression in vertebrate development [144]. The majority of CpG sites in the vertebrate genome are methylated, and the spontaneous deamination of these methylated cytosines into thymidine is thought to be responsible for the underrepresentation of CpG dinucleotides in the mammalian genome. However, certain regions of the genome, called CpG islands, remain largely unmethylated and retain the expected CpG frequency. These clusters of CpGs are often associated with the promoter regions of ubiquitous housekeeping genes and some tissue-specific genes and generally remain unmethylated regardless of their transcription status [145]. However, regulated methylation of CpG islands does occur on the inactive X chromosome, as well as in imprinted regions, and contributes to stable silencing of the associated genes [146].

Methylation is catalyzed by DNA methyltransferases (Dnmts), and three active DNA cytosine methyltrans-

ferases have been identified in mammals: *Dnmt1, Dnmt3a* and *Dnmt3b* (reviewed in [147]). *Dnmt1* was the first to be cloned and is believed to be a maintenance methylase; however, there is evidence that *Dnmt1* may also have de novo methyltransferase capabilities. *Dnmt3a* and *Dnmt3b* are required for de novo methylation during mammalian development [148, 149]; however, they have also been shown to interact and functionally cooperate with *Dnmt1* to maintain methylation patterns in human cancer cells and in mouse ES cells [150–152]. Developmentally specific isoforms of both *Dnmt1* and *Dnmt3* have been characterized and are likely responsible for the establishment and maintenance of the distinct methylation patterns observed at different stages in development [147].

DNA methylation can regulate gene expression by different mechanisms [153]. Methylation at CpG dinucleotides may directly interfere with the binding of transcription factors to their recognition sequences. Alternatively, methyl-CpG-binding proteins may directly compete with transcription factors for binding sites at the promoter or may initiate the recruitment of corepressors to alter the chromatin structure. The methyl-CpG-binding protein MeCP2 can recognize and bind DNA sequences containing a single methylated CG dinucleotide [154], and through its association with the transcriptional repressor Sin3A and histone deacetylases HDAC1 and HDAC2, it can alter chromatin structure and silence transcription in a methylation-dependent manner [155]. In addition to directing chromatin remodeling through histone deacetylation, MeCP2 is also associated with histone methylation activity specific for lysine 9 of histone H3. Therefore, MeCP2 can direct chromatin modifications not only through the recruitment of histone deacetylase activity, but also the recruitment of histone methylation [156]. Similarly, another methyl-CpG-binding protein, *MBD2*, which requires >10 methylated CpGs to bind DNA, forms a complex with the ATP-dependent chromatin remodeling protein Mi-2 and histone deacetylases *HDAC1* and *HDAC2* [157, 158]. Therefore, methylation at CpG sites can target repressor complexes to specific regions in the genome, directing deacetylation and methylation of histones and formation of silent chromatin structures.

DNA-mediated gene transfer experiments provided the first evidence that DNA from the inactive and active X chromosomes was differentially modified and functionally inequivalent (reviewed in [159]). A role for DNA methylation in at least some of the functional differences between the active and inactive X was supported by early observations that unlike most CpG islands which remain unmethylated regardless of the transcriptional status of the gene, CpG islands associated with genes on the X chromosome tend to be heavily methylated on the inactive X, but unmethylated on the active X, and genes that escape inactivation are often unmethylated. In addition, treatment of interspecific somatic cell hybrids with the demethylating agent 5-azacytidine resulted in reactivation of the silenced genes on the inactive X, demonstrating a direct role for methylation in the maintenance of inactivation.

Targeted disruptions of the DNA methyltransferase loci in mouse have further defined the role of methylation during both the initiation and maintenance stages of X inactivation. Methylation during initiation of inactivation appears to be important for regulation of *Xist* expression since hypomethylation at the *Xist* promoter in *Dnmt1* mutants results in ectopic *Xist* expression and inactivation [42, 43]. In addition, methylation by *Dnmt1* appears to be important for maintaining the silenced state in the embryonic lineage since transgenes integrated into the X often reactivate in mutant embryos. However, methylation does not appear to play as crucial a role in imprinted inactivation in extraembryonic tissues since inactivation appears to be properly maintained in *Dnmt1* mutant embryos [160]. The *Dnmt3* family of methyltransferases also appears to be involved in maintaining X inactivation. Mutations in the *DNMT3B* gene underlie ICF immunodeficiency syndrome (immunodeficiency, centromeric decondensation, facial anomalies), which results in hypomethylation of CpG islands, advanced replication timing and destabilization of the silenced state of X-linked genes normally subject to inactivation [161]. Therefore, the methylase activities of *Dnmt1* and *Dnmt3b* are required for both initiation and maintenance of the inactive state on the X chromosome.

DNA hypermethylation does not, however, appear to be an essential aspect of X inactivation in all mammals. Imprinted inactivation in marsupials, as well as inactivation in extraembryonic tissues, occurs without extensive methylation [162, 163]. However, inactivation in these cases is less stable and is often incomplete, which suggests that DNA methylation may be a recent acquisition in eutherians associated with more stable, random inactivation [164].

Other changes associated with an inactive X

The presence of the Barr body in female cells was one of the key observations upon which Lyon based her original hypothesis of X-chromosome inactivation [165]. The Barr body stains brightly with DNA stains, which suggests it is more condensed than the active X chromosome. However, more recent cytological examinations suggest that the inactive X chromosome is not dramatically reduced in volume relative to the active X chromosome; rather, it shows a differential shape [166]. The Barr body was originally described as being adjacent to the nucleolus [167], and is also often found at the nuclear periphery [168], consistent with its heterochromatic state. Therman suggested that because isodicentric X chromosomes

formed 'bipartite' Barr bodies, there was a region in proximal Xp that escapes inactivation [169], which has been supported by recent studies of human genes that escape inactivation [118].

The inactivated X chromosome is also seen to be 'folded' in metaphase spreads at an unusually high frequency [170, 171]. This bend is found in several species, and appears to be located at the site of the X inactivation center [172]. Such a bend could result from the telomeres of the inactive X being closer to each other than those of the active X, suggesting a looped structure in interphase [173], although this has not been supported by multiprobe analysis [174]. Finally, the inactive X chromosome is less sensitive to DNase I than the active X chromosome [175, 176], consistent with active chromatin being preferentially sensitive to this enzyme [177]. In addition, general DNase sensitivity and the presence of DNase hypersensitive sites at individual genes corresponds to their activity (e.g. [178–180]); however, there does not appear to be a difference between the active and inactive X chromosomes in their scaffold-associated regions [181].

Evidence for complementary and interactive roles of chromatin changes in X inactivation

Once established, the inactivated state is extremely stable due to the interplay of several different repressive mechanisms. These interactions are shown schematically on figure 1. Expression of *Xist* seems to be necessary and sufficient for the induction of X inactivation; however, it is not clear how expression results in the acquisition of heterochromatin and gene silencing. *Xist* expression does recruit mH2A independently of silencing; however, the formation of an MCB has not been associated with other features of X inactivation. *XIST* localization is lost in mouse/human somatic cell hybrids [46, 182], which have also been shown to be more susceptible to gene reactivation with demethylating agents [159]. Furthermore, hybrids are reported to lose the peripheral localization of the inactive X [168]. While *XIST* is not essential for maintenance of an inactive X [32–34], loss of *Xist* does result in a slightly elevated reactivation frequency, which is enhanced by inhibition of DNA methylation or histone deacetylation [31].

Replication timing appears to be closely linked with DNA methylation. Changes in replication are induced by treatment with 5-azacytidine, an inhibitor of DNA methylation, in a cell-dependent manner [183–186]. 5-aza-deoxycytidine acts late in S phase to inhibit condensation of late replicating X [187], and also affects the constitutive heterochromatin of centromeres [188]. Furthermore, ICF cells that are mutated for *DNMT3B*, in addition to being hypomethylated, also display altered replication patterns, with many X-linked loci replicating earlier in S phase. Hypomethylation of satellite sequences is also observed in ICF cell cultures, and some sequences tend to show advanced replication [189]. Inhibitors of HDACs also disrupt replication timing (reviewed in [86]). In somatic cell hybrids, induction of gene expression by 5-azacytidine has been demonstrated to result in early replication of the reactivated gene; however, an advance in replication timing for *XIST* and *F9* could be detected after treatment even when transcription was not induced [110]. The nature of the relationship between DNA methylation and replication timing is not clear; however if CpG-rich sequences serve as origins of replication [88], methylation of these islands might alter origin use or delay the time of firing. There is growing support for a model in which a 'timing decision point' early in G1 establishes replication timing, and that association with the nuclear periphery at this decision point delays initiation of replication at origins, perhaps through association with chromatin regulators such as chromodomain proteins [86].

A link between DNA methylation and histone methylation has also been observed in several species. In *Neurospora crassa* histone methylation is required for DNA methylation, and similarly in *Arabidopsis thaliana* mutations in histone methylation disrupt DNA methylation [190, 191]. In mammals, many of the methylated DNA-binding proteins recruit HDACs [147], and chromatin-remodeling complexes influence DNA methylation [192, 193]. In addition, MeCP2 has recently been shown to be associated with histone methylation activity specific for lysine 9 of histone H3 and can therefore direct chromatin modifications to sites of DNA methylation not only through histone deacetylation, but also through the recruitment of histone methylation [156].

Figure 1 demonstrates that rather than being a simple stepwise accumulation of chromatin changes that lead to silencing, X-chromosome inactivation involves a complex interaction amongst the various events. Indeed, the inactive X can be retained after loss of *XIST* expression [33, 34], changes in replication timing [101], altered histone modifications [194], loss of the MCB [32] or the absence/reduction of DNA methylation (as in marsupials [3], ICF syndrome [189] and extraembryonic tissues [162]). Reactivation of individual genes occurs at a higher frequency when a feature is disrupted, such as in somatic cell hybrids that have delocalized *XIST* [159] or marsupials that lack DNA hypermethylation [3]. This reactivation frequency can be elevated synergistically when multiple features are lost [31]. Reactivation of an entire X chromosome is rarely observed – it occurs naturally during oogenesis and is also observed upon somatic cell fusion of extraembryonic cells [195]. While there is strong evidence that stable expression and localization of *Xist* is the initiating event in X-chromosome silencing, it is not yet clear how all the other heterochromatic features are assembled to result in the extremely stable inactivation of

the X chromosome. The recent identification of additional players in the process, such as Eed/Ezh2, macroH2A and BRCA1 may start to provide insights into the initiation and maintenance of silencing.

X-chromosome inactivation is clearly essential for the normal development of mammalian females. The choice of chromosome to inactivate is normally random in humans, and thus females tend to be protected from X-linked disease. Skewing of inactivation can occur due to chance, or more commonly due to selective advantages or disadvantages of certain cell populations, as seen with X-chromosome rearrangements or some X-linked mutations. Primary skewing of inactivation is seen in mice, and may be associated with variants in humans, possibly of the *XIST* gene [37, 38], but it is not known how common such variants may be. Skewed inactivation has also been reported in individuals who experience recurrent spontaneous abortions [196, 197], or are at risk of ovarian or breast cancer [198–200], but no mechanistic association has yet been conclusively demonstrated. In addition to answering questions of clinical importance, the study of X-chromosome inactivation can serve to address general mechanisms of epigenetic silencing. While the *XIST* RNA is unique to mammals, silencing by RNAs may be a more common event than previously thought. The study of epigenetic silencing has gained much attention recently as its role in expression changes associated with cancer [201] and somatic reprogramming [202] has been detailed, but recent reports also suggest roles in diverse areas such as development [203], circadian clocks [204], long-term memory [205] and quantitative traits [206]. In the over 40 years since X-chromosome inactivation was first hypothesized [165], much has been learned, but there is still much more to learn about the silencing of one X chromosome in mammalian females.

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