

SILENCING OF THE MAMMALIAN X CHROMOSOME

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■ **Abstract** Mammalian X chromosome inactivation is one of the most striking examples of epigenetic gene regulation. Early in development one of the pair of ~160-Mb X chromosomes is chosen to be silenced, and this silencing is then stably inherited through subsequent somatic cell divisions. Recent advances have revealed many of the chromatin changes that underlie this stable silencing of an entire chromosome. The key initiator of these changes is a functional RNA, *XIST*, which is transcribed from, and associates with, the inactive X chromosome, although the mechanism of association with the inactive X and recruitment of facultative heterochromatin remain to be elucidated. This review describes the unique evolutionary history and resulting genomic structure of the X chromosome as well as the current understanding of the factors and events involved in silencing an X chromosome in mammals.

OVERVIEW

X inactivation, as first hypothesized by Lyon in 1961 (80), results in the silencing of one of the pair of X chromosomes in mammalian females. This achieves dosage equivalency with males who have a single X chromosome and the sex-determining Y chromosome. Once an X is chosen to be active or inactive this state is stably inherited through subsequent somatic mitotic divisions, resulting in females being mosaics for cells with each parental X active (as seen in the patches of a tortoiseshell or calico cat). Thus, X inactivation is a remarkable example of epigenetic inheritance, silencing more than a thousand genes and able to distinguish between one of an essentially identical pair of chromosomes. The process also exemplifies the plasticity of such epigenetic events, as the X reactivates during oogenesis, permitting random inactivation in the next generation.

Research in the last 40 years has shown that the inactive X acquires many of the features of heterochromatin, including DNA methylation, histone modifications, late replication timing, and a peripheral nuclear location. The identification and characterization of the *XIST* gene suggests that the critical initiator of the

process is a large, functional RNA. With sequencing of the human genome complete, we now know virtually the entire sequence of the X chromosome (105a), and sequencing of additional mammalian and vertebrate genomes has yielded considerable insight into the unique evolutionary history of the X chromosome. But how XIST orchestrates the *cis*-limited heterochromatinization of the X chromosome in mammalian females remains a topic of investigation and speculation. We review the unique biology of the X chromosome and the *cis*- and *trans*-acting factors and regions that interact to achieve silencing of the approximately 155-Mb X chromosome.

ORIGIN OF MAMMALIAN SEX CHROMOSOMES

Comparative mapping in the three major classes of mammals—eutherians, metatherians (marsupials), and prototherians (monotremes)—has identified the region distal to Xp11.23 as a potentially more evolutionarily recent autosomal addition specific to eutherians (135; reviewed in 46) (see Figure 1). Because genes proximal to human Xp11.23 are present on the X chromosome in all three mammalian classes, this X conserved region (XCR) must be at least 170 million years old. The genes on human Xp distal to the Xp11.23 fusion point, however, are autosomal in both marsupials and monotremes, implying a more recent addition of autosomal material [coined X added region (XAR)] onto the eutherian X, after divergence of the marsupials from eutherians. Hence, this addition must have occurred between 130 million years ago (mya), after the eutherian-marsupial divergence, and 80 mya, before the eutherian radiation.

Recently, origins of the X chromosome were revealed by comparative mapping between mammals and the more distant vertebrate genomes of fish and chicken (58, 67, 105a). Because the sex chromosomes of mammals appear completely unrelated to those of birds, mammalian sex chromosomes are thought to have evolved from a pair of autosomes between 300 and 350 mya, shortly after the avian-mammalian divergence. Synteny of the XCR to 4p in chicken compared to synteny of the XAR to chicken 1q13–q31 supports derivation of the eutherian X from separate autosomal counterparts (67). However, it appears that parts of these regions map together to the same chromosome in pufferfish and zebrafish (48, 67), so it is possible that fission of the XAR occurred in monotremes and marsupials, followed by refusion in eutherians.

The mammalian sex chromosomes probably differentiated from an autosomal pair when a male sex-determining mutation arose on the Y, likely changing the X-linked gene *SOX3* into a new penetrant allele *SRY* (reviewed in 70). Lahn & Page (69) used synonymous mutation rates (*K_s* values), considered to be selectively neutral, to gauge evolutionary time of the sex chromosome's divergence (69). By measuring *K_s* for X-Y gene pairs in humans, they found that the genes fell into four distinct groups, which they hypothesized to reflect the existence of four evolutionary “strata” that were caused by large-scale rearrangements on the ancient Y. These were likely major inversions that led to recombination suppression and

divergence of the Y through the sequential accumulation of mutations (69), stratum 1 being the earliest (oldest) and stratum 4 being the most recent group to undergo X-Y differentiation. Based on the locations of genes across species, they also determined that stratum 4 is 30–50 million years old, stratum 3 is 80–130 million years old, stratum 2 is 130–170 million years old, and stratum 1 is 130–350 million years old. Further analysis of the recently completed X chromosome sequence suggests that stratum 4 can be further subdivided (105a). Furthermore, gene order on the X chromosome was conserved, whereas the corresponding genes on the Y showed scrambled Ks groups, consistent with chromosomal rearrangement of the Y. In addition, the Ks values indicated that *SOX3* and *SRY* are the oldest known X-Y pair in humans, supporting the idea that mutation of *SOX3* was the first step in X-Y differentiation (69).

The pseudoautosomal regions (PARs), at distal Xp and Xq, undergo meiotic recombination, enabling exchange of material between the sex chromosomes. PAR pairing between the X and Y is critical for meiotic segregation in many mammals. However, the absence of PARs in marsupials and some rodents implies that pairing at the PAR is not crucial in all mammals and that no recent additions occurred in the marsupial lineage (reviewed in 45). Furthermore, the genes on the PAR in mouse are distinct from those on the human PAR, and many lineage-specific rearrangements have occurred in the PAR (see Figure 1). The complexity of the rearrangements that have occurred is demonstrated by the PAR2, which appears to have been added to the eutherian X and then rearranged at least four times before it was transposed to the Y chromosome (20).

Because lack of recombination reduces the chance of countering harmful mutations by recombination, Muller's ratchet dictates that inversions on the Y would lead to erosion of Y gene function. Consistent with this, there are a high proportion of nonfunctional pseudogenes on the Y chromosome (reviewed in 70). Because the Y is highly enriched for large palindromic repeats, gene conversion could provide an alternative mechanism to compensate for the lack of recombination on the Y (108). However, this type of "recombination," although increasing the variation of the Y in a whole population, might not lead to a more functional Y. If the Y continues to degenerate a new means of sex differentiation may eventually be required (47). As discussed below, the X chromosome also shows a predominance of large, well conserved, inverted repeats (133).

DOSAGE COMPENSATION

Loss of functional genes on the Y chromosome would result in a dosage imbalance between females with two X chromosomes and males with a single X chromosome. Therefore, a compensatory mechanism is necessary to deal with expression levels of genes that are hemizygous in males and disomic in females. It is likely that during the evolutionary transition to dosage compensation a first event would involve upregulation of genes on the single male X chromosome to maintain expression levels. Consistent with this hypothesis, studies in mice show that some genes

might be compensated for by an increase in expression (1). However, this aspect of gene dosage compensation is relatively unexplored, whereas the process of X chromosome inactivation is better elucidated.

In mammals, all but one X chromosome is silenced early in embryogenesis, except in the rare situations of triploids and tetraploids, which demonstrate that one X per diploid set is retained active (90). Silencing can spread into autosomal material in X/autosome translocations (e.g., 74, 81), which may result in functional gene imbalances. Therefore, Susumo Ohno predicted that genes that are X linked in one mammalian species would be X linked in all other mammalian species (95). Genomic mapping and sequencing have validated Ohno's Law (131), with the exceptions noted above with respect to the XAR and PAR. Similarly, there are also exceptions to the rule of inactivation—some genes continue to be expressed from the inactive X chromosome—and these are discussed in detail below for the clues they can yield about the genomic features leading to X silencing.

THE UNIQUE NATURE OF THE X CHROMOSOME

The evolutionary history of the X has resulted in a unique genomic structure for the chromosome (see Figure 2). The X now spends two thirds of its time in females, enabling differential evolutionary pressures resulting from sex-specific selection or mutation frequencies. The human X chromosome is approximately 155 Mb in size and contains 1098 annotated genes (105a). Thus, the X chromosome is among the larger of the human chromosomes, but it has a slightly lower gene density than the autosomes. The lower gene density is accompanied by a slight excess of repetitive elements relative to the rest of the genome.

Repetitive Elements on the X

The abundance of repetitive elements on the X is due almost exclusively to enrichment in long interspersed nuclear elements (LINEs)—in particular the L1 subclass—with a significant clustering around the X inactivation center (3). Compared to the autosomes, the X chromosome has nearly twice the number of L1 elements (30% compared to 17%; see Figure 2). The over-representation of L1 elements on the X led Lyon to propose that the prevalence of L1s on the X might reflect an active role in dosage compensation (82; see discussion below). Another common type of repeat is the Alu sequence. Alu repeats are the most abundant retroposon in humans, with a predominance in GC-rich, gene-rich environments (68). This bias appears to be significant on all chromosomes but is most pronounced on the X chromosome, resulting in the X having a comparatively increased density of recently retroposed Alu families (61). This is in sharp contrast to an otherwise overall lower density of SINEs on the X chromosome compared to the rest of the genome (see Figure 2) (3). LTR elements and inverted repeats are also enriched on the X (see Figure 2). The inverted repeats tend to have a preponderance of testis-expressed genes contained within them (133).

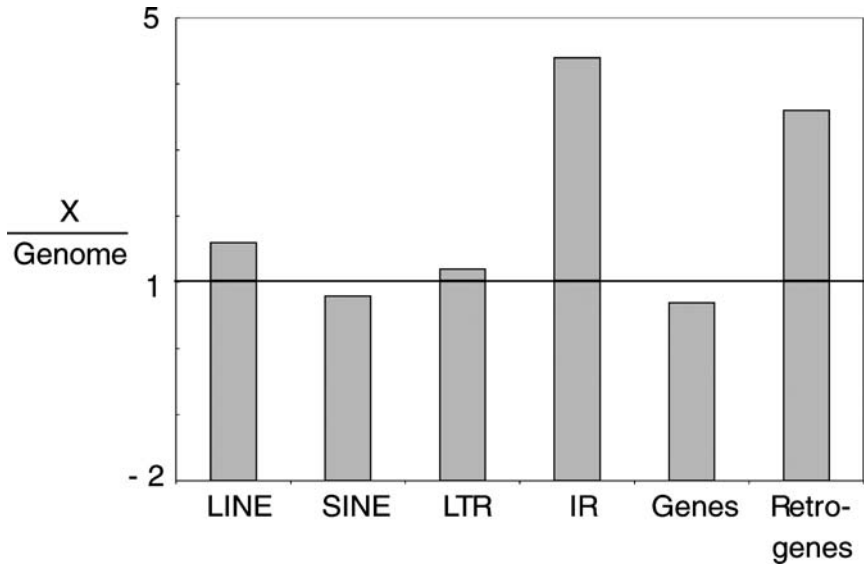


Figure 2 Relative density of repeats, genes, and retrogenes on the X chromosome compared to the whole genome. LINE (long interspersed nuclear element), LTR (long terminal repeat), IR (inverted repeat), and retrogenes are over-represented, whereas SINE (short interspersed nuclear element) and genes are under-represented on the X chromosome (see text for references).

In contrast to its wealth of retrotransposons, the X chromosome has a density of microsatellites that is comparable to that of the autosomes (122). However, the X chromosome microsatellite loci are less polymorphic than the autosomal loci, possibly as a result of different mutation rates (most mutations occur in the male germline, where the X resides only one third of the time) or selective pressures. Because of their high mutability, microsatellites play a significant role in genome evolution by creating and maintaining quantitative genetic variation (62). A subset of simple sequence repeats—trinucleotide repeats—are of particular interest because of the role they play in some human disorders. Of 14 documented genes containing trinucleotide repeats that can expand to cause disease, three are actually X linked, including *FMR1* and *FRAXE* (both causing Fragile X), and *AR* (which causes spinal and bulbar muscular atrophy).

Pseudogenes and Retrogenes

It has been assumed that the number of retroposed copies of genes that chromosomes accept is proportional to their size and, furthermore, that they generate retrogenes in proportion to the number of genes located on them. A recent analysis of chromosomal locations of “parental” genes revealed that in comparison

to the autosomes, the mammalian X chromosome has generated and recruited a disproportionately high number of functional retroposed genes (41). Several X-originated retrogenes have been identified and implicated in reproduction by their testis-specific expression pattern. Silencing the X chromosome during male meiosis (discussed further below) may be the driving force behind the evolution of X-originated autosomal retrogenes. Retrogenes entering the X chromosome tend to avoid female expression (41). Expression inhibition in females might permit the spread of such insertions into the chromosome and thus reflect a positive role for the elements during the evolution of mammalian X chromosomes.

GENES

Twenty years ago Rice (101) predicted that X chromosomes might contain an excess of genes that are sex specific in their expression. There is increasing evidence that X chromosomes have an unusual complement of genes, particularly genes that have sex-specific expression. Recent work indicates that there are functional themes in the gene content of the X chromosome, with certain classes of genes systematically over-represented and other classes under-represented (56). Genes expressed in muscle are especially abundant on the X chromosome (5), as are genes involved in reproduction and genes expressed in the brain (132, 138). For example, nearly half of all genes related to the earliest stages of sperm production reside not on the male sex chromosome, as might be expected, but rather on the X chromosome. In contrast, placentally expressed genes are unusually rare on the X chromosome (65).

THE PROCESS OF X INACTIVATION

The XIC Region

The process of X chromosome inactivation presents a problem unique to mammals in that females inactivate only one of a pair of homologous X chromosomes present in the same nucleus. In contrast, dosage compensation in fruit flies is achieved by the upregulation of the single X in males, whereas there is a downregulation of both Xs in female nematodes (reviewed in 4, 88). Mammalian cells retain one active X per diploid autosome set and inactivate all other X chromosomes in a *cis*-limited fashion. Models often break the inactivation process into different stages including “count,” where the number of X chromosomes relative to autosomes is determined, and “choice,” whereby one X per diploid set is chosen to remain active. Silencing is then established on the other X chromosome and spreads in *cis*, recruiting various heterochromatic features to maintain this silent state through subsequent cell divisions.

One key to the inactivation process is a multifunctional domain present on the X to be inactivated called the X inactivation center (*XIC*), whose presence was first

hypothesized when it was observed that specific X-linked sequences were required in *cis* for inactivation in X/autosome translocations (109). From the analysis of X rearrangements, the *XIC* was mapped to Xq13 in humans and the syntenic region in mouse (Figure 3) (8, 99). Much of the current understanding of the processes of X inactivation has come from studies in mice, and thus much of this summary applies to mouse; the locus names are fully capitalized when referring to human. Various transgenes and knockouts in mouse encompassing different portions of this *Xic* region have further identified essential elements in the region (reviewed in 106). A critical component of the *XIC* is the *XIST* gene, which encodes a more than 17-kb untranslated RNA that coats the inactive X chromosome and is essential for silencing. *Xist* is also sufficient to initiate inactivation because transgenes can silence genes in the surrounding chromatin; however, the *Xist* RNA itself does not appear to be sufficient to recapitulate the entire X inactivation process because elements involved in count and choice lie outside the *Xist* gene. The element(s) responsible for count appears to be contained within a 20-kb bipartite domain 3' of *Xist* whose removal in XO embryonic stem (ES) cells results in inappropriate inactivation of the single X chromosome (25, 92). Although the mechanism of count is currently unclear, it may involve histone H3 lysine 4 methylation within the *Xist* gene, which is altered in these deletions.

The mechanism of choosing which X chromosome will remain active and which will be inactivated appears to be complex and regulated by multiple elements in the *XIC*. In general, inactivation is random in the cells of the eutherian embryo; however, there are examples of skewed choice resulting in nonrandom inactivation patterns. For example, in marsupials and in the extraembryonic tissues in mouse, a parental imprint leads to preferential inactivation of the paternal X chromosome (27, 123). Although the nature of the imprint is unclear, it likely affects one of the many elements involved in X chromosome choice. In mouse, an X controlling element (*Xce*) maps 3' of *Xist* (119) and can skew X-inactivation patterns from the normal 50:50 ratio. Four different strain-specific alleles of the *Xce* locus have been identified and display differing "strengths" such that the weakest allele, *Xce^a*, is the most likely to be inactivated (16, 17). Elements that resulted in skewing of choice during inactivation in humans have also been proposed (93). A second element, *Tsix*, has a main transcriptional start 12-kb downstream of *Xist* and gives rise to spliced and unspliced RNA transcripts antisense to *Xist* that span the entire length of the mouse gene (71, 111, 116). Mouse knockouts, transgenes, and transcript truncations demonstrate that *Tsix* negatively regulates *Xist* through a mechanism requiring overlapping transcription and blocks inactivation on the future active X in both imprinted and random X chromosome inactivation (72, 73, 111, 117). Alterations in *Tsix* expression lead to skewed inactivation patterns, with deletions of the *Tsix* promoter resulting in preferential silencing of the mutant chromosome (72, 73, 111) and upregulation of *Tsix* expression inhibiting inactivation of the altered X (78, 120). Although an antisense to *XIST* has been observed in humans, the functionality of this transcript, which is reduced in size and abundance relative to the mouse, is unclear (11). Another modifier of X chromosome

choice, *Xite*, lies upstream of *Tsix* and appears to act through modulation of *Tsix* expression (94). The *Xite* region contains developmentally regulated DNaseI hypersensitive sites and transcriptional start sites that give rise to low-level intergenic transcripts. Deletions of the *Xite* region implicate it as a positive regulator of *Tsix* expression and it has been hypothesized to be a candidate locus for the previously described *Xce*.

Trans-Acting Factors Involved in X-Inactivation

To retain one active X, current models suggest that autosomal, *trans*-acting factor(s) interact with an X-linked “counting” element and block inactivation of a single X chromosome in a diploid cell. The presence of CTCF binding sites at the *Tsix* promoter region suggests a role for the ubiquitous transcription factor and chromatin insulator in count and choice, possibly through transcriptional regulation at the *Tsix* and/or *Xist* loci (19). CTCF has also been implicated in the regulation of the *XIST* promoter (98a). In addition, mouse mutagenesis screens have identified two autosomal dominant mutations that skew X inactivation patterns early in development (97). Characterizing these loci will help to elucidate their roles in X inactivation.

Trans-acting factors are also involved in interpreting the *XIST* signal and establishing the heterochromatic state. The Polycomb group proteins Eed/Enx transiently associate with the inactivating X soon after *Xist* expression and are responsible for H3 lysine 27 methylation, a mark important for recruiting factors required to create stable, inactive heterochromatin. Other chromatin changes also occur soon after the appearance of *Xist*, including demethylation at lysine 4 and deacetylation at the lysine 9/27 of histone H3, possibly in preparation for subsequent marks that will be deposited on these residues in setting up the heterochromatic state (53, 98, 118). The enzymes responsible for these modifications have yet to be definitively established but may be part of a larger Polycomb-related complex that includes Eed/Enx (7). The Polycomb proteins Ring1A/B also transiently associate with the inactivating X chromosome, mediating histone H2A ubiquitinylation (42). In addition, localized *Xist* is essential for the accumulation of the histone variants macroH2A1 and 2 on the inactive X chromosome to form the macrochromatin body (MCB) (28, 30, 32). Because MCB formation is a relatively late event, appearing several days after the first appearance of *XIST* expression and the first signs of inactivation, it is important for maintaining rather than initiating heterochromatinization (89). BRCA1, the breast and ovarian tumor suppressor, also localizes to the inactive X and is especially interesting because it is the first protein found to be necessary for proper *XIST* localization and stabilization of the inactive state (44) (see Figure 3).

The Establishment of Facultative Heterochromatin

Establishing the inactive state involves acquiring many features of heterochromatin in a sequential and interdependent manner (reviewed in 54) (see Figure 4). In mouse

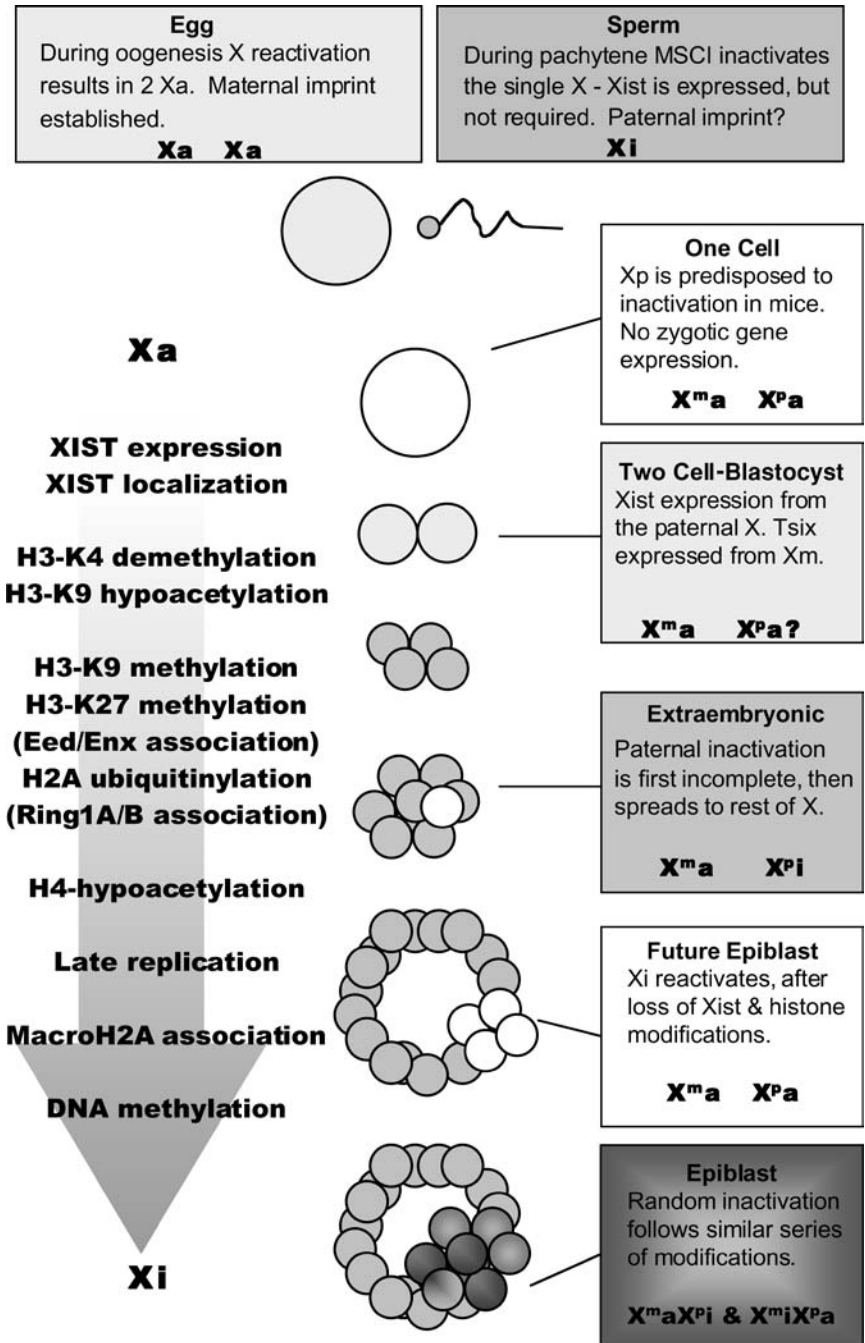
female ES cells, the appearance of these features was monitored upon induction of differentiation and one of the first events in the inactivation cascade is the upregulation and coating of the X chromosome with the *Xist* transcript, which occurs within the first two days of induction. *Xist* coating is followed closely by various histone modifications including hypoacetylation of histone H3; loss of methylation at histone H3 lysine 4; methylation at histone H3 lysine 9, lysine 20, and lysine 27; and ubiquitylation of histone H2A (42, 53, 66, 98, 118). These early changes lead to transcriptional silencing and this inactive state is then “locked in” through other chromatin modifications such as hypoacetylation at histone H4, macroH2A recruitment, and DNA methylation, which act redundantly and synergistically to maintain the silent state (10, 33).

The early appearance of *Xist* expression suggests a role in the initial recruitment of factors required for silencing. However, *Xist*-mediated silencing appears to depend on developmentally regulated factors because *Xist* expression at different times in development leads to variable spread and stability of inactivation. In undifferentiated ES cells, silencing can be initiated by *Xist* expression from an inducible cDNA transgene; however, inactivation at this stage is reversible, requiring continuous *Xist* expression, and is not associated with other features of inactivation such as histone hypoacetylation and late replication (137). Conversely, inducing *Xist* expression after two days of differentiation is not associated with any extensive inactivation in mouse ES cells. Surprisingly, ectopic *XIST* expression is associated with inactivation in human HT1080 somatic cells, indicating that the extent of silencing that can be induced by *XIST* may also be affected by species differences or perhaps cellular transformation (49).

In mouse preimplantation embryos *Xist* expression from the paternally inherited X has been detected as early as the two-cell stage and is associated with transcriptional repression, as evidenced by the absence of elongating RNA Polymerase II and Cot-1 staining, which marks heteronuclear RNA transcripts (96). However, silencing at this stage does not seem to spread to the ends of the X chromosome, with genes close to the *Xic* showing the greatest amount of silencing and more distal genes showing more variability and escape from silencing (57). Silencing at this stage is associated with histone modifications like H3 methylation at lysines 9 and 27 and macroH2A association. These marks are lost in the cells of the inner cell mass, which will give rise to the embryo proper as they lose *Xist* expression and reactivate the X chromosome in preparation for subsequent random X inactivation (83, 96).

X Inactivation During Spermatogenesis

There has been some controversy regarding whether the paternal X arrives in the zygote in a preinactivated state (57) or whether the X is reactivated and then inactivated again at around the four-cell stage (96). It is certainly possible that the paternal X retains epigenetic marks from the transient inactivation of the single X chromosome that is observed during the pachytene stage of meiosis in males,



forming the densely staining sex chromatin body or X-Y body. The molecular basis of this meiotic sex chromosome inactivation (MSCI) is not well understood, and although the final outcome of heterochromatinization and transcriptional silencing is similar to somatic inactivation, the mechanisms are quite distinct. Expression and localization of the *Xist* transcript is clearly necessary for inactivation in female somatic cells; however, in male spermatogenesis, *Xist* is expressed (85, 102, 112), but not required, for spermatogenesis and MSCI because mice with *Xist* deletions are fertile and can form a condensed X-Y body (84, 86, 128). Like the inactive X in female somatic cells, the X-Y body in spermatocytes has an accumulation of the histone variant macroH2A1.2 and BRCA1 (44, 55, 103). The sex chromatin is not, however, hypoacetylated at histone H4 (2). Another protein that accumulates at the X-Y body is the phosphorylated form of H2AX, which is essential for heterochromatinization and transcriptional silencing during spermatogenesis (43).

In addition to differences in the mechanism of MSCI and somatic X inactivation, the role of the two processes differs. Whereas X inactivation achieves dosage equivalence between XX and XY cells, MSCI results in silencing of the single X chromosome, creating a larger imbalance between males and females where reactivation during oogenesis results in two active X chromosomes. The role of MSCI is unclear but several hypotheses have been suggested. Lifschytz & Lindley hypothesized that MSCI may silence genes on the sex chromosomes that are inhibitory to spermatogenesis (75). Unlike autosomes, the heteromorphic X-Y pair can only pair over a limited region, and heterochromatinization may ensure efficient synapsis (129) or prevent recombination between unpaired regions of the two chromosomes (87).

Differences Between Mice and Men

Studies of the developmental process of X inactivation are more tractable in the readily manipulated mouse model system, and these have revealed surprising complexity to the early events of X inactivation (see Figure 4). However, it is important

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Figure 4 The developmental acquisition of X inactivation in mouse. Shown on the left (and as described in the text) are a series of chromatin modifications, protein associations, and DNA modifications that are sequentially observed during X inactivation. The order of events is compiled from studies of early mouse development as well as ES and trophoblast stem cell differentiation (reviewed in 96). Although there are some differences in componentry (35) or timing between the systems (29), the order of events is generally similar. K indicates the lysine that is modified on the histone. Inactivation of the paternal X occurs first, in cells that will give rise to the extraembryonic tissues. This is due to a maternal imprint resisting inactivation (124) as well as potential paternal marks. Reactivation followed by random inactivation results in cells of the epiblast being mosaic for either the paternal or maternal X active. See text for discussion and further references.

to bear in mind that there are differences between inactivation in humans and mice, and thus it remains to be seen how much similarity there will be in the early stages of the inactivation process. Inactivation in the extraembryonic tissues of humans does not appear to involve a strict parent-of-origin mark (77, 139). Expression of human *XIST* is observed in early embryos and, consistent with a lack of imprint, includes maternally derived transcripts (34, 100). However, in human ES cells that had been differentiated toward extraembryonic lineages, there was a nonrandom inactivation suggestive of an imprint in human cells (36). Not unexpectedly for a functional RNA, there are considerable sequence differences between the human and mouse *XIST* genes (6, 9), some of which may account for the failure of the human RNA to bind to metaphase X chromosomes, although it does in mouse (24, 40). Another notable difference between human and mouse inactivation is in the number of genes that escape inactivation.

GENES THAT ESCAPE INACTIVATION

Although most genes on the inactive X are stably silenced, as many as a quarter of X-linked genes are expressed from the otherwise silent chromosome in some females (14). The study of such genes is important both for the insights they may provide into the process of inactivation as well as for their potential role in dosage differences in individuals with X chromosome aneuploidies, and perhaps even between males and females.

Which and Y—Location of Genes that Escape Inactivation

The X inactivation status along the current human X broadly reflects the events of Y decay that occurred at different time points during evolution (see Figure 1). The oldest stratum shows the most complete inactivation, and the most recent stratum shows near complete escape, whereas all genes examined from the PAR1 in the distal Xp of the X chromosome region escape inactivation (14). Thus “escapees” provide a snapshot of the ongoing evolutionary processes, and to some extent reflect the retention of functional homologs on the Y chromosome so that X inactivation would not be necessary to maintain equal dosage between males and females. Some genes that retain Y homologs and escape inactivation occur outside the PARs at considerable distances in both the short and long arm, thus being located in the XCR found in all mammals. This highlights that escapees cannot simply be predicted by their evolutionary history.

Not all genes that escape inactivation in humans have Y homologs. Several models have been proposed to account for the lack of dosage compensation between such genes. Perhaps gene dosage differences are inconsequential between males and females at certain X-linked loci. Alternatively, these genes could confer female-specific functions. Furthermore, differences in gene expression at the RNA level may not translate into differences at the protein level. Transcriptome analysis between males and females did not detect female overexpression for many of the

genes determined to escape inactivation by alternate methods (31). Thus, there may be additional mechanisms regulating transcriptional equivalence between the sexes. However, the importance of X inactivation for dosage compensation is apparent when examining the Xq PAR. Although the two most distal genes escape inactivation as expected, the two more proximal genes in this region (*SYBL1* and *HSPRY3*) are inactive on both the X and the Y (23). Hence, this scenario renders one dose in males but similarly leads to one dose in females.

It is important to emphasize that the inactivation situation in mouse appears to be considerably different than in human (see Figure 1). An extensive survey of more than 600 X-linked genes and ESTs has revealed that 15% of them escape inactivation in human and an additional 10% show variable silencing (14). In mouse, of all loci investigated to date, only *Enox*, *Utx*, *Mid1*, *Smcx*, *Dbx*, *Eif2s3x* (non-PAR), and *Sts* (mouse PAR) appear to escape silencing, and five of these seven genes possess Y homologs (<http://www.informatics.jax.org/>). Because fewer genes, and a more biased selection, have been examined in mice, it is difficult to directly compare the percentage of escapees in the two species. However a more complete silencing of the mouse inactive X would explain the absence of a marked phenotype in 39,X mice. This is in contrast to humans, where 45,X results in Turner syndrome, likely owing to the absence of critical genes expressed from the inactive X (and the Y chromosome).

What accounts for the apparently more complete inactivation of the mouse X chromosome? The presence or absence of human and mouse Y homologs seems to partly explain this discrepancy. For example, *Zfx* and *Ube1x*, which escape inactivation in humans but are subject to inactivation in mouse, have Y homologs that are expressed solely in the mouse testis, but ubiquitously expressed in human tissues. Hence, dosage compensation requires that only one copy be expressed in female mice because only one copy is expressed in male somatic tissues, whereas human males and females would require two doses each (38). Studies by Jegalian & Page also support the importance of Y homologs on silencing pattern. By examining the inactivation status in multiple mammalian species, they found that the escape from inactivation for *RPS4X* seemed to be unique to primates who retained their Y homologs, whereas *Zfx* appeared to be uniquely inactive in rodents who did not possess a Y homolog (59). Thus, some of the differences in X-inactivation status may reflect evolutionary processes that have differentially shaped mammalian sex chromosomes, and a survey of more X-linked genes in other eutherian mammals will help to evaluate the distinct paths of sex chromosome evolution. However, as mentioned above, numerous genes that escape inactivation do not possess Y homologs and it is currently unknown why or how these genes manage to escape silencing within a larger inactivated region.

Escape—Regional or Gene-Specific Control?

Regulation of inactivation may reside at the level of the chromosomal domain or individual genes. The degeneration of X/Y homology in evolutionary steps implies

that a regional mechanism might have been acquired, whereas the study described above showing dependence on retention of a Y homolog for escape from silencing might suggest a gene-specific mechanism. Discordant X-inactivation profiles are observed between closely mapped genes, implicating regulation at the gene level. For example, *UBE1* escapes inactivation, but *RMB10*, which is less than 20 kb away, does not (26). In mouse, the *Utx* and *Smcx* genes escape inactivation despite being surrounded by genes subject to inactivation, suggesting an individual gene mechanism. However, analyzing the promoter of mouse *Smcx* failed to reveal individual gene characteristics that explain its unique escape status, and sequence comparisons between the 5' CpGs of the human and mouse *ZFX/Zfx* gene revealed no significant difference in sequence (38, 79, 126, 127).

In humans, the presence of blocks of adjacent genes that escape inactivation suggests chromosomal domain regulation. One such region is the Xp11.1–11.3 region that maps to a distinct chromosomal origin (chicken chromosomes 1, 4, and 12) compared to the rest of the XCR (chicken 4p) (67, 105a). A comparative analysis of the corresponding segment in mouse reflected a decrease in composition of long terminal repeats (LTRs) in the human escape region but not in the mouse, and thus these may represent elements that enhance silencing in mouse (127). Differential modifications that distinguish genes that escape from those that are subject to inactivation (reviewed in 21) seem to be observed as regions on the inactive X (e.g., 60). Fine mapping of these features shows surprising heterogeneity along the inactive X (130), and further correlation of these features with the X sequence and expression status will provide additional insight into both inactivation and escape from inactivation.

Is the Escape from Silencing or Maintenance of Silencing?

Lack of spread of silencing, perhaps by failure of *XIST* association, may be avoided in regions of escape due to lack of an appropriate binding element, or reactivation may occur due to the presence of limiting factors that prevent stable inactivation, or enrichment of elements that enhance stable silencing in regions of inactivation regions (127). Although an earlier study suggested that CpG islands seem to be less abundant at the 5' end of genes escaping inactivation (63), this seems to be true only for those genes showing variable inactivation patterns (14), which might suggest that the absence of methylation at certain loci fails to stabilize silencing in the region, rendering it prone to reactivation. The mouse *Smcx* gene is initially subject to inactivation and then reactivates early in development, supporting that, for at least some genes, escape reflects reactivation, which is variable among tissues (13, 76, 115). In humans, absence of methylation of the *TIMP1* promoter correlated with stable expression from the inactive X, whereas methylation contributed to low-level and unstable expression in hybrids. Females with ICF syndrome, associated with hypomethylation at 5' CpG islands due to mutations in the *DNMT3B* gene (coding for a DNA methyltransferase), sometimes show abnormal escape from X-inactivation, implicating methylation in stable silencing and also demonstrating

an important role for late replication timing in maintaining the inactive X (51). Boundary elements may be one factor involved in protecting domains of escape from the surrounding heterochromatic context, and CTCF insulators are suggested to be enriched at transition points between genes that escape and are subject to inactivation (38).

Whether there is a precommitment of regions to stable or unstable *Xist* localization and/or subsequent modifications guided by *cis*-acting elements is uncertain. There is evidence that monoallelically expressed genes, including imprinted genes and genes that are subject to X inactivation, display H3 lysine 4 di-methylation restricted to their promoters, whereas genes that escape or are biallelically expressed show additional H3 lysine 4 di-methylation in exonic regions (107). These differences are most drastic in undifferentiated ES cells as opposed to differentiated fibroblasts, highlighting that some modifications could mark the gene status prior to inactivation. Studies of X/autosome translocations have further raised questions as to whether intrinsic *cis* factors prevent the initial spread of silencing or affect subsequent maintenance.

GENOMIC ELEMENTS AND X/AUTOSOME TRANSLOCATIONS

The distinct evolutionary history of the X necessitates X inactivation and may also lead to differences in genomic composition that are only indirectly associated with inactivation. Because dosage compensation by X inactivation appears to continue to adapt to X/Y divergence, each mammalian lineage may show differences in the process. Thus, examining differences between regions of better inactivation or escape within a species is a powerful approach to identify *cis*-acting regions positively associated with the propagation of the inactivation signal. In particular, natural X/autosome translocations may bear inherent sequence differences that allow for comparison of X-inactivation spread.

Studies of X/autosome translocations in mice have demonstrated that *Xist* is excluded from autosomal material in the derivative chromosomes and that a high proportion of autosomal genes escape inactivation (40). Normally, *Xist* RNA shows preference for gene-rich G-light bands (R-bands) but is excluded from constitutive heterochromatin such as the centromere. In humans, as well, a large proportion of autosomal genes escape inactivation (136), and late replication, deacetylated H4, and the *XIST* RNA are often lacking from the autosomal portion (50, 64). In some cases the autosomal material retains some features of heterochromatin and, in general, the histone modifications H3 lysine 4 di-methylation, H3 lysine 14 acetylation, and H4 lysine 8 acetylation correspond well to autosomal regions that are expressed in X/autosome translocations (113, 114). Because the unbalanced translocations studied tend to be from viable individuals and show variable extents of late replication it has been suggested that *XIST*'s coverage of the autosomal portion was initially more extensive, followed by recession of the RNA (50).

Thus, the fundamental difference in *XIST* affinity for DNA of the X compared to autosomal sequences may become more evident over time.

Studies using both human and mouse X/autosome translocations show that the same autosomal segment can either escape or inactivate, and the spread of inactivation can be continuous or discontinuous, depending on the rearrangement (110, 114). Better spread of inactivation seems to correlate with proximity to the *XIC* and lack of transit through constitutive heterochromatin (12). Consistent with this latter finding, the presence of intercalary constitutive heterochromatin between the autosome and X seems to have been selected for in species that have genome rearrangements involving the X, presumably because it prevents silencing from spreading to the autosome (39). Thus, chromatin environment is an important effector of the spread of silencing. Ongoing research will elucidate which sequence or structural components encourage or discourage the binding or spread of *XIST* and what factors influence the maintenance factors of inactivation. Recently, Lyon proposed that LI elements may be involved in the spread of silencing (82).

THE LYON “REPEAT” HYPOTHESIS

Given the abundance of LINE elements on the X, but not on chromosomes with limited spread of inactivation in X/autosome translocations, Lyon postulated that L1 LINE elements could be the way stations or booster elements proposed by Riggs (104). This theory is supported by observations that regions that escape inactivation on the X are significantly reduced in L1 content compared with those that are subject to inactivation (3). It has been observed that L1 methylation on both the active and inactive X is mediated by different methyltransferases, with L1 methylation on the inactive X following that of the active X (52). Thus, L1s are unmethylated at the time of X inactivation and may be responsible for X chromosome inactivation spreading.

Some observations argue against Lyon's LINE hypothesis. For example, the *Xist* RNA preferentially localizes to gene and GC-rich R bands (40), whereas L1 elements display a nonrandom distribution and accumulate primarily in AT-rich G-positive bands (68). It is also possible that the L1 enrichment on the X chromosome may be a result of low recombination rates on the X compared to autosomes, irrespective of the X-inactivation mechanism. Consistent with this idea, the LINE content on the Y seems to be even greater than the X, and enrichment of L1 content on chromosome 21 seems to occur at the regions of low recombination (134). However, after examining the L1 content in many eutherian mammals, Waters et al. (134) found that L1 enrichment on the X was conserved across all eutherian clades, as would be expected for a way station involved in inactivation. L1s may not be the only repeat element constitutively enriched on the X chromosome. LTRs are also more abundant on the X (see Figure 2) but are decreased in regions of genes that escape inactivation (127), whereas Bkm (GATA) repeats are enriched on both sex chromosomes in many organisms (121). Given the involvement of Polycomb

proteins in the silencing cascade, elements such as the Polycomb response element, which is best defined in fruit flies where they were first described (105), could be candidate way-station elements involved in the spread of silencing.

Lyon's repeat hypothesis, in a manner reminiscent of the 1961 Lyon hypothesis, which was widely debated before achieving validation and general acceptance, provides the research community with a testable model requiring further study. With the new genomic databases (both of single nucleotide polymorphisms and sequences of additional species, including marsupials), new technologies (such as quantitative PCR and chromatin immunoprecipitation), and new model systems (such as human somatic cells that undergo inactivation), we are well equipped to continue unraveling the *cis*- and *trans*-acting factors that mediate Lyonization.

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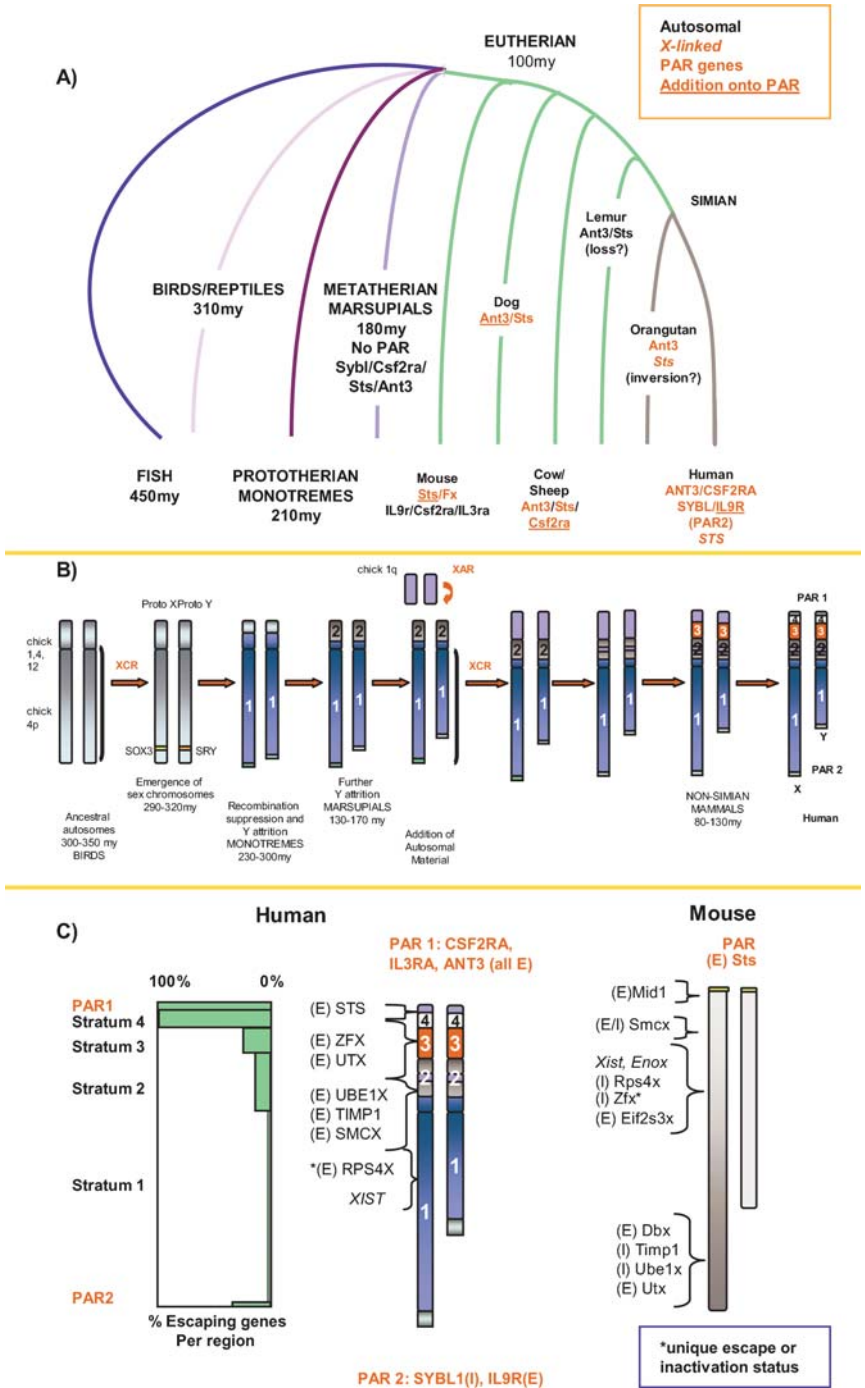
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Figure 1 (A) Divergence of the pseudoautosomal regions in various mammalian lineages. *Sybl*, *Csf2ra*, *Sts*, and *Ant3* are autosomal in the tammar wallaby but *Sts* is located at the mouse PAR, *Ant 3* at the carnivore PAR, and *Csf2ra* at the artiodactyl PAR, implying addition onto the X chromosome in these lineages (45, 125). In lemurs, *Ant3* and *Sts* are autosomal whereas these genes are still found in some simian X chromosomes, suggesting a lineage-specific loss from the X in certain prosimian lineages (45). *Sts* appears to be relocated from the PAR to another location on the X in orangutans, perhaps due to an inversion event (45). Humans seem to have gained *SYBL* and *IL9R* onto PAR2 (20). For simplification, only PAR genes whose locations are known across the various species in the tree are included in the figure. (B) Proposed mechanism of mammalian sex chromosome evolution from an ancestral pair of autosomes. Emergence of a sex-determining factor on the Y would favor rearrangements such as inversions that would lead to recombination suppression and allow genes beneficial to male sex determination to remain on the same chromosome. Without recombination, mutations would accumulate in noncrucial genes on the proto Y and deletion of these already disrupted genes would lead to ongoing decay of this chromosome. X and Y differentiation likely involved four major and other minor rearrangements, indicated by red arrows, occurring sequentially at different time points in mammalian lineages, resulting in four evolutionary strata. Points of rearrangements during different mammalian lineages are indicated (69, 70, 105a, 135). The X conserved region (XCR) is the portion of the human X also found in monotremes and marsupials, predominantly derived from chicken chromosome 4. The X added region, corresponding to strata 3 and 4, possibly evolved from a syntenic chicken 1q region found in an avian or reptilian ancestor (46, 58, 67, 69). (C) Genes that escape inactivation in human compared to mouse. An estimated 15% to 25% of the human X-linked genes or expressed sequence tags (ESTs) investigated to date escape inactivation as opposed to 5% in mouse (14); (E) = escapes inactivation, (I) = subject to inactivation, * denotes unique gene status observed in one of the two species. The percentage of escapees on the human X is indicated on the left graph for regions from different chromosomal origins, as described in the text. Note that the percentage of genes that escape silencing decreases from youngest to oldest region. All genes in human PAR1 show complete escape, 100% (13/13), whereas in PAR2, *SYBL1* and *HSPRY3* are subject to inactivation on both the X and Y, 33% (1/3) (14). The definition of escape, as presented in the graph, is defined as the expression at a locus in at least 7/9 X chromosomes tested (data from 14). The seven genes that escape inactivation in mouse are indicated (37, 38) (<http://www.informatics.jax.org>).

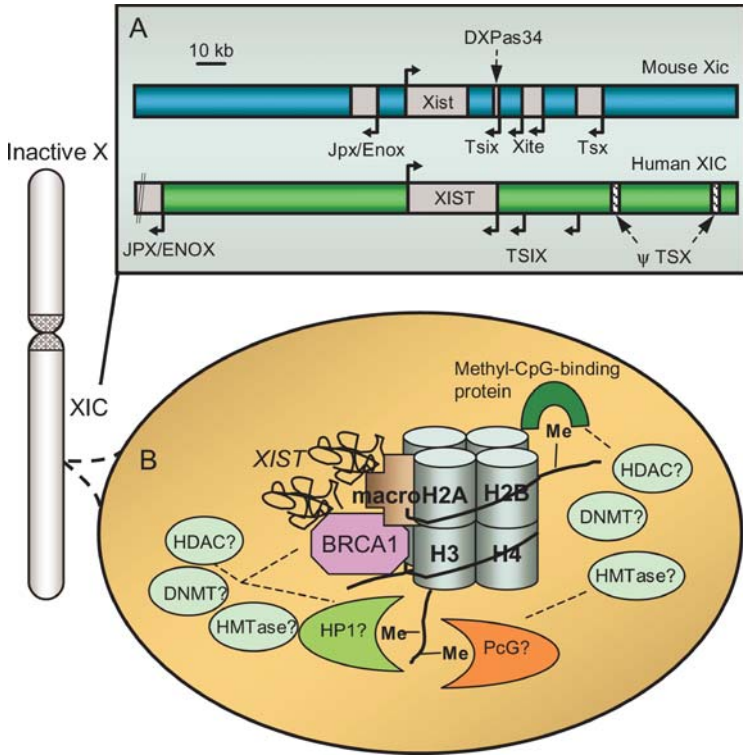


Figure 3 (A) The *XIC* region in human and mouse. *XIST* encodes an untranslated, functional RNA essential for recruiting factors required for inactivation. *Xist* expression is regulated by an antisense transcript, *Tsix*, which initiates 3' of *Xist*. In mouse, *Tsix* has multiple initiation sites and splice variants, but the main transcriptional start is associated with the mini-satellite repeat *DXPas34*. *Tsix* negatively regulates *Xist* expression and is involved in X chromosome choice. Like mouse, *TSIX* transcripts have variable initiation sites, with transcription starting ~27-kb and 10-kb downstream of *XIST* (22, 91). The *Xite* region in mouse contains initiation sites for low-level transcripts as well as DNaseI hypersensitivity sites and appears to be involved in X chromosome choice through the modulation of *Tsix* expression (94). (B) The inactive X chromatin is characterized by several features of heterochromatin that work together to maintain the silent state. BRCA1 associates with the inactive X and is required for localizing the *XIST* transcript and for stably maintaining the inactive state. In turn, localized *XIST* is required for initially recruiting the Polycomb group proteins Eed/Enx1 for histone H3 lysine 27 methylation and for continually associating macroH2A with the inactive X. The heterochromatin protein HP1 does not appear to be enriched on the inactive X in mouse; however, it does appear to associate with the Barr body in human somatic cells (18). Many other factors have yet to be identified and probably include DNA methyltransferases (DNMT), histone methyltransferases (HMTase), and histone deacetylases (HDAC).

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