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Mechanisms of X-chromosome inactivation

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1. ABSTRACT

Mammalian X-chromosome inactivation is an impressive example of epigenetic gene regulation, whereby the majority of genes on the ~ 160 Mb X chromosome are silenced in a strictly *cis*-limited fashion. In this review we will discuss the important players involved in the silencing process. The process is initiated by transcription and cis-localization of the non-coding XIST RNA, which then recruits many of the epigenetic features generally associated with heterochromatin, including histone modifications, histone variants and DNA methylation.

2. INTRODUCTION TO X-CHROMOSOME INACTIVATION

X-chromosome inactivation occurs early in the development of mammalian females. Once the initial choice of which X to inactivate is made, the same X is stably silenced through subsequent mitotic divisions. Thus, females are mosaics for two cell populations: one in which the paternal X chromosome is active, and one in which the maternal X chromosome is active. This mosaicism is observable when the product of an X-linked gene can be visualized, as in the case of the coat colour of calico cats. Indeed, the patchy or mosaic expression of X-linked genes was one basis for the original Lyon hypothesis in 1961 (1).

2.1. Dosage compensation

X chromosome inactivation is believed to occur in order to achieve dosage equivalence between mammalian females who have two X chromosomes and males, who have a single X chromosome and the sex-determining Y chromosome. In other organisms with sex

chromosomes there is a similar need for dosage compensation; however, the mechanism of achieving equivalent sex chromosomal gene expression is remarkably varied. For example, in *Drosophila,* genes on the single X in XY males are hyper-transcribed, while in *Caenorhabditis,* genes on both X chromosomes in XX hermaphrodites are down-regulated. Despite the very different outcomes, these processes all involve chromatin modifications and both the *Drosophila* and mammalian processes involve a functional RNA.

Since the need for dosage compensation arises from the divergence of the X and Y chromosomes, it was anticipated that genes shared between the two chromosomes, particularly those in the pairing regions of the X and Y (the pseudoautosomal regions - PAR), would not be subject to X inactivation (2). This is indeed the case, and these genes are said to escape inactivation. Surprisingly, there are also a large number of additional genes on the human X chromosome that escape inactivation (3,4).

2.2. Genes that Escape X Inactivation

Females are normally mosaic for populations of cells with each X active, complicating the determination of whether a gene is subject to inactivation. It was not until 1979 that definitive evidence was provided that a gene, the human steroid sulphatase (*STS*) locus, could escape inactivation. Individual clones of fibroblasts were isolated from female carriers for X-linked icthyosis due to *STS* deficiency, who were also heterozygous for *G6PD.* Clones expressing either allele of *G6PD* continued to express *STS*, demonstrating that even when the *STS* mutation was on the active X, the inactive X must have been contributing STS (5). To assess inactivation status of genes without the need for clonal populations and polymorphisms or mutations to distinguish the two X chromosomes, many studies have utilized somatic cell hybrids, which can be selected to retain either the active or inactive human X chromosome (6). Using a panel of hybrids retaining the inactive X, Carrel *et al.* have surveyed over 600 X-linked transcripts, and demonstrated that a surprising 15% are expressed from the inactive X (4). These genes show a non-random distribution, with the majority of 'escapees' found on the short arm of the X, which is an evolutionarily more recent addition to the eutherian X being autosomal in marsupials and monotremes (7). Surveying inactivation of three X-linked genes across many eutherians, Jegalian and Page hypothesized that it is the deterioration of the Y homologue that drives inactivation of the X-linked copy (8). Thus it may be that genes on the short arm have lost their functional Y copy more recently and have not yet acquired complete inactivation.

Ongoing expression of genes from the inactive X in females can result in dosage differences between males and females. Microarray comparisons between males and females have identified such dosage differences for some genes (9, 10). The genes identified to escape inactivation by microarray and somatic cell hybrid studies are not completely concordant, perhaps due to: additional levels of gene regulation (such as translational control); other differences between males and females that influence X-linked gene expression (such as hormonal differences, or regulation by an X-linked gene that escapes inactivation (11)); tissue-specific variability in inactivation (as has been observed for the mouse *Smcx* gene (12, 13)); or reduced stability of inactivation in the hybrid system (perhaps due to loss of *XIST* localization (14, 15)). Genes that are expressed from the inactive X chromosome are the likely culprits in the phenotypes associated with X chromosome aneuploidies (16), but may also be the basis for some differences between males and females (*e.g.* (17)).

2.3. Differences Between Humans and Mice

Lyon first hypothesized inactivation based on her studies of mice, and since then the mouse has been a powerful model system for the study of X inactivation. Murine female embryonic stem (ES) cells undergo inactivation upon *in vitro* differentiation. Manipulation of these cells by transgene insertion and homologous gene targeting, combined with the ability to reconstitute mice allowing *in vivo* studies, have made mouse ES cells a highly tractable model system for the study of inactivation. However, there are several known differences between inactivation in humans and mice, two of which are particularly relevant. First, it appears that more genes escape inactivation in humans. Although not as many genes have been examined in mouse, the idea that more human genes are expressed from the inactive X is consistent with the development of Turner syndrome in human females lacking a sex chromosome in contrast to the apparently normal phenotype of such mice (16). Second, imprinting of inactivation varies amongst species. In marsupials the paternal X chromosome is preferentially inactivated in all tissues (18), while in mice preferential paternal inactivation is observed only in the extraembryonic tissues (19). In humans there have been reports of non-random inactivation in extraembryonic tissues, however it is clear that in general this is not a stringent imprint (*e.g.* (20)). The extraembryonic tissues are the first tissues to undergo inactivation in mice, and the imprint is lost by the time the somatic tissues undergo inactivation (21-23). Thus the absence of imprinting in humans may reflect a delay in timing of inactivation until imprint loss has already occurred, however it might also reflect differences in the initial events of inactivation, which are currently only well-studied in the mouse model.

3. THE PROCESS OF X INACTIVATION

3.1. The X Inactivation Center (*XIC*)

In diploid mammals all X chromosomes in excess of one are inactivated, suggesting an initial marking of a single active X. Early studies of X chromosome rearrangements identified a region required in *cis* for inactivation of the chromosome, and demonstrated that inactivation can spread from this region into translocated autosomal material (24). Studies of human X chromosome rearrangements refined the *XIC* to an approximately one Mb region of Xq13 (25), while mouse transgene studies have identified a 450 kb region that can recapitulate the features of the *Xic* (26) (standard nomenclature dictates that the lower case naming is for the mouse locus, while the

upper case name is the designation for the human). The *Xic* region (see Figure 1) contains seven protein-encoding genes, none of which have been shown to be involved in the X-inactivation process. All the genes identified in this region in mouse are conserved in human, except *Ppnx* and *Tsx* (which has become a pseudogene in human). The region encodes a number of non-coding RNAs (see Figure 1) and also has a high density of pseudogenes (22 in 714 kb), which may in part be related to the transcriptionally active nature of this genomic region in the testis, as three (*Tsx*, *Ppnx*, and *Cnbp2*) of eleven genes in the region are specifically expressed in the testis (27). The most striking of the genes in the region is the X-Inactive Specific Transcript gene (*Xist*/*XIST),* which is required for inactivation. The region includes elements that are required for the marking of an active X chromosome and the stable expression and localization of *XIST* from the inactive X chromosome.

3.1.1. The *XIST* Gene

Xist/*XIST* is a large (>15 kb) alternatively processed, poly-adenylated, untranslated RNA that is the only gene known to be transcribed from the inactive but not from the active X chromosome in somatic cells. The gene lacks any conserved open reading frame, and presumably functions as an RNA. In interphase nuclei, the co-localization of the Xist/XIST RNA with the inactive X territory as part of the heterochromatic Barr body is suggestive of an involvement in X inactivation. A direct requirement for *Xist* in X inactivation was demonstrated by 'knock out' of *Xist* in female ES cells which abolished X inactivation potential in *cis*. Furthermore, transgenes of *Xist*, integrated as multi-copy arrays, are able to induce inactivation of autosomes, identifying *Xist* as the principle component of the *Xic* (reviewed in (28)). Prior to inactivation, *Xist* expression is detected as a small pinpoint of expression from both X chromosomes, until the transcripts accumulate and localize on the future inactive X, mediated at least in part by stabilization of the transcript (29, 30). While localization and stability are developmentally concurrent, they have been experimentally separated, with stable transcripts failing to localize (14, 15, 31-33). The puzzle of how one of two apparently equivalent X chromosomes can be chosen to express *Xist,* and thus be inactivated, remains to be solved. It is clear, however, that components of the *Xic* are involved, and it has been suggested that the levels of Xist RNA may influence which X undergoes inactivation (34).

3.1.1.1. Xist Gene Regulation

The *Xist*/*XIST* promoter regions in mouse and human (see Figure 1) are constitutively active, containing binding sites for ubiquitous transcription factors and lacking sex-specific activity (35-37). Thus, additional elements must be responsible for the silencing of *XIST* in males and expression from a single X in females. DNA methylation has been implicated in the regulation of *Xist*/*XIST* in differentiated cells since the promoter region of the transcriptionally active allele on the inactive X chromosome is unmethylated, whereas that of the transcriptionally inactive allele on the active X chromosome is methylated (38, 39). Loss of *XIST*/*Xist* methylation results in expression of the gene in somatic (14, 40, 41), but not embryonic cells (41), suggesting that methylation is a late event in the control of *XIST* expression. Elements both 5' and 3' of the *Xist* gene appear to regulate the function of *Xist* early in development, and are thus important components of the *Xic*.

3.1.2. Additional Components of the Xic

A series of elements downstream of *Xist* have been identified in mouse (see Figure 1). Among these elements is *Tsix*, an RNA antisense to *Xist*, which has been implicated in both random and imprinted X inactivation (41-45). *Tsix* is expressed in undifferentiated cells prior to inactivation and seems to oppose *Xist* expression in *cis* (46, 47). The mechanism of *Tsix* action is not resolved, but requires both the *Tsix* RNA and the process of antisense transcription itself (48). The major *Tsix* transcription start is associated with a CpG island and a 34 bp minisatellite termed DXPas34, and analysis of this region identified several binding sites for the DNA-binding protein CTCF (49). CTCF binding to these motifs was demonstrated *in vitro* and an association of CTCF with the DXPas34 element was detected in female fibroblasts. CTCF functions as a boundary element (50), leading to the hypothesis that DXPas34 could act as an insulator element involved in 'choice' (49). *XIST* is conserved among examined eutherians, particularly in the region of repeats at the 5' end of the gene (38); however, neither *Tsix* exons, nor the *Tsix* promoter region display significant levels of homology between mouse, human and cow (27). Nonetheless there is a large region of antisense transcription initiating 30 kb 3' of the human *XIST* gene. Compared with the mouse antisense, the human counterpart shows less extensive overlap with the *XIST* locus and is not expressed as abundantly relative to *XIST* (33, 51, 52). These differences, as well as the lack of imprinted inactivation in humans leave it unclear whether the human *TSIX* is a functional ortholog of mouse *Tsix* (53). Cows also have an antisense transcript that differs from the mouse as it appears to be expressed during fetal development in *cis* with the sense transcript (54).

The X controlling element (*Xce*), a locus mapping 3' of the *Xist* gene, skews inactivation based on allelic combinations (55). A series of transcription start sites and DNaseI hypersensitive elements upstream of *Tsix* were named *Xite* and hypothesized to correspond to the *Xce* locus (56). *Xite* lies within a 6 kb intergenic region close to the *Tsx* gene (see Figure 1). Deletion of the region downregulates *Tsix* in *cis* and skews inactivation, suggesting that *Xite* may promote *Tsix* persistence on the future active X. Truncating Xite RNA is inconsequential, indicating that Xite action does not require intact transcripts (56). Ogawa and Lee (2003) propose that allele-specific *Xite* action promotes *Tsix* asymmetry and generates X chromosome inequality (56). While there is some evidence for inheritance of skewed inactivation in humans (57), no ortholog to *Xce* (or *Xite*) has been identified.

Several lines of evidence point to the importance of elements upstream of the *Xist* locus in controlling *Xist*

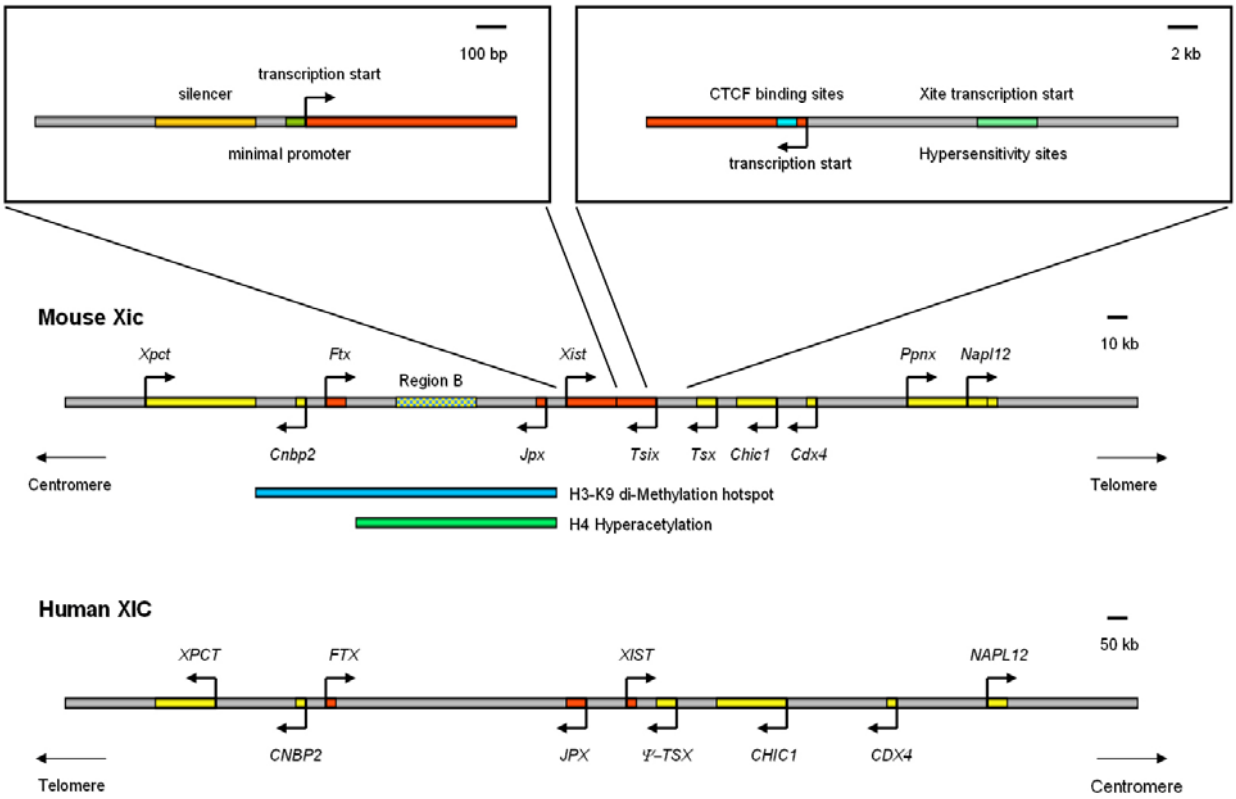

Figure 1. Transcription maps of the *Xic*/*XIC* regions in mouse and human (27). There are 11 genes in the mouse *Xic* region: *Xpct, Xist, Tsx*, *Tsix, Chic1* (formerly, *Brx*), *Cdx4*, *NapIl2* (formerly, *Bpx*), *Cnbp2, Ftx, Jpx*, and *Ppnx*. Protein coding genes are represented by yellow boxes. Four of the 11 genes, *Xist, Tsix, Ftx*, and *Jpx*, are untranslated RNA genes and represented by red boxes. Region B, a non-coding expressed domain, is represented by a striped box. All the genes identified in mouse are conserved in human, except *Ppnx* and *Tsix*. In human, however, *Tsx* has become a pseudogene. The human region is approximately three times larger than the mouse. Despite this major change in size, the order and orientation of genes is conserved in human and mouse, except for *Xpct*, which is at the same location but in the inverse orientation. A histone H3 lysine 9 dimethylation hotspot and H4 hyperacetylation are represented by blue and green boxes below the transcription map of the *Xic* region in mouse. Pillet *et al.* showed that the region -1157 to +917 has no *in vitro* sex-specific promoter activity (35). A minimal constitutional promoter was assigned to a region from -81 to +1. Deletion of the segment -441 to -231 is associated with an increase in CAT activity and may represent a silencer element. The choice/imprinting center contains tandem CTCF binding sites. Chao *et al.* proposed that *Tsix* and CTCF together establish a regulatable epigenetic switch for X-inactivation (49). Ogawa and Lee showed that *Xite*, located 10 kb from the *Tsix* transcription start, harbours two clusters of DNase hypersensitive sites (56).

and X inactivation. An 80 kb *Xist* transgene, including 30 kb of upstream sequence, was able to cause upregulation of the endogenous or the transgenic *Xist* locus in male cell lines upon differentiation (58). The absence of the 30 kb upstream region abolished the ability of the transgene to induce inactivation suggesting that the region upstream of *Xist* is critical for *Xic* function (58). In addition, the region 5' to *Xist* is highly enriched in histone H3 (H3) methylated at lysines 9 and 27 in ES cells (59, 60). This hotspot of H3 lysine 9 hypermethylation extends more than 100 kb upstream, including the non-coding RNAs *Ftx* and *Jpx*, as well as region B (see Figure 1). Region B is a non-coding, expressed domain that extends over an ~50 kb region (60). The H3 lysine 9 methylation hotspot is detected in both male and female ES cells on the endogenous X chromosomes as well as in single-copy and multi-copy YAC transgenes inserted on autosomes (59). Although this hotspot is also found in single copy transgenes in undifferentiated ES cells, it disappears when the cells are induced to differentiate, coincident with the inability of *Xist* to be stably upregulated. In contrast, the hotspot of H3 lysine 9 methylation in multicopy transgenic lines is maintained during differentiation, as *Xist* coats the autosome carrying the transgene insertion. Whether the hotspot of H3 lysine 9 methylation is necessary for Xist RNA to accumulate, or is reflective of the accumulation of *Xist*, modification of this region is an early event in inactivation, preceding the global enrichment of H3 lysine 9 methylation at the chromosomal level. Thus it has been suggested that this region functions as a nucleation center to trigger the Xist RNA-dependent association of a ribonucleoprotein complex responsible for the spreading of heterochromatinization onto the presumptive inactive X chromosome (59).

Interestingly in tetraploids two X chromosomes are kept active, while triploids have either one or two active X chromosomes, but the 'choice' is stable (61), indicating that the autosomal complement is involved in this marking. The autosomal factor has not been determined, however the presence of most single chromosomes (in trisomies) does not have a similar effect (62). Clues to autosomal factors may be provided by a screen for non-random inactivation in mice that has uncovered autosomal regions that impact the initial choice of chromosome to inactivate (63), or from the variety of proteins that have been shown to interact with the Xist RNA.

3.2. Factors Interacting with Xist RNA

Expression of *Xist* is the initiating step in X inactivation, but it remains to be determined how the RNA localizes to the chromosome from which it is transcribed, and how localized *Xist* initiates the cascade of heterochromatinizing events that lead to a stably silent inactive X chromosome. Analysis of partial deletions of the Xist RNA have shown separable domains, with the capacity to associate with the inactive X chromosome involving three spatially distinct and apparently redundant regions of the *Xist* RNA, while deletion of the conserved 5' repeat eliminates the ability to silence without disrupting the ability to localize (32). These repeats show sequence conservation in all species in which *Xist* has been analyzed and are predicted to form an RNA hairpin (32, 38). Only RNAs that are capable of coating the chromosome are able to silence (32). While no specific interactions with the various domains of the Xist RNA have been shown, proteins have been identified that associate with the inactive X, and thus might interact with XIST/Xist. These include the variant histone macroH2A (discussed later), the Eed/Ezh2 polycomb complex that modifies histones (also discussed later), and *BRCA1,* which has been reported to be necessary for *XIST* localization (64). Many mammalian homologues of the PRC1 protein complex that maintains homeotic gene silencing in *Drosophila* have also been shown to associate with the inactive X in human and/or mouse somatic cells (65).

It is also worth considering that the *Xist*/*XIST* RNA may form a protein complex similar to the two non-coding RNAs, *roX1* and *roX2* (RNA on the X) involved in the dosage compensation complex (DCC) in *Drosophila*. Protein components of the DCC include: the MSL (male-specific lethal) proteins, MSL1, -2 and –3; MLE (maleless) a helicase; MOF (males absent on the first) a histone acetyltransferase; and JIL1, a histone H3 kinase ((66, 67); reviewed in (68)). Initial transcription of the roX1 RNA is independent of the MSL proteins and transcription is also observed in females, although the RNA soon requires MLE for stability (69). It appears that *roX* RNAs are required for the proper binding of the DCC to the X chromosome, as low levels of DCC can be found ectopically bound to autosomal sites in the absence of *roX* RNA (68). Recently in *C. elegans*, a two-step model of dosage compensation has been suggested, involving the recruitment of a DCC to defined sites on the X chromosomes, followed by "spreading" into the neighbouring chromatin (70).

In mammals it appears that the autosomes contain DNA sequences necessary to support a substantial degree of inactivation, but are not fully competent to propagate or maintain inactivation. This led to the suggestion that there were 'way-stations' or 'booster' elements that might spread the inactivation signal (71). A selective affinity of the Xist RNA for particular regions of chromatin could account for the compromised spread or unstable maintenance of inactivation in autosomal regions (for example 72-75). Way-stations could be similar to the proposed chromatin entry sites of *Drosophila*, and might include the previously discussed H3-lysine 9 hotspot lying 5' to *Xist*.

3.3. L1 Elements as Way-Stations

Mary Lyon put forth the idea that 'way stations' could be L1 LINE elements that enable spreading by intrachromosomal pairing of L1 repeats (76). L1 elements are transposable elements comprising about 30% of the X chromosome, approximately two times the average genome abundance. They are also enriched in the *XIC* region relative to the short arm (which contains more genes that escape X inactivation) (77), although the abundance of L1 elements is not observed in the mouse *Xic* region (27). L1 elements have also been implicated in establishing monoallelic expression of autosomal genes in mouse and humans. Allen *et al* found that ~20 kb flanking some imprinted genes and random monoallelically expressed genes had a higher density of L1 elements, fewer SINE elements and fewer CpG islands compared to biallelically expressed genes (78). L1 elements are repressed in the human genome by methylation of the 5' internal promoter (79) and have been shown to be hypermethylated on both the active and inactive X chromosome in rodent/human somatic cell hybrids, however they seem to be modified by different processes, and at different times (80). Thus the intrachromosomal pairing suggested by Lyon may be possible because of hypomethylation of L1 elements on the inactive X chromosome at the time of inactivation (80).

4. FEATURES OF HETEROCHROMATIN ON THE INACTIVE X

Association of the Xist RNA with the future inactive X results in the acquisition of a number of chromatin modifications. While *Xist* is essential for the initiation of inactivation, once inactivation has occurred it works synergistically with the chromatin modifications acquired by the inactive X (discussed below) to maintain full stability of the inactive state (81). In developing mouse embryos, and particularly in differentiating mouse ES cells it has been possible to determine the order of events during inactivation (reviewed in (82)), and the features will be discussed in the order of their appearance, as shown in Figure 2.

4.1. Histone Modifications

Histones are subject to a wide variety of covalent modifications, some of which are directly involved in transcriptional regulation (83) and may also contribute to the chromatin memory of a specific state of activity through mitosis (84). Known modifications are concentrated in the tails of the histones, and include post-translational lysine acetylation, lysine and arginine methylation, serine phosphorylation, and lysine

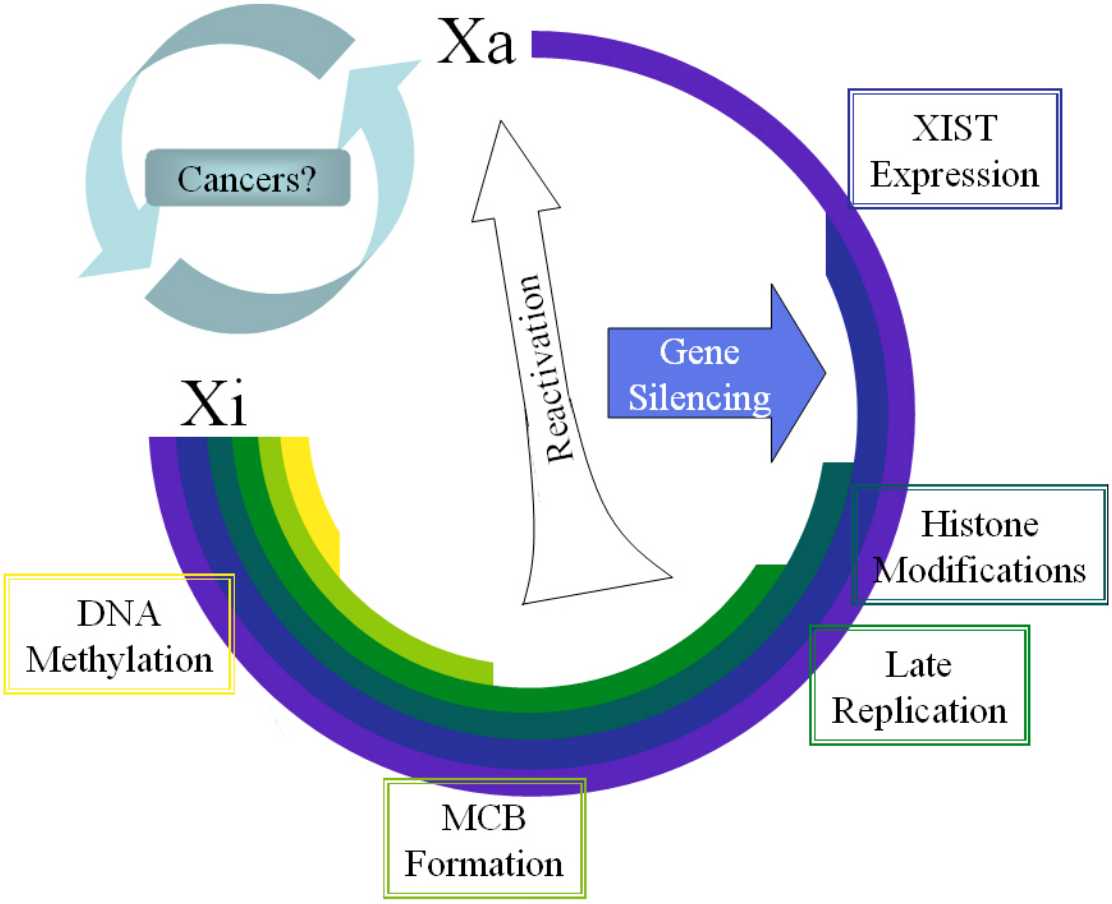

Figure 2. Accumulation of chromatin changes during X inactivation. The timing of changes to the inactive X are ordered as observed in studies of early mouse development and ES cell differentiation. Silencing can result from Xist expression, but stabilization of the silencing requires additional changes. Reactivation occurs rarely as discussed in the text, and in general the inactivation status is very stably maintained once established.

ubiquitylation (85, 86). In addition to the complexity of different modifications, the number of methyl groups, and not just the particular site, appears to play an important role in the functional consequences of histone methylation (87, 88). Furthermore, the modification of one site may alter the modification of another site (*e.g.* (89)). Histone tail modifications are thought to affect chromosome function through two distinct mechanisms. First, nearly all modifications alter the electrostatic charge of the histone, changing the structural properties of the histone or its affinity to DNA. Second, modifications impact protein recognition modules and can recruit specific functional complexes to their sites of action. Two well-characterized chromatin recognition modules are the bromodomain, which can recognize acetylated lysine, and the chromodomain, which can recognize methylated lysine (90, 91).

Over the past decade, a growing number of histone modifications have been associated with the inactive X chromosome (see Figure 3), and many of these are generally associated with silent chromatin in the genome. These include hypoacetylation of histones H3 and H4 (92-94), di-methylation and tri-methylation of H3 lysine 9 (59, 95, 96), tri-methylation of H3 lysine 27 (97, 98) and absence of di- and tri-methylation of H3 lysine 4 (95, 99, 100). These modifications are generally detected by antibodies, and cross-reaction of antibodies, particularly between H3 lysine 9 and lysine 27, has been a problem in identifying specific modifications for the inactive X (60). Recently methylation at H4 lysine 20 and ubiquitinylation of H2A lysine 119 have also been shown to mark the inactive X (101, 102). Modifications do not appear to be evenly distributed across the X, with many predominantly associated with the promoters of genes (*e.g.* (103)). At the chromosome level many of these modifications show regionality or 'banding' (e.g. 92, 104). It has recently been proposed that there are in fact distinct regions of heterochromatin on the inactive X that are differentially associated with different combinations of modifications (96).

Many of these modifications appear early during the X-inactivation process, and, as discussed previously, a hotspot of histone H3 lysine 9 methylation 5' to *Xist*, is constitutively present in male and female ES cells before

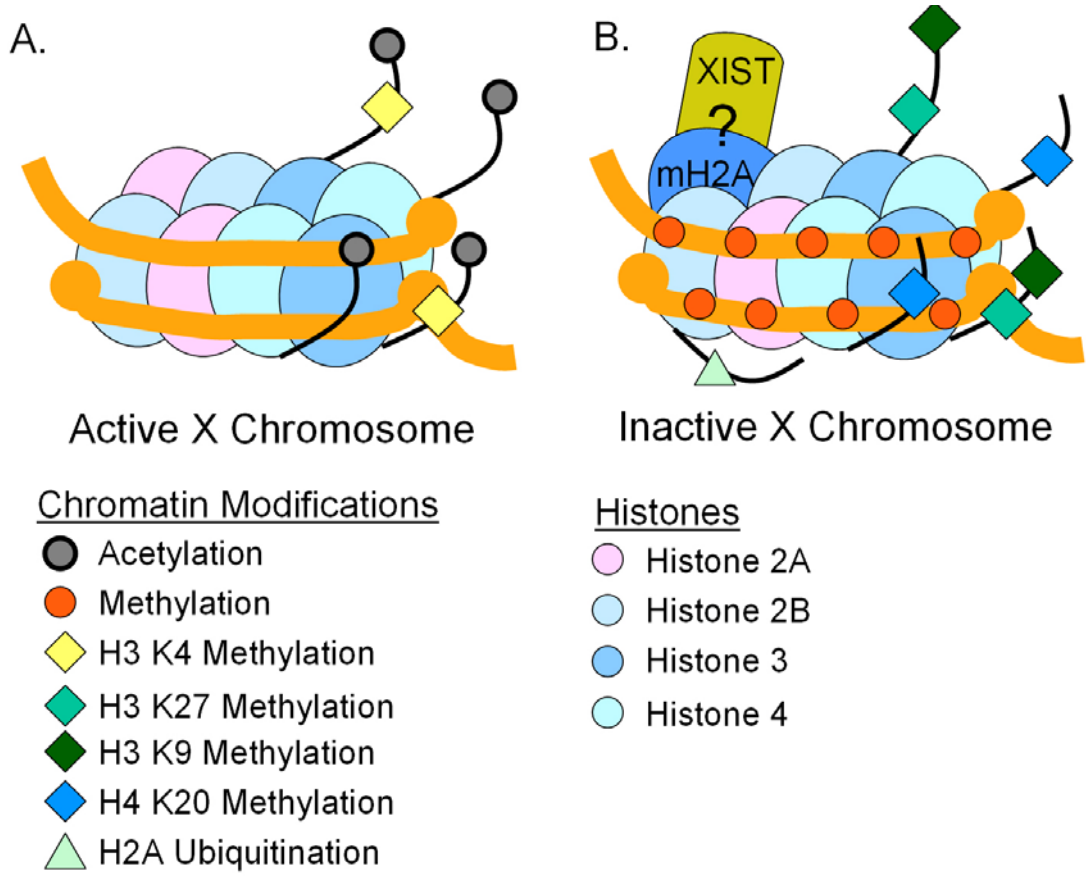

Figure 3. Chromatin remodeling on the inactive X chromosome. A. Active X chromatin is characterized by acetylation of H3 and H4 of the core nucleosome. There is also methylation of H3 lysine 4. B. Inactive X chromosome. Upon expression and localization of *Xist* there is macroH2A recruitment. It is unclear if these are bound together physically or are associated in some yet unidentified ribonuclear protein complex. The histone tails on the inactive X become hypoacetylated and methylated at H3 lysine 9 and 27, and H4 lysine 20. In addition, ubiquitination of H2A lysine 119 within the histone body is observed. DNA methylation is a late event in the inactivation process to lock in the inactive state. Note: it is not known to what extent the histone modifications are occurring on the same histone or within the same nucleosome, but at least some appear to be found in alternate domains (96).

and after differentiation (59, 60). O'Neill *et al.* (2003) also reported a hotspot of H4 hyperacetylation, overlapping the methylation of H3 lysine 9 domain, present only in female ES cells prior to differentiation (100). Intriguingly, they also report that before the onset of X inactivation, the two X's of female ES cells show hyperacetylation of all core histones, hypermethylation of H3 lysine 4 and hypomethylation of H3 lysine 9 compared with autosomal genes or genes on the single active X in male cells. This suggests that X-linked genes may be selectively marked in female ES cells in a way that distinguishes them from the equivalent genes in males. Thus, despite recent advances in identifying the different types of histone modifications associated with inactive X chromatin, there remain large questions about how these marks are established and their subsequent impact.

Some advances have been made towards identifying the enzymes involved in establishing these chromatin modifications. The Ring1A/B proteins have recently been implicated in the ubiquitinylation of H2A (102). The Polycomb group proteins Eed and Enx1 are involved in both imprinted and random X inactivation (97, 98, 105, 106). Transient association of the Eed/Enx1 complex with the X chromosome is observed shortly after Xist RNA coating, and is accompanied by H3 lysine 27 methylation, consistent with a role for Enx1 as the histone methyltransferase. Interestingly, coating of the X chromosome with the silencing-deficient Xist RNA can still recruit the Eed/Enx1 complex and induce H3 lysine 27 methylation on the X chromosome, even though gene silencing is not induced (98). Similarly, both methylation of H3 lysine 27 and H4 lysine 20 have been shown in the absence of transcriptional repression (101). This suggests that such modifications alone are insufficient to trigger silencing, and that *Xist* may play a direct role in recruiting these modifications. It is still unclear which histone methyltransferase causes H3 lysine 9 di-methylation on the inactive X chromosome, although a recent study has shown that G9a is necessary for this modification in the

constitutive hotspot region lying 5' to Xist (60), although X inactivation is stably initiated and maintained in embryos lacking this enzyme (107). The proteins that bind the modified chromatin to lock in the inactive state are currently unknown. These unidentified proteins could potentially recruit factors that maintain these histone modifications, and/or induce other epigenetic changes, such as a shift in replication timing, incorporation of macroH2A and DNA methylation of promoter regions, resulting in a combination of epigenetic marks that act synergistically to maintain the inactive state.

4.2. Late Replication Timing

The late replicating pattern of the inactive X was recognized early in the study of X inactivation (*e.g.* (108)). There are regions of the inactive X that are not late replicating, and although these can be variable across tissues, they generally correlate with regions of genes that escape inactivation (*e.g.* (109)). Genes that escape inactivation tend to replicate synchronously (reviewed in (110)); however domains of replication will generally include several genes. It appears that the same origin of replication is used on both chromosomes (111), but what accounts for the differential time of firing of the origins between the active and inactive X is currently unknown. The latest replicating regions of the inactive X appear to be associated with chromatin containing histone H3 trimethylated at lysine 9 (96).

4.3. Macrohistone H2A

In addition to modifications of the histones that epigenetically mark chromatin, the incorporation of core histone variants has been identified as a means of changing chromatin state. The centromere protein, CENP-A is a histone H3 variant thought to replace H3 in specialized nucleosomes of the centromere region, whereas other variants such as H3.3 and H2A.Z have been found to be associated with active chromatin (reviewed in (112)). Of interest to X inactivation are the macroH2A variants, which have been shown to mark the chromatin of the inactive X. There are two macroH2A genes, macroH2A1 and macroH2A2, located on different chromosomes yet showing 80% amino acid identity (113). Furthermore, macroH2A1 has two isoforms 1.1 and 1.2 resulting from alternative splicing. Each gene consists of two regions, the N-terminal histone-like region which shares ~65% homology with H2A and occupies one third of the gene and the C-terminal non-histone region of uncertain function (113).

The macroH2A family is evolutionary conserved throughout vertebrates (113). MacroH2A is likely to have a basic cellular function in addition to its role in X inactivation, as levels are similar in male and female cells (114, 115), and the genes are found in species where the dosage compensation mechanism is different from mammals (116). Estimates of macroH2A1 protein content suggest there could be up to one macroH2A per 30 nucleosomes (117). The exact mechanism of how macroH2A incorporation affects the surrounding chromatin remains a mystery, however, the basic structure of the nucleosome does not appear to be altered when macroH2A is substituted (118).

Immuno-staining of macroH2A shows a diffuse, "speckled" pattern in the nucleus and a predominant nuclear macroH2A-rich region only in female cells (117). This macroH2A dense region in female nuclei colocalizes with the inactive X and is referred to as the macrochromatin body or MCB (113, 117, 119). There also appears to be a microtubule-dependent concentration of macroH2A1 at the centrosome that is sensitive to the method of cellular fixation (120-122). It is unclear whether the centrosome acts as a storage center for macroH2A or if there is a functional significance to the association. Regardless, macroH2A localization to the inactive X is a relatively late event, following the initiation and propagation of X inactivation (120, 123). Antibodies to macroH2A immunoprecipitate XIST, suggesting that the two are physically associated, either directly, or in a ribonuclear protein complex (124). Induction of *Xist*, even in the absence of silencing, recruits macroH2A (125), while loss of *Xist* from the inactive X also results in loss of macroH2A1.2 and the MCB (126). Therefore, expression and localization of *Xist* appear to be required for macroH2A recruitment to the inactive X and MCB formation.

Analysis of the macroH2A gene for domains specific to MCB formation and association with XIST is crucial to establish a functional role for macroH2A in X inactivation. Chadwick *et al* utilized GFP fusion proteins that expressed various truncated macroH2A1 protein regions and determined the protein's ability to form MCBs (127). Two macrochromatin domains were identified, one located within the H2A-like core globular region and a second located within the non-histone tail (127). Both domains were independently capable of forming MCBs at the inactive X, although with differential ability to target the nucleosome. The large non-histone region of macroH2A renders it structurally distinct from conventional H2A. Clues to a role for this domain may be found in other proteins with a similar domain, which has been termed the macro domain (128). The macro domain can occur once or up to three times within a protein (129), and has been found in proteins from bacteria, archaea and eukaryotes (128). Several types of RNA viruses have non-structural proteins with a similar domain (114, 128). Interestingly, the sindbis virus contains this domain in a protein that associates with RNA and is required for RNA synthesis (114). This observation supports the theory that macroH2A variants can bind XIST RNA. However, other proteins with this domain have been found to have a role in protein-protein interactions, nucleic acid recognition and ADP-ribosylation (128). Bycroft and colleagues even suggest a possible enzymatic role for macro domains based on crystal structures (128). The idea is intriguing, hinting that a histone incorporated into the nucleosome core could act to regulate the ADP-ribosylation of local chromatin. A chromatin influence is also suggested by evidence that macroH2A interferes with transcription factor binding and SWI/SNF nucleosome remodeling (130).

4.4. DNA Methylation

DNA methylation is important for gene regulation in mammals and is necessary for normal embryonic development, genomic imprinting, and X chromosome inactivation. DNA methylation occurs when a DNA methyltransferase (DNMT) catalyzes the post-replicative addition of a methyl group from S-adenosyl-L-methionine to the 5' carbon position of cytosine forming 5-methylcytosine (m^5C). The mammalian genome contains ~$3x10^7$ m^5Cs, predominantly located in CpG dinucleotides. While the deamination of m^5C has resulted in the depletion of CpG dinucleotides in general, CpG rich areas, referred to as CpG islands, are often found within the 5' or promoter region of a gene (reviewed in (131)). There are three active mammalian Dnmts – Dnmt1, Dnmt3a and Dmnt3b. Dnmt1 is necessary for embryonic development (132) and maintains methylation patterns in proliferating cells by targeting replication foci and methylating hemi-methylated CpGs in the nascent strand of DNA (133). Dnmt3a and Dnmt3b are involved in the initiation of *de novo* methylation and the establishment of new DNA methylation patterns during development (134).

Gene-specific methylation patterns and gene activity are often inversely correlated (135), and the importance of methylation for transcriptional silencing is further demonstrated by *in vitro* methylation of promoter sequences or artificial demethylation of gene sequences resulting in repression and activation of gene activity respectively (reviewed in (136)). In addition, CpG methylation has been shown to be associated with heterochromatic regions in the mammalian genome (reviewed in (137)). While the addition of a methyl group to cytosine changes the major groove of the DNA helix and thus can directly influence the interaction with proteins, it also recruits multiprotein repression complexes including histone deacetylases mediated by binding of methylated-DNA binding proteins (138).

DNA hypermethylation is observed at the 5' region of genes on the inactive X, but not on the active X or genes on the inactive X that escape inactivation. Methylation is generally assumed to be a late event in the X inactivation process and is thought to play a more significant role in the maintenance of the inactive state rather than the initiation (139, 140). This is supported by studies showing that *Dnmt1* and *Dnmt3a/Dnmt3b* deficient cells demonstrate proper *Xist* expression and silencing of X linked genes (141, 142). On the other hand, the later importance of DNA methylation for X chromosome inactivation is supported by studies that have shown reactivation of some genes after treatment with demethylating agents, particularly in conjunction with disruption of other features of a stable inactive X chromosome.

4.5. Synergism of Features of Heterochromatin on the Inactive X

Deletion of *Xist* results in an inactive X chromosome that is prone to re-expression of inactivated X-linked genes upon treatment with demethylating agents (81). The frequency of reactivation was further increased by treatment with a histone deacetylase inhibitor (trichostatin A) demonstrating a synergistic rather than a simply additive interaction between these modifications (81). In fact, the ability to reactivate genes from the human X in somatic cell hybrids (*e.g.* (143)), but not human cells (144) may be due to delocalization of human *XIST* in mouse/human hybrids (15). The importance of CpG methylation in maintenance of X inactivation is observable in patients with ICF syndrome, which is caused by mutations in *DNMT3B* (134). While females are not more severely affected than males, X inactivation is unstable in cell lines from females, with reactivation of some loci, often accompanied by loss of late replication (145). The recent results of Chadwick and Willard (96) identify distinct domains of chromatin modifications, suggesting that not all modifications might act in concert with one another. They also demonstrate variability amongst cell lines, suggesting that the relative importance of modifications in maintaining silencing may vary between cells.

The interplay between histone modifications and DNA methylation (as well as other features of heterochromatin) is complex, and not yet completely understood. Methylated DNA binding proteins have been shown to recruit histone modifying proteins (146), and DNMTs themselves are associated with histone methyltransferases and their interacting proteins, supporting the concept of a mutually-reinforcing repressive chromatin environment (147). Consistent with this, mouse cells lacking the Suv39h histone methyltransferases show altered pericentromeric satellite DNA methylation (148). Mutations in the methylated DNA binding protein MECP2 cause Rett syndrome and cells from individuals with Rett syndrome show histone hyperacetylation (149). Using fibroblast cell lines derived from females with Rett syndrome, Gartler *et al.* demonstrated that despite a lack of MeCP2 in these cells, the inactive X chromosome had normal H3 and H4 acetylation, and H3 methylation (150). In this same study, the authors looked at cells from ICF females and found that despite the hypomethylated inactive X, the above chromatin modifications were also normal.

5. PERSPECTIVES

Once the X has acquired these various features, silencing is extremely stable. Indeed, clonality for X inactivation is often used as a means of testing the monoclonal origin of a tumour (151). However, reactivation does occur naturally – during the process of oogenesis (152), and recently it has been shown that reactivation of the paternally inactivated X occurs early during mouse development, prior to the random inactivation that occurs in the epiblast (22, 23). Interestingly, in both of these cases the full lockset of heterochromatic modifications may not have been assembled, as DNA methylation, which seems to be a late event in the process, may not yet have occurred. Although somatic inactivation is generally considered very stable, there are reports of inactivation occurring in testicular germ tumours (153), and that loss of *BRCA1* expression can lead to reactivation (64). While the former may reflect the plasticity of the germ-cell progenitors, the involvement of the

X in breast and ovarian cancer clearly needs further study. There are reports of non-random somatic inactivation in some breast and ovarian cancer patients (154, 155), and overexpression of X-linked genes in ovarian cancers (156).

Lack of a second sex chromosome results in Turner syndrome, presumably largely due to the lack of gene expression for genes that are shared between the X and the Y and expressed from the inactive X. The expression of genes from the inactive X that are no longer shared with the Y chromosome may contribute to differences between males and females, and this is a topic of study, particularly in sexually dimorphic expression in the brain (157). While considerable progress has been made in documenting the genes that escape inactivation (4), it remains to be understood how some genes continue to be expressed from an otherwise inactive chromosome. Nuclear compartmentalization, a concept increasingly encountered in studies concerning transcriptional silencing and barrier elements (158), might ensure that identical loci be submitted to different regulatory environments, and a recent study suggests that binding of CTCF demarcates boundaries between domains of active and silent genes on the inactive X (159).

In addition to differences in expression for genes that escape inactivation, females also differ from males in being mosaics for X-linked gene expression. While this heterozygosity is generally protective against X-linked recessive diseases, it can also result in X-linked dominant diseases, as was recently demonstrated for mutations in *EFNB1* resulting in craniofrontal nasal syndrome (160, 161). Additionally, males have only a maternal X chromosome (having inherited the sex-determining Y chromosome from their father), thus females are unique in having paternally inherited X-linked genes, and there are reports of imprinted genes on the X chromosome (reviewed in (162)).

Recent work in mice has shown that the meiotic sex chromosome inactivation (MSCI) that occurs during spermatogenesis has many similarities to somatic X inactivation, as well as some important differences, including a non-essential role for the *Xist* gene (163). Additional work will be necessary to identify the similarities and differences between the processes, and whether there are marks carried forward from MSCI that serve as the imprint for the very early paternal inactivation seen in mice (21). As this imprint is absent or less stringent in humans new model systems will be necessary to understand the early events of inactivation in humans, perhaps including differentiating human ES cells (164) or somatic cells which show features of inactivation (31). The development of RNA interference (RNAi) as a tool to knock-down expression of specific genes (165) should assist in such studies. The process of RNAi itself has been implicated in epigenetic silencing in various organisms, and mouse ES cells deficient in the RNAi pathway show transcription, hypomethylation and altered histone modifications at centromeric repeats (166).

While the evidence is clear for a critical role for the XIST RNA in the initiation of X inactivation, it remains to be discovered how the RNA finds the chromosome from which it is expressed, and then how association with XIST causes silencing. With the discovery of miRNAs and an unexpected abundance of intergenic transcripts, non-coding RNAs are emerging as important regulatory mechanisms for gene expression. Thus resolution of the many outstanding questions about mammalian X-chromosome inactivation are likely to continue to yield insights into general questions of gene regulation by epigenetic processes.

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