Review

X-chromosome inactivation in development and cancer

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Abstract

X-chromosome inactivation represents an epigenetics paradigm and a powerful model system of facultative heterochromatin formation triggered by a non-coding RNA, Xist, during development. Once established, the inactive state of the Xi is highly stable in somatic cells, thanks to a combination of chromatin associated proteins, DNA methylation and nuclear organization. However, sporadic reactivation of X-linked genes has been reported during ageing and in transformed cells and disappearance of the Barr body is frequently observed in cancer cells. In this review we summarise current knowledge on the epigenetic changes that accompany X inactivation and discuss the extent to which the inactive X chromosome may be epigenetically or genetically perturbed in breast cancer.

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1. Introduction

In mammals, dosage compensation for X-linked gene products between the sexes is achieved by X-chromosome inactivation (XCI) in females [1]. This process leads to the highly regulated transcriptional silencing of one of the two X-chromosomes during early development, leading to the formation of the heterochromatic Barr body. X inactivation is an outstanding example of chromosome-wide epigenetic regulation involving the developmental silencing of approximately one thousand genes. XCI shares many of the features of other epigenetic mechanisms such as a mosaic cellular phenotype; mitotic heritability but developmental reversibility of X-chromosome inactivity; asynchronous DNA replication timing compared to the rest of the genome; and finally a combination of several epigenetic mechanisms – including DNA promoter methylation, histones post-translational modifications, and an unusual nuclear organization. These various features are believed to act synergistically to maintain the X inactive state. Thus, the inactive X chromosome represents a remarkable illustration of the numerous epigenetic mechanisms that can underlie the formation and maintenance of facultative heterochromatin throughout the lifetime of mammals. Pathologists have long noted that the heterochromatic structure of the Barr body was frequently absent in breast cancer cells, particularly in the most aggressive tumors ([2,3]). This observation was later found to be due to the frequent genetic loss of the Xi, with reduplication of the Xa also occurring in some cases in cancer cells resulting in a double dose of X-linked genes. However, another mechanism for Barr body loss in cancer that has been proposed involves the decompaction of its heterochromatic structure, which may be accompanied by X-linked gene reactivation.

Here we provide a brief overview of the current knowledge pertaining to the establishment and maintenance of X inactivation, as well as highlights of some of the known perturbations of the inactive X chromosome's state in a cancer context. This review does not provide an in depth analysis, for this, the reader is referred to other recent reviews [4–6].

2. Setting up inactivation of the X chromosome in mammals

The X-inactivation center (Xic) is a key locus that is required for the initiation of X inactivation. Studies involving X-chromosome deletions and X-autosome translocations in mice and humans mapped the precise region which contains the essential gene for triggering the silencing process, the non-coding RNA Xist (X-inactive-specific-transcript) (for review [7]). Xist is a 17 000 nucleotide (19 kb in Human), spliced, untranslated regulatory transcript that coats the X chromosome from which it is expressed in cis [8,9]. Perhaps surprisingly, Xist is present only in eutherian mammals, with no orthologous described in marsupials or monotremes to date. However in marsupials, another non-coding RNA Rsx (RNA on the silent X) was recently discovered, that appears to have similar properties to Xist even though its sequence is unrelated to Xist [10]. In the mouse, deletions and transgenes have demonstrated that Xist is required for both the imprinted and
random forms of X inactivation [11–13]. Nevertheless, Xist alone cannot recapitulate all the roles of the Xic which include “sensing/competence”, whereby a cell initiates XCI only when more than one Xic is present; “counting”, where only one X chromosome stays active per diploid autosome set; and “choice”, whereby one of the two X chromosomes is chosen for inactivation while the other remains active. Some of these functions seem to be ensured by other elements of the Xic, in the neighborhood of Xist. For example, in mice the cis-acting Xist antisense transcription unit, Tsix [14], plays a key role in the choice of which chromosome will be inactivated. However, several additional cis-acting elements and trans-acting factors are involved in the dosage-sensitive, monovalent regulation of Xist expression during early differentiation. For example, the Rnf12 gene lies upstream of Xist and its protein product, an ubiquitin ligase that leads to the degradation of the Xist repressor protein Rex1, has been shown to have a key role in the XX-dosage dependent activation of Xist [15,16]. A recent study also shed light on the precise chromosomal folding of the Xic region in ESCs, revealing that the Xist and Tsix promoters lie in separate Topologically Associated Domains (TADs) [17]. The Xist promoter and all of Xist’s known positive regulators, including Rnf12, lie in a single 500 kb TAD, while the Xist repressor Tsix and its regulators lie in the neighboring 220 kb TAD, with the Xist/Tsix overlapping gene bodies across the boundary between the two TADs. The spatial separation between the Xist and Tsix promoters is likely to be essential for the finely tuned reciprocal regulation and expression of these two keys players in the XCI process [18–19].

So far, most studies of XCI during early development have been carried out in mice, which are more amenable to a genetic approach and where access to embryonic material is easier. In this species, XCI occurs in two phases during embryogenesis, an initial imprinted form and a later random form. In the imprinted form, Xist itself is imprinted such that only the paternal allele is expressed from two cell stage, whereas the maternal allele is repressed by an as yet undefined maternal imprint that is established during oogenesis. The paternal X chromosome (Xp) becomes silenced during pre-implantation development and remains inactive in extra-embryonic tissues. However the Xp is reactivated in the inner cell mass (ICM) of the mid-stage blastocyst, that will give rise to the embryo proper [20]. XCI is then reinitiated and occurs randomly by up-regulation of Xist from either the paternal or the maternal X chromosome. This second wave of XCI, can be recapitulated in vitro in differentiating mouse ESCs which provide a powerful model system for the study of the molecular mechanisms underlying random XCI. Remarkably, the differentiation process is delayed or paused in female ES cells until one of the two X chromosomes is chosen for inactivation, suggesting that a double dose of X-linked genes interferes directly with differentiation, thus creating a coupling between XCI and development [21]. Unexpectedly, in other mammals recent studies have revealed that the timing and pattern of XCI initiation observed in rodents is rather different to that seen in other eutherian mammals such as humans and rabbits [22,23], where XIST is not imprinted but is expressed from all X chromosomes in males and females from the 4 or 8 cells stages [24]. In rabbits, a significant proportion of blastomeres display XIST RNA coating of both X chromosomes in early blastocysts. This situation is rapidly resolved at slightly later stages, where most cells display only one XIST RNA-coated chromosome. In humans, the onset of XCI appears to be much slower than in mice and rabbits, as the two X chromosome remain active both in the ICM and the trophectoderm even though XIST coating is present. By contrast, early XCI appears to be absolutely essential in mice, in order to ensure proper dosage compensation given their fast embryonic development [25]. The exact reasons for this evolutionary diversity are still not clear but the rapid evolution of rodent genomes may mean that they have acquired mechanisms for finely regulating Xist, such as an early maternal imprint to prevent premature XCI, as well as reactivation of the Xp to allow for random XCI (see [21,24] for discussion).

The mechanisms underlying the early events during XCI are still not fully understood. Nevertheless, gene silencing is clearly triggered by Xist RNA coating of the future inactive X chromosome. It has been proposed that the Xist transcript might bind to high affinity sites on the chromosome, thereby inducing local heterochromatinisation, which can then facilitate the spread of XCI. LINE1 (Long Interpersed Nuclear Elements 1) repeat elements, which are highly enriched on the X chromosome compared to autosomes, have been proposed to facilitate the spread of XCI, by participating in the local propagation of inactivation and facilitating the recruitment of genes into the silent nuclear compartment formed by Xist RNA [26]. However, direct genetic evidence proving that L1s are required for efficient spreading or gene silencing is still lacking. More recent findings, based on mapping of Xist RNA along the X chromosome and of the nuclear organization of the Xist RNA coated inactive X, suggest that Xist propagation and coating are highly related to the three-dimensional conformation of the inactive X chromosome, and possibly also to the binding of Polycomb Repressive Complex 2 (PRC2) protein complex [27–29]. However, although studies comparing PRC2/H3K27me3 density and Xist RNA mapping suggest co-localisation [27,28], this is not in agreement with super-resolution Xist RNA FISH/immunofluorescence studies, where PRC2 and Xist RNA are found to be spatially segregated [30]. Furthermore, these studies do not demonstrate that the regions of the X associated with Xist RNA are directly involved in its spreading or silencing functions. Indeed, the mechanisms underlying Xist’s cis-limited chromosome coating capacity, as well as its ability to inactivate genes, remain mysterious.

Several studies have focused on the early changes in chromatin structure that occur during the initiation of XCI with the hope of providing insight into the role of Xist and the establishment of the silent state of the inactive X. Loss of euchromatin-associated histone modifications (such as H3K9ac, H3K4me2 and H3K4me3) are amongst the earliest chromatin changes that occur following Xist RNA coating [20,31,32] (Fig. 1). Global H4 hypoacetylation occurs shortly afterwards [33]. Passive histone-loss during replication or else active removal (either by enzymatic activity, proteolytic activity or histone exchange) may underlie the early disappearance of those histones modifications. In addition to these early chromatin changes, the disappearance of factors associated with transcription, such as RNA polymerase II and loss of nascent transcripts are observed on the Xi immediately after Xist RNA coating. One or two cell cycles later, several new histone modifications appear on the Xist-coated chromosome. These include H3K27me3, H3K9me2, H4K20me1 and H2A/K119ub1 (for review [22]) which all become enriched with rather similar kinetics of Xi enrichment during random XCI in differentiating ESCs. In pre-implantation embryos, H3K27 tri-methylation precedes H3K9 di-methylation on the X chromosome undergoing inactivation [20]. Intriguingly, H3K27me3 and H3K9me3 seem to be enriched in different regions of the human inactive X chromosome in somatic cells, suggesting the existence of two different types of heterochromatin [34]. In murine differentiating female ESCs and somatic cells, H3K27me3 and H3K9me2 are enriched rather uniformly along the Xi, though slightly more in gene-rich regions [35]. The Xi profile of H2Aub1 has not yet been described.

The factors that lay down or bind to the different chromatin marks present on the Xi are being unravelled in mice. Polycomb repressive complex 1 (PRC1) mediates mono-ubiquitination of histone H2A lysine 119 (H2Aub1) [36,37] and PRC2 catalyzes the tri-methylation of H3K27 [38,39]. Xist RNA is believed to have a role in the targeting of both PRC1 and PRC2 to the Xi, though whether this is direct or indirect is still unclear. In the case of PRC2, the cofactor
Jarid2 was recently found to be essential for the targeting of PRC2 to Xist RNA coated chromatin [40] (Fig. 1). In the case of PRC1, the canonical complex may be recruited through binding of the chromodomain region of the Cbx7 protein to H3K27me3 enriched chromatin; while the non-canonical (Rybp containing) PRC1 complex appears to be Xist RNA dependent. The mono-ubiquitination of histone H2A is induced thanks to the ring1b protein, although the exact role of this mark in X inactivation is not clear. At later stages of differentiation, in the maintenance phase, the PRC2 and PRC1 complexes no longer appear to be present on the Xi. However, macroH2A becomes associated with the inactive X at the final differentiation stage. The latest mark to appear is DNA methylation of promoters of X-linked genes. DNA methylation is deposit thanks to Dnmt proteins. Smchd1 may play a role in deposition or maintenance of DNA methylation. (B) During the XCI process, Xist RNA coating on the inactive X chromosome induces the formation of a nuclear silent compartment. The genes escaping silencing are excluded from this nuclear domain and then preferentially interact with each other in a long-range manner.

Fig. 1. The kinetics of X-chromosome inactivation. Schematic view of the kinetics of events on the X chromosome based on findings of many laboratories, performed mainly in differentiating female embryonic stem cells. (A) During the initiation of XCI, Xist is expressed in cis from the future inactive X chromosome. Then Xist RNA exerts its repressive effect through unknown factors. One of the earliest events following Xist RNA coating is loss of euchromatic histone marks, H3K4me2/3, H3K9Ac and H4Ac. During this same time window, X-linked gene-silencing initiates. Several histone modifications also become enriched on the Xist RNA-coated chromosome at this time. These include H3K27me3, H3K9me2, H2Aub1 and H4K20me1. The PRC2 complex proteins Eed, Pcl2, Suz12, EzH2 and Jarid2 are also detectable on the Xi at this stage. EzH2 is responsible for the appearance of H3K27me3 on the X. Jarid2 plays a role in the targeting of PRC2 to the chromatin. The histone methyl-transferases responsible for H4K20 mono- methylation is PRSet7 but that for H3K9 di-methylation is not yet clear. By binding H3K27me3/H3K9me2 enriched Xi, Cdy1 may also participate in propagating heterochromatin marks such as H3K9me2 through G9a recruitment. PRC1 canonical complex may be recruited through binding of the chromodomain region of the Cbx7 protein to H3K27me3 enriched chromatin; while the non-canonical (Rybp containing) PRC1 complex appears to be Xist RNA dependent. The mono-ubiquitination of histone H2A is induced thanks to the Ring1b protein, although the exact role of this mark in X inactivation is not clear. At later stages of differentiation, in the maintenance phase, the PRC2 and PRC1 complexes no longer appear to be present on the Xi. However, macroH2A becomes associated with the inactive X at the final differentiation stage. The latest mark to appear is DNA methylation of promoters of X-linked genes. DNA methylation is deposit thanks to Dnmt proteins. Smchd1 may play a role in deposition or maintenance of DNA methylation. (B) During the XCI process, Xist RNA coating on the inactive X chromosome induces the formation of a nuclear silent compartment. The genes escaping silencing are excluded from this nuclear domain and then preferentially interact with each other in a long-range manner.
with PRC complexes, in the context of an independent transcript, RepA [47,48]. However, the exact role of RepA remains unclear and the A-repeat region of Xist is clearly dispensable for Polycomb factor recruitment to the Xi, whereas another Xist region containing the conserved B and F repeats was recently identified as being critical for inducing PRC2 recruitment, via Jarid2 [40]. The multiple roles of Xist RNA in X inactivation are still poorly understood and seem to be at several levels, involving chromatin, nuclear compartmentalization, as well as chromosomal organization. Although the formation of a silent nuclear compartment by Xist RNA and the loss of euchromatic marks correlate well with the onset of transcriptional inactivity [44], whether these changes are cause or consequence of XCI remains to be seen.

In addition to the recruitment of protein complexes such as PRC2, PRC1, as well as Cdfy and its partners, some further changes occur at later stages during the onset of XCI in differentiating ESC. These include enrichment of histone variant macroH2A [49] which has been shown to interfere with transcription factor affinity and SWI-SNF nucleosome-remodeling in vitro [64,65]; the Trithorax group protein Ash2L [45]; and the scaffold factor SAF-A [50], which is thought to participate in restricting or recruiting Xist RNA to the inactive X. Most X-linked genes on the Xi also acquire DNA methylation at their CpG islands, with different genes having rather different kinetics of hypermethylation [51]. The mechanism through which DNA methylation is recruited to X-linked genes remains unclear, although the Smc2d1 and Dnmt1 DNA methyltransferase proteins have been proposed to participate [52,53] (Fig. 1). Smc2d1 was originally identified in a genetic screen for epigenetic modifiers, as a factor involved in the maintenance of X inactivation and the hypermethylation of CpG islands associated with the inactive X [51,52]. Smc2d1 has also been shown to participate in the compaction of the Xi in somatic human cells, through an interaction with Hb1X1 [66]. These two proteins act in a PRC2-independent manner.

The inactive X chromosome also displays a shift to asynchronous replication timing during development. Asynchronous replication is in fact one of the most conserved features of the inactive X (see review [22]) and may provide a temporal segregation that minimizes the exposure of the inactive X to transcription factors, thus improving the maintenance of transcriptional silencing. This change in replication timing of the Xi occurs downstream of chromatin changes such as loss of euchromatic marks and PRC recruitment [31]. It is still not known whether asynchronous replication has a role in XCI, or is simply a consequence of the heterochromatinisation of the X. Thus several independent pathways seem to participate in the heterochromatinisation of the X. Establishing the mechanisms of recruitment and action and the possible links between them represents an important challenge for the future.

3. Developmental plasticity of the inactive state and synergy of epigenetic marks in the maintenance of X-chromosome inactivity

In somatic cells, the inactive X chromosome is in a highly stable state of transcriptional silence, with sporadic reactivation of X-linked genes estimated to occur at frequencies of less than $10^{-8}$ [54]. However, during mouse development different lineages display rather different degrees of X-linked gene silencing, and this may be associated with slightly different epigenetic signatures. During early development, the paternal X becomes associated with Xist RNA, macroH2A, PRC2 and the histone modification it deposits, H3K27me3. At the blastocyst stage, these marks are reversed in the inner cell mass where the inactive paternal X (Xp) is reactivated [20,55]. However these marks are maintained on the inactive Xp in extra-embryonic cells of the trophectoderm lineage. In trophectoderm giant cells (TGC) that are derived from this extraembryonic lineage, high rates of escape from XCI are observed for some, but not all, X-linked genes [56]. The current hypothesis is that there is a need for longer-term, more stable silencing in embryonic lineages as these give rise to the embryo-proper, where cellular memory must be maintained throughout the lifetime of the animal. On the other hand, there is only a short-term requirement for XCI in extraembryonic tissues which are dispensable after birth. In particular, DNA (cytosine) methylation of X-linked CpG islands appears to be present in embryonic but not extra-embryonic lineages in eutherians mammals and its absence correlates with reactivation [57]. Indeed, epigenetic marks involved in maintaining the inactive state appear to vary considerably in both embryonic and extraembryonic lineages [58]. In TGCs, the Xi has an highly unusual chromatin content, not only lacking DNA methylation at CpG islands but presenting both heterochromatic marks such as H3K27me3 and euchromatic marks such as histone H4 acetylation and H3K4 methylation. Moreover, Xist RNA does not form an overt silent nuclear compartment and reactivation of Xi-linked has been observed [56]. This may be partly due to the fact the trophectoderm cells undergo endoreplication resulting in an unusual chromosome organization and possibly less stable propagation of the silent chromatin state. Nevertheless, the Xi shows a substantial degree of gene reactivation in these cells it remains globally silent. The presence of PRC2 and H3K27me3 on the Xi may be sufficient to maintain silence in this context. Indeed mouse embryos mutant for the PRC2 component, Eed, show much higher reactivation rates of an X-linked GFP transgene specifically in the trophectoderm [59]. In the same mutants, no impact was found in the epiblast, presumably due to the presence of other epigenetic factors, including PRC1 complexes and DNA methylation. Although a direct role of PRC1 complex proteins in the maintenance of X inactivity has not so far been demonstrated, two different complexes have been found associated with the Xi [41]. The timing of recruitment of PRC1 complexes to the Xi during the same time window as PRC2 in developing embryos [36,37,60] suggests that this complex is likely involved in the maintenance of inactivity. This is potentially by SWI-SNF complexes inhibition, blocking transcriptional activity or by recruiting silencing components, as described in other systems [61–63].

The extent that different epigenetic marks participate in the maintenance of the inactive state has been addressed in experiments aiming to prevent or reverse them. Studies have disputed several marks on the Xi simultaneously – such as DNA methylation (by 5-azacytidine treatment or in DNA methyltransferase mutant cells, Dnmt1–/–), histone hypoacetylation (by pan-histone deacetylase inhibitor treatment, TSA) and Xist RNA (by a conditional KO) – can lead to a significant increase of Xi-linked genes expression [54]. However disruption of each of these marks alone has little effect. This clearly demonstrates that several epigenetic modifications lock in the inactive state of the Xi. Nevertheless, given the relatively low reactivation frequencies even after the combined removal of these three marks, other epigenetic players are likely to be involved in the maintenance of silencing. Indeed, so far, full reactivation of the Xi has never been achieved experimentally other than via fusion with ES cells, which have the reprogramming potential of the inner cell mass [20,37], or by nuclear transfer where the reprogramming potential of the oocyte is involved [67,68], or through the expression of the ‘Yamanaka cocktail’ pluripotency factors (Oct4, Klf4, c-Myc and Sox2) that can drive somatic cells into an induced pluripotent state resembling ES cells [67,69–71]. The reactivation of the Xi is thought to be one of the later events during reprogramming, presumably due to the need to reverse several layers of epigenetic marking, some of which may require active mechanisms (specific transcription factors or chromatin enzymatic activities) and others may require
passive DNA replication to enable gradual removal. Further studies on the key players involved in the X-chromosome reactivation process during reprogramming, both during development (in the ICM and in the germ line) and also experimentally (via nuclear transfer or IPS formation) should provide insights into the nature and extent of the maintenance mechanisms that are at play in different cellular states and for different parts of the X chromosome (for review [5]).

4. Variability in X-inactivation status and escape from XCI

In eutherian mammals, such as humans and mice, the majority of X-linked genes are subject to transcriptional repression during XCI, although a variable subset of X-linked genes can escape silencing (escapees) and are bi-allelically expressed – showing higher expression in females when compared to males [72–75]. Only Xist appears to be expressed exclusively from inactive X in female somatic cells. Two main groups of escapees are present on the X chromosome. One group comprises escapees that lie within the pseudo autosomal region (PAR) and have an exact homolog on the Y chromosome, and show equal expression levels between the sexes [73]. A second group consists of genes that are located outside the PARs. These include genes such as Kdm5c (Smx or Jarid1c) and Utx, which have Y-chromosomal homologs lying outside of the PAR and presumably represent evolutionary remnants from the proto-sex chromosomes. Some other escapees have no obvious Y-linked homologs. The actual degree of escape from XCI for such genes can vary considerably and depend on the tissue or the species [74,76,77]. Such escapees usually display a lower level of expression from the inactive X compared to the active X, thus leading to female expression that is less than twice the dose compared to males [78]. The lower expression may be due to the gene only being expressed from the Xi in a proportion of cells and/or to lower transcription levels from the Xi. Allele-specific analyses of genes that escape X inactivation reveal that they occupy an unique chromatin configuration, with no/low enrichment of heterochromatin-associated marks and no coating by Xist RNA [27,29,35,74,76]. RNA FISH studies have shown that escapees tend to be located outside the Xist RNA coated territory [28,44]. More recently, chromosome conformation capture analyses have revealed that Xi escapees tend to interact with each other preferentially in the nucleus [29]. The ability to escape may require a specific genomic context, as it has been shown that Kdm5c gene has a capacity to “escape” from XCI that is independent of its location on the inactive X chromosome [79]. This raises the question of how genes that are subject to XCI, can resist the influence of nearby escapees. Recent studies suggested that regions of the X chromosome that are subject to XCI appear to be protected from the spreading of transcriptionally active “escape” domains thanks to CTCF insulator elements [80,81] and the presence of young active LINE-1 elements has also been speculated to be involved in facilitating XCI of genes in regions that are otherwise prone to escape [28].

Surprisingly, many more escapees have been found in humans when compared to mice, with an estimation of up to 15% of X-linked genes escaping XCI to some extent in humans. An additional 10% of human X-linked genes show variable degree of escape, based on investigation in cell lines [82,83]. Many of these human escape genes lie on the more recently added short arm of the X chromosome. Indeed, it has been hypothesized that their incomplete silencing could be due to a barrier effect caused by the centromeric heterochromatin that separates the XIC on the long arm (Xq13) from the short arm where most escapees are located [for review [78]]. It should be noted however, that to date, only a few of these human Xi escapees have been confirmed in vivo given the difficulties in obtaining human tissue samples and in accurately quantifying allelic expression of X-linked genes. Nevertheless, comparative analysis of CpG island DNA methylation has been successfully used to determine indirectly the XCI status of X-linked genes between different tissues, supporting the idea of substantial tissue-specific escape from XCI [84]. There is also evidence for human individual-specific genes that escape from X inactivation. TIMP1 is good example of this phenomenon where it has been shown that H3 acetylation status correlates well with females in which escape of this gene is found [85].

Apart from a small number of genes for which escape from XCI appears to be evolutionarily conserved (such as KDM5C/JARID1C), for most escapees on the human X, escape from XCI is not necessarily consistent between individuals or between tissues or even between cells within the same individual. Nevertheless, the existence of escape from X inactivation, particularly in human remains largely unexplained [78]. Escape genes may play essential roles for normal development. Indeed, Turner syndrome is very deleterious to patient with a single X chromosome, perhaps due to haploinsufficiency of escape genes, even though XO mice are largely not affected [86–88].

A comprehensive study of X-linked allelic expression in hundreds of human lymphoblastoid cell lines has revealed that 5% of X-linked genes show increased expression level in females when compared to male. Based on this in vitro system, it seems that only a few escape genes have a role in compensating sex biases even though this 5% could have a strong impact when they are lost [83]. A recent in vivo study using RNA sequencing done on post-mortem human brain tissues revealed that X-linked genes show gene expression dimorphisms. Sex-biased gene expression was widespread in terms of gene number, chromosomes and range of brain regions involved. However, the percent of X- and Y-linked genes involved in the sex-bias seem highly related to particular regions of the brain [89]. For a more comprehensive view of the biological roles of genes that escape X inactivation in mammals, and particularly in the context of human health and disease, more primary tissue-specific and developmental analyses of XCI status will be required. Further investigation of the DNA sequence context, chromatin environment, nuclear organization and chromosome conformation, as well as developmental regulation will be needed to assess the mechanisms underlying escape from XCI. More detailed investigation of some of the known factors that ensure the maintenance of Xi gene silencing, for example SmcHd1 which results in partial escape from XCI when deleted in the mouse [93], will also be needed to understand the molecular basis for developmental and tissue-specific escape from XCI. Finally, there are some examples of X-linked gene reactivation from the Xi that have been associated with the aging, for example as shown for the OtC gene in mice [94]. The mechanism for this age-related escape is unclear although it may be due to epigenetic "erosion" due to inefficient maintenance of chromatin states over extended cell division or in adult stem cells. Although loss of the Barr body in ageing females has been reported, the degree to which age-related epigenetic relaxation of the Xi occurs in humans and mice has not so far been investigated systematically.

5. Unstable X-chromosome inactivation in cancer?

As described above, Xi reactivation is observed in a developmental context in mouse extra-embryonic lineages and in the germ line, and escape from XCI is observed for a subset of X-linked genes in somatic cells particularly in humans. However, the Xi is globally stably silent in somatic tissues suggesting that dosage compensation is important, although the impact that failure to maintain Xi inactivity in adult tissues would have, has not so far been assessed in any systematic way. The synergy and partial redundancy of
heterochromatin-associated marks that cooperatively maintain the integrity of heterochromatic inactive X were discussed above. The loss of Xist from the Xi in terminally differentiated cells does not lead to global reactivation of the inactive X, as genes remain repressed presumably via the above-mentioned synergistic epigenetic modifications [54]. Thus although Xist is required for initiation of X inactivation, it was thought not to be necessary for the maintenance of transcriptional silencing on the Xi up until recently [90,91]. However, a recent study showed that the conditional knock-out of Xist in the hematopoietic compartment of the mouse, led to an aggressive form of hematologic cancer in females [92]. Although allele-specific analyses were not performed in this study and thus Xi reactivation could not be demonstrated directly, the authors hypothesized that X-linked gene over-expression could be the result of X inactivation relaxation in tumor cells and somehow trigger cancer.

In humans, there is still only limited evidence available for the status of the Xi in cancer to date, even though it was described more than 50 years ago that the Barr body is frequently lost in breast cancers [2,95] (Fig. 2), leading to the proposal that Xi reactivation may be a common event in some cancers. More recent studies suggest that the disappearance of the Barr body is sometimes associated with over-expression of X-linked genes, suggesting a potential role of the X chromosome in cancer progression [96]. In some cases, the tumors lacking an inactive X chromosome, also present a duplication of the active X chromosome [96–98]. Thus, at least two mechanisms could explain the loss of Barr body in cancer cells (Fig. 2). On one hand, epigenetic instability of Xi may occur, with decondensation of heterochromatic Barr body, leading to Xi-linked gene reactivation. Although this mechanism has been frequently hypothesized, so far no evidence supports a model of global Xi reactivation and heterochromatin loss in cancer [99–101]. In some cases, XIST RNA mislocalisation and sporadic Xi reactivation has been observed [102,103]. For example, one study on an ovarian cancer cell line, showed a disruption of XIST expression and potential reactivation of the MPP1 (p55) gene [97]. In the context of breast cancer, bi-allelic expression of a single X-linked gene, VBP1, associated with promoter DNA hypomethylation was reported in one primary breast tumor sample [96]. However, without global analyses of expression status and chromatin structure of the inactive X chromosome, it is difficult to assess the precise extent of Xi instability in a cancer context. On the other hand, Barr body disruption can be due to physical loss of the Xi in female cancers [103,104]. Indeed, recent evidences show that the inactive X chromosome is genetically unstable in cancer as this study reporting an higher mutations rate on the inactive X compare to rest of the genome [105]. The consequence of both models would result with double dose of some or all X-linked genes. In early embryos, the presence of two active X chromosomes is ultimately lethal [12]. However in somatic cells the increased dose of a subset of genes on the X chromosome could potentially provide a selective advantage and promote cancer development, considering that even a rare cell expressing a gene with a proliferative advantage could contribute to cancer progression. Future studies on the causes or consequences of Xi reactivation in different situations, during normal development and in cancer, will be needed. Indeed, the emerging role of aberrant gene dosage in diseases, whether of the X chromosome or for autosomes, brings with it the possible application of drugs that impact on epigenetic regulators in potential therapeutic strategies [106–108].

A direct role for BRCA1, a tumor suppressor protein that is frequently mutated in familial cases of breast and ovarian cancer, was proposed some years ago to explain loss of the Xi [109]. This study proposed a direct action of BRCA1 on XIST RNA coating of the Xi

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**Fig. 2.** Genetic and epigenetic instability of the Xi in cancer. Schematic view of genetic and epigenetic alterations that can affect the X chromosome in breast cancer cells. The left panel lists the different genetic situations that have been observed concerning the X chromosomes in cancer cells i.e. Xi loss and Xa reduplication (for example [2]); Xi hypermutation [105]; and no Xi loss (for example [104]). On the right, we represent potential epigenetic instability that has been hypothesized to affect the Xi in some cancer cell. So far few studies have evaluated X-linked gene reactivation in breast cancer (for example [96]). Further studies are needed to explore to what extent epigenetic instability is present on the inactive X chromosome in cancer cells and what could be their impact in carcinogenesis.
and suggested that in BRCA1 mutated tumors, the Xi might be epigenetically relaxed. However subsequent studies [102,104] revealed that cells of BRCA1-mutated primary breast tumors can contain one or several XIST RNA coated chromosomes. Nevertheless, this does not invalidate a role for BRCA1 in XIST expression [102], as, among its many roles it may act as a transcription regulator [110]. Another chromatin regulator frequently over-expressed in cancer, Aurora B Kinase (AURKB), has also been proposed to regulate the association of XIST to the Xi [111,112]. However, what the exact consequences are on Xi status are not clear. Thus, the disappearance of the Barr body in aggressive breast tumors noted more than 50 years ago, still remains a fascinating but poorly understood phenomenon. Further investigation will be required to elucidate the nature and extent of the inactive X chromosome's genetic and epigenetic instability in cancer, with obvious clinical interest.

6. Conclusion

The inactive X chromosome provides a remarkable example of chromosome-wide gene repression that reveals the diversity of processes that can contribute to the formation of facultative heterochromatin. XCI also highlights the complex issue of gene dosage. The repeated inactivation and reactivation of the X chromosome during development illustrates the importance of a fine regulation of all the mechanisms involved. Recent studies have revealed that a combination of chromatin factors, chromosome conformation and nuclear compartmentalization together ensure maintenance of the inactive state. The mechanism that triggers gene silencing in the first place, via XIST RNA remains tantalizingly obscure. Once XCI is established, silencing appears to be rather stable in terminally differentiated cells, although XCI is less complete in terminally differentiated cells, although XCI is less complete in terminally differentiated cells [108]. Once XCI is established, silencing appears to be rather stable in terminally differentiated cells, although XCI is less complete in terminally differentiated cells [108].

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