Establishment of X chromosome inactivation and epigenomic features of the inactive X depend on cellular contexts

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X chromosome inactivation (XCI) is an essential epigenetic process that ensures X-linked gene dosage equilibrium between sexes in mammals. XCI is dynamically regulated during development in a manner that is intimately linked to differentiation. Numerous studies, which we review here, have explored the dynamics of X inactivation and reactivation in the context of development, differentiation and diseases, and the phenotypic and molecular link between the inactive status, and the cellular context. Here, we also assess whether XCI is a uniform mechanism in mammals by analyzing epigenetic signatures of the inactive X (Xi) in different species and cellular contexts. It appears that the timing of XCI and the epigenetic signature of the inactive X greatly vary between species. Surprisingly, even within a given species, various Xi configurations are found across cellular states. We discuss possible mechanisms underlying these variations, and how they might influence the fate of the Xi.

Keywords: development; differentiation; heterochromatin landscape; non-coding RNA; pluripotency; stem cells; X chromosome inactivation

Introduction

Dosage disequilibrium introduced by the heteromorphic nature of the X and Y sex chromosomes is compensated by mechanisms that specifically target the X chromosome and modulate its transcriptional competency. The first hint of male/female chromatin difference in mammals was cytological evidence of a nuclear body specific to female somatic cells, referred to as the Barr body [1], and identified as a condensed X chromosome [2]. The formation of this sex chromatin was proposed by Mary Lyon to arise early during development and to result in the transcriptional silencing of one the two Xs, in females only [3]. Balancing X-linked gene products in females through X chromosome inactivation (XCI) is essential for proper development, and failure to do so blocks embryogenesis shortly after implantation [4, 5]. In adults, presence of two active X (Xa) chromosomes in somatic cells has been observed mainly in deleterious cell states such as cancer cells [6, 7], and loss of X-linked gene dosage compensation is believed to cause aggressive hematological cancers [8].

XCI is triggered by chromosome-wide coating of the long non-coding RNA (lncRNA) Xist and characterized by profound changes of the epigenetic landscape of the inactive X chromosome (Xi) with respect to its active counterpart. Molecular events downstream of XIST accumulation and leading to stable silencing include eviction of RNA polymerases, accumulation of specific histones modifications and...
variants, changes in DNA methylation profiles, switch of replication timing, and reallocation of the Xi to specific nuclear compartments.

XCI is a striking example of facultative heterochromatin formation. In this case, the facultative nature of the heterochromatin, as opposed to the constitutive one, refers primarily to the fact that one X chromosome is packaged into condensed chromatin while its homolog is organized into open, transcriptionally active euchromatin. As XCI is random in most species, the parental origin of the inactive X can vary from cell-to-cell, so that the maternal X can be inactive in one cell and active in the other. In addition, although the inactive status is remarkably stable throughout adult life, it fluctuates during the early life cycle, where the X chromosome undergoes waves of inactivation and reactivation. In fact, XCI depends on a highly wired and dynamic network that connects the process of inactivation to the cellular state. The epigenetic landscape of the inactive chromatin may also vary from one developmental stage to another, and from one species to the next, influencing the stability of XCI and the probability of X-linked genes to escape from this inactivation. Here, we will discuss the developmental regulation of XCI and how this process is connected to cellular states, both within and across organisms. We will also review the variety of epigenetic signatures of the inactive X chromosome, and how they relate to cellular contexts and to the stability of the inactive state in eutherians as well as non-eutherian species.

**X chromosome inactivation and reactivation are dynamically regulated in physiological and pathological contexts**

**XCI initiates early in development, concomitantly to cellular differentiation**

Dosage compensation of sex chromosomes is absolutely required for mammalian development to progress properly. The presence of two active X chromosomes appears to be tolerated in some contexts, which may vary from one species to the other, but these usually correspond to undifferentiated or poorly differentiated states. The first hint that XCI was coupled to cell differentiation came from biochemical analysis of X-linked enzymatic activities in early mouse embryonic stages, which highlighted different XCI kinetics in extraembryonic versus embryonic tissues that follows the differentiation timing of these lineages [9].

Further analysis of early cleavage stages revealed that XCI could initiate prior to implantation, at least in the mouse (Fig. 1A). This inactivation, which begins around the four-cell stage, is imprinted: the paternal X (Xp) is preferentially inactivated. Inactivation of the Xp persists up to the early blastocyst stage and is preserved in throphectoderm (TE), and primitive endoderm (PrE) derivatives [10–14]. Imprinted XCI is, however, reversed in the pluripotent cells of early to mid-blastocysts, leading to both X chromosomes being active in those cells [9, 15–18]. As cells of the inner cell mass (ICM) progress to the epiblast stage and enter differentiation, random XCI (rXCI) is rapidly triggered, and leads to the inactivation of either the maternal or paternal X chromosome. This second wave of inactivation is completed in most cells of the E6.5 epiblast, and possible timing differences depend on the cell lineage [13, 19, 20]. Once established, rXCI is stably maintained in somatic cells.

In contrast to the mouse, rabbit, and human pre-implantation development proceeds in the absence of XCI (Fig. 1A). Assessing the allelic expression of candidate X-linked genes by RNA-FISH on rabbit embryos, Okamoto et al. showed that XCI initiates at the blastocyst stage, and may progress through a transient state where both Xs are inactivated, a situation that is rapidly resolved. In humans, both X chromosomes are also active from the zygotic genome activation up to peri-implantation stages, when rXCI is believed to be initiated [21]. This confirms earlier studies showing that human, and also cat and macaque embryos, display the first cytological signs of an inactive X (Xi) at the late blastocysts to epiblast stage [22, 23]. Intriguingly however, in humans XIST is expressed and accumulates on X chromosomes several divisions prior to the onset of XCI, so that XIST coating and XCI are transiently uncoupled in this species.

Thus, although the different mammals appear to display variable tolerance for X chromosome activity at pre-implantation stages, XCI is likely robustly and stably established soon after implantation, concomitantly to cellular differentiation. Further investigation in a broader range of species is, however, necessary to assess the consistency of this rule.

**To what extent is XCI coupled to differentiation in vitro?**

Ex vivo cellular models have been very powerful to decipher mechanisms of XCI initiation, and cell lines derived from embryonic contexts further confirmed the coupling of XCI to cell differentiation. The first cell line used was embryonal carcinoma (EC) female cells, derived from teratocarcinomas, which bear characteristics of blastocyst cells [24, 25]. In these cells, the activity of X-linked enzymes is reduced twofold after differentiation, an indication that cell differentiation is accompanied by XCI. Mouse pluripotent, embryonic stem cells (ESC), however, rapidly became the model of reference for the study of rXCI (Fig. 1B). Female mESC display two active X chromosomes and recapitulate rXCI when differentiation is triggered, similarly to the cells of the ICM from where they are derived. Multiple metastable states of pluripotency have been described, namely ground/naive and primed, depending on the embryonic stage from which cells are derived, and related to their capability to give rise to blastocyst chimeras [26]. ESCs in the mouse are found in ground/naive states. In contrast, mouse epiblast stem cells (EpiSC), derived from epiblast stage embryos, where XCI has already been triggered, are in a XaXi status, and are considered to be in the primed state (Fig. 1B) [27]. Based on studies in the mouse, ground pluripotency has been defined by the presence of two active X chromosomes, while primed cells have undergone XCI.

In contrast to the mouse, most human ESC (hESC) are found in the primed state, in which XCI has already been initiated (Fig. 1B). Their transcriptomic profiles and the
signaling pathways ensuring their maintenance indeed positioned them closer to mouse EpiSC than to mouse ESCs [27–29]. Female hESCs, however, display a surprising XCI instability, which has not been reported so far for hEpiSC. Female hESCs undergo a process of erosion of XCI, characterized by the loss of some of the hallmarks of the inactive state. Erosion of XCI also leads to a certain degree of X chromosome reactivation (XCR) [30, 31], which could argue in favor of the necessity for double dose of certain X-linked genes to sustain pluripotency in humans.

Numerous studies have tackled the issue of stably propagating hESC in the ground/naive state, which could favor retention of the pre-XCI status in female cells. Precise assessment of XCI status in human pluripotent cells is, however, complicated by the possible confusion between pre-inactive and eroded state, and further work is needed to characterize the pre-inactive state in humans and to establish robust protocols for the maintenance of pre-XCI female hESC. However, in the few cases in which pre-inactive hESC were reported, their differentiation was accompanied by inactivation of one of the two Xs [32]. ESCs are thus, a powerful model to study XCI and to assess its link to cell differentiation. No doubt that the availability of ESC lines from other mammalian species will prove extremely useful to further explore this connection and to unravel the underlying mechanisms across mammals.

**Figure 1.** Contexts for X chromosome in- and reactivation in vivo and ex vivo. A: Developmental timing of X chromosome inactivation and reactivation in human, mouse, and rabbit embryos of different stages (active and inactive X chromosomes are depicted in green and red, respectively). XIST RNA expression is schematized by the colored background. Imprinted XCI (iXCI) is observed in pre-implantation stages and extraembryonic tissues in the mouse, while random XCI (rXCI) is observed at later stages of mouse development, and in human and rabbit. The X chromosome is reactivated in primordial germ cells (green halo in embryo and fetus). The X chromosome is inactive in the sperm due to meiotic sex chromosome inactivation (MSCI). B: Patterns of XCI in mouse (top) and human (bottom) ex vivo cellular models. C: Genetic and epigenetic instability leading to abnormal XCI figures in cancer cells.

**X chromosome reactivation**

The progression to a differentiated state is accompanied by the inactivation of one X chromosome in female. Hence reprogramming toward pluripotency generally leads to the reactivation of the inactive X, both in vivo and ex vivo [33]. The first occurrence of developmental XCR takes place in the zygote, after the fusion of the highly specialized germ cells, the spermatocytes and oocyte, and concomitantly to zygotic genome activation (Fig. 1A). Indeed, the paternal X is inactive in the sperm, an effect that results from meiotic silencing of un-synapsed sex chromosomes that occurs during spermatogenesis (meiotic sex chromosome inactivation, MSCI). However,
the paternal X undergoes a transient phase of activity in the zygote before imprinted XCI is established [34–36]. A second wave of XCR is observed in the early mouse blastocyst, when the paternal X is reactivated prior to the establishment of rXCI, but this might be specific to some mammalian species undergoing imprinted XCI, such as rodents, and possibly bovines (Fig. 1A). XCR also occurs later in a subset of epiblast cells that dedifferentiate to give rise to primordial germ cell (PGCs), before meiosis and prior to sex specific differentiation of the germline (Fig. 1A) [37–41].

XCR can also be induced ex vivo during reprogramming of differentiated cells toward pluripotency. Nuclear transfer, cell fusion, or ectopic expression of reprogramming factors is accompanied by the reactivation of the inactive X, at least in mouse cells [42–46]. Whether Xi reactivation occurs when human differentiated cells are reprogrammed is still a matter of debate, but this might be linked to the difficulty of deriving and maintaining human pluripotent stem cells with two active X chromosomes. This emphasizes the importance of understanding the mechanisms governing both pluripotency and XCI reactivation in human, as further clinical applications of disease modeling in female human induced pluripotent stem cells might be influenced by this incomplete reprogramming of the Xi and/or by erosion of XCI [30, 47].

X chromosome inactivation may be altered in pathological contexts

While XCI is extremely stable in normal differentiated cells, abnormal XCI patterns are a hallmark of some pathological contexts, including aging and cancers. For example, female cells without a Barr body have been observed in breast and ovarian tumors [7], and X chromosome aneuploidies were reported in several tumor types [48]. Although genetic instability due to mitotic segregation errors commonly accounts for the loss of the Xi and duplication of the Xa, recent work has identified major epigenomic remodeling of the Xi in some cancer cells associated with abnormal dosage of some X-linked genes (Fig. 1C) [69]. Impairing Xist expression in the hematopoietic lineage results in the development of aggressive hematological cancers in females only [8], further suggesting that loss of XCI could play a causative role in tumorigenesis. In cancers also, the presence of more than one Xa is linked to the differentiation status of the tumor cells, because the loss of the Xi has been observed in aggressive, poorly differentiated, tumor cells [7, 50, 51]. The instability of X chromosome activity in cancer cells might be related to the fact that the X chromosome harbors many genes (tumor suppressors, chromatin remodelers) that are related to cancer progression. Abnormal X-linked gene dosage might thus, confer a selective advantage to the cell, a situation that is reminiscent of human pluripotent stem cells in which erosion of XCI spontaneously occurs in culture.

X chromosome activity is thus, connected to the differentiation status of the cell, and only undifferentiated or poorly differentiated cells in physiological, or pathological contexts cope with the presence of two active X chromosomes. A recent study proposed that XCI is required for differentiation to proceed, because double dosage of X-linked genes prevents the cells from exiting pluripotency [52]. This study, conducted in mouse, identified signaling pathways critical for differentiation that are blocked by the presence of two Xa. This would ensure that differentiation would progress only after XCI has been achieved. A tight coordination between dosage compensation and early developmental transition is further secured by direct control of XCI regulators by the pluripotency network. Whether X-linked gene dosage also controls differentiation in human is currently unknown. There is, however, no evidence so far that XCI profiles of female hESCs influence their differentiation potential; cells with eroded XCI, in which X-linked genes reactivation is irreversible [30], appear to differentiate properly. In that case, however, X chromosome dosage compensation might be achieved through a different mechanism, as recently reported in human pre-implantation embryos [53].

What are the molecular mechanisms coupling XCI to the cellular state?

Multiple cis- and trans-acting factors cooperates to regulate Xist and XCI

Regulatory mechanisms of XCI initiation and maintenance have mainly been studied in the mouse. The complex interplay that controls the expression of Xist, the master coordinator of the process, is integrated at a region physically mapped on the X chromosome, referred to as the X inactivation center (Xic). In the mouse, this region is topologically organized in two broad domains (Topologically Associated Domains, TAD) [54], which segregate factors that promote XCI from repressors of the process (Fig. 2). The boundary between the two Xic TADs lies between Xist promoter and that of Tsix, an antisense transcript whose expression is anti-correlated with Xist, and which blocks Xist accumulation in cis [55, 56]. Within the Xist TAD lie the non-coding loci Jpx and Ftx, as well as the protein-coding gene Rnf12, which altogether function as activators of Xist [57–61] (Furlan and Rougeulle, unpublished results). Conversely, the Tsix TAD encompasses loci such as Xite and Linx, which regulate Tsix and thus, further act as blocker of XCI [54, 62, 63]. Pluripotency-associated transcription factors are involved in the transcriptional regulation of those two TADs at multiple levels to ultimately repress Xist in pluripotent stem cells. OCT3/4, NANOG, SOX2, and REX1 have been suggested to directly repress Xist expression by binding within the Xist locus or its regulatory regions [64, 65] while REX1, KLF4, and c-MYC act indirectly through activation of Tsix [66]. Rnf12/Rlim is also regulated by pluripotency factors [67], and the ubiquitin ligase that it encodes in turn targets REX1 protein for degradation, contributing to the activation of Xist upon differentiation [65, 68]. Altogether, the multiple interactions of pluripotency factors and XCI regulators ensure a tight connection of XCI to differentiation, at least in the mouse.

The extent to which this holds true in other species remains to be investigated. It appears from the analysis of XIST expression profiles in human and rabbit blastocysts, and in human pluripotent cells, that the link between XIST expression and pluripotency might not be straightforward in
non-rodent species [21, 69–72]. More generally, very little is known about XIST regulation in species other than mouse. TSIX, for instance, is poorly conserved, and is unlikely to fulfill extensive repressive function on XIST [73]. The possibility exists that additional factors participate in the regulation of XIST or XCI in a species-specific manner. The IncRNA XACT might be such a candidate in the human. XACT is transcribed from a genomic region located more than 40 Mb downstream of the XIC, in the form of a very long non-coding transcript that remains associated to the chromosome from which it is expressed [74]. In contrast to XIST, XACT coats active X chromosomes, and its expression is restricted to early human embryonic contexts preceding, or concomitant with, the establishment of XCI. XACT is downregulated upon differentiation of hPSC and re-repressed upon reprogramming. Studies of XACT expression in the context of XCI erosion in hPSC suggested that XACT could serve as a modulator of XIST localization and/or activity [31]. XACT might thus, to some extent, connect XCI to specific developmental contexts and to pluripotency in the human.

**XIST-mediated silencing initiates in defined developmental contexts**

XIST is essential for triggering XCI, yet, it can only do so in certain cellular environments. Elegant experiments using inducible XIST cDNA transgenes indeed defined developmental contexts favorable for the establishment of XIST-mediated silencing. In pluripotent ESCs, for instance, overexpression of XIST causes long-range transcriptional silencing, but this repression is reversible, and depends on continuous expression of XIST. The repression becomes stable and maintained independently of XIST when differentiation is triggered. In contrast, forcing XIST expression in cells differentiated for more than 48–72 hours fails to induce silencing. The early stages of ESC differentiation thus, provide a window of opportunity for the establishment of stable silencing induced by XIST [75].

Similar experiments performed in vivo indicated that XIST is able to initiate silencing from the blastocyst stage up to mid gestation (E9.5–12.5), from which XIST competency to cause inactivation is gradually lost [76]. A notable exception was found specifically in adult hematopoietic progenitor cells, in which ectopic XCI can be initiated. This is not the case in the stem or the mature cell compartment [76]. These results suggest the existence of competency factors for XIST-mediated silencing that are downregulated as cells commit to differentiation in most somatic lineages, except in hematopoietic progenitors. SATB1 was identified as such a competency factor in a mouse T-cell lymphoma model [77]. Its overexpression in cultured mouse embryonic fibroblasts restored the ability of XIST to induce silencing in this differentiated context. SATB1 is, however, unlikely to be the only factor for XCI initiation, because its mutation is compatible with female development [78]. Other proteins that bind to XIST RNA and are necessary for various aspects of gene silencing have been systematically identified, and some of these XIST interactors could also function as competency factors [79–83]. These proteins may contribute to provide the proper context for XIST to be functional, but they are also directly involved in shaping the inactive X chromosome and its particular heterochromatin epigenetic signature.

**Are all inactive X chromosomes equivalent?**

The X chromosome in females can thus, be found in an active or an inactive form, and the transition between states is closely related to development and to the cellular context. In addition, the inactive status in itself is far from being fixed and constant; although it is generally characterized by the accumulation of repressive epigenetic marks, the Xi epigenetic signature can vary, both across and more strikingly within species, in a context-dependent manner.

**Imprinted and random XCI lead to distinct Xi signature in the mouse**

In mice, the molecular bases of imprinted inactivation are set during pre-implantation embryogenesis, and have been studied in vivo in early mouse embryos [16]. Epigenetic marks
of inactivation accumulate as early as the four-cell stage: loss of H3K4 methylation and H3K9 acetylation immediately follows the accumulation of Xist, and the exclusion of the RNA Polymerase II on the future inactive X. Accumulation of members of the PRC2 complex, such as EED and EZH2, and the resulting H3K27me3 enrichment, takes place subsequently, at the morula stage (16-cell), concomitantly to that of the histone variant macro-H2A (mH2A). Enrichment on the Xi of H3K9me2 is only detected from the blastocyst stage (32-cell).

The kinetics of rXCI initiation has been inferred from in vitro studies using ES cell differentiation. Similarly to imprinted XCI, the process is initiated with Xist coating, immediately followed by loss of RNA Polymerase II and of active histone marks from the future inactive X [84–86]. Dynamics of downstream histone modifications during rXCI, however, differ from iXCI; PRC1 and PRC2 complexes are rapidly recruited, and H3K27me3 and H2AK19Ub accumulate early in the process, concomitantly to the loss of histone acetylation, at around day 2 of differentiation [87]. H3K9me2 enrichment is also observed early in differentiation [88] and simultaneously with H3K27me3 [89, 90]. In contrast, association of the histone variant macro-H2A only occurs several days after Polycymb recruitment. Are these differences in the timing of epigenetic transformation of the Xi linked to differences in the kinetics and stability of gene silencing? Studies in pre-implantation embryos have shown that if most X-linked genes are efficiently silenced upon iXCI, some genes are only partially inactivated (Atp6ap2 and Mecep2) or escape inactivation (Huwe1) up to the blastocyst stage [91]. These genes are ultimately subjected to iXCI, as observed later on in extraembryonic tissues, suggesting that iXCI might not be complete in the pre-implantation embryo. This delayed inactivation might be related to the slow kinetics of repressive histone marks enrichment during iXCI compared with rXCI. The partial nature of the iXCI process at this developmental stage might also facilitate its reversal in cells of the ICM, prior to rXCI.

The inactive state of the Xp is, however, passed on and maintained, at least for the period of in utero life, in cells of the extra-embryonic lineage. The maintenance and stability of iXCI in this post-implantation context has mainly been addressed in vitro, through the analysis of cells derived from the trophectoderm (TS, trophoblast stem cells, and differentiated derivatives such as TGC, trophoblast giant cells) and from the primitive endoderm (XEN cells) – and in rare cases, from in vivo analyses [92–94]. These studies revealed peculiar features of imprinted X chromosome and lower stability of iXCI. Enrichment of H3K27me3 on the Xi has been observed in all cells from the extra-embryonic lineages [93–95], yet, to a lower extent compared to a randomly inactivated X: approximately 76–80% of X-linked genes display female-specific enrichment of H3K27me3 in XEN and TS cells, while this number reaches 93% in liver cells [93]. This is in agreement with the observation that levels of H3K27me3 are globally lower in TS and XEN cells compared with post-implantation embryonic stages [96]. Extra-embryonic tissues also display reduced levels of DNA methylation, especially at promoters of X-linked genes [97, 98]. Dnm1t deficient mice show reactivation of transgenes on the Xi in the embryonic lineage, but not in the extra-embryonic annexes [98], which further suggests that DNA methylation does not play a major role in the maintenance of iXCI. Other epigenetic marks further distinguish the imprinted Xi from its random counterpart. Notably, while the Xi is globally depleted from H3K4me2 and H4 acetylation in somatic cells [16, 84, 85, 99], TGCs, which are derivatives of TS cells, display only partial or no hypoacetylation of H4, and only partial hypomethylation of H3K4 of the Xi [92]. Moreover, these cells do not show enrichment in another Xi characteristic repressive mark, H4K20me1.

It remains to be fully understood to what extent this peculiar epigenetic Xi signature in extra-embryonic lineages is directly linked to partial lack of XCI and/or frequent reactivation events from the Xi. Indeed, more genes escape XCI in placent al progenitor cells (around 13% in TS cells) than in various adult tissues [93, 100–102]. Relaxation of XCI has been observed in up to 40% of TGCs for X-linked genes (Huwe1 and Atrx), which do not escape XCI in other lineages [92]. This confirms earlier results showing spontaneous reactivation of X-linked GFP and LacZ transgenes in cells from the trophectoderm lineage [19, 103]. These findings taken together highlight the plasticity of imprinted XCI, which might be linked to reduced epigenetic lockdown of the Xi silenced state in this lineage [104]. It must be noted, however, that in some cases, the epigenetic status of X-linked genes might not fully correlate with their transcriptional state: the gene G6dp displays low promoter DNA methylation in TGCs, yet, it remains tightly regulated and solely expressed from the Xa [92].

**Heterochromatin signature of the Xi varies across cellular states**

Pioneer immunofluorescence (IF) studies on human metaphase chromosomes have defined two major subtypes of facultative heterochromatin on the human Xi: one resembling constitutive heterochromatin, characterized by H3K9me3, H1F1 [105–108], and H4K20me3 enrichment [105] and a second distinguished by H3K27me3, macroH2A, and XIST RNA accumulation [105]. These signatures were shown to be mutually exclusive, each being associated with different cytogenetic bands on the Xi in hTERT-RPE1 cells [105]. Further analysis of these epigenomic marks on metaphase spreads from additional cell lines; however, highlighted a variability in this organization, with substantial cell-to-cell, and line-to-line differences in H3K27me3 and H3K9me3 distribution on the Xi [109]. The banding pattern of H3K27me3 appears to be a consistent feature of all cell lines examined; however, only one region, Xq22–23, remained commonly enriched in H3K27me3 in all cells and across all cell lines. In contrast, H3K9me3 was rather found restricted to Xi centromeres and telomeres. Although confined to the organization of metaphase chromosomes, these initial observations already suggested the existence of an epigenetic plasticity of the Xi in somatic cells, and implied that the X chromosome inactive state might not be propagated with a single epigenetic signature.

We recently addressed the diversity of the Xi heterochromatin landscape across various human cellular contexts, by ChIP-seq analysis of H3K9me3 and H3K27me3 distribution in
pluripotent stem cells, as well as in primary and immortalized differentiated cell lines [31]. While the inactive X is overall enriched in H3K9me3 and H3K27me3 in all cases, integrated bioinformatics analysis revealed a specificity of the Xi chromatin organization for each context (Fig. 3, left panels). First, the distribution along the Xi of each of the two marks varies from one state to the other. Some regions, however, display a common pattern in all conditions. In particular, ChIP-seq analysis of multiple datasets confirms the observation made by IF on metaphase chromosome [109]: that is that the Xq22–23 region, located between 103 and 116.5–Mb (hg19 assembly), is consistently enriched in H3K27me3. Intriguingly,
this domain encompasses several loci potentially involved in
the initiation or maintenance of XCI. DXZ4 is a macrosatellite
that produces non-coding transcripts and displays unusual
chromatin features: the allele on the Xi is enriched in
euchromatin marks and bound by CTCF, while the Xa copy is
packaged into heterochromatin [110]. The DXZ4 locus has
recently been proposed to play a role in the organization of
the Xi chromosome into megadomains [111, 112]. The
locus producing the XACT lncRNA also maps to within this
domain [74], raising the hypothesis that H3K27me3-mediated
repression of the region might be important for proper XCI.

In addition, the relative organization of H3K27me3 and
H3K9me3 vary across cellular states, and these two marks are
anti-correlated specifically in pluripotent cells and immortal-
ized differentiated cells. This is in agreement with what was
previously found by immunofluorescence on hTERT-RPE1, an
immortalized cell line [105]. In surprising contrast, we found
overlap between H3K27me3 and H3K9me3 enrichment zones
in all the primary differentiated cell lines that were included in
our analysis. Notably, in this context H3K9me3 was no longer
organized into domains (Fig. 3, “Xi in primary differentiated
cells,” left panel), but rather into focal loci dispersed all over
the chromosome.

Finally, the relative enrichment in H3K9me3 and
H3K27me3 on the Xi compared with autosomes also differs
between cellular states (Fig. 3, right panels). In pluripotent
cells, the Xi is the only chromosome to be highly enriched in
both H3K9me3 and H3K27me3. In contrast, it is only
H3K27me3 that distinguishes the Xi from other chromosomes
in primary differentiated cells; while in immortalized cells
the Xi is selectively marked by H3K9me3. Taken together,
the combination of H3K9me3 and H3K27me3 distribution
distinguishes the Xi from a pluripotent to a differentiated
cell type, and between primary versus immortalized cell types.
The consequences of such plasticity remain unclear. In the
case of hESCs, where XCI has been shown to be highly
unstable [32, 69], we provided evidence that the heterochro-
matin organization of the Xi might underlie gene-specific
predisposition to reactivation, in which transcriptional
reactivation of the Xi is limited to H3K27me3 domains [31].
Following this line of reasoning, the overlapping distribution
of H3K9me3 and H3K27me3 in primary differentiated cells
might protect the Xi from reactivation, and ensure the stable
maintenance of the inactive state across cell divisions,
although it remains to be shown whether these marks co-
exist on the same nucleosome.

We also explored the variability of the Xi chromatin
landscape in the mouse by analyzing published H3K27me3
ChIP-seq and ChIP-chip datasets (Fig. 4). This preliminary
investigation revealed striking differences in the relative
distribution of this mark on the X and autosomes, compared
with the human. In pluripotent cells, for instance, many
autosomes display substantial levels of H3K27me3, while the
Xs emerge as the less-enriched chromosomes (Fig. 4B, upper
panels). Most chromosomes overall maintain their methyla-
tion levels in differentiated cells, but the X undergoes a
massive transition to become the most enriched chromosome,
specifically in female cells, reflecting the inactivation of one of
the two homologues. The active X, as seen in male cells,
remains mostly depleted in H3K27me3, and this might be
linked to the up-regulation of the Xa, which takes place to
compensate for X-autosome dosage imbalance [113].

In primary differentiated female cells, H3K27me3 is
distributed into large domains over the Xi, and the enrichment
of the Xi in H3K27me3 is around twice that of autosomes, just
as in human cells. The presence and relative distribution of
H3K9me3 remain to be addressed through similar ChIP-seq
analysis, and so far few data sets characterize H3K9me3
distribution. Evidence suggests that H3K9me2 accumulates on
the Xi in the mouse [88, 90, 114]. One study provided ChIP-
chip datasets for H3K27me3 and H3K9me2 in mouse ES
cells and cells differentiated for 14 days [115]. We reanalyzed
the data to compare the relative positioning of H3K9me2
and H3K27me3 on the X chromosome (Fig. 4C), and found
anti-correlated distribution of the two marks on the Xi in
differentiated cells. Accordingly, a recent study analyzing
H3K9 di- and tri-methylation, and H3K27me3 in mouse
embryonic fibroblasts also revealed anti-correlated distribu-
tion of the K9me2/3 and K27me3 marks on the Xi [116]. These
results contrast with what we observed in human primary
differentiated cells, where these marks overlap significantly
on the Xi.

Drivers of this epigenomic variability might lie in the
mechanisms targeting these repressive modifications to the
Xi. First of all, what triggers H3K9me2/3 accumulation on
the Xi is still unclear. We have shown, however, that
H3K9me3 maintenance on the Xi is independent of XIST
and of H3K27me3, at least in hESCs [31]. In contrast, it is
well described that Xist is the main trigger for H3K27me3
deposition and maintenance on the Xi, and the genomic
regions contacted by Xist RNA correlate with those occupied
by H3K27me3, at least in the mouse [117]. Such link has not
been investigated in depth in human. In addition, we know
that during early human development, XIST accumulation
on X chromosomes in male and female embryos does not
trigger H3K27me3 deposition [21]. What uncouples XIST from
H3K27me3 is unknown, and it will be of great interest to assess
the involvement of the lncRNA XACT in this process, as XIST
and XACT accumulate simultaneously on the X chromosome at
these stages (unpublished data).

The divergence in Xist sequences across species [118] could
impact on its associated factors and on its activity. While Xist
interactome is beginning to be deciphered in the mouse [79,
80, 82], it is yet unknown whether Xist partners are the same
in other species. Recently, JARID2 has been implicated in
bridging PRC2 complex with Xist RNA, and thus, in targeting
H3K27me3 to the Xi in the mouse [119]. Changes in JARID2/
XIST interaction in human could explain why XIST RNA
coating does not lead to immediate H3K27me3 deposition
present in early embryos.

Dynamics of expression of factors involved in the
deposition of H3K27me3 and/or H3K9me2/3 may as well
contribute in setting up the Xi landscape, and should be
revisited in that context. This concerns H3K9 and H327
histone methyltransferases (HMT), as well as co-factors. CDYL
in particular is a chromodomain-containing transcriptional
corepressor whose recruitment to the Xi depends on both
H3K9me2 and H3K27me3. CDYL interacts with the HMT G9A,
which may be responsible for triggering H3K9me2 in the
vicinity, or across H3K27me3 marked regions [120].
Species differences in the organization of the Xi chromatin might also be promoted by difference in the genomic content of the X chromosome. Repeats in particular, which differ between mouse and human [121], could influence the global epigenomic landscape. On mouse autosomes, H3K27me3 broad domains correspond to gene-rich regions enriched in SINE repeats, and alternate with H3K9me3/H4K20me3-marked regions enriched in LINE/LTR repeats [122]. Such organization appears to be maintained on the mouse Xi [116]. In contrast, in hESC we did not detect preferential accumulation of either H3K9me3 or H3K27me3 over specific repeat- or gene-rich regions (data not shown).

It is important to bear in mind that the H3K9-K27 methylation differences observed in the various cellular contexts and across species are likely only glimpses of the epigenomic differences that exist on the various Xi. Additional epigenetic marks, such as DNA methylation and other histone modifications, should be investigated and integrated to fully grasp the variability of the epigenetic organization of the Xi across cellular contexts, and the influence of this epigenetic signature on the stability of XCI. This in-depth characterization of Xi epigenetic signatures, as well as the higher order chromatin organization will be necessary to further characterize the tenants of this potential heterogeneity observed across species and cellular states.

Figure 4. Heterochromatin signature of the inactive X chromosome in mouse. A: ChIP-seq profiles (log2 enrichment over input) and chromosomal density maps of peaks detected for H3K27me3 (green tracks) for male and female ES cells, and male and female differentiated cells. B: Same plots as in Fig. 3 for each mouse chromosome, representing cumulative H3K27me3 occupancy versus chromosome size in bp. C: Superposition of H3K9me2 and H3K27me3 ChIP-chip profiles in LF2 mESC, and LF2 differentiating cells at day 10 [115]. Log2 ratios were compared using Pearson correlation test and the associated p-value was calculated using random permutations of the data sets. Datasets are from GSE23943, GSM1754897, GSM769032, and GSE15883.

in SINE repeats, and alternate with H3K9me3/H4K20me3-marked regions enriched in LINE/LTR repeats [122]. Such organization appears to be maintained on the mouse Xi [116]. In contrast, in hESC we did not detect preferential accumulation of either H3K9me3 or H3K27me3 over specific repeat- or gene-rich regions (data not shown).
Conclusions and outlook

The connection between the activity status of the X chromosomes and the level of differentiation was among the first observations made in the exciting field of XCI. Since these early days, we have advanced greatly in our description of the process and our understanding of the molecular network that sustain this intimate relationship – at least in the mouse, which has, till now, been the leading model species in the field. Many questions remain unanswered, however. The necessity of such coupling is, for instance, not so clear: why are two active Xs tolerated only in poorly differentiated contexts? This is of importance not only because we, researchers, aim to disentangle fundamental issues in biology. It will also likely impact our comprehension of pathological states linked to dedifferentiation. In addition, the variation in the strategies and kinetics of XCI between species that is being brought to our attention raises questions as to the extent to which the discoveries that have been made in the mouse in that matter hold true in other mammals.

XCI is considered as the paradigm of epigenetic regulations, in which the status of the inactive X chromosome is stably transmitted to daughter cells upon cell division, for the duration of the individual’s life, in mammals. We are now facing the possibility that this might be a bit of an illusion, and that the nature of the Xi might also change depending on cellular contexts. For sure, a simple epigenetic signature does not characterize the inactive status of the X chromosome, and differences have been found in the kinetics and the quality of the marks that form the Xi facultative heterochromatin between species. Even within a given species, the mouse, the Xi differs depending on whether it has been inactivated in an imprinted or a random manner, prior to or after implantation. Such a difference is not so unexpected, however, because a transient phase of reactivation during which the marks are erased separates those two states, allowing for new remodeling to take place. More striking is recent evidence of a certain plasticity of the Xi described in humans, in which variation in the distribution of at least some chromatin marks occurs between cell lines and between cellular states that are, at least in theory, not disconnected by a reactivation phase. Transmission of the inactive state across cell divisions might thus not be as faithful as initially postulated, and while the inactive state is maintained, flexibility is tolerated in the internal organization of the Xi chromatin. Determining what governs these changes and their consequences will require further investigation.

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