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Recent advances in X-chromosome inactivation

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X inactivation is the silencing one of the two X chromosomes in XX female mammals. Initiation of this process during early development is controlled by the X-inactivation centre, a complex locus that determines how many, and which, X chromosomes will be inactivated. It also produces the Xist transcript, a remarkable RNA that coats the X chromosome in *cis* and triggers its silencing. Xist RNA coating induces a cascade of chromatin changes on the X chromosome, including the recruitment of Polycomb group proteins. This results in an inactive state that is initially labile, but may be further locked in by epigenetic marks such as DNA methylation. In mice, X inactivation has recently been found to be much more dynamic than previously thought during early pre-implantation development. The paternal X chromosome is initially inactivated in all cells of cleavage-stage embryos and then selectively reactivated in the subset of cells that will form the embryo, with random X inactivation occurring thereafter.

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Abbreviations

Eed	embryonic ectoderm development
Enx1	Enhancer of zeste (EZ) mouse homolog
ES	embryonic stem
ESC	extra sex combs
HMTase	histone methyltransferase
ICM	inner cell mass
MMSI	male meiotic sex chromosome inactivation
Xi	inactive X chromosome
Xic	X-inactivation centre
Xist	X-inactive-specific-transcript
Xm	maternal X chromosome
Xp	paternal X chromosome

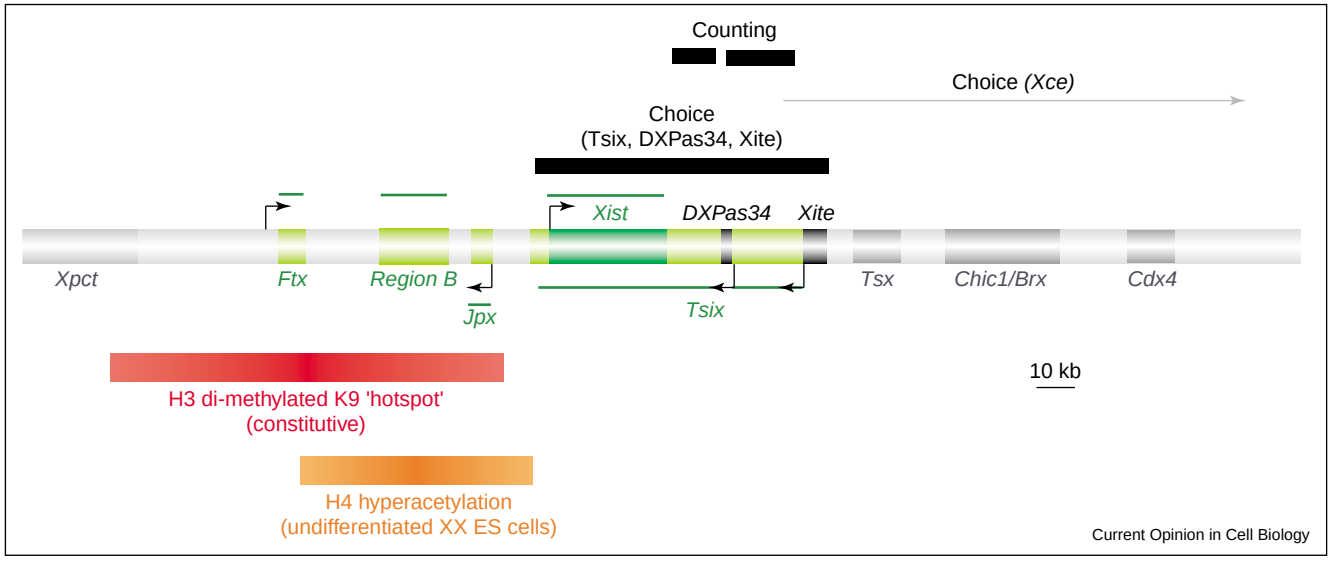
Introduction

Different dosage compensation strategies are employed across the evolutionary spectrum to ensure that levels of X-linked gene products are equal between XY males and XX females. This review deals with the form of dosage compensation adopted in female mammals, X-chromosome inactivation. For dosage compensation strategies in other organisms such as *Drosophila* and *Caenorhabditis elegans*, the reader is referred to recent reviews [1,2]. The mammalian strategy of X-inactivation is unique in that, within the same nucleus, two X chromosomes are differentially treated: one is transcriptionally silenced while the other remains active. This process takes place during early development and, once established in the soma, the inactive state is stably maintained through countless cell divisions. X-chromosome inactivation thus represents one of the great paradigms of epigenetics, defined by Holliday as the study of changes in gene function that are mitotically or meiotically heritable but do not entail a change in DNA sequence [3]. The mechanisms that trigger X inactivation and the changes that are induced to transform an active X chromosome into facultative heterochromatin are subjects of intense research. This review will provide a brief overview of some of the most recent advances in the X-inactivation field over the past two years. A number of in-depth reviews can be consulted for more information [4–6].

Regulation of the initiation of X inactivation

The initiation of X inactivation is dependent on a master control locus, the X-inactivation centre (Xic) [4]. At its heart lies the *Xist* (X-inactive-specific transcript) gene which, when the decision to inactivate has been made, produces a 17 kb-long, untranslated RNA that coats the X chromosome in *cis* and triggers its inactivation. The Xic is also implicated in critical steps upstream of *cis*-inactivation, such as 'counting', whereby only a single X chromosome will remain active per diploid autosome set, with inactivation of supernumerary X chromosomes, and 'choice', whereby one of the two X chromosomes is selected for inactivation (or to stay active). Deletion and transgenesis analyses over the years have revealed that the Xic is a highly complex locus (Figure 1). The Xic's functions have been extensively reviewed recently [7–9] and only selected highlights will be presented here. The Xic elements required for counting have recently been narrowed down to a 20-kb bipartite domain within a 37-kb region (Figure 1) lying 3′ to *Xist* [10•]. In embryonic stem (ES) cells with only one X chromosome, deletion of this region leads to the unique phenotype of inappropriate X inactivation upon differentiation. This study also demonstrates that counting is assured solely by the Xic region of the X chromosome and that it is not a phenomenon specific to cells with two X chromosomes. The choice function of the Xic appears to be influenced by multiple elements spread across the Xic region. In addition to the genetically defined, but so far elusive, *Xce* locus, several targeted deletions lying 3′ or 5′ to or within *Xist* lead to non-random X inactivation [8]. Antisense transcription of *Xist*, in the form of *Tsix*, has been the

Figure 1



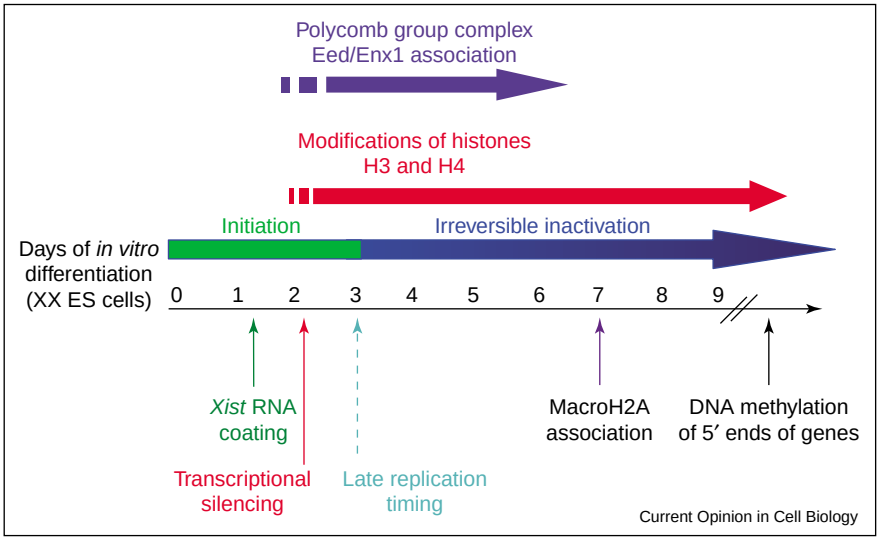
Functional elements within the X-inactivation centre. The Xic region, as defined by deletion and transgenesis analyses, is shown, together with important functional elements. For more details the reader is referred to references [7] and [8]. Genes shown in green produce non-coding transcripts (green lines). Genes shown in grey produce protein-coding transcripts and have no known Xic related function. Above the map, regions shown in black encompass elements defined by targeted deletions involved in counting and choice. Counting is the process whereby a cell senses the number of X chromosomes that are present and ensures that only a single X chromosome remains active per diploid autosome set, all supernumerary X chromosomes being inactivated; choice concerns the selection of the X chromosome that will remain active or that will be inactivated. The candidate region for the genetically mapped *Xce* locus involved in choice is shown in grey. Below the map, the red bar represents a hotspot domain of histone H3 K9 dimethylation, which is constitutively present in male and female ES cells before and after differentiation [16,36]; the orange bar represents a hotspot of H4 hyperacetylation, present only in female ES cells prior to differentiation [30•].

focus of much attention as a potential regulator of choice over recent years [5,8,9]. Deletions of the major *Tsix* promoter (Figure 1) result in *Xist* accumulation and complete non-random X inactivation of the targeted allele in differentiating XX, but not XY, ES cells, as expected for an element involved in choice but not in counting. The consensus opinion, based on several different types of study, is that *Tsix* transcription exerts a repressive effect on Xist RNA accumulation before and during the initiation of X inactivation [5,8,9,10•,11,12]. Whether this repression is RNA-dependent or transcription-dependent is still not known, however. Furthermore, recent studies suggest that the actual *Xic* choice elements within *Tsix* may even be independent of its promoter activity [12].

Intriguingly, several additional non-coding RNAs have recently been discovered in the Xic region. Intergenic transcripts have been found associated with a regulatory element of *Tsix* (*Xite*) [13•] and three new genes, producing apparently untranslated transcripts, have been identified in the region 5′ to *Xist* [14,15]. Whether these represent functional RNAs, like Xist, or whether they are involved in, or symptomatic of, a specialized chromatin status is not known. The latter possibility is particularly attractive in the context of the unusual chromatin characteristics in the region 5′ to *Xist*. A 'hotspot' of constitutively enriched H3 K9 di-methylation has been identified in a >150 kb domain [16] and part of this region has also been found to be enriched, in undifferentiated female ES cells, in acetylated histone H4 [17]. This unusual combination of histone modifications, coupled with the clustering of non-coding transcripts, may signify one or more functional roles for this region in X inactivation, for example as a regulatory domain controlling *Xist* or as a nucleation centre for the *cis*-action of the Xic [16].

A better understanding of how X inactivation is regulated will clearly come from identification of the factors that bind to the Xic and mediate counting and choice. For counting, most models evoke the existence of an autosomal factor produced in limited quantity that binds to the Xic of a single X chromosome per diploid cell, protecting it from inactivation. As the 'counted' region, which should contain the target sequence of this putative blocking signal, has now been refined [10•], identification of the signal itself may be in sight. In the case of choice, the CTCF transcription factor, which is known to be involved in chromatin insulation at imprinted loci, has been proposed as a candidate for regulating *Tsix* expression [18], although functional proof of this is lacking. Recently, two autosomal mutations affecting the random nature of X inactivation early in development have been identified using a phenotype-driven genetic screen [19•].

Figure 2



Kinetics of X inactivation in differentiating female ES cells. The timing of the various events that characterise random X inactivation in differentiating female ES cells are shown. This summary is compiled based on findings reviewed here and elsewhere. The earliest time point at which each of these characteristics is first detected is indicated. The time periods corresponding to a transition from a Xist RNA dependent 'initiation' phase (green) and a Xist RNA independent 'irreversible' phase (blue) are indicated and are based on the study of Wutz *et al.* [20].

Identification of the corresponding genes should shed new light on the molecular basis of choice.

Xist RNA and the establishment of the inactive state

Targeted deletions at the *Xist* locus have demonstrated the absolute requirement for this gene in the initiation of X inactivation in *cis* [4]. In cells with more than one Xic, *Xist* expression is up-regulated on the future inactive X chromosome within one to two days following induction of ES cell differentiation. Gene silencing along the length of the X chromosome rapidly ensues, within one or two cell cycles [20] (Figure 2). This suggests that Xist is directly responsible for mediating transcriptional shut-down. Its action is developmentally restricted, as at later stages of differentiation induction of Xist RNA coating cannot trigger inactivation [20]. One of the most tantalising current questions in the field concerns how Xist RNA actually induces X inactivation. A recent study, involving an inducible *Xist* cDNA in ES cells, has taken us one step closer to the answer by showing that the chromosomal association and silencing abilities of Xist RNA are functionally separable [21••]. Silencing requires a conserved repeat region located at the 5′ end of the transcript. Association of Xist RNA with chromatin is mediated by other sequences, which are functionally redundant and dispersed throughout the remainder of the transcript. Identification of the proteins that bind to the different functional regions of the Xist transcript will undoubtedly provide important insights into its exact mechanism of action. A recent exciting finding is that the BRCA1 protein, mutations of which are associated with breast and ovarian cancer, may be required for XIST RNA coating of the inactive X chromosome (Xi), at least in somatic cells [22•]. How BRCA1 targets XIST RNA to chromatin, whether it does so during the initiation of X inactivation, and what its exact role might be in the epigenetic stability of the inactive state, particularly in the context of cancer, are important questions for the future.

Early chromatin changes during X inactivation and the role of Polycomb group proteins

Evidence from multiple organisms suggests that histone tail modifications are directly involved in transcriptional regulation [23] and can also provide the chromatin memory of a specific state of activity through mitosis [24]. The inactive X chromosome has been associated with characteristic patterns of histone modifications, such as hypoacetylation of histones H3 and H4, di-methylation of H3 Lys-9 [16,25,26], tri-methylation of H3 Lys-27 [27••,28••] and a lack of di- and tri-methylation of H3 Lys-4 [25,26,29•,30•], many of which appear early on in the X-inactivation process, just after Xist RNA coating [26,27••,28••,30•] (Figure 2). Most work has focused on the inactive X chromosome rather than its active counterpart. A new perspective on the status of X-linked chromatin has now been provided by recent findings, which suggest that the X chromosome carries early and specific epigenetic marks (some of which are also found at imprinted loci) that could be responsible both for its monoallelic expression [31•] and for the restriction of X inactivation to female cells [30•]. This raises the

possibility that monoallelic status may be epigenetically defined, even before monoallelic expression patterns are established.

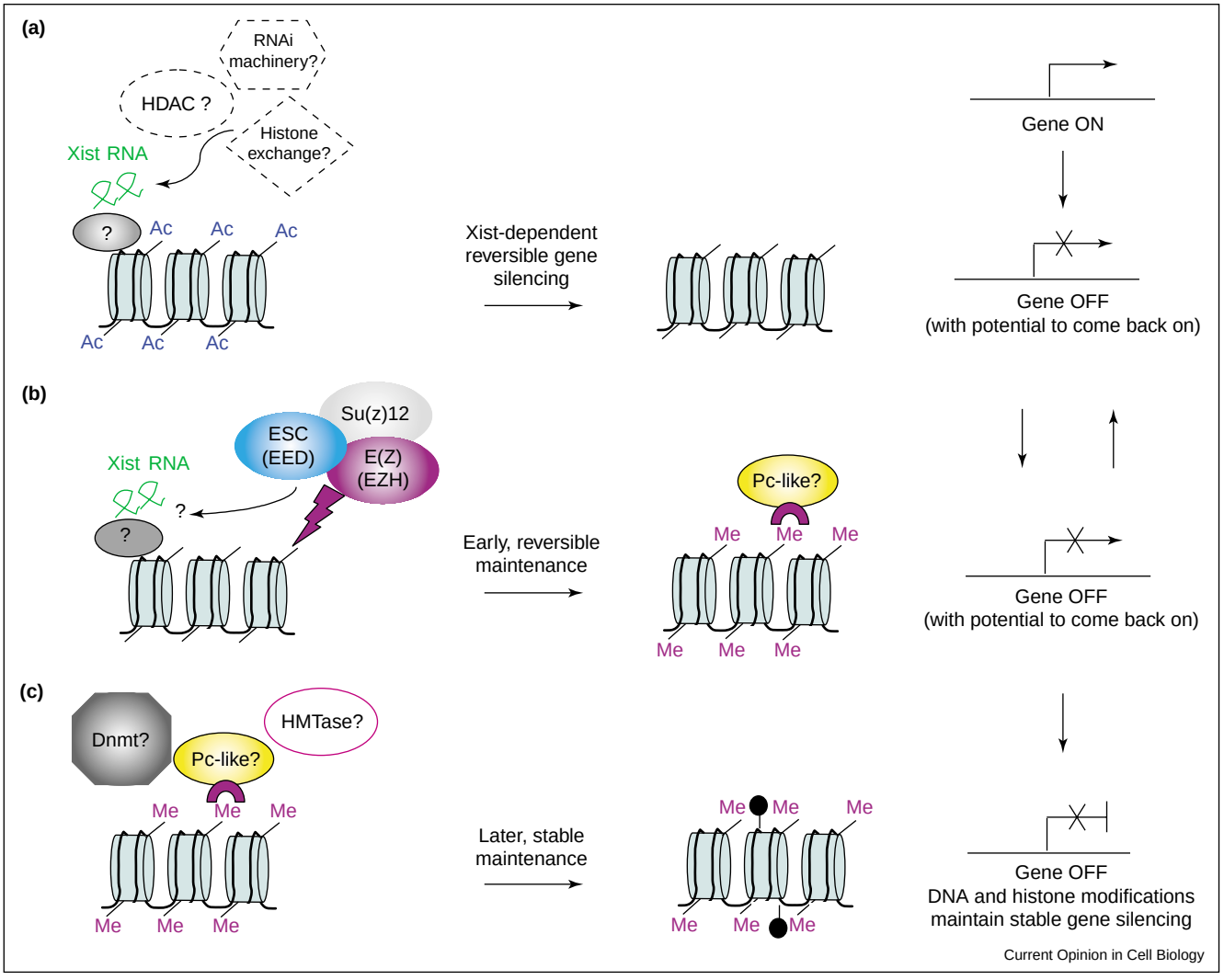
The enzymes responsible for the chromatin modifications that are induced during X inactivation are just starting to be identified. Indeed, one of the most exciting findings in the past year has been the discovery that the Polycomb group proteins Eed (embryonic ectoderm expression, also known as extra sex chromosomes or ESC) and Enx1 (the mouse homologue of Enhancer of zeste [EZ]), the latter being a H3 Lys-9/27 histone methyltransferase (HMTase), may be implicated in both imprinted and random X inactivation [27••,28••,32,33•]. This seems to parallel the situation in *Drosophila*, where the ESC/EZ complex is involved in the initial maintenance of repressed states during early development [34,35] (see also review in this issue by Lund and van Lohuizen). Transient association of the Eed/Enx1 complex with the X chromosome is observed shortly after Xist RNA coating and is accompanied by H3 K27 methylation (Figure 2), consistent with a role for Enx1 as the HMTase responsible. It is still unclear which HMTase causes H3 K9 di-methylation on the inactive X chromosome, although a recent study has shown that G9a is the enzyme responsible for this modification in the constitutive hotspot region lying 5′ to Xist [36]. This study also demonstrated that both the H3 K9 di-methylation and H3 K27 tri-methylation marks are present on the inactive X, with overlapping but distinct distributions, thus clarifying recent confusion that arose due to K9/K27 cross-hybridisation problems with some antibodies. In terms of mutant analysis, Silva *et al.* found that in *eed* mutant mouse embryos, no recruitment of Enx1 or H3 K27 methylation can be seen on the Xist-coated X chromosome [27••]. Although this does not cause global X-reactivation, it does cause an increase in sporadic X-linked gene re-expression and an increase in H3K9 acetylation and H3 K4 methylation on the X chromosome in a proportion of cells. Plath *et al.* used a *Xist* cDNA transgene to show that the coating of the X chromosome with the silencing mutant of Xist RNA can still recruit the Eed/Enx1 complex and induce H3 K27 methylation on the X chromosome, even though gene silencing is not induced [28••]. Taken together, these findings show that the Eed/Enx1 complex is important for the early maintenance of the inactive state, but it probably cannot account for the initial silencing induced by Xist. This implies that the Xist transcript functions in parallel pathways during the initiation time window, inducing both silencing, via an unknown mechanism which possibly involves histone deacetylation, histone exchange or other processes (Figure 3a), and early maintenance, via Eed/Enx1 and associated histone H3 K27 methylation (Figure 3b). In conclusion, Polycomb group proteins and associated chromatin-modifying enzymes may be mediators of the critical transition from the reversibly inactive, Xist-dependent phase of inactivation, to the irreversible, Xist-independent phase defined by Wutz and Jaensich [20]. As the Eed/Enx1 complex is only present transiently during development, what accounts for the later maintenance of the inactive state? In *Drosophila*, another Polycomb group complex, PRC1, appears to ensure the later maintenance of repressed states [34,35]. However, PRC1 homologues have so far not been detected on the Xi at any stage. This suggests that other, so far unidentified, Polycomb group proteins may be involved. Alternatively, the late replication timing of the Xi, incorporation of the histone variant macroH2A, and recruitment of DNA methylation to promoters of genes on the Xi may, together with certain histone modifications, be sufficient to maintain the inactive state [37] (Figure 3c).

New insights into the developmental regulation of X inactivation

In rodents, X inactivation is initially subject to imprinting, with the paternal X chromosome (Xp) being preferentially inactivated in those cells of the blastocyst that will form the extra-embryonic tissues (Figure 4). Random inactivation of either the paternal or maternal X chromosome is observed shortly afterwards in cells derived from the inner cell mass (ICM) that go on to form the embryo proper (Figure 4). Imprinted X inactivation is also found in marsupials, where it affects all tissue types. Indeed, imprinted X inactivation has been proposed to represent the ancestral form of this process, with random X inactivation evolving only in placental mammals.

One long-standing question is the nature of the parent-specific imprints underlying preferential Xp inactivation. In mice, a powerful maternal imprint, acquired during the oocyte growth phase of meiosis, prevents the maternal X chromosome (Xm) from being inactivated in early embryos, even when two Xm chromosomes are present [38]. This Xm imprint prevents the maternal *Xist* gene from being expressed during early cleavage stages [39], and may act via *Tsix* [40], although its exact nature and location remain elusive. In addition to this resistance of the Xm to inactivation, the Xp may also carry a predisposition to becoming inactivated. One oft-cited model for imprinted X inactivation suggests that partial inactivity of the Xp and/or a predisposition to inactivation might be carried over from its passage through the male germ line [41,42]. Here, the Xp and Y chromosomes together form the highly condensed, heterochromatic XY body following male meiotic sex chromosome inactivation (MMSI). In fact, Xist is expressed at low levels in the male germ line. It has even been proposed that the Xp arrives in the zygote in a pre-inactivated state because of its history in the male germ line [43,44••]. However, several X-linked genes are known to be re-expressed in post-meiotic spermatids, indicating that the Xp is actually reactivated just prior to fertilisation [45]. Furthermore, recent data have shown that unlike female imprinted X inactivation,

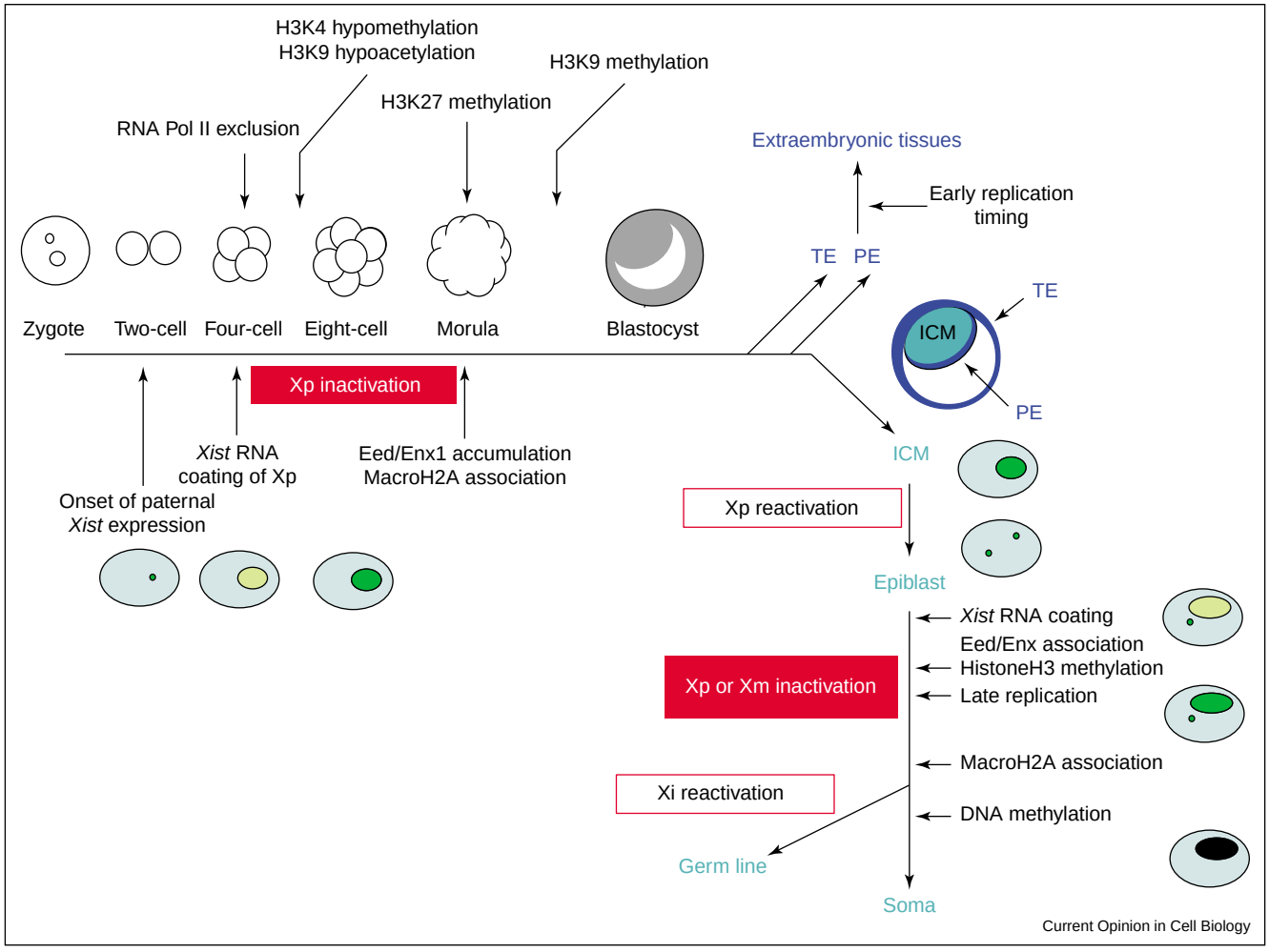
Figure 3



Xist RNA may have multiple roles acting in parallel pathways. A schematic model showing possible mechanisms that function during the establishment and maintenance of the inactive state is shown, based on findings in differentiating ES cells [16,26,27••,28••] and early mouse embryos [44••,49••,51••]. **(a)** Xist RNA associates with the X chromosome in cis, through unknown factor(s), and recruits an initial silencing activity (possible candidates include HDACs, histone exchanging/remodelling complexes and/or the RNAi machinery). This leads to rapid transcriptional shut-down, which is easily reversible and Xist RNA-dependent. **(b)** Xist RNA also recruits the Eed/Enx1 PcG complex (directly or indirectly), which introduces histone modifications such as H3 K27 or possibly H3 K9 methylation that help to lock in the inactive state. The proteins that bind the H3 K9 and K27 methyl marks to mediate this function are currently unknown. At this stage, the inactive state is still reversible and Xist RNA-dependent. The Eed/Enx1 PcG complex is only transiently present during development. **(c)** Specific histone modifications on the Xi and the unknown proteins that bind to them could in turn 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. The combination of some/all of these epigenetic marks acts synergistically to maintain the inactive state.

which is dependent on *Xist* [39], MMSI is *Xist*-independent [46,47] and occurs through a very different process involving the histone variant H2AX [48•]. Consistent with *Xist* expression being the critical determinant for imprinted Xp inactivation, rather than MMSI, silencing of paternal X-linked genes appears to spread from the *Xist* locus during early female development [44••]. Therefore, although in this study it is claimed that the Xp arrives in the zygote in a pre-inactivated state [44••], overall, the paternal X-linked gene expression data points to the Xp being active at the two-cell stage but becoming rapidly inactivated following Xist RNA coating from the four-cell stage onwards [45,49••]. Thus, in summary, the only imprint required for Xp inactivation to occur in mice may be the elusive 'resistance' imprint on the Xm that prevents the maternally inherited Xist gene from being expressed, and, consequently, the Xm from being inactivated. The expression of the paternal allele of *Xist* at

Figure 4



Kinetics of X inactivation during pre-implantation mouse development. The dynamic X-inactivation events that occur in pre-implantation embryos are summarised, based on new studies [44••,49••,51••]. At the two-cell stage, Xist expression has just begun and is localised to its site of transcription (shown as a small spot). At the four-cell stage, Xist RNA is accumulated over the Xp chromosome (shown as a domain) [44••,49••]. Although the initial silencing event triggered by Xist RNA coating occurs prior to Eed/Enx1 recruitment and H3K27 or H3K9 methylation of the X chromosome, it does appear to be linked with H3 hypoacetylation and loss of H3K4 methylation. The former could be explained by recruitment of an HDAC (see Figure 3a), but the latter requires loss of the histone (or its tail). Given the absence of a histone demethylase to date, this would appear unlikely. Another possibility is that a histone exchange activity could be recruited by Xist RNA, which would replace the histones present in active chromatin with unmodified histones. In the blastocyst, essentially all cells contain an inactive Xp associated with the PcG proteins Eed and Enx1. In the trophectoderm and primitive endoderm (extraembryonic lineages), this inactive state is maintained and perhaps further locked in by a shift in replication timing (early replication in this case). In the ICM, however, the Xp becomes reactivated, losing its Xist RNA coating and the Polycomb group proteins, and the histone methylation marks are gradually reversed. In this way, cells that will subsequently contribute to the epiblast (embryo-proper) contain two active X chromosomes, prior to the random inactivation of either the Xp or Xm. In the female germ line, which is set aside subsequently, the inactive X becomes reactivated just prior to meiosis. PE, primitive endoderm; TE, trophectoderm.

zygotic gene activation is thus likely to lead to the rapid silencing of the Xp by default. It is striking to note that the exclusively paternal expression of Xist in the early embryo parallels the situation at other imprinted loci, where non-coding RNAs are systematically found to be paternally expressed and maternally repressed [50].

The recent discovery that Xp inactivation takes place much earlier than was previously thought, in cleavage-stage embryos [44••,49••] (Figure 4), means that the long-standing idea that this process is normally associated with cellular differentiation no longer holds. The very early timing of imprinted Xp inactivation raises the question of how random X inactivation can occur subsequently in cells of the embryo proper. This has been addressed in two studies, which have shown that all cells indeed have an inactive Xp by the early blastocyst stage but that this inactivity is reversed during ICM growth as cells rapidly lose Xist RNA coating, Eed/Enx1 enrichment and histone modifications on the Xp [49••,51••]. In trophectoderm

and primitive endoderm cells of the blastocyst, on the other hand, the silent state of the Xp is maintained (Figure 4). These findings reveal the highly labile epigenetic state of the Xp during early development, and demonstrate the critical role of the ICM in reactivating the Xp. This is also consistent with past studies involving nuclear transfer [52] or fusion of a somatic cell nucleus with ES cells [53]. The evolutionary reasons for such a dynamic cycle of paternal X inactivation, followed by reactivation, followed by random X inactivation in the mouse are unclear. However, it demonstrates the epigenetic plasticity of facultative heterochromatin during development and raises our awareness that the silent state of the X is not infallible — an issue that needs to be more closely examined in pathological situations such as cancer, where epigenetic marks can be found to be relaxed and/or inappropriate.

Conclusions

The numerous findings over the past two years have ensured that X inactivation continues to live up to its reputation as a paradigm of epigenetics, helping us understand how gene silencing can be established and then heritably maintained. The grey area in our knowledge of what lies between the fully active and stably silent states is starting to be filled by important and evolutionarily conserved repressive systems such as the Polycomb proteins and histone modifications. What has also become clear, however, is that there are many ways to silence an X chromosome, as illustrated by the very different mechanism of X inactivation and associated histone modifications observed during male meiosis [48•]. The future of X-inactivation research will undoubtedly continue to excite epigeneticists, as the answers to several important questions unravel; these questions include which factors control Xic function, the mechanism by which Xist RNA brings about silencing and the role of unexpected proteins such as BRCA1 in X inactivation.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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An important study that characterizes the functional domains of the Xist transcript. The strategy involves the use of 48 inducible Xist cDNA transgenes carrying different deletions, all targeted into the same site on the X chromosome to avoid variation due to position effects. Xist RNA chromosome coating and silencing activities are found to be distinct. The silencing domain corresponds to a conserved region at the 5′ end of the Xist transcript and consists of 7.5 repeated units, each predicted to contain two RNA stem loops. Domains involved in chromosome coating are dispersed throughout the Xist transcript, are functionally redundant and show no common sequence homology. Some of these localisation regions are also found to be important for macroH2A.

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This study demonstrates that recruitment of Eed–Enx1(Ezh2) to the Xi occurs during random X inactivation in the embryo proper. Localization of Eed–Enx1 complexes to Xi occurs very early, at the onset of Xist expression, but then disappears as differentiation and development progress. In Eed mutant mice, Enx1 can no longer be recruited to the Xi and Eed–Enx1 is found to be required to establish methylation of histone H3 at lysine 27 on Xi. This, in turn, appears to be required to stabilize the Xi chromatin structure, as histone re-acetylation and sporadic X-linked gene reactivation on the Xi is observed in some cells.

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Like [27••], this study demonstrates the transient recruitment of the Eed–Enx1(Ezh2) complex to the Xi during initiation of X inactivation in both extra-embryonic and embryonic cells, accompanied by H3-K27 methylation. Evidence is also provided that recruitment of the complex and methylation on the Xi depend on Xist RNA coating, but are independent of Xist's silencing function. Together, these studies demonstrate a role for Eed–Enx1(Ezh2)-mediated H3-K27 methylation during the early stages of both imprinted and random X inactivation and show that although H3-K27 methylation is not sufficient for silencing of the Xi, it may be involved in the early maintenance of the inactive state.

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This study shows that the paternal X chromosome is inactive in pre-implantation mouse embryos much earlier than was previously thought

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During meiotic prophase in male mammals, the X and Y chromosomes condense to form a macrochromatin body, termed the sex, or XY, body, within which X- and Y-linked genes are transcriptionally repressed. A phosphorylated form of H2AX, a histone H2A variant implicated in DNA repair, accumulates in the sex body. This paper shows that the X and Y chromosomes of histone-H2AX-deficient spermatocytes fail to condense to form a sex body, do not initiate male meiotic sex chromosome inactivation, and exhibit severe defects in meiotic pairing. H2AX is thus required for the chromatin remodelling and associated silencing in male meiosis.

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