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Lessons from comparative analysis of X-chromosome inactivation in mammals

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Abstract In most mammals, X-chromosome inactivation is used as the strategy to achieve dosage compensation between XX females and XY males. This process is developmentally regulated, resulting in the differential treatment of the two X chromosomes in the same nucleus and mitotic heritability of the silent state. A lack of dosage compensation in an XX embryo is believed to result in early lethality, at least in eutherians. Given its fundamental importance, X-chromosome inactivation would be predicted to be a highly conserved process in mammals. However, recent studies have revealed major mechanistic differences in X inactivation between eutherians and marsupials, suggesting that the evolution of the X chromosome as well as developmental differences between mammals have led to diverse evolutionary strategies for dosage compensation.

Keywords X inactivation · dosage compensation · Eutheria · marsupial · evolution

Abbreviations

ICM	Inner cell mass
MSCI	Meiotic sex chromosome inactivation
XCI	X-chromosome inactivation
Xic	X-inactivation centre
Xist	X-inactive-specific-transcript

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Introduction

The difference in X-chromosome number between heterogametic and homogametic individuals requires some form of dosage compensation to ensure equal levels of X-linked gene activity. In placental mammals (marsupials and eutherians) this is achieved through X-chromosome inactivation: the developmentally regulated transcriptional silencing of one of the two X chromosomes in females. Since its proposal by Mary Lyon (1961), X inactivation has remained an outstanding example of chromosome-wide epigenetic regulation. However, its function and the exact mechanisms are still poorly understood. Furthermore, recent studies have shown that the mechanisms underlying X inactivation, as well as the developmental dynamics of the process, vary considerably between mammals. The choice of which X chromosome is inactivated is usually random in eutherians, and the inactive state is then stably inherited, giving rise to adults that are mosaics for two cell types expressing one or the other X chromosome. However in marsupials and during early embryogenesis of some eutherians such as rodents, only the paternal X chromosome is inactivated. In mice, this imprinted

form of X inactivation is subsequently reversed in cells of the embryo-proper and random inactivation of either the paternally or maternally derived X chromosome occurs. Although these two examples of imprinted XCI appear superficially similar, in fact they seem to be controlled differently. X inactivation in eutherians is controlled by a complex locus, the X-inactivation centre (Xic), which produces the non-coding Xist RNA responsible for triggering silencing in cis. *Xist* appears to be a eutherian invention however, having evolved after the divergence between eutherians and marsupials. In rodents, random X inactivation relies on random, monoallelic *Xist* up-regulation, while imprinted X inactivation appears to rely on imprinted paternal-specific *Xist* expression. Marsupials, on the other hand, may exploit meiotic sex chromosome inactivation (MSCI), which occurs in the male germ line (Namekawa et al. 2007, see review by Turner 2007) as a means of faciliating the silencing of the paternally-inherited X chromosome following fertilization. Thus marsupials and eutherians may have evolved quite different strategies of X inactivation to achieve dosage compensation in the 130 million years since they diverged. Such differences in X-inactivation strategies would be consistent with the major differences also found between these mammals in their sex determination and genomic imprinting pathways (Graves 2006). The nature of sex chromosome dosage compensation in monotremes, which diverged from eutherians and marsupials 170 million years ago, remains even more enigmatic, and may not require X inactivation.

In this review we will focus on recent advances in our understanding of the developmental regulation of X inactivation, the role of the Xic locus and the epigenetic marks associated with the inactive X chromosome, paying particular attention to what is known in different species.

The X-inactivation centre and the *Xist* gene

Over several decades of work on X-chromosome deletions and X-autosome translocations, a locus that is essential for inducing X inactivation , the Xic, was mapped to a multi-megabase region of the X chromosome in mouse and man (see Heard and Avner 1994 for review). The discovery of the *Xist* gene within this candidate region (Fig. 1) represented a breakthrough in the X-inactivation field (Brown et al. 1991). *Xist* produces a 17 000 nucleotide, spliced, untranslated regulatory RNA which coats and silences the X chromosome from which it is produced (Brown et al. 1992; Clemson et al. 1998). In the mouse, deletion and transgenesis studies demonstrated that Xist RNA is essential for the initiation and spread of X inactivation in cis (Penny et al. 1996; Marahrens et al. 1997; Wutz and Jaenisch 2000). However, *Xist* does not recapitulate all of the functions of the Xic, notably "sensing", whereby a cell triggers XCI when more than one Xic is present; "counting", whereby only one X chromosome will stay active per diploid autosome set; and "choice", whereby one of the two X chromosomes is chosen for inactivation or to remain active. These functions are assured by other elements, lying 5′ and 3′ to *Xist* although their molecular basis remains mysterious. Nevertheless, the key outcome of sensing, counting and choice appears to be the monoallelic up-regulation of *Xist*, at least during random X inactivation in the mouse. The analysis of *Xist* regulation during random XCI using mouse embryonic stem (ES) cells as a model system has been covered in several recent reviews (Navarro and Avner 2009; Starmer and Magnuson 2009). In summary, in female ES cells one of the two alleles of *Xist* is up-regulated upon differentiation and Xist RNA coats the chromosome that will then be inactivated (Fig. 2) (Sheardown et al. 1997; Panning et al. 1997). The events leading up to the switch from low-level biallelic *Xist* expression, to high level monoallelic expression seems to involve a complex interplay of different factors and multiple elements within the Xic, including the *Xist* antisense transcript, *Tsix*, an antisense repressor of *Xist* during the onset of X inactivation (Lee and Lu 1999; Lee 2005; Luikenhuis et al. 2001; Morey et al. 2001). Up-regulation of *Xist* in the mouse appears to require the down-regulation of pluripotency factors such as Oct4, Nanog and Sox2 during differentiation (Navarro et al. 2008), together with a "sensing" step which ensures that only cells with two or more copies of the Xic trigger *Xist* up-regulation. Sensing may involve a competence factor that is expressed only when two or more copies of the Xic are present (Lee 2005), and/or transient pairing between the two Xics, via a long–range region known as the *Xpr* (Augui et al. 2007) or a combination of such mechanisms. Repression of *Xist* on the active X chromosome requires Tsix transcription — although

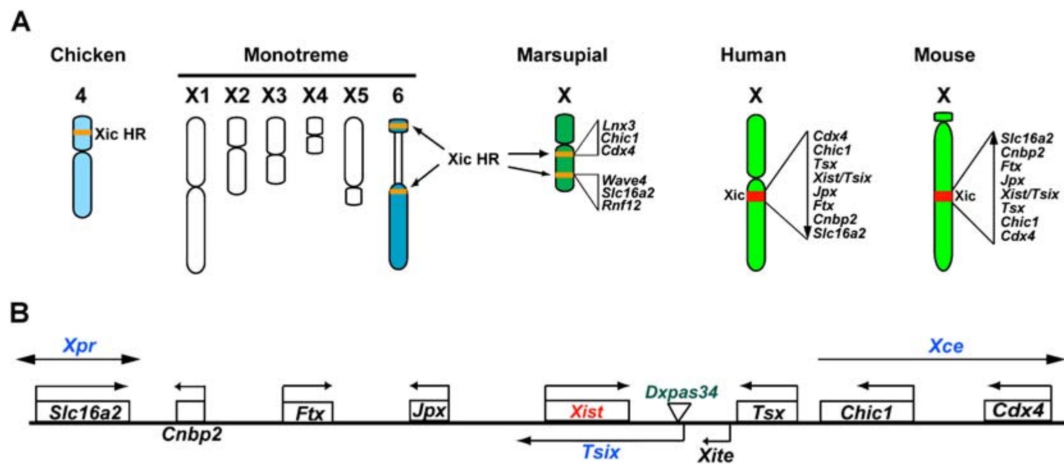

Fig. 1 **a**. Comparative maps of X-inactivation centre and its homologouse region in different mammals, based on recent studies by Duret et al, 2006; Hore et al, 2007; Davidow et al, 2007 and Shevchenko et al. 2007. Homologous regions to the eutherian X-inactivation centre are clustered in chicken, but lie in two clusters in marsupials and monotremes. The *Xist* gene is believed to have evolved from the protein coding Lnx3 gene. **b**. Genomic structure of the *Xist* gene in the mouse

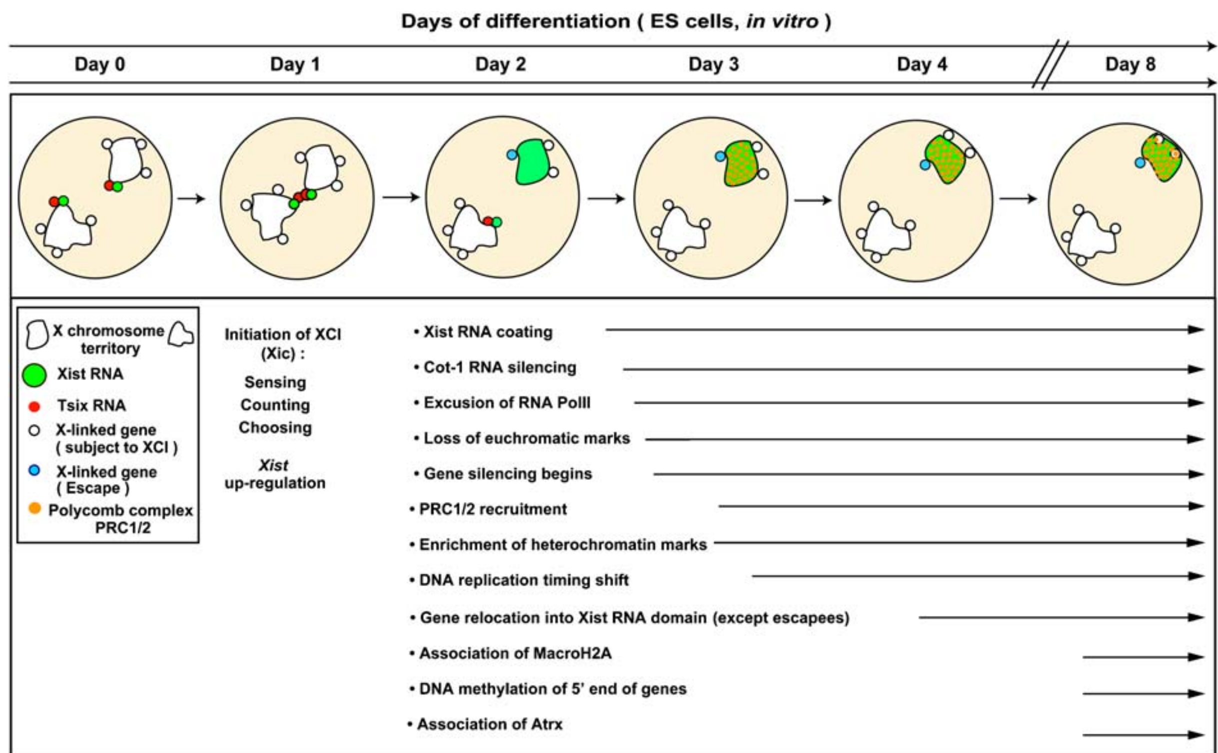

Fig. 2 Nuclear dynamics and kinetics of epigenetic changes associated with X-chromosome inactivation during ES cells differentiation in mice. Random X inactivation involves a series of steps that result first in *Xist* up-regulation and then in gene silencing and stable propagation of this silenct state through epigenetic marks. Xic trans-interactions (green) take place just prior to monoallelic up-regulation of *Xist* during X inactivation and are believed to participate in the initiation of XCI. The subsequent kinetics of chromatin changes as well as the relocation of genes into the transcriptionally inert Xist RNA compartment are shown. Only genes that escape XCI, and the *Xist* gene itself, remain outside or at the periphery of the Xist RNA compartment

how exactly this is achieved in a monoallelic fashion is rather unclear. Chromatin changes at the *Xist* gene are linked to this function of *Tsix* (Navarro et al. 2006; Sado et al. 2005; Sun et al. 2006), although these alone cannot explain the asymmetric regulation of the *Xist/Tsix* loci in a female cell. It has been proposed that the transition from biallelic to monoallelic *Xist* and *Tsix* expression may also depend on transient pairing events at the *Tsix* locus (Bacher et al. 2006; Xu et al. 2006). Pairing may enable symmetry of *Tsix* and *Xist* expression patterns to be broken (Nicodemi and Prisco 2007), such that one X down-regulates *Tsix* and up-regulates *Xist* (the future Xi), while the other maintains *Tsix* expression and prevents *Xist* up-regulation (the future active X). Recent studies have shown that Tsix pairing is dependent on the presence of two Xpr regions at the Xic (Augui et al. 2007), as well as the CTCF factor and transcription (Xu et al. 2007). The molecular basis of the asymmetry that might be generated by pairing remains unknown however.

Once the choice of which X chromosome will up-regulate *Xist* has been made, the RNA coats the chromosome in cis and induces its silencing. Accumulation of Xist RNA over the X chromosome represents the earliest manifestation of the X-inactivation process, both in embryos (Fig. 3) and in differentiating ES cells (Fig. 2). Gene silencing begins within one or two cell cycles of *Xist* up-regulation (Kay et al. 1994; Sheardown et al. 1997; Panning et al. 1997; Wutz and Jaenisch 2000). Xist RNA coating induces gene silencing only during an early developmental time window (Wutz and Jaenisch 2000). The basis for this developmental restriction was unclear until recently, when it was shown that the SATB1 and SATB2 proteins appear to be involved in capacitating Xist's function during early development (Agrelo et al. 2009). The exact mechanisms underlying Xist RNA's cis-association and its capacity to induce silencing are still being unraveled. The emerging idea is that it probably has multiple roles in the X-inactivation process (Fig. 2). First, it induces the rapid silencing of a repetitive core of the X chromosome, which manifests itself as a silent nuclear compartment (Chaumeil et al. 2006). Second, it induces the silencing of genes and their relocation into this silent compartment (Wutz et al. 2002; Chaumeil et al. 2006). Third, it induces a chromosomal memory that facilitates the action of subsequent chromatin-modifying proteins including Polycomb group complexes and associated histone modifications (Fig. 2) (Kohlmaier et al. 2004). Deletion analysis of the Xist RNA has revealed that a highly conserved domain (the A repeats) appears to be critical for gene silencing during X inactivation (Wutz et al. 2002), although this region is not required for creation of a silent nuclear compartment (Chaumeil et al. 2006). More recently this region has also been shown to be critical for appropriate regulation of the *Xist* gene itself as its deletion at the endogenous locus prevents *Xist* up-regulation (Hoki et al. 2009). This region also produces a 1.6 kb non-coding RNA (the RepA transcript) which has been proposed to associate with PRC2 (Zhao et al. 2008). However, RepA's association with PRC2, cannot be essential for the recruitment of Polycomb group proteins to the Xi, since this still occurs in *Xist* mutants lacking the A-repeat region (Wutz et al. 2002; Kohlmaier et al. 2004). In summary, despite its key role in the X-inactivation process, the molecular mechanism of the non-coding Xist RNA still remain obscure.

Evolutionary conservation of the Xic

Even in the mouse, where extensive studies have been performed to define and dissect Xic function, the extent and exact sequence content of the Xic still remain to be defined. Studies have centered on *Xist*, but long range regulatory sequences are clearly required for full Xic function (Heard et al. 1999; Monkhorst et al. 2008). Until these elements are identified functionally it will be difficult to assess the overall conservation of the Xic. At the level of *Xist*, given its essential functions in X inactivation, it might be expected that this gene would be highly conserved across mammalian species. However, its sequence is quite poorly conserved between eutherians, apart from its exon-intron structure and a series of well-conserved repeat motifs (including the A-repeats) within the gene (Hendrich et al. 1993). This relative lack of sequence conservation made it difficult to deduce whether *Xist* was actually present in other mammalian species such as marsupials. Recent genomic sequencing efforts enabled the region of the X chromosome expected to contain the Xic to be sequenced in marsupial species, including Monodel-

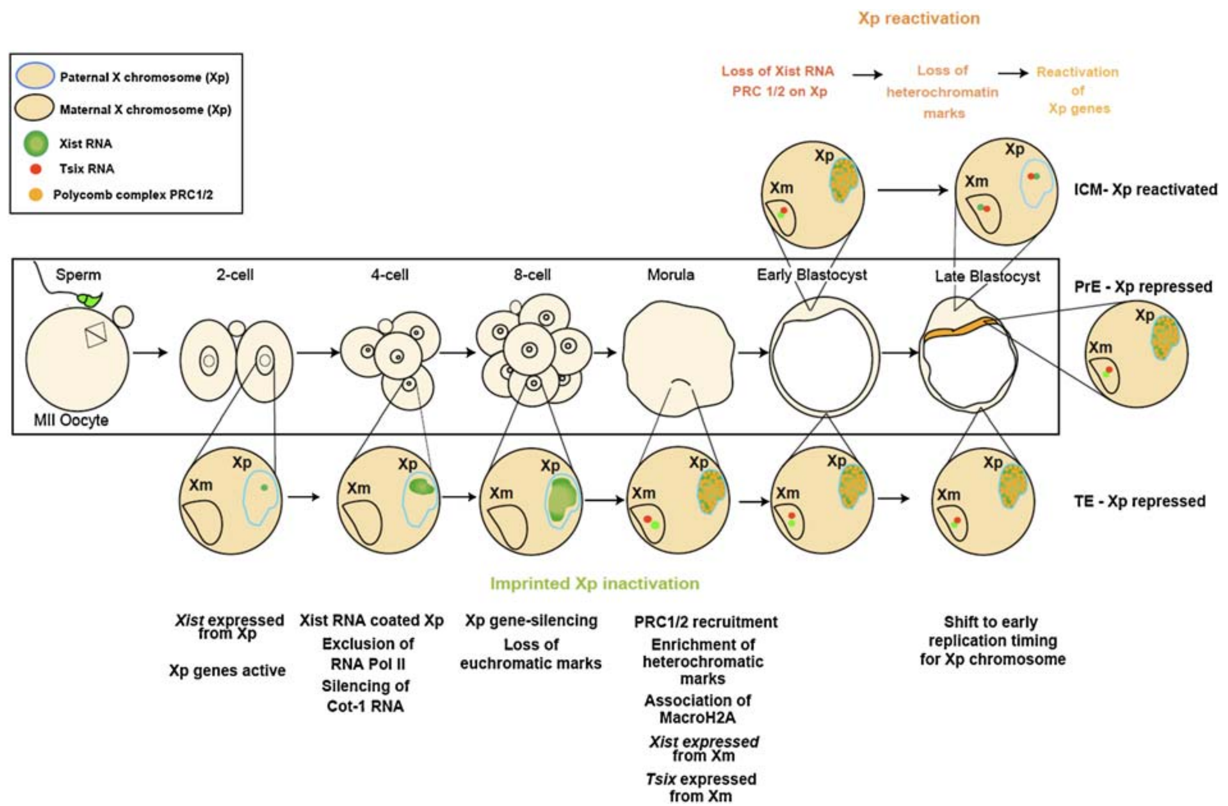

Fig. 3 Kinetics of epigenetic changes associated with imprinted paternal X inactivation and reactivation during pre-implantation development in mice. The kinetics of paternal X inactivation during pre-implantation development are shown based on studies by Huynh and Lee (2003); Mak et al (2004) and Okamoto et al (2004, 2005). All of the changes shown affect the Xp in all blastomeres of pre-implantation embryos except for early replication timing which is found only in the trophectoderm (TE) at the blastocyst stage. In the inner cell mass of the blastocyst, the Xp becomes reactivated (Mak et al, 2004; Okamoto et al, 2004). The loss of Xist RNA coating and Polycomb complex proteins seem to occur concomitantly, followed shortly afterwards by loss of histone modifications such as H3K27me3. The exact timing of gene reactivation is not known but by the late blastocyst stage the Xp is thought to be genetically active and equivalent to the Xm, prior to the onset of random X inactivation in the epiblast

phis domestica and Didelphis virginiana. The syntenic Xic candidate interval expected to contain *Xist* was found to be retained in chicken and in Xenopus (Duret et al. 2006). However, this region was disrupted independently in marsupials and monotreme lineages (Fig. 1) (Hore et al. 2007; Davidow et al. 2007). A detailed sequence comparison of the Xic homologous region in the opossum revealed an evolutionary precursor of *Xist* in the form of *Lnx3*, that is linked to *Chic1* and conserved in all vertebrate classes (Fig. 1) (Duret et al. 2006). Unlike *Xist*, *Lnx3* expression is not sex-specific and it appears to be a bona fide protein-coding gene. *Xist* is thus believed to have arisen by peudogenisation of *Lnx3*. Recent bioinformatics studies suggest that *Xist* exons may have originated from mobile elements which gave rise to simple tandem repeats (Elisaphenko et al. 2008). This event must have occurred after the divergence between eutherians and marsupials, as *Xist* is found in all eutherians, suggesting that it was probably present in the eutherian ancestor (Fig. 4). Thus, although dosage compensation in both marsupials and eutherians involves chromosome-wide silencing, the two lineages may exploit very different strategies for triggering the onset of this process (Fig. 4). Marsupials must exploit a *Xist*-independent pathway involving either another non-coding RNA or an alternative mechanism that relies on germ-line imprinting as it is always the paternal X that is inactivated in these mammals (see below). In monotremes, which have a remarkable set of five X and five Y chromosomes (Fig. 1) (Grutzner et al. 2004), the Xic homologous

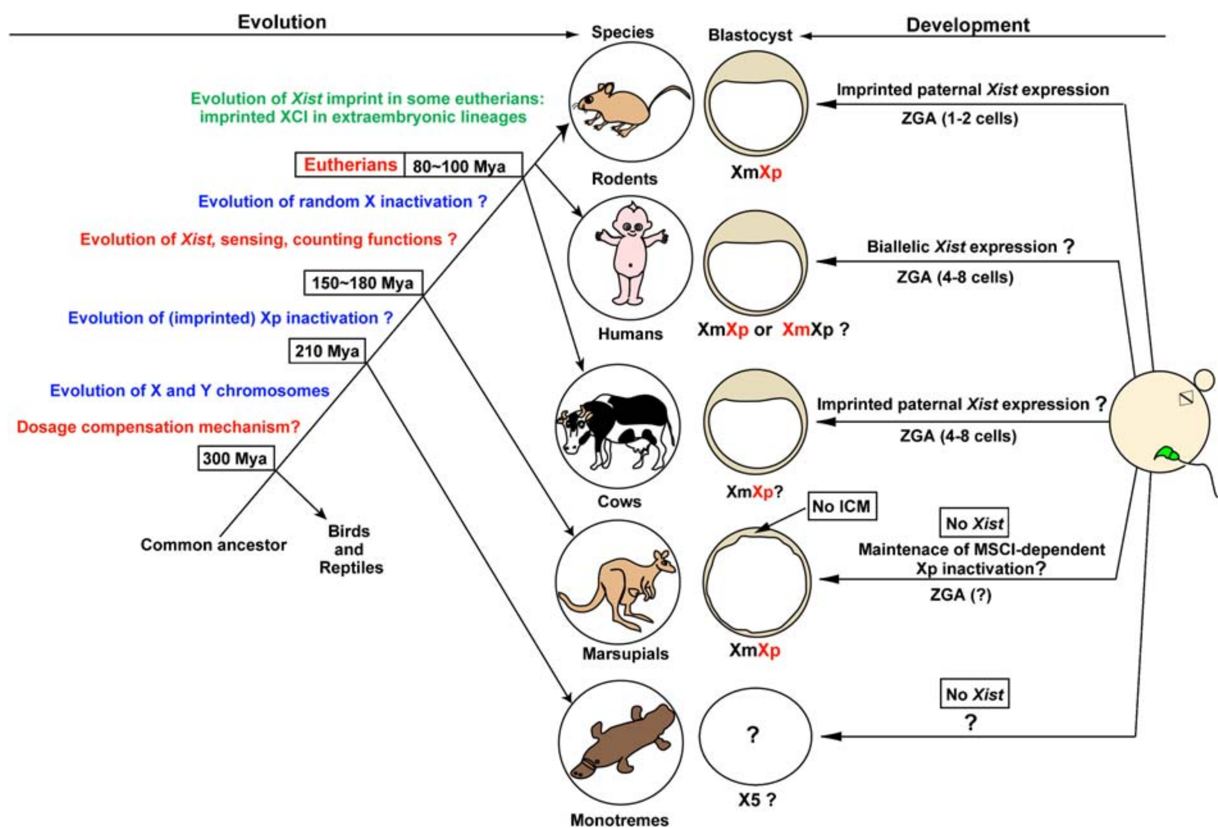

Fig. 4 Developmental and evolutional timing of X inactivation in mammals. In monotremes it is unclear whether X inactivation is used as a means of dosage compensation. In marsupials, imprinted inactivation of the Xp occurs only; the exact timing of ZGA is unclear and the activity status of the Xp during early embryogenesis is also obscure. The favored hypothesis is that there is a carry over of meiotic silencing that persists following fertilisation and could either be maintained throughout development and in the soma, or else trigger other epigenetic marks. Marsupials develop as unilaminar blastocysts, and do not have an inner cell mass as found in eutherians. This configuration of the epiblast may mean that there is no requirement for reprogramming and that the Xp thus remains silent throughout blastocyst stage, unlike in eutherians. The situation is believed to be the ancestal form of X inactivation. During human development, the onset of *XIST* expression is believed to occur around the time of ZGA, at the 4–8 cell stage. *XIST* expression is predicted to be biallelic initially and subsequently to become randomly monoallelic. The activity status of the two X chromosomes has not been examined during early embryogenesis, although it appears that X inactivation is random in both extraembryonic and embryonic tissues. In the mouse, the onset of *Xist* expression is exclusively of paternal origin. The two X chromosomes are genetically activte at the time of ZGA and paternal X inactivation is triggered by Xist RNA coating from the 4–8 cell stage onwards. In bovine embryos, *Xist* expression may initiate after the time of ZGA, at the 4–8 cell stage. The activity status of the two X chromosomes has not been examined during early embryogenesis, although it has been suggested that X inactivation may be non-random in extraembryonic tissues (placenta)

region is actually located on an autosome rather than being X-linked, indicating that dosage compensation does not require this locus.

In eutherians, it is tempting to speculate that the evolution of *Xist* was the basis for the evolution of random X inactivation. It is therefore interest to consider the conservation of some of the known regulatory elements of *Xist*, such as *Tsix*. In fact, although there are some sequence homologies to *Tsix* present in the human XIC, this region is actually rather poorly conserved (Chureau et al. 2002; Duret et al. 2006). Furthermore, antisense transcription does not cover the whole *XIST* gene and in particular it does not extend up to the *XIST* promoter (Migeon et al. 2001), unlike the situation in the mouse. This raises the interesting possibility that in humans, and presumably also in other eutherians, *XIST* is in fact regulated in a *TSIX*-independent fashion and that *Tsix* may have evolved for specific regulatory requirements of *Xist* during mouse development. Other

potential Xic regulatory sequences include the recently discovered *Xpr* locus(Augui et al. 2007). Although the exact sequence elements within this large region have still to be defined, it contains several highly conserved sequences suggesting that this locus may be important for Xic function across species. Thus, in eutherians the appearance of the *Xist* gene through the pseudogenisation of *Lnx3*, may have been the first event in the evolution of this locus as “Xic”, or master regulator of X inactivation. This may have occurred in the context of a regulatory landscape including sensing elements such as *Xpr* and other as yet undiscovered sequences. Subsequently, further control elements (such as *Tsix*) may have evolved in some eutherians to enable the fine tuning and appropriate developmental regulation of *Xist* Indeed, little is known about the developmental regulation of the initiation of XCI in mammals other than the mouse.

Thus, contrary to the idea that a single X-inactivation process evolved from a common mammalian ancestor, the emerging picture is that multiple strategies have evolved to ensure dosage compensation in different mammals and that even within eutherians, the Xic is rather an evolutionarily labile locus.

Keeping the silence: epigenetic marks underlying the inactive X chromosome

What is known about epigenetic marks on the inactive X chromosome and their actual role in maintaining the inactive state? Our knowledge in eutherians is largely based on studies in mice, where Xist RNA coating is the first event in a cascade of changes on the X chromosome during development. The detailed kinetics of these changes during ES cell differentiation and early mouse development are outlined in Figs. 2 and 3. One of the earliest events to occur following Xist RNA coating is the exclusion of transcription factors from the Xist-coated core of the X chromosome (Chaumeil et al. 2006). Subsequently, the induction of chromatin modification, such as H3K27 tri-methylation, via Ezh2 of PRC2 complex (Plath et al. 2003; Silva et al. 2003), H2A K119 ubiquitination, via Ring1a/b of the PRC1 complex (de Napoles et al. 2005) and H4K20 mono-methylation, via Prset7 (Oda et al. 2009), as well as the incorporation of the histone variant macro-H2A take place on the inactive X chromosome.

The recruitment of both PRC1 and PRC2 complexes to the Xi is at least partly dependent on *Xist* (Plath et al. 2003; Kohlmaier et al. 2004; Schoeftner et al. 2006). However, this is independent of Xist-mediated silencing. PRC1 complex recruitment may also involve binding, via its chromodomain, to the H3K27me3 mark induced by Ezh2 of PRC2 (Bernstein et al. 2006). Although Polycomb group proteins are known to be generally involved in altering chromatin structure and perpetuating repressed states during development, their exact role in XCI still remains unclear. Certain studies suggest that the PRC2 complex might be important for the establishment of a chromosomal memory induced by Xist RNA, but may not participate in the memory itself (Kohlmaier et al. 2004; Schoeftner et al. 2006). It is nevertheless clear, that a lack of PRC2 activity (and associated H3K27me3) results in some degree of reactivation of X-linked genes, particularly in extra-embryonic tissues (Wang et al. 2001; Silva et al. 2003) as they differentiate (Kalantry et al. 2006). In the embryonic lineage, the inactive state seems to be less perturbed by a lack of PRC1 and PRC2 (Eed) (Wang et al. 2001; Schoeftner et al. 2006). A logical explanation for this could be that the lack of promoter DNA methylation in extraembryonic lineages renders them more susceptible to perturbations in Polycomb-related epigenetic marks.

In the embryonic lineage of eutherians, DNA methylation eventually becomes recruited to the promoters of genes on the inactive X chromosome. As this is a relatively late event during the onset of X inactivation, it does not seem to be an immediate consequence of gene silencing. DNA methylation is clearly important for the stable maintenance of gene silencing on the Xi in embryonic tissues, as Dnmt1-/- mutant embryos display increased reactivation rates of X-linked genes (Sado et al. 2000). Recently, some new proteins that appear to be involved in maintenance of XCI have been identified. One of these, appears to act upstream of DNA methylation as SmcHD1 mutants show a loss of DNA methylation at the promoters of some X-linked genes. The relatively late lethality of *Scmchd1* female embryos suggests a defect in maintenance rather than initiation of XCI. Intriguingly, SmcHD1 contains a structural maintenance of chromosome hinge domain (Blewitt et al. 2008), suggesting parallels with cohesin-like proteins in X chromosome dosage compensation in C. elegans (Chuang et al. 1994). Another new factor

possibly involved in propagating or maintaining the inactive state is the alpha thalassemia/mental retardation X-linked (ATRX) protein, which is a chromatin remodeling protein belonging to the SWI/SNF2 ATP-dependent helicase family (Baumann and De La Fuente 2009). ATRX binds to the Xi during late differentiation and it appears to affect maintenance of imprinted XCI although its exact role in XCI remains to be found.

In addition to epigenetic marks at the chromatin level, spatial segregation (nuclear compartmentalization) of the inactive X chromosome, as well as its temporal segregation (asynchronous replication timing), may be two rather universal features of the inactive X chromosome and both may be involved in the stable maintenance of the inactive state and its cellular memory. Late (or asynchronous) replication timing correlates well with the presence of an inactive X in both eutherians and marsupials (Sharman 1971). It has recently been demonstrated that the inactive X (Xi) is targeted transiently to the nucleolus in an *Xist*-dependent manner during mid-to-late S phase (Zhang et al. 2007). Thus spatial and temporal segeregation of the inactive X chromosome, together with various types of chromatin modifications could provide multiple alternative layers of stability, depending on species, lineage or developmental contexts.

In marsupials, little is known about the epigenetic marks that are associated with the inactive X chromosome other than that are particularly late replication timing, global histone hypoacetylation and loss of active histone modification marks (H3K9 acetylation and H3K4 dimethylation) (Cooper et al. 1993; Wakefield et al. 1997; Koina et al. 2009) as well as X-linked promoters appear to be unmethylated in marsupials. This may be one reason why much higher frequencies of sporadic reactivation in these mammals compared to eutherians (for review, see Heard et al. 1997). The lack of Xist RNA and the chromosomal modifications that it can induce might also be a cause of this lability. However, clearly there must be some mechanisms- whether via an imprint or another non-coding RNA — that allow for overall heritable gene silencing in marsupials.

X inactivation during early embryogenesis

An important issue concerns potential differences between mammals in the exact timing and mechanism of X-inactivation during early embryogenesis. Recent studies have revealed that X-linked gene silencing happens in a far more heterogeneous manner than originally thought, with some genes being silenced rapidly and others being silenced much more slowly during pre-implantation mouse development (Patrat et al. 2009) and during ES cell differentiation (Lin et al. 2007). This suggests regional differences across the X chromosome in the onset of XCI. Several genes also show a certain degree of escape from XCI. This was believed to be an intrinsic property of some X-linked loci although it seems to be lineage dependent in some cases.

Although random X inactivation is the norm in the embryonic lineages of eutherians, X inactivation is imprinted in pre-implantation embryos and extraembryonic tissues of mice (Takagi & Sasaki 1975; Okamoto et al. 2005; Patrat et al. 2009,) (Fig. 4). However, the paternal X chromosome does not undergo imprinted inactivation in human embryos and extra embryonic tissues (Daniels et al. 1997; Ray et al. 1997; Zeng and Yankowitz 2003). In bovine embryos, some studies suggest that *Xist* expression is imprinted and that Xp inactivation may be found in placenta (Fig. 4) (Xue et al. 2002), although definitive studies have not been performed. In marsupials, where XCI is always imprinted and where there is no *Xist* gene, it has been proposed that this may be a carry-over effect on the Xp chromosome of meitotic sex chromosome inactivation (MSCI) (Namekawa et al. 2007). Indeed a popular idea has been that meiotically induced inactivity of the Xp could somehow remain associated with the Xp after fertilization and that this might have been selected for in ancestral mammals thanks to the dosage compensation it could confer in an XX embryo (Monk and MacLaren 1981; Huynh and Lee 2003). However, there is no evidence for this to date in marsupials. Indeed, the degree to which chromatin marks on the paternal genome can be transmitted to the fertilized egg without being lost during the remodeling step at the end of spermatogenesis, whereby protamine replace histones (Fig. 4) remains obscure. In rodents it seems that imprinted XCI is *Xist*-dependent (Marahrens et al. 1997) and is largely regulated via *Xist* imprinting (Tada et al. 2000; Okamoto et al. 2005). The situation in other eutherians remains to be discovered.

In mice, following the initiation of imprinted XCI in early cleavage stages, the Xp is maintained in the

inactive state in the trophectoderm of blastocysts but is reactivated in the inner cell mass (ICM) (Mak et al. 2004; Okamoto et al. 2004). The mechanism of reactivation is not clear but in Nanog-expressing cells, *Xist* appears to be down-regulated, chromatin marks such as H3K27me3 are reversed and X- linked genes are reactivated in mice (Fig. 3). Whether such reactivation of the inactive X occurs in the ICM of other eutherian mammals remains to be found. Indeed, it is not clear at what stage X inactivation initiates during early development in other mammals. In marsupials, the Xp is also believed to be silenced by the blastocyst stage (Johnston and Robinson 1987). However there is no ICM in marsupial blastocysts suggesting that there may not be a reactivation step in these mammals and that this could account for the maintenance of imprinted inactive Xp in all tissues of marsupials.

In conclusion, given the diversity of situations for imprinted X inactivation found between marsupials, mice, cows and humans, it is now evident that it will be critical to examine the Xp activity status during early embryonic develeopment; In marsupials this will require an analysis of X-linked gene activity and X-chromosome chromatin changes during early embryogenesis and particularly at the time of zygotic genome activation, in order to assess whether MSCI may underlie XCI in these mammals. Studies of *Xist* expression patterns and X-linked gene expression in other eutherians should also help us to understand whether *Xist*-dependent X inactivation initially evolved in eutherians in a random or imprinted form and also how imprinted X inactivation evolved in mammals.

Conclusions

In this review we have discussed early developmental aspects of X-chromosome inactivation, putting particular emphasis on the evolutionary diversity that recent findings have uncovered in this process. The discovery that in marsupials, X inactivation must be initiated in an Xist-independent manner is a clear illustration that X inactivation can be triggered in very different ways in different mammals. It has been suggested that in marsupials there is a link between meiotic sex chromosome inactivation and early female X inactivation, although this still needs to be proven. In eutherians, both imprinted and random X inactivation are clearly *Xist*-dependent processes, and the underlying imprint is at the level of the Xist gene. The Xic, in addition to containing *Xist*, is a complex regulatory locus, ensuring several functions of random X inactivation, including sensing, counting and choice, as well as *Xist*-dependent cis-inactivation. There are even further species-specific differences in the content and regulation of the Xic. The emerging picture is that the evolutionary origins of X inactivation are multiple, which is perhaps not surprising given the complexity of the selective pressures that are likely to act on X inactivation during early development. Clearly studies investigating the early events and players in X inactivation in species other than mouse will be important to address this issue in the future.

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