REVIEW

# Dosage compensation in mammals: fine-tuning the expression of the X chromosome

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**Mammalian females have two X chromosomes and males have only one. This has led to the evolution of special mechanisms of dosage compensation. The inactivation of one X chromosome in females equalizes gene expression between the sexes. This process of X-chromosome inactivation (XCI) is a remarkable example of long-range, monoallelic gene silencing and facultative heterochromatin formation, and the questions surrounding it have fascinated biologists for decades. How does the inactivation of more than a thousand genes on one X chromosome take place while the other X chromosome, present in the same nucleus, remains genetically active? What are the underlying mechanisms that trigger the initial differential treatment of the two X chromosomes? How is this differential treatment maintained once it has been established, and how are some genes able to escape the process? Does the mechanism of X inactivation vary between species and even between lineages? In this review, X inactivation is considered in evolutionary terms, and we discuss recent insights into the epigenetic changes and developmental timing of this process. We also review the discovery and possible implications of a second form of dosage compensation in mammals that deals with the unique, potentially haploinsufficient, status of the X chromosome with respect to autosomal gene expression.**

In mammals, dosage compensation for X-linked gene products between XX and XY individuals is achieved by silencing one of the two X chromosomes in female cells (Lyon 1961). A second form of dosage compensation maintains a balanced expression between X-linked and autosomal genes by doubling the transcriptional output of the active X. These distinctive regulatory processes derive from the unique evolution of the sex chromosomes. In eutherians, X-chromosome inactivation (XCI) affects the paternal or maternal X chromosome randomly during early development, 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. The initiation of X inactivation is controlled by the X-inactivation center (Xic), which produces the noncoding *Xist* transcript responsible for triggering silencing in *cis*. In marsupials and in the extraembryonic tissues of some placental mammals such as rodents, XCI is imprinted, with the paternal X chromosome (Xp) being inactivated. Imprinted XCI has been proposed to represent the ancestral form of X inactivation. Furthermore, it has been proposed that imprinted XCI may have arisen as a carryover effect from meiotic sex chromosome inactivation (MSCI) in the male germline, a process found in several species that results in silencing of the X and Y chromosomes. We discuss data suggesting that in mice, MSCI may not be essential for imprinted Xp inactivation, raising the possibility that imprinted XCI may have arisen more than once during evolution. We also discuss new insights into the phenomenon of escape from XCI. Although XCI affects most of the X chromosome, several X-linked genes are known to escape. Such escapees can provide important epigenomic insights into how a gene embedded in heterochromatin can overcome, or avoid, its repressive neigborhood. Escapees also have important implications for potential differences between the sexes. Finally, we extend the concept of dosage compensation from that concerned with X inactivation, which ensures that sex chromosome expression levels are similar between XX and XY individuals, to that concerning the dosage of gene products from the single, active X chromosome compared with autosomes, present as two copies. New evidence showing that the X chromosome is globally up-regulated compared with autosomes is discussed.

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## Genetic content and evolution of the mammalian X chromosome

The content, regulation, and evolution of the mammalian X chromosome are intimately related to the evolu-

tion of the Y chromosome, its partner. The sex chromosomes differ significantly in their gene content: The human X contains ~1100 genes, whereas the Y contains ~100 genes. This striking divergence results from evolutionary forces that progressively altered an ancestral homologous pair of autosomes or proto-sex chromosomes (Fig. 1A; Ohno 1967). The Y chromosome accumulated male-advantageous genes around the testis-determinant gene *SRY* and lost many genes by suppression of recombination between the X and the Y in the heterogametic male sex to avoid the production of abnormal sexual phenotypes (Charlesworth 1996; Rice 1996; Charlesworth et al. 2005).

Comparisons between the sex chromosomes of eutherian mammals and those of marsupials (metatherians) and monotremes (prototherians) have illuminated surprising evolutionary pathways. Gene mapping has led to the hypothesis that the marsupial X is the "ancestral" mammalian X chromosome, while the eutherian X results from successive translocations of autosomal material onto the pseudoautosomal region (Graves 1991). More recent studies in chicken and fish reveal a more intricate pattern, since some genes from the human X long arm ("ancestral") and short arm are linked on fish chromosomes (Grutzner et al. 2002; Kohn et al. 2004). Amazingly, the sex chromosomes of a more distant mammal, the platypus (a monotreme), include five X and five Y chromosomes, probably generated by repeated translocations between sex chromosomes and autosomes (Grutzner et al. 2004). The sex chromosomes of eutherian mammals apparently diverged by a stepwise mechanism that progressively suppressed recombination by means of large Y inversions (Lahn and Page 1999). The human X contains at least five evolutionary strata, based on sequence comparisons between residual X/Y gene pairs, the more recent strata representing material added to the pseudoautosomal region (PAR) of the "ancestral" X (Fig. 1B; Lahn and Page 1999; Ross et al. 2005). Attrition of the Y chromosome by suppression of recombination has driven the progressive incorporation of X-linked

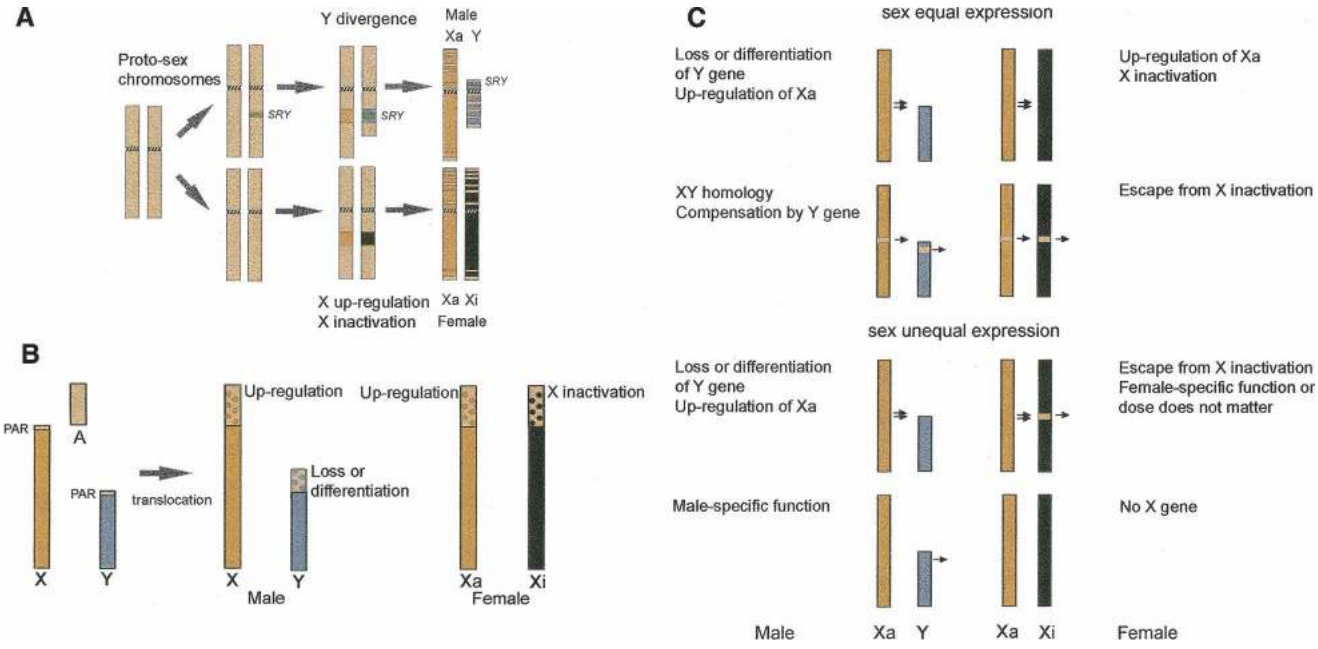


**Figure 1.** Evolutionary pathways of the sex chromosomes. (*A*) The sex chromosomes derived from a homomorphic pair of chromosomes (proto-sex chromosomes). Once sex was determined by SRY (testis determinant gene) on the Y chromosome, recombination was suppressed between the sex chromosomes. In males, the Y diverged from the X by gene loss and accumulation of male-advantageous genes (blue) around SRY (green), and the X became up-regulated (dark orange). In females, the active X (Xa) became up-regulated (dark orange), and the inactive X (Xi) became subject to X inactivation (black). Some regions of present-day sex chromosomes remain homologous between the X and Y (light orange). (*B*) Translocation of autosomal material to the sex chromosomes. Translocation of autosomal material (A) to the pseudoautosomal region of the sex chromosomes (PAR) was followed by loss or differentiation (blue dots) of the added region on the Y, and progressive up-regulation (dark-orange dots) and inactivation (black dots) of the added region on the X. (*C*) Dosage of sex-linked genes. (*Top* row) Dosage compensation between the autosomes and the sex chromosomes and between males and females is achieved for most genes that have lost their Y paralog by a combination of up-regulation of the active X in males and females (Xa, dark orange) and X inactivation in females (Xi, black). When the X/Y gene pair persists, equal dosage results from expression from the Y-linked gene and escape from X inactivation of the X-linked gene on the Xi (light orange). (Second row) The allele on the Xa may (not shown) or may not be up-regulated already (light orange). (Third row) Unequal expression between the sexes will arise when the Y paralog is lost or differentiated in a male-specific gene but the X-linked gene still escapes X inactivation on the Xi. Note that expression from the up-regulated gene on the Xa may be higher than that on the Xi. For such genes, higher expression in females may play a role in ovarian or other female-specific functions or may not cause any phenotypic differences. (*Bottom* row) In rare cases of autosome-to-Y translocations, the male would have selective expression of the newly acquired Y-linked gene.

genes in the X up-regulation/X-inactivation systems (Fig. 1B; Charlesworth 1996). Because of these unique regulatory mechanisms, the gene content of the eutherian X chromosome is highly conserved between species, except for the rare X;autosome translocation (Ohno 1967; Rugarli et al. 1995).

The X chromosome is enriched in genes related to sexual reproduction and brain function, as well as cancer-testis antigen genes (e.g., melanoma-associated antigen genes, MAGE), which encode proteins that are immunogenic in cancer patients, making them potentially useful for immunotherapy (Zechner et al. 2001; Khil et al. 2004; Vallender and Lahn 2004; Ross et al. 2005; Simpson et al. 2005). Genes that enhance male sexual reproduction are thought to have accumulated on the X because recessive mutations expressed in males due to hemizygosity of the X could give rise to novel functions. The X chromosome is enriched for genes expressed in spermatogonia (Wang et al. 2001), but not for genes expressed in later stages of spermatogenesis, likely because of silencing at meiosis (MSCI) (see below) (Khil et al. 2004). Cancer-testis antigen genes are common on the X based on the recent annotation of its entire sequence (Ross et al. 2005). These genes, which are predominantly expressed in normal tests and cancer, will probably fall in the category of genes advantageous to male sexual reproduction. An added peculiarity of the X chromosome is that it is unusually active in retrotransposition (Emerson et al. 2004). Genes that have been retrotransposed from the X to autosomes often retain their function and may serve as a protection from MSCI in males or may have been selected for their meiosis-specific function (Khil et al. 2005). Genes involved in female sexual reproduction are also enriched on the X (Khil et al. 2004); some of these genes could have a dosage-dependent function in females by escaping from X inactivation (see below).

What is the cause of enrichment for brain-expressed genes on the X? This question has led to speculation about mechanisms for selection of genes that confer enhanced cognitive functions (Zechner et al. 2001). Such genes may provide a selective advantage to males in sexual reproduction. Our own studies have shown a higher expression of X-linked genes in brain tissues compared with others (Nguyen and Disteche 2006). This has implication for human diseases, especially X-linked forms of mental retardation, which are common and for which causative mutations have started to be identified (Ropers and Hamel 2005). Imprinting of X-linked genes expressed in brain may explain differences in behavior between XO mice that inherited their single X from their mother or father (Davies et al. 2005; Raefski and O'Neill 2005). In turn, these findings could potentially explain differences in mental function between patients with a single maternal or paternal X and Turner syndrome (Skuse et al. 1997).

In summary, the gene content of the X chromosome reflects its role in sexual reproduction due to divergence between the X and the Y. These evolutionary processes not only influenced which functions would be specifically performed by the X, but also resulted in unique regulatory mechanisms, including X inactivation and X up-regulation, to overcome the presence of a single X in males.

## Initiation of X inactivation: counting, choice, and *cis* inactivation

We next discuss mechanistic aspects of X inactivation. The initial differential treatment of the two X chromosomes during early mammalian development is controlled by the Xic, which produces the noncoding *Xist* transcript responsible for triggering silencing in *cis*. Classic cytogenetic studies of deleted or rearranged X chromosomes defined this ~1-Mb region of the X chromosome to be critical for X inactivation (for review, see Avner and Heard 2001). Only chromosomes carrying the Xic are able to induce XCI, although the Xic sequence requirements for the imprinted and random forms of XCI may differ (Okamoto et al. 2005). Mechanistic insights into the role of the Xic have come from studies of early mouse embryos and embryonic stem (ES) cells, the latter representing a useful tissue culture system, in which differentiation is accompanied by random XCI. Studies in ES cells have shown that, for X inactivation to occur, cells must have at least two Xics (Rastan 1983; Rastan and Robertson 1985). Furthermore, this locus is at the heart of the process that senses, or "counts," the number of X chromosomes, and ensures that only a single X will remain active per diploid autosome set, all extra copies being inactivated. Autosomal ploidy is thought to be important for counting (Jacobs and Migeon 1989), and the most popular model invokes the existence of an autosomal factor produced in limiting quantity, that is sufficient to block one Xic per diploid cell (for review, see Alexander and Panning 2005). The Xic is also involved in the choice of which X chromosome will remain active/be inactivated. The signal produced by the Xic, that triggers *cis* inactivation of the X chromosome, or even of an autosome, in X–autosome translocations, appears to be the noncoding *Xist* transcript (Brown et al. 1991). *Xist* is expressed only from the inactive X chromosome, producing a 19-kb-long, untranslated RNA that coats the X chromosome from which it is produced in *cis* (Brown et al. 1992; Clemson et al. 1998). Deletions and transgenes demonstrated that *Xist* is essential for both imprinted and random X inactivation in mice (Penny et al. 1996; Marahrens et al. 1997; Wutz and Jaenisch 2000).

However, *Xist* alone does not account for the multiple functions of the Xic. For example, *Xist* does not appear to be involved in counting, as deletion of one *Xist* allele does not prevent the cell from registering the presence of >1 Xic and triggering XCI via the wild-type *Xist* allele (Penny et al. 1996). Multiple elements 3′ to *Xist* (Fig. 2) appear to be involved in counting and choice functions. Deletions have defined a 37-kb bipartite region, the absence of which results in aberrant inactivation of the single X chromosome in differentiating XO or XY ES cells (Clerc and Avner 1998; Morey et al. 2001, 2004). Furthermore, certain sequences from within this region,

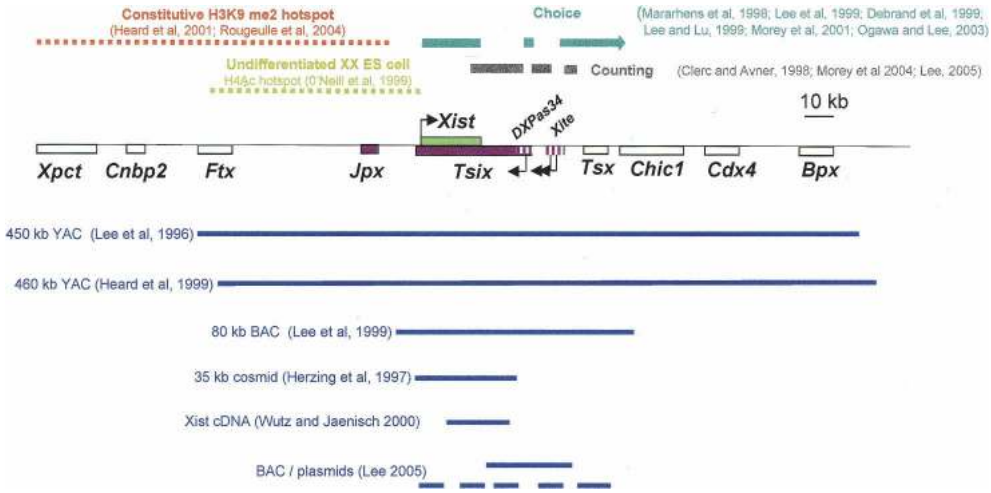


**Figure 2.** Features of the Xic. A map of the mouse Xic region is shown, with *Xist* and its antisense unit, *Tsix* (shown in color, with other genes not known so far to be involved in X inactivation shown as clear boxes). *Above* the map the elements involved in counting and choice, including *Tsix* and *Xite*, as defined by deletion, are shown. Regions of unusual chromatin enrichment are also indicated (see text for details). Transgenes used to test for Xic function are shown *below* (in blue). The full extent of the region capable of ensuring autonomous Xic function remains to be defined, as transgenes of up to 460 kb in length are unable to induce counting, choice, and *cis* inactivation when present as a single copy on an autosome (Heard et al. 1999).

when used as transgenes in XX ES cells, can interfere with the normal counting process and block XCI (Lee 2005). Monoallelic regulation of *Xist*, at least in mice, involves a complex combination of antisense transcription to *Xist* in the form of *Tsix* and *Xite* (Stavropoulos et al. 2001, 2005; Ogawa and Lee 2003) as well as *cis*-regulatory sequences located in the 3′ region of *Xist* (for review, see Clerc and Avner 2003). In undifferentiated ES cells, *Tsix* is expressed, along with low-level *Xist* transcription, from the active X chromosome(s) (Debrand et al. 1999; Lee et al. 1999). Upon differentiation of XX cells, the disappearance of *Tsix* is accompanied by the accumulation of *Xist* RNA in *cis*. The basis for this sudden reciprocal behavior of Xist and Tsix remains unknown. However, two recent studies have revealed that the Xics transiently colocalize, via the *Tsix* region, during the onset of X inactivation, at the time when counting and choice are thought to occur (Bacher et al. 2006; Xu et al. 2006). This apparent "cross-talk" between the Xics may be required for an exchange of information between *Xist*/*Tsix* homologs that ultimately leads to monoallelic down-regulation of *Tsix* and up-regulation of *Xist* on one X chromosome and not the other. The *Tsix* expression pattern, and the fact that targeted deletions/insertions that abolish *Tsix* transcription result in *Xist* RNA accumulation demonstrate that *Tsix* has a repressive effect on *Xist* during the initiation of X inactivation (Lee et al. 1999; Luikenhuis et al. 2001; Morey et al. 2001; Sado et al. 2001). However, it is still unclear whether it is the *Tsix* transcript, or the act of transcription, or both, that are involved (Nesterova et al. 2003; Shibata and Lee 2004). Recent studies point to *Tsix* transcription having a role in modifying the chromatin of the *Xist*/*Tsix* locus in ES cells, by participating in the formation of a domain highly enriched in H3K4 methylation, a histone modification associated with open chromatin (Navarro et al. 2005). *Tsix* has also been shown to participate in generating the silent chromatin status at the *Xist* promoter on the X chromosome that remains active once XCI has occurred (Sado et al. 2005). In a more recent study, *Tsix* has been proposed to prevent the recruitment of H3K27 methylation at the *Xist* locus, as its absence (in a *Tsix* mutant) results in the appearance of this mark across the *Xist* locus just prior to *Xist* up-regulation (Sun et al. 2006). Other loci that affect choice include the X-chromosome controlling element (Xce), which leads to skewed patterns of XCI (for review, see Avner et al. 1998). This has been genetically mapped 3′ to *Xist*, although its exact location and molecular nature remain to be found (Simmler et al. 1993; Chadwick et al. 2006).

The region 5′ to *Xist* consists of an unusual stretch of chromatin (or "hotspot") spanning 250 kb upstream of *Xist* that is highly enriched in repressive histone marks, namely, H3K9 dimethylation and H3K27 trimethylation, detectable by immunofluorescence and chromatin immunoprecipitation (Heard et al. 2001; Rougeulle et al. 2004). This hotspot is constitutively present at the Xic in undifferentiated ES cells and throughout early differentiation on both X chromosomes. Intriguingly, part of this region is also enriched in a mark usually associated with active chromatin, H4 acetylation, in XX and not in XY ES cells (O'Neill et al. 1999). Several noncoding RNAs (*Ftx*, "*region B*," and *Jpx*) also lie in this region 5′ to *Xist* (Fig. 2; Chureau et al. 2002; Nesterova et al. 2003). It is unclear to date whether these noncoding RNAs and the unusual stretch of chromatin in which they lie have any role in X inactivation. However, transgenesis studies suggest that the large H3K9me2/H27me3 hotspot 5′ to *Xist* may be critical for correct Xic function, as large *Xist*-containing transgenes that contain all of the critical

elements 3′ to *Xist* but only about half of this H3K9me2/H27me3 hotspot region cannot function autonomously as ectopic Xics to trigger random X inactivation when present as single copies (Heard et al. 1999, 2001). On the other hand, when present in multiple tandem copies, these transgenes do form a H3K9me2/H27me3 hotspot (Heard et al. 2001), and can function as ectopic Xics (Lee et al. 1996; Herzing et al. 1997; Heard et al. 2001), implying that two or more of these transgenes can reconstitute a functional Xic in some way. One possibility is that the H3K9me2/H27me3 region acts as a nucleation center that participates in the spread of *Xist* RNA-mediated silencing (Heard et al. 2001). The recent finding that single-copy transgenes lacking an intact H3K9me2/H27me3 region 5′ to *Xist* do not display the transient Xic cross-talk that accompanies counting and choice during early ES cell differentiation (Bacher et al. 2006) suggests that this region may be involved in the Xic sensing process that ensures that X inactivation is triggered if more than one Xic is present in the cell.

The nature of the protein factors that mediate Xic counting and choice functions have remained elusive. A mutagenesis screen for alleles that affect randomness of XCI has uncovered several possible candidates (Percec et al. 2002). Two studies have pointed to CTCF as being important in the choice function of the Xic. One study reported that CTCF binds the region close to the 5′ end of *Tsix*, and could therefore be important in regulating monoallelic *Tsix* transcription (Chao et al. 2002). Another study has reported that CTCF binds the promoter region of the human *XIST* locus (Pugacheva et al. 2005). Thus mutations in CTCF-binding sites could well be implicated in skewing of X inactivation. Furthermore, CTCF may be involved in transient Xic cross-talk during initiation of random X inactivation, as it has been shown to be important for nuclear *trans*-interactions between other loci (Ling et al. 2006). Clearly CTCF mutants will be helpful in deciphering its exact role in the initiation of XCI.

## The epigenetics of the inactive X chromosome

Once the decision to trigger X inactivation has been taken, what is the basis of the differential transcriptional regulation of the two X chromosomes? The inactive X chromosome can be distinguished from its active counterpart in several ways, including *Xist* RNA coating, chromatin changes such as histone modifications and DNA methylation, as well as asynchronous replication timing. The kinetics of these changes during imprinted and random XCI has been studied in mouse embryos and differentiating ES cells, respectively. *Xist* RNA accumulation over the X chromosome to be inactivated is the earliest known event in XCI. Gene silencing across the chromosome rapidly ensues, within one or two cell cycles (Kay et al. 1993; Panning et al. 1997; Sheardown et al. 1997; Wutz and Jaenisch 2000; Okamoto et al. 2004). Within *Xist*, a highly conserved repetitive region (the A-repeats) has been defined as being critical for the silencing function of this RNA (Wutz et al. 2002), but so far its binding partners and its mechanism of action remain a mystery (for review, see Wutz 2002). Using inducible *Xist* cDNA transgenes, Wutz and Jaenisch (2000) have also shown that *Xist* RNA-induced silencing can only occur during early ES cell differentiation. This implies either that a developmentally regulated factor must exist to mediate *Xist*'s action, or else that chromatin is not competent for XCI at later stages of development. This study also showed that during the initial phases of ES cell differentiation, X inactivation can be reversed by switching off the *Xist* gene, but subsequently the repressed state becomes locked in and is no longer dependent on *Xist* (Fig. 3). What causes this irreversibility? The earliest chromatin changes observed are the loss of histone modifications associated with active chromatin, such as H3K9 acetylation and H3K4 methylation (Heard et al. 2001; Goto et al. 2002; Okamoto et al. 2004), although whether this is a cause or consequence of the silencing induced by *Xist* remains to be defined. Subsequently, the X chromosome becomes hypoacetylated for histone H4 (Keohane et al. 1996; Heard et al. 2001; Chaumeil et al. 2002) enriched in H3 Lys-27 trimethylation (Plath et al. 2003; Silva et al. 2003; Okamoto et al. 2004), H3 Lys-9 dimethylation (Heard et al. 2001; Okamoto et al. 2004), and H4 Lys-20 monomethylation (Kohlmaier et al. 2004), as well as H2A K119 monoubiquitylation (de Napoles et al. 2004; Fang et al. 2004; Smith et al. 2004; Hernandez-Munoz et al. 2005). The successive appearance of these histone modifications on the X following *Xist* RNA coating may underlie the progressive stability and the heritability of the inactive state (Kohlmaier et al. 2004).

What is known about the mechanisms that bring about these histone modifications on the X chromosome, or about their possible roles in X inactivation? The Polycomb group protein Ezh2 appears to be the histone methyltransferase responsible for trimethylation of H3K27 on the X (Plath et al. 2003; Silva et al. 2003). Ezh2 is a member of the PRC2 complex, which also includes the Eed and Suz12 proteins, necessary for the stability of the complex and for HMTase activity of Ezh2 (for review, see Cao and Zhang 2004). These three proteins are recruited to the inactive X chromosome early on in XCI, at the time of appearance of the H3K27me3 mark, both in differentiating ES cells and early embryos, in what appears to be an *Xist* RNA-dependent fashion (Plath et al. 2003; de Napoles et al. 2004; Kohlmaier et al. 2004; de la Cruz et al. 2005). However, an *Xist* transcript deleted for the A-repeats and thus unable to induce silencing can still recruit the PRC2 complex to the X chromosome and lead to H3K27 methylation, implying that this mark is not involved in, or at least not sufficient for, silencing (Plath et al. 2003; Kohlmaier et al. 2004). Instead, genetic evidence points to a role in maintenance of the inactive state of the X, as in *Eed* mutant embryos, reactivation of X-linked genes is observed, particularly in the trophectoderm (extraembryonic) lineage (Wang et al. 2001; Silva et al. 2003). This is consistent with the well-known function of Polycomb group proteins in the heritability of silent states in *Drosophila* (for a recent review, see Cao and Zhang 2004).

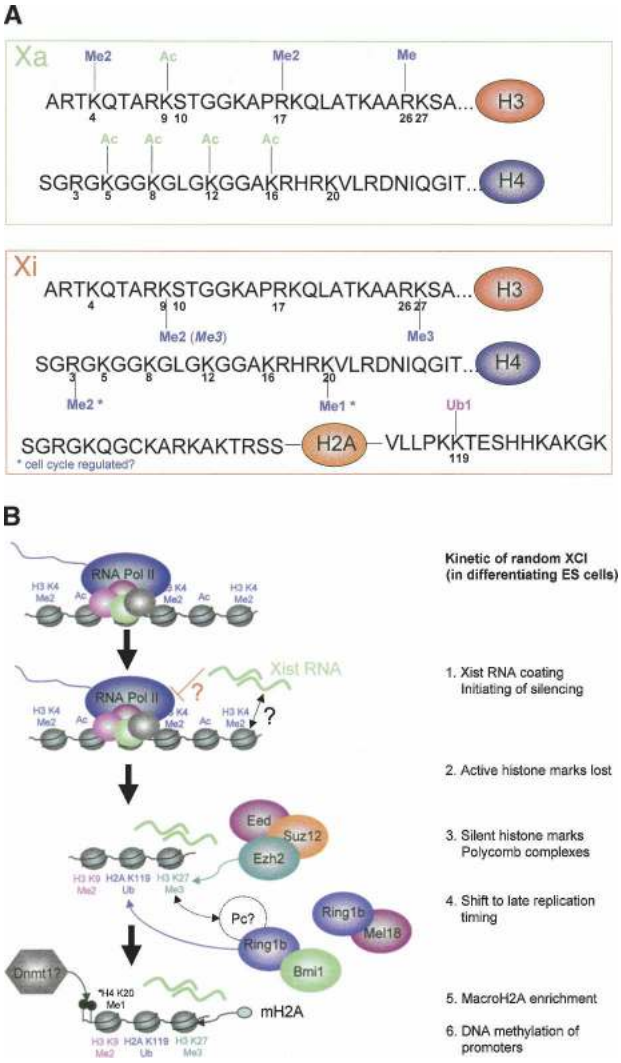


**Figure 3.** Epigenetic marks associated with X inactivation. (*A*) The current status of histone modifications known to be globally associated with the inactive X chromosome (Xi) or its active counterpart (Xa) are shown on amino acid maps of the N (and C) termini of the core histones indicated. (*B*) A schematic representation of the timing of appearance of epigenetic marks that accompany X inactivation during female ES cell differentiation is shown (see text for details). First, Xist RNA coats the chromosome from which it is transcribed in *cis* and induces silencing, through unknown mechanisms. Second, histone marks associated with transcriptional activity, such as acetylation or dimethylation of H3K4, are lost either actively or passively. Third, the PRC2 complex is recruited, and H3K27me3 appears in the Xi. A shift to late replication timing appears to follow these early events. MacroH2A becomes enriched on the inactive X from around day 4 onward. Finally, DNA methylation is recruited to the promoters of X-linked genes at later stages.

In the case of the dimethylation of H3 Lys-9 of the Xi, the HMTase responsible has not yet been identified. The knockout of one candidate, the G9a HMTase, does not appear to disrupt X inactivation, although the H3K9me2 mark on the Xi was not actually examined in these mutants (Ohhata et al. 2004). Ezh2 has been reported to have some H3K9 methylation activity; however, the appearance of H3K9me2 on the Xi shows different kinetics from H3K27me3 when examined by immunofluorescence (Okamoto et al. 2004; Rougeulle et al. 2004). Furthermore, the distributions of these two marks on the Xi, as examined by chromatin immunoprecipitation on mouse embryonic fibroblasts, are overlapping but distinct. H3K27me3 is enriched both at the promoter and within the body of genes on the Xi, but not on the Xa, whereas H3K9me2 is enriched only at promoters on the Xi, and is present within the body of X-linked genes both on the active and inactive X chromosomes (Rougeulle et al. 2004). Thus if Ezh2 is the HMTase of H3K9me2, it is likely to involve different partners for this activity compared with H3K27me3. Interestingly, it has been noted that in some human cell lines, trimethylation of H3K9, a modification associated with HP1 binding, particularly at constitutive heterochromatin, characterizes domains of the Xi that are negative for H3K27me3 and *XIST* RNA (Chadwick and Willard 2004). Thus different states of H3K9 methylation may characterize different chromatin regions on the Xi, illustrating the fact that this large block of facultative heterochromatin is by no means a uniform entity. Furthermore, the exact combination of histone modifications in any one region of the Xi may vary during development, in different lineages (see below) and cell types, and even across the cell cycle. The H4K20 monomethylation mark is one example of cell cycle regulation as it only appears to be enriched in a proportion of cells (Kohlmaier et al. 2004). The enzyme(s) responsible for this mark has not been identified, although PR-Set7 is a strong candidate given its participation in modifying this histone within facultative heterochromatin in other species (Karachentsev et al. 2005).

Recently, several groups reported that the Xi is ubiquitinated, and that this is at least partly due to the monoubiquitination of histone H2A at Lys 119. Recent work by de Napoles et al. (2004) demonstrated that this histone modification is dependent on the Ring1a and Ring1b proteins, particularly the latter, which is also a component of the polycomb PRC1 complex. Several members of the PRC1 complex have now been found associated with the Xi, including Mel18, Bmi1, and HPC2, although there are differences between reports probably due to the antibodies and cell types used (for review, see Heard 2005 and references therein). Whether or not PRC1 members or H2A K119 ubiquitination are critical for the initiation or maintenance of XCI, remains unclear. Indeed, issues of redundancy, not only between epigenetic marks on the inactive X chromosome, but also between mammalian Polycomb group proteins, render functional studies challenging. For example, several potential mouse homologs exist for each of the PRC1 components. Nevertheless, the finding that PRC1 is recruited to the Xi provides an important parallel to the situation in *Drosophila*, as it has been shown that the H3K27me3 mark deposited by the PRC2 complex could act as a binding site for the chromodomain of the Polycomb protein (Fischle et al. 2003; Min et al. 2003); in this

way, PRC2 could result in the recruitment of PRC1 as a further step in maintaining repressed states. In mammals, multiple Pc homologs exist, and the recruitment of several of them to the Xi does appear to be facilitated by the H3 K27me3 mark, although this mark alone may not be sufficient in vivo, and an RNA component may also be required (Bernstein et al. 2006).

Two other changes in chromatin constitution that occur during XCI are the association of the histone H2A variant, macroH2A (Constanzi and Pehrson 1998), and DNA methylation of promoters of X-linked genes (Fig. 3; for review, see Heard et al. 1997). Both of these marks appear to be relatively late events during XCI. The recruitment of macroH2A to the X chromosome appears, despite its late timing, to be *Xist* RNA dependent (Csankovszki et al. 1999), although no direct association has been demonstrated so far. The role of macroH2A in XCI has remained a puzzle in the absence of mutants. It has been suggested that the apparent recruitment of this histone variant may simply be a reflection of the compaction of the Xi (Perche et al. 2000), but given its role in inhibiting chromatin remodeling associated with activation (Angelov et al. 2003), it seems logical that it could participate in maintenance of the inactive state. MacroH2A is enriched on the Xi in a cell cycle-dependent fashion (S phase and G1), suggesting that its repressive role on the Xi, if any, is only exerted during a certain time window (Chadwick and Willard 2002). This may imply that it participates in ensuring silencing and/or the transmission of epigenetic information, at a time when certain histone modifications are lost.

DNA methylation, on the other hand, has been clearly shown to play an important role in stabilizing the inactive state of the Xi, at least in somatic cells (see Heard et al. 1997 and references therein). Analysis of *Dnmt1*$^{-/-}$ mutant embryos has shown that methylation is required for stable maintenance of gene silencing on the Xi in the embryonic lineage (Sado et al. 2000). In extraembryonic lineages, on the other hand, the 5′ ends of X-linked genes do not appear to be hypermethylated. Although this correlates with higher rates of sporadic reactivation of X-linked genes (Kratzer et al. 1983), the maintenance of the inactive state, at least in some extraembryonic tissues such as the visceral endoderm, seems to tolerate extensive demethylation in vivo (Sado et al. 2000). As mentioned above, Polycomb group proteins appear to have a more important role in the maintenance of X inactivation in extraembryonic lineages (Wang et al. 2001), which is also consistent with recent findings for some imprinted autosomal loci (Lewis et al. 2004; Umlauf et al. 2004). Nevertheless, it should be noted that even between extraembryonic tissues, differences can be found in the epigenetic marks on the Xi. For example, in cells derived from the primitive endoderm, despite the fact that the inactive state of the X appears to be very stable in this lineage, no, or very low levels of PRC2 and H3K27 methylation are detected on the Xi (albeit at the immunofluorescence level), unlike in the trophectoderm and embryonic ectoderm (Kunath et al. 2005). Furthermore, the role of Eed in maintaining the inactive state in trophoblastic tissues appears to be differentiation dependent (Kalantry et al. 2006).Thus, the Xi may carry different combinations of epigenetic marks in different cell lineages and differentiation stages. The situation in marsupials, where no promoter DNA methylation is observed, will clearly be of interest (Kaslow and Migeon 1987). Examination of other epigenetic marks on the Xi in marsupials, such as the Polycomb group proteins, will provide important insights into the evolution of cellular memory mechanisms in mammals.

In addition to epigenetic modifications, temporal and spatial segregation of the inactive X chromosome may be important means of ensuring the differential activity of the inactive X chromosome. The shift to asynchronous replication timing that accompanies XCI is likely to be important in maintaining the inactive state during each cell cycle, as it provides a temporal segregation of the two X chromosomes (Hansen et al. 1996). This may minimize the exposure of the Xi to transcription factors, and optimize its exposure to the appropriate chromatin-modifying enzymes, thus facilitating the maintenance of transcriptional silence. A striking, but so far unexplained observation is that during random XCI, the Xi becomes late replicating (Takagi et al. 1982), whereas during imprinted XCI it is early replicating (Sugawara et al. 1983). In both cases, the shift in replication timing appears to follow the onset of the histone modifications (Chaumeil et al. 2002; Okamoto et al. 2005), suggesting that it might be a consequence of changes in chromatin structure of the X. Intriguingly, there is also evidence that the silent *Xist* locus on the active X chromosome may control asynchronous replication timing of the X chromosome, although the mechanistic basis for this remains unclear (Diaz-Perez et al. 2005). The functional importance of asynchronous replication is underlined by the fact that it seems to be one of the best-conserved characteristics of the Xi in mammals (Sharman 1971). However, the role of replication asynchrony in XCI cannot be easily tested without the accompanying perturbation of other epigenetic marks.

The spatial segregation of the inactive X chromosome in the nucleus may also be important in the maintenance, and perhaps even in the initiation, of XCI. The idea that the inactive X chromosome could represent a repressive nuclear compartment has been proposed in the past (Clemson et al. 1996) and is supported by the recent finding that the nuclear scaffold protein SAF-A (Fackelmayer 2005) associates with the inactive X chromosome. Such a compartment may be nucleated through the action of epigenetic marks together with the *Xist* transcript at every cell cycle. In support of this, a recent study has shown that Xist RNA defines a silent nuclear compartment early on in the X-inactivation process (Chaumeil et al. 2006). Whether distinct domains of heterochromatin define its organization and position in the nucleus (Chadwick and Willard 2004) remains an important question for the future.

The epigenetic marks and segregation mechanisms described above are likely to act in synergy to maintain the inactive state and to provide the cellular memory that

enables its heritability through successive cell divisions. Disruption of a single mark, such as DNA methylation, histone hypoacetylation, or *Xist* RNA coating in somatic cells seems to barely affect the stability of the inactive state, while the combined absence of several marks results in increased rates of sporadic gene reactivation on the Xi (Csankovszki et al. 2001). Nevertheless, even under these conditions, global reactivation of the X chromosome is never seen, suggesting that we may still be missing some critical mark or else that spatial–temporal segregation of the Xi alone can ensure maintenance of its inactive state. Although reactivation of X-linked genes rarely happens in vivo, it can increase with aging and in cancer cells (Wareham et al. 1987; Spatz et al. 2004), suggesting a loosening of epigenetic marks in these situations. Potential new insights into the reasons underlying such sporadic reactivation have come from studies suggesting that perturbation of factors such as BRCA1, ATM, or ATR, which are known to be involved in DNA repair and to act as genome "caretakers," can lead to disruption of *XIST* RNA coating in the case of BRCA1 (Ganesan et al. 2002) or perturbations in gene silencing in the case of ATM and ATR (Ouyang et al. 2005). Although the molecular basis for this is unclear, the epigenetic stability of the inactive state may be linked to genomic stability in general. The mechanistic nature of this link and its possible implications for cancer represent exciting questions for the future.

## Escape from X inactivation

Although X inactivation is thought of as a chromosome-wide phenomenon, in fact, some genes can escape X inactivation; that is, they are biallelically expressed in female cells. Escapees with a Y paralog may represent evolutionary remnants from the proto-sex chromosomes (Fig. 1). In human, escape genes are numerous (15% of X-linked genes) and are concentrated in the more recent evolutionary strata, supporting the notion that acquisition of X inactivation is dependent on the loss or differentiation of Y-linked genes (Fig. 4; Jegalian and Page 1988; Lahn and Page 1999; Carrel and Willard 2005). As expected, genes located in the recombining pseudoautosomal region(s) of the sex chromosomes escape X inactivation. One fascinating exception comprises a few genes located in the PAR2 in human that are subject to silencing both on the inactive X in females and on the Y in males. When on the Y, one of these genes, *SYBL1*, bears epigenetic marks characteristic of genes silenced on the inactive X including DNA methylation, histone modifications, and chromatin condensation, suggesting a Y-specific silencing mechanism independent of *Xist* (Matarazzo et al. 2002). Since fewer remaining X/Y gene pairs with similar (usually ubiquitous) expression have been found in mouse, the scarcity of mouse escapees is seemingly easy to explain (Disteche et al. 2002). However, many human genes that have lost their Y paralog still escape X inactivation (Carrel and Willard 2005). Thus, the acquisition of X inactivation in the face of Y degeneration/differentiation has proceeded at a different

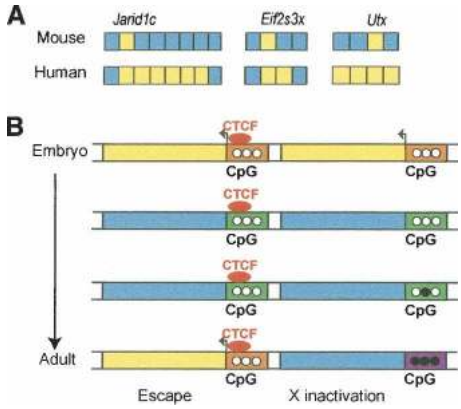


**Figure 4.** Escape from X inactivation. (*A*) Domains of escape from X inactivation are smaller in mouse than in human, as shown for three domains of escape in mouse (one escape gene, *Jarid1c*, *Eif2s3x*, *Utx*) and human (multiple escape genes). (Blue) Genes subject to X inactivation; (yellow) escape genes. (*B*) Model for the possible role of CTCF at the boundary of escape domains. CTCF (red ovals) binding to the CpG island of escape genes may prevent the establishment of DNA methylation (black circles), resulting in reactivation of the escape genes during development. Histone modifications characteristic of inactivated genes (green box) may be associated with the transient silencing of escape genes during development. At reactivation, such genes would then acquire marks characteristic of active genes (orange). (Blue) Genes subject to X inactivation; (yellow) escape or noninactivated genes. Adapted from Filippova et al. (2005) with permission from Elsevier © 2005.

rate in each species. Incomplete silencing of the human X may be due to a barrier effect caused by the centromeric heterochromatin that separates the XIC from the short arm where most escape genes are located (Disteche 1999). In some rodent species with a large block of heterochromatin on the X chromosome, *Xist* RNA does not spread in this region (Duthie et al. 1999). Nevertheless, the paradoxical persistence of escape genes, especially in human, remains largely unexplained (Disteche et al. 2002; Brown and Greally 2003).

Escape genes play an important role in ensuring a normal phenotype in humans. Indeed, Turner syndrome is largely lethal in fetuses with a single X chromosome, due to haploinsufficiency of escape genes, while XO mice are largely unaffected (Adler et al. 1991; Ashworth et al. 1991). Although no specific gene has been implicated yet, escapees may have a dosage-sensitive role in female-specific functions; for example, ovarian function, which is compromised in Turner syndrome (Fig. 1C). The level of expression of escapees is usually lower from the inactive X, resulting in modest differences in transcription levels between the sexes (Carrel and Willard 2005; Nguyen and Disteche 2006). This was first recognized for the steroid sulfatase gene, expressed on the inactive X at 30% of the active X (Migeon et al. 1982). Lower expression of the allele of an escapee on the Xi suggests partial silencing, perhaps due to the proximity of inactive chromatin, or conversely, selective up-regulation of the allele on the Xa (see below). Interestingly, the Y partner of an X/Y pair also appears to have a lower expression as com-

pared with the X partner (for review, see Disteche et al. 2002). However, X and Y partners often differ in their function despite apparent similarities, and complementation studies using knockout systems will be necessary to sort out the roles of X/Y gene pairs.

For a given gene, escape from X inactivation is not necessarily consistent between individuals or between tissues and/or cells within an individual. A comprehensive survey in human confirms the original observation that some genes only escape X inactivation in subsets of cells (Anderson and Brown 1999; Carrel and Willard 2005). Interestingly, many genes (~10% of X-linked genes) behave in this manner, resulting in potentially variable expression levels between female tissues and individuals. Whether, in turn, this generates female phenotypic variation is an interesting possibility that remains to be explored. Partial or variable escape from X inactivation is in agreement with progressive incorporation of genes into the X up-regulation/X inactivation systems once the Y paralog degenerated (Fig. 1B).

Molecular mechanisms of escape can be derived from genomic and epigenetic analyses of chromosomal domains containing escape genes. Comparisons of multiple homologous genomic domains in human and mouse indicate that diminishing escape domains typify the mouse X chromosome (Fig. 4A; Tsuchiya and Willard 2000; Tsuchiya et al. 2004; C.M. Disteche, unpubl.). Among factors potentially associated with the shrinking of escape domains on the mouse X are differences in DNA repeat expansion. Escape domains appear to be depleted in long terminal repeats (LTR) (Tsuchiya et al. 2004). Surprisingly, differences in the distribution of L1 elements, which have been proposed as way-stations for the propagation of silencing along the X (Lyon 1998), were not found (Tsuchiya et al. 2004). However, other studies report a correlation between a low density of L1 elements, especially young (recently expanded) L1 repeats in regions that escape inactivation (Bailey et al. 2000; Carrel and Willard 2005).

Genes that escape X inactivation are actively expressed within the context of silenced chromatin. In adult tissues their chromatin structure, including histone modifications and lack of DNA methylation, is characteristic of that of active genes (Goodfellow et al. 1988; Gilbert and Sharp 1999; Boggs et al. 2002; Filippova et al. 2005). Yet, in ES cells, escape genes are marked by specific histone modifications characteristic of biallelically expressed genes (Rougeulle et al. 2003). The existence of actively transcribed domains within inactive chromatin suggests that boundary elements are positioned between domains of escape and inactivation. Accordingly, binding sites for a chromatin insulator element, CTCF, have been discovered at the 5′ end of two mouse genes (*Jarid1c* and *Eif2s3x*) and one human gene (*EIF2S3X*); all three genes escape X inactivation and are each adjacent to an inactivated gene (Filippova et al. 2005). CTCF-specific binding between domains of inactivation and escape, but not in a region between two escape genes, suggests that protection from stable silencing may operate at the level of domains that contain one or several escape genes. CTCF binding to a specific DNA sequence both depends on and regulates DNA methylation (Fedoriw et al. 2004; Pant et al. 2004). In the context of X inactivation, which is associated with CpG island methylation, a role for CTCF in escape may involve interference with this methylation process. Insulation of escape genes from adjacent inactive chromatin could be mediated by interactions between CTCF and the establishment and/or cooperative spreading of DNA methylation (Fig. 4B). This is supported by findings of near-complete absence of methylation during early development at the CpG dinucleotides contained within CTCF-binding sites at the 5′ end of *Jarid1c* (Filippova et al. 2005). Interestingly, *Jarid1c* is thought to be transiently silenced during development (Lingenfelter et al. 1998). A possible explanation for such transient silencing could be that labile chromatin modifications are induced at the locus during the initiation of X inactivation, but that these do not persist and the inactive state cannot be locked in due to lack of CpG island methylation because of CTCF binding. Thus, escape genes may be susceptible to some of the molecular layers that control X inactivation, but they may be protected from certain modifications, resulting in unstable silencing. Incomplete inactivation of some X-linked genes could also be due to low affinity for *XIST* RNA and its accompanying silencing complex. X;autosome translocations show patchy spreading of silencing in the autosomal regions, apparently dependent on different affinity for *XIST* RNA (Hall et al. 2002; Sharp et al. 2002). The heterogeneous heterochromatin domains on the human X may also play a role in the distribution of escapees (Chadwick and Willard 2004). Finally, genes that escape may occupy a different nuclear compartment from that of inactivated genes. Chromatin loop structure possibly mediated by chromatin elements such as CTCF could help separate domains on the X chromosome (Fig. 4B). Additional analyses of their chromatin structure as well as their nuclear localization during development will be informative.

## Sex chromosome inactivation in spermatogenesis

X inactivation in females is not the only form of X-chromosome silencing in mammals. Silencing of the sex chromosomes during male meiosis (MSCI) has been recognized for a long time (Monesi 1965; Lifschytz and Lindsley 1972; McCarrey et al. 1992). However, recent studies have uncovered significant differences between MSCI and somatic X inactivation established in the embryo. Unlike female X inactivation, which is dependent on *Xist*, MSCI is *Xist* independent (McCarrey et al. 2002; Turner et al. 2002) and occurs through a very different process, involving ATR-mediated phosphorylation of the H2AX histone variant (Fernandez-Capetillo et al. 2003). The MSCI process may prevent deleterious, illegitimate recombination events between the unpaired regions of the sex chromosomes and other chromosomes during meiosis (Jablonka and Lamb 1988). The most interesting aspect of meiotic silencing is that this proc-

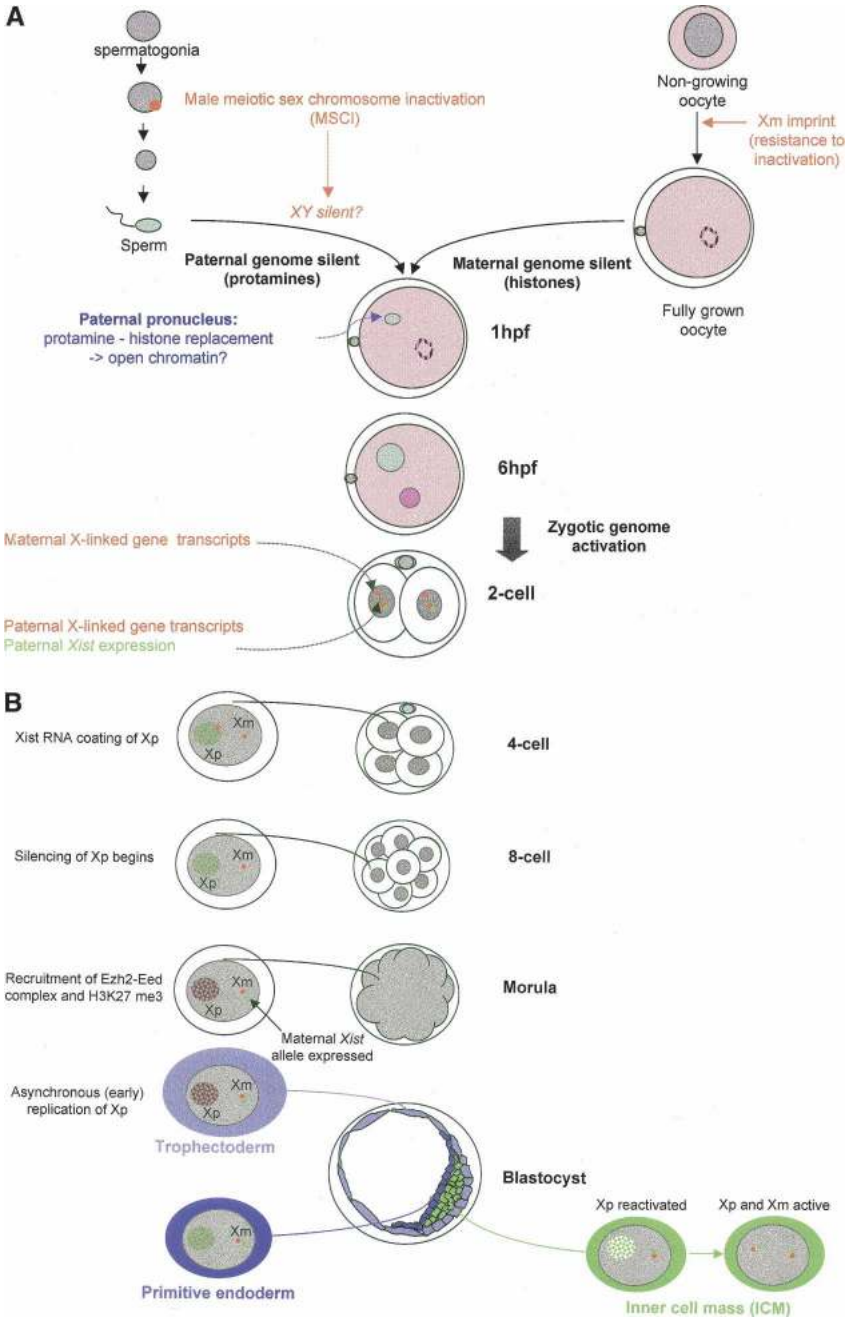


**Figure 5.** Imprinted X inactivation in mice. Maternal and paternal germline events that are possibly relevant to the imprint(s) underlying paternal X inactivation in mouse embryos are shown in the *upper* part of the figure. At 1 h post-fertilization (1 hpf), the genome of the paternal pronucleus (blue) becomes remodeled, and this may facilitate the early activation of the paternal *Xist* gene. The maternal allele of *Xist* remains silent through to the morula stage, as a result of an imprint laid down during oocyte growth. The maternal pronucleus is shown in pink. Minor zygotic gene activation occurs at the one-cell stage; major zygotic gene activation occurs at the two-cell stage. X-linked gene primary transcripts are shown as red spots. The onset of Xist RNA accumulation (green) of the Xp occurs from the four-cell stage in every blastomere. The first signs of gene silencing on the Xp can be detected from the eight-cell stage and the recruitment of polycomb group proteins, H3K27 trimethylation, and H3K9 dimethylation occur subsequently. Inactivity of the Xp, albeit leaky, is maintained in the trophectoderm and is found in the primitive endoderm, but the Xp becomes reactivated in the inner cell mass (Mak et al. 2004; Okamoto et al. 2004).

ess is not unique to the sex chromosomes but affects all unpaired regions at pachytene (Turner et al. 2005, 2006). This may act as a checkpoint mechanism against aneuploidy by arresting meiosis with faulty pairing. In a normal male, the sex chromosomes are sequestered into the sex vesicle and silenced, except for the paired pseudoautosomal regions. The unpaired regions of the sex chromosomes become associated with BRCA1, ATR, and phosphorylated histone H2AX (Baarends et al. 2005; Turner et al. 2005). The protein complex triggers a series of histone modifications and gene silencing. Unpaired autosomes are subject to the same chromatin modifications. Following meiosis I, several X-linked genes reactivate (McCarrey et al. 1992; Wang et al. 2005; Nguyen and Disteche 2006) and histone modifications associated with transient silencing of the X/Y pair at pachytene revert to at least partially active chromatin (McCarrey et al. 1992; Khalil et al. 2004). However, recent studies suggest that the X and Y chromosomes do retain some heterochromatic marks acquired during meiotic inactivation (Namekawa et al. 2006; Turner et al. 2006). Indeed, it has been proposed that the silence induced during meiotic inactivation and that is maintained, to some extent, throughout later stages of spermatogenesis, may result the in transmission of a preinactivated paternal X chromosome to the zygote and form the basis of imprinted X inactivation in some mammals (Huynh and Lee 2003). However, as will be discussed

below, recent work in mice suggests that meiotic inactivation is not actually required for imprinted Xp inactivation.

## Imprinted X inactivation and reactivation

Imprinted inactivation of the paternal X chromosome is found in all tissues of marsupials (Sharman 1971; Cooper et al. 1993), and in the extraembryonic tissues of some eutherians, such as mice (Takagi and Sasaki 1975; West et al. 1977). Classical cytogenetic studies in mouse embryos had suggested that the paternal X only became inactivated at the blastocyst stage, accompanying cellular differentiation in the trophectoderm and primitive endoderm (Takagi et al. 1982). However, recent studies have revealed that that the paternal X has already begun to inactivate by the eight-cell stage (Fig. 5; Huynh and Lee 2003; Mak et al. 2004; Okamoto et al. 2004). Following fertilization, the Xp is transcriptionally active at zygotic gene activation (two-cell stage). This was revealed by the chromosome-wide presence of RNA Polymerase II (using immunofluorescence) and by the detection of nascent transcripts of X-linked genes and of Cot-1 repeat-specific transcription, using RNA FISH (Okamoto et al. 2005). Inactivation of the Xp initiates following *Xist* RNA coating at the four-cell stage (Okamoto et al. 2004, 2005). The chromatin changes induced subsequently on the Xp are similar to those found in differentiating ES cells, with two notable differences: macroH2A is recruited early on, by the morula stage, at a similar time to Ezh2 and H3K27me3 (Constanzi et al. 2000); furthermore, DNA methylation is not found at the promoters of X-linked genes in the trophectoderm (Fig. 2). Indeed, the repressed state of the Xp is much more unstable or "leaky" during imprinted XCI compared with random XCI (Huynh and Lee 2003; Mak et al. 2004; Okamoto et al. 2004).

By the early blastocyst stage, the Xp appears to be globally inactive in all cells of normal female (XmXp) embryos. In the trophectoderm, this inactivity of the Xp is maintained, and presumably further locked in by the shift to asynchronous (early) replication timing (Sugawara et al. 1983). In the ICM of early blastocysts (3.5 days post-coitum [dpc]), the Xp is also inactive. Strikingly, however, during blastocyst growth, the Xp becomes reactivated in the ICM, with cells rapidly losing their *Xist* RNA coating, Eed/Enx1 enrichment, and the histone modifications characteristic of X inactivation (Fig. 5; Mak et al. 2004; Okamoto et al. 2004). This reactivation precedes subsequent random inactivation of either the maternal or paternal X chromosome in epiblast cells following implantation. The reactivation of the X chromosome is likely to be symptomatic of a more genome-wide erasure of epigenetic marks. These findings are relevant to nuclear transfer experiments, where correct genome-wide erasure of epigenetic marks is critical for the establishment of a normal pattern of development, and the reprogramming activity of the ICM likely participates in this. Eggan et al. (2000) have shown that in cloned mouse embryos, the inactive X chromosome derived from a somatic cell carried over its inactive state to the extraembryonic tissues, but was subject to random X inactivation in the embryo proper, presumably due to the reprogramming events in the ICM (Mak et al. 2004; Okamoto et al. 2004). More recent work on embryos following nuclear transfer, which examined the early *Xist* RNA and chromatin patterns on the X, revealed that although the Xi derived from a somatic cell loses its *Xist* RNA coating very rapidly following transfer into the ooplasm, it may nevertheless retain some marks, such as H3K27me3, which could predispose it to early inactivation (Bao et al. 2005). Strikingly, the kinetics and pattern of XCI events in embryos after nuclear transfer appear to be very different from those for imprinted inactivation of the Xp at equivalent stages (Bao et al. 2005; Nolen et al. 2005). For example, the *Xist* gene becomes biallelically repressed early on, in some blastomeres—a situation that is never found in a normal female embryo owing to the maternal imprint that prevents maternal *Xist* expression. The incomplete erasure of some epigenetic marks on the Xi and the aberrant patterns of *Xist* expression may be maintained in the trophectoderm but are presumably overridden in the embryo itself owing to the reprogramming event that occurs in the ICM.

## The imprint(s) underlying preferential paternal X inactivation

The molecular basis and evolutionary origins of imprinted X inactivation have been the subject of much debate. On the one hand, the maternal X may be imprinted to remain active; on the other hand, as discussed above, the paternal X may carry a predisposition to inactivate. Important mechanistic insights into imprinted XCI in mice have come from studies on embryos showing uniparental disomy for the X. The failure to develop extraembryonic tissues and the early death of mouse embryos carrying two Xm chromosomes (Goto and Takagi 1998, 1999) suggest that there is initially a powerful maternal mark that prevents the Xm from being inactivated during early embryogenesis (Lyon and Rastan 1984). Mice with an XpO genotype, on the other hand, are fully viable and normal (showing only a slight growth retardation early development) (Papaioannou and West 1981), which demonstrates that the paternal X is not irrevocably destined to inactivate during early development. Direct evidence for a maternal imprint that prevents the Xm from being inactivated early on in embryogenesis has come from an elegant study on embryos that were derived by combining maternal genomes from a fully grown (fg) oocyte and from an early nongrowing (ng) oocyte (Tada et al. 2000). In such ng/fg embryos, only the X chromosome derived from the ng oocyte was inactivated in extraembryonic lineages, consistent with acquisition of an Xm mark on the fg X chromosome during oocyte maturation. The nature of this maternal mark remains unclear, but is very likely to act at the level of *Xist*, as maternal *Xist* expression is completely repressed until the morula stage. The exact location of this maternal imprint is not known, but recent transgenesis studies

have limited it to a 210-kb region that includes *Xist* (Okamoto et al. 2005).

Although there is strong evidence for a maternal mark that prevents Xm inactivation in the early mouse embryo, this does not exclude the possibility that the Xp carries an imprint, or predisposition to inactivate, perhaps as a result of its silent status at meiosis, in the XY body, during spermatogenesis (see above). However, this hypothesis was recently ruled out as autosomal *Xist* transgenes were shown to induce imprinted *cis* inactivation with identical kinetics to paternal X inactivation, without any meiotic inactivation or passage through the XY body (Okamoto et al. 2005). Thus if the paternal X does bear a mark that predisposes it to inactivation, this is unlikely to be due to MSCI. Instead, early monoallelic *Xist* expression appears to be the critical determinant for imprinted Xp inactivation, at least in mice. Why is the paternal allele of *Xist* expressed so early on in mouse development? Hypomethylation of the *Xist* promoter during spermatogenesis (Norris et al. 1994) presumably facilitates this. Another predisposing event may be that just after fertilization, the paternal genome undergoes a massive chromatin remodeling phase, when the protamines with which it is packaged in the sperm are replaced by maternal histones. This dramatic remodeling may provide an opening in which the paternal genome as a whole (Aoki et al. 1997; van der Heijden et al. 2005), and perhaps the paternal *Xist* gene in particular, are transiently more highly transcribed than their maternal counterparts (Fig. 5). It will be interesting to determine what happens to the Xp chromosome in a female embryo where the maternal *Xist* allele is not imprinted to be silent. Would X inactivation during early development still initiate only on the Xp chromosome, or on both X chromosomes simultaneously, or would it affect the Xp or the Xm randomly? This latter situation would, of course, imply that counting can occur in preimplantation embryos. There is some evidence that counting can occur from the morula stage onward, based on studies using XpXp androgenotes (Okamoto et al. 2000) and nuclear transfer embryos (Bao et al. 2005). However, it is not clear that counting occurs at earlier stages, and, as discussed below, this might be one of the reasons why it is critical that one of the two *Xist* alleles is initially imprinted to be silent, at least in mice. It is interesting to note that in humans, where evidence for imprinted Xp inactivation is much less clear, the *XIST* gene does not appear to be imprinted during early development, as the maternal allele can be detected in both male and female human embryos from the five- to 10-cell stage (Ray et al. 1997). In mice, the very early timing of zygotic gene activation may have imposed a necessity to silence one of the two *Xist* alleles, in order to prevent the triggering of inactivation of both X chromosomes, prior to the onset of "counting" at the morula stage. In marsupials, on the other hand, where no *Xist* gene has been identified so far, the evolution of imprinted X inactivation could indeed be due to a carryover effect from the male germline, but this possibility needs to be addressed (see below).

## The evolutionary origins of X inactivation

The evolutionary origins of monoallelic silencing by X inactivation have been widely discussed and speculated over. Comparisons between eutherians and more distant mammals, marsupials, and monotremes provide some clues about evolutionary aspects of the silencing mechanism. *Xist* may have evolved specifically on the X of eutherian mammals, since there is no evidence of *Xist* in marsupials (Graves and Westerman 2002). Although poor conservation of the gene (evident between human and mouse *XIST/Xist* genes) may have precluded its identification in marsupials so far, the availability of a complete marsupial DNA sequence may still reveal the presence of *Xist*. Marsupial X inactivation is imprinted and specific to the paternal X chromosome (Sharman 1971; Cooper et al. 1993). The silencing does not use DNA methylation of CpG islands of genes (Kaslow and Migeon 1987), but does use histone modifications similar to those found in eutherian mammals (Wakefield et al. 1997). Thus, DNA methylation may be an added step designed to provide a locking mechanism and more stable X inactivation in eutherians. Nothing is known about platypus X inactivation, except for late replication of the portion of the X chromosome homologous to the marsupial X (Wrigley and Graves 1988). Given the presence of five X chromosomes in platypus, their inactivation pattern is anyone's guess (Grutzner et al. 2004)!

The mechanics of X inactivation could have either originally evolved on the X or derived from existing silencing processes that modify autosomes. X inactivation and autosomal imprinting both use noncoding RNA to silence one allele (O'Neill 2005). Furthermore, as described above, early X inactivation is imprinted at least in mouse, where the paternal X becomes specifically silenced at the four- to eight-cell stage. Marsupial X inactivation is similarly imprinted. Because of these similarities, it has been proposed that autosomal imprinting may be derived from imprinted X inactivation as species with a placenta evolved (Huynh and Lee 2005). Alternatively, it has been proposed that these processes may have coevolved originally (Reik and Lewis 2005), with random X inactivation only evolving later, in the eutherian lineage, probably as a more favorable process. Yet another hypothesis is that imprinted X inactivation and imprinting of autosomal gene clusters arose independently, as a result of parental-specific epigenetic marks that became imposed on a pre-existing state of stochastic, low-level, monoallelic gene expression (Ohlsson et al. 2001). Indeed, random monoallelic expression of autosomal genes is being recognized as a widespread phenomenon. Genes subject to this type of regulation include immunoglobulin, T-cell receptor, olfactory receptor, and vomeronasal genes. Interestingly, inactivation of either allele of these genes appears constrained at the chromosome level. Indeed, the replication timing of monoallelic genes on a given pair of autosomes is coordinated on the same homolog (Singh et al. 2003). This suggests that nuclear compartments may exist for each autosome, just as there

is a specific compartment (Barr body) for the inactive X (see above).

Despite their similarity, imprinted X inactivation in rodents and other eutherians may not necessarily share the same mechanisms as marsupial imprinted X inactivation. Indeed, these mammals show fundamental differences in their early development and extraembryonic tissue formation (Selwood 2001), in their sexual differentiation and determination strategies (Watson et al. 2000), and possibly even in their regulation of X inactivation, since no homolog of *Xist* has so far been identified in marsupials (Graves and Westerman 2002). The selection forces acting on X-linked gene expression during the divergence of mammals are probably diverse, and thus, imprinted X inactivation may have evolved independently in marsupials and eutherians (Ohlsson et al. 2001). Taking into account this available information, the following possible sequence of evolutionary events in mammals could be that an imprinted form of XCI originally arose, perhaps as a carryover effect of MSCI (Fig. 6). Subsequently, in eutherians, random XCI arose, perhaps with the arrival of the *Xist* gene as a control element that took over the initiation of XCI, and so the ancient form of imprinted XCI was gradually lost in eutherians (such as humans). In some eutherians such as rodents, the arrival of *Xist* may have led to a new need for imprinting: There may have been some evolutionary pressure to control this gene—and XCI—very early on in development by silencing one of its two alleles (Fig. 6). The early onset of zygotic gene activation in rodents, on the one hand, and the timing of X chromosome up-regulation in early embryos (see below) are two possible sources of selective pressure for the "reinvention" of imprinted X inactivation. This time, an imprint preventing the maternal *Xist* allele from being expressed early on may have been the chosen way.

## X chromosome hyperactivation: dosage compensation between the X and autosomes

Although dosage compensation between the sexes has been the focus of attention, one neglected question is the inequality between X-linked and autosomal gene dosage. Loss and differentiation of Y-linked genes imply that most X-linked genes have been retained as single copy in males. Haploinsufficiency for a whole chromosome is not well tolerated in most organisms. In order to avoid such deleterious effects and to maintain balanced expression between the autosomes (two copies) and the X chromosome (one copy in males), the overall transcription level of genes on the active X must have doubled (Fig. 7A; Ohno 1967; Adler et al. 1997). This phenomenon is well known in *Drosophila*, where the male X becomes associated with the MSL proteins and its expression level is doubled through chromatin modifications, including acetylation of histone H4K16 and replacement of histone H3 by the variant histone H3.3 (Akhtar 2003; Mito et al. 2005). Recent studies have shown that up-regulation of the X chromosome can be detected not only in *Drosophila* but also in several mammalian species, including human, primates, rat, and mouse, by measuring the global transcriptional output from the X compared with the autosomes, using microarray analyses to demonstrate that the X:autosome expression ratio is close to 1 in most somatic tissues from males and females (Nguyen and Disteche 2006). This balanced expression between the X and autosomes is maintained in male and female germ cells. Since haploid germ cells

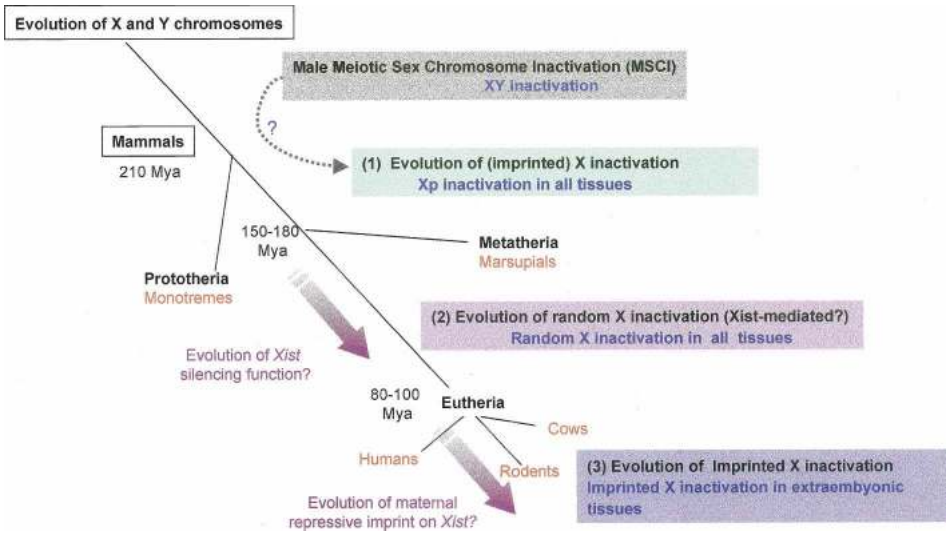


**Figure 6.** Hypothetical sequence of events underlying the evolution of X-chromosome inactivation. (I) The evolution of meiotic sex chromosome inactivation may underlie the evolution of paternal X inactivation in ancestral mammals and could be the source of imprinted Xp inactivation still found in marsupials, although this remains to be proven. (II) Random X inactivation evolved subsequently in eutherians and may be linked to the evolution of *Xist* as the mediator of *cis* silencing. (III) In some eutherian mammals, such as rodents, imprinted paternal X inactivation may have arisen for a second time, through the evolution of a repressive imprint on Xist in the female germline.

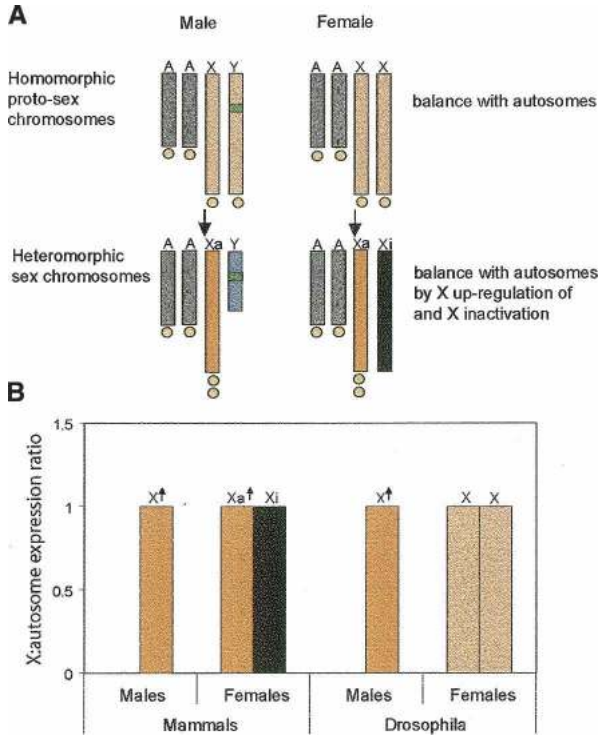


**Figure 7.** Regulation of X-chromosome expression by up-regulation. (*A*) Evolution of mammalian dosage compensation. Once the sex chromosomes diverged, X-linked genes must have become up-regulated (dark orange) in both sexes, and subject to X inactivation (black) in females in order to maintain balance of gene expression between the X (one copy) and the autosomes (two copies). Yellow dots *under* the chromosomes indicate units of gene expression dosage. (*B*) Comparison of dosage compensation in mammals and *Drosophila*. Dosage compensation is achieved in mammals by a combination of doubling of the expression level of X-linked genes in both sexes and of X inactivation in females, whereas in *Drosophila*, the X is selectively up-regulated in males only. All achieve an X:autosome expression ratio of 1.

only have one set of autosomes, up-regulation of the X does not occur in these cells. However, a balanced expression between the X and autosomes was found in early mouse embryos, suggesting that up-regulation occurs early. The interplay between up-regulation and X inactivation in early embryos remains to be deciphered.

The ultimate role of mammalian X inactivation may be to avoid "functional tetrasomy" of X-linked genes due to up-regulation. Indeed, embryos with two active X chromosomes are severely impaired, with a phenotype resembling that of autosomal tetrasomy, not the milder phenotype of autosomal trisomy (Mizuno et al. 2002). Furthermore, XX ES cells with two active X chromosomes display hypomethylation of their genome, perhaps in an attempt to increase autosomal gene expression to counterbalance the presence of two active X chromosomes (Zvetkova et al. 2005). While *Drosophila* achieves dosage compensation by selective up-regulation of the X in males, mammals employ a combination of up-regulation of the X in both sexes together with X inactivation in females (Fig. 7B). In *Caenorhabditis elegans*, the X is also up-regulated in hermaphrodites (XX) and in males (X) (Gupta et al. 2006), making it necessary to reduce its expression in hermaphrodites by decreasing expression of both X chromosomes via an epigenetic mechanism (Csankovszki et al. 2004). Although regulatory processes vary, the finding of X-chromosome up-regulation in mammals, *Drosophila*, and *C. elegans* unifies the concept of dosage compensation between the X and autosomes and between the sexes in multiple species (Gupta et al. 2006; Nguyen and Disteche 2006). Up-regulation of the mammalian X chromosome may have evolved on a gene-by-gene basis, as the Y degenerated (Charlesworth 1996). If this was the case, X up-regulation may result from permanent changes in the DNA sequence of genes at their promoter–enhancer regions or in regions that affect mRNA stability, rather than from chromatin modifications similar to those found in *Drosophila*. Permanent changes in the sequence of X-linked genes may have affected only a portion of genes for which balanced expression with autosomal genes was critical. Such a process would also imply specific repressive mechanisms in haploid germ cells to reduce expression from the X chromosome in the presence of a single set of autosomes. Alternatively, X up-regulation may be mediated by chromatin modifications of the active X in early embryos, these modifications being removed in haploid germ cells. Further studies will be required to distinguish between these possibilities.

## Conclusions

Mammals use a panoply of regulatory processes to ensure the correct dosage of the X chromosome. This complexity of dosage compensation strategies is inextricably linked to the evolution of the sex chromosomes. As can be seen from this review, the constraints and selective pressures imposed on the expression of genes that reside on the X chromosome are numerous. The mechanisms underlying the onset of X inactivation still remain mysterious at many levels, particularly with respect to *Xist*'s action. Furthermore, although the chromatin modifications associated with the inactive X chromosome are gradually being unraveled, we are still a long way from understanding exactly how these modifications intervene in the X-inactivation process. Even less is known about the mechanisms involved in the up-regulation of the X chromosome, which may act at the genetic or epigenetic level. Studies in marsupials and monotremes will be key for our understanding of the evolutionary origins of X-chromosome inactivation, on the one hand, and X-chromosome up-regulation, on the other.

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## Note added in proof

A recent study has shown that the Xist gene does not exist in marsupials, but arose from pseudogenisation of a protein coding gene (Duret et al. 2006).

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