X Chromosome Dosage Compensation: How Mammals Keep the Balance

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Abstract

The development of genetic sex determination and cytologically distinct sex chromosomes leads to the potential problem of gene dosage imbalances between autosomes and sex chromosomes and also between males and females. To circumvent these imbalances, mammals have developed an elaborate system of dosage compensation that includes both upregulation and repression of the X chromosome. Recent advances have provided insights into the evolutionary history of how both the imprinted and random forms of X chromosome inactivation have come about. Furthermore, our understanding of the epigenetic switch at the X-inactivation center and the molecular aspects of chromosome-wide silencing has greatly improved recently. Here, we review various facets of the ever-expanding field of mammalian dosage compensation and discuss its evolutionary, developmental, and mechanistic components.

INTRODUCTION

Sex chromosomes: Specialized chromosomes which determine the sex of an individual with genetically determined sex

"All things are poison and nothing is without poison, only the dose permits something not to be poisonous." This quotation attributed to Theophrastus Bombastus von Hohenheim (better known as Paracelsus, 1493–1541) describes a fundamental principle valid not only for his field of toxicology but also for how cells need to keep the dosage of gene expression in check. While diploid organisms usually can cope with variations in copy number of single genes, this does not hold true to larger portions of the genome like entire chromosomes. Aneuploidies during human development, for example, usually result in abortion, with the remaining survivors displaying birth defects such as developmental abnormalities and mental retardation (75). The few live-born babies with aneuploidies (0.3%) have either abnormal numbers of one of the four gene-poorest chromosomes Y (344 genes), 21 (386 genes), 18 (480 genes), or 13 (611 genes), or of the relatively gene-rich (1529 genes) X chromosome (75) (gene counts retrieved from NCBI MapViewer, Build 36.3: **http://www.ncbi.nlm.nih.gov/mapview/**). In addition, X chromosome aneuploidies like those in XO females (Turner syndrome) or XXY males (Klinefelter syndrome) show considerably milder phenotypes than autosomal aneuploidies do. What sets the X chromosome apart from autosomes such that abnormal numbers of it are sometimes tolerated, despite its high gene content?

The answer can be found in the diverse dosage compensation mechanisms that mammals and other organisms have developed to equalize sex chromosome-linked gene expression between the sexes with unequal sex chromosome constitution (120, 222). In most model organisms studied thus far, dosage compensation seems to be an essential requirement for successful development, and failure in dosage compensation leads to embryonic lethality. This review provides a general outline of the latest findings in vertebrate dosage compensation and in particular of mammalian X-inactivation. We first explain why and how dosage compensation might have been established during the evolution of sex chromosomes. We then summarize what is known about the different types of mammalian dosage compensation and focus on recent advances in our understanding of the underlying mechanisms. For more in-depth information on each topic, we refer the reader to the more specialized review literature cited here.

ONE DOES NOT FIT ALL: THE VARIOUS MODES OF DOSAGE COMPENSATION AND THEIR EVOLUTION

The Development of Sex Chromosomes and Dosage Compensation is Linked

Sex determination in the animal kingdom is achieved by surprisingly diverse ways and can be dependent either on chromosomal constitution or environmental factors (**Figure 1**). In some fish and reptile species, e.g., turtles or crocodiles, sex is determined by the egg incubation temperature after fertilization (see Reference 42 for a review). This environmental sex determination has the advantage that offspring of the better-adapted sex can be preferentially produced if conditions like temperature favor the reproductive fitness of either sons or daughters (26, 254). As males and females are chromosomally identical and no specific sex chromosomes exist, this system does not need any form of dosage compensation. A major disadvantage of environmental sex determination is that the existence of a species can be threatened by sudden changes in the environment such as global warming. According to one theory, climate changes caused the extinction of the dinosaurs, because their temperature-dependent sex-determination system might have forced them to produce predominantly male or female offspring (145).

In contrast to environmental sex determination stands genetic or chromosomal sex determination in which the adult sex is predetermined during fertilization. It has been proposed

that the ancestors of today's reptiles, birds, and mammals initially determined sex by using environmental cues (42). Sex chromosomes were then developed from a pair of autosomes by acquisition of a sex-determining gene or mutation on one of the two autosomes (153, 170; see References 25, 63 for reviews). That this happened independently more than once during vertebrate evolution is apparent from the remarkably diverse sex chromosome constitutions and sex-determination mechanisms in different species. Two major systems can be distinguished depending on which of the two sexes has two identical sex chromosomes (homogametic sex) and which one has two different sex chromosomes (heterogametic sex). In the first system used by birds and some reptiles like snakes, females are heterogametic and have a Z and a W chromosome, whereas males are homogametic and have two Z chromosomes. The second system is the familiar XY-based system, which most mammals employ with heterogametic XY males and homogametic XX

Homogametic: the member of any one species that makes only one type of gamete. In mammals, it is the XX female; in birds, it is the ZZ male

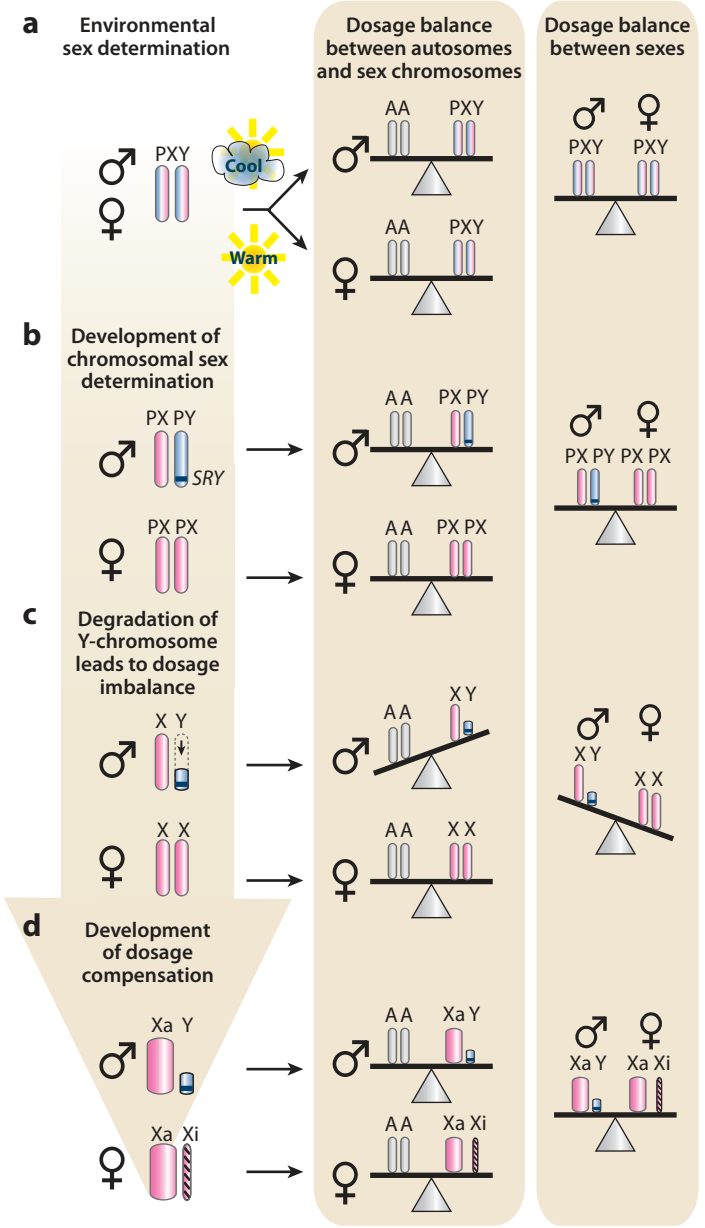


Figure 1

Simplified overview of the interlinked development of chromosomal sex determination and the need for dosage compensation mechanisms. (*a*) Initially males and females are chromosomally indistinguishable and sex is determined by environmental cues such as breeding temperature. An autosomal pair is the predecessor of the future sex chromosomes (PXY, Proto-XY). (*b*) The acquisition of a sex-determining gene (e.g., *SRY*) on one copy of the Proto-XY pair establishes distinct sex chromosomes (PX, Proto-X and PY, Proto-Y) leading to a chromosomal sex-determination system. As PX and PY otherwise share most of the genes with each other, no gene dosage imbalance yet exists. (*c*) Additional accumulation of male-specific genes on the PY leads to a suppression of meiotic recombination with PX and to progressive degradation to the Y chromosome in its current form. Many X-linked genes are now present in only one copy in males compared to two copies of genes on autosomes (A) creating a gene dosage imbalance between the X and the autosomes. Another imbalance emerges between the X-linked genes in males and females as females have two copies of X-linked genes. (*d*) To counteract these imbalances, mammals developed two dosage compensation mechanisms. Genes on the Xa (active X) are upregulated about twofold by an unknown mechanism, reestablishing the balance between the X and autosomes in males. Xa upregulation in females is counteracted by inactivation of the Xi (inactive X), to avoid imbalance with the autosomes and to create X chromosome balance between the sexes. Which mechanism (Xa-upregulation vs. Xi-inactivation) was developed first, or if the mechanisms coevolved, is unknown.

females. Over time the sex chromosomes diverge more and more from each other by accumulating further sex-linked mutations and genes. The part still shared between both sex chromosomes is called the pseudoautosomal region (PAR) and can be traced back to their autosomal ancestor pair. The PAR is the only region where crossover in meiosis can occur between the different sex chromosomes. Recombination of the sex-specific alleles outside the PAR is suppressed and therefore these regions accumulate mutations and deletions and get progressively lost on the sex chromosome specific to the heterogametic sex (W in birds, Y in mammals). This makes the W or Y chromosome increasingly smaller until it might eventually disappear (63, 170).

Heterogametic: the member of any one species that makes two types of gamete. For example, in mammals, it is the XY male; in birds, it is the ZW female

PAR: pseudoautosomal region

A consequence of the loss of genes on the W or Y chromosomes is that their equivalents remaining on the Z or X chromosomes suddenly face a dosage problem (**Figure 1**). First, these genes are present only in one copy in the heterogametic sex (ZW or XY). As a result, they are only at half of their levels before having disappeared from the W or Y chromosome. This disturbs their balance in comparison to autosomal genes, which are present in two copies each. Second, these genes are present twice in the homogametic sex (XX or ZZ), which means dosage inequality between the two sexes. As a consequence of the evolution of chromosomal sex determination, a number of different dosage compensation mechanisms have been developed to get around the problems arising from the invention of heteromorphic sex chromosomes.

Sex Determination and Dosage Compensation in Nonmammalian Species

Dosage compensation in invertebrates. Genetic sex determination has also been developed in invertebrate species; these found their own ways to compensate for the resulting dosage imbalances. Despite using different mechanisms, both the roundworm *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* determine their sex by measuring the ratio between X chromosomes and autosomes. In both species the molecules translating the X:A ratio into the appropriate sex are at the same time instrumental in triggering the respective dosage compensation mechanisms, demonstrating how tightly linked those two processes are (see References 35, 120 for reviews). In *Drosophila*, females have an XX and males an XY karyotype. Male flies upregulate gene expression on their single X chromosome by twofold to reach the same levels as females. This upregulation is controlled by the *roX* RNA-containing protein complex MSL, which assembles at the transcription site of *roX* genes on the male X chromosome. From there, the MSL complex spreads along the X chromosome to binding sites of variable affinity and boosts X-linked transcription by modifying its chromatin status (see References 120, 222 for reviews). The opposite approach is taken by *C. elegans*. Depending on the X to autosome ratio, *C. elegans* embryos develop either into XX hermaphrodites (X:A = 1) or XO males (X:A = 0.5). Hermaphrodites achieve equal X-linked gene dosage with males by downregulating expression from both X chromosomes by half (140). This downregulation is controlled by the Dosage Compensation Complex (DCC), which binds specific DNA elements on the hermaphrodite X chromosomes (55, 136).

Sex determination and dosage compensation in birds. The sex-determining mechanism used by birds is still under debate (218). A likely candidate for a sex-determining gene is *DMRT1*, which is located on the Z but not on the W chromosome, resulting in females having one *DMRT1* copy and males having two. This causes a double dose of *DMRT1* expression in gonads of males compared to females during sexual differentiation, which might initiate male-specific development (192, 234). Intriguingly, *DMRT1* homologues also play different roles in male sex differentiation in *Drosophila*, *C. elegans*, and many vertebrates including mice and humans (193). Even during the temperature-dependent

sex determination in turtles and alligators, *DMRT1* is expressed higher in male than in female gonads, suggesting *DMRT1* to be an evolutionary link between environmental and genetic sex-determining mechanisms (102, 217).

In birds, Z chromosome dosage compensation is incomplete, and many Z-linked genes are expressed at higher levels in males than in females (53, 64, 89). Genes that are dosage compensated belong to functional groups other than noncompensated genes, suggesting selective recruitment of genes to the dosage-compensation machinery depending on how critical their expression levels are (138). A majority of the compensated genes are localized within the so-called male hypermethylated region (MHM) of the Z chromosome. The MHM locus is coated specifically in females by the noncoding MHM RNA and is rich in acetylated lysine 16 on histone H4 (H4K16ac) (13, 234). This bears a striking resemblance to the situation in *Drosophila*, where the male X chromosome is also coated by noncoding *roX* RNA that recruits the histone H4 acetyltransferase MOF responsible for H4K16ac modification, which in turn causes transcriptional upregulation of the X (see Reference 120 for a review). Thus, a hypothetical model for dosage compensation in birds could be that MHM-RNA recruits a histone acetyltransferase, which promotes local hypertranscription of key genes on the single female Z chromosome. Furthermore, *DMRT1* is immediately adjacent to the MHM locus (234), a fact that would support the idea that through evolution the region close to the sex-determining *DMRT1* gene first became differentiated between the sex chromosomes and therefore needed to be dosage compensated. However, *DMRT1* itself is not marked by H4K16ac, which possibly explains how it can escape from dosage compensation, which is critical for its function as a dosage-dependent sex determinant (13). Further work is needed to elucidate the exact mechanisms of avian sex determination and dosage compensation.

Meiotic Sex Chromosome Inactivation and Ancient Roots for Imprinted X-Inactivation in Mammals

Monotreme sex chromosomes. Extant mammals can be categorized into three major groups depending on their divergence during evolution: The monotremes (prototherians) are the closest mammalian relatives to birds and reptiles and branched off from other mammals 165 million years ago (mya). They were followed by the two therian mammalian groups, the marsupials (metatherians) 150 mya and eutherians (placental mammals), which emerged around 100 mya (246, 255). Although all three groups have XY sex chromosome systems with males being the heterogametic sex, substantial differences exist regarding sex determination and dosage compensation. Studies on the sex chromosomes of the egg-laying, duck-billed platypus, which belongs to the monotremes, initially suggested a link between the mammalian and avian sex chromosome systems (68, 195). Platypus has a very peculiar set of sex chromosomes consisting of 5 different Xs and 5 different Y chromosomes. Platypus females are of $X_1X_1X_2X_2X_3X_3X_4X_4X_5X_5$ karyotype, whereas males are $X_1Y_1X_2Y_2X_3Y_3X_4Y_4X_5Y_5$. During male meiosis, the X and Y chromosomes pair with each other in this alternating order and form a chain ensuring proper segregation between the 5 Xs and 5 Ys. X_1 and Y_1 on one end of the chain share the highest similarity with each other, whereas X_5 and Y_5 basically diverged completely. This suggests that X_1Y_1 might be the evolutionarily youngest sex chromosome pair and X_5Y_5 the oldest. Strikingly, the X_5 contains the *DMRT1* gene, which implies a common history with the bird's Z chromosome. For that reason, a bird-like ZW sex chromosome system might have been ancestral to mammals before it was gradually replaced by a XY system. However, it has become evident recently that the platypus sex chromosomes share no homologies with the sex chromosomes of therian (marsupial and eutherian) mammals (246). Therefore, the

Monotremes: a mammalian subgroup belonging to the Prototherian clade, which evolved ~165 mya. An example is the egg-laying, duck-billed platypus. There is an absence of placenta

Marsupials: a mammalian subgroup belonging to the Metatherian clade, which evolved some 150 mya. Marsupials make a rudimentary placenta and give birth to very immature offspring, which then attach to external tits of the mother

Eutherians: placental mammals, evolved ~100 mya

Xp: paternally inherited X chromosome

Xm: maternally inherited X chromosome

MSCI: meiotic sex chromosome inactivation

MSUC: meiotic silencing of unsynapsed chromatin

therian XY system was newly developed after the monotreme lineage branched off, a finding that was independently confirmed by a study looking at the movement of retrogenes from X chromosome to autosomes (186). Still unclear is which gene determines sex in platypus and to which degree and how its sex chromosomes are dosage compensated (67, 251).

Therian sex chromosomes. Therian mammals have taken a completely different avenue of sex determination from that of the proposed dosage-based mechanism in birds. These species have a key maleness-determining gene on the Y chromosome named *SRY* (sex-determing region Y), which encodes a high mobility group (HMG)-box transcription factor (216). *SRY* has most likely evolved from the *SOX3* gene, which is autosomal in nonmammalian vertebrates and in monotremes but X-linked in therian mammals (251, 255). Hence a possible scenario might be that *SOX3* on an ancestral proto-sex chromosome mutated into a dominant testis-determining switch, which in turn could have initiated the divergence between X and Y chromosomes. The ensuing erosion of the Y chromosome resulted in the need for X-dosage compensation, which in therian mammals is achieved through X-inactivation. In imprinted X-inactivation, it is always the paternal X chromosome (Xp) that is silenced while the maternal X (Xm) stays transcriptionally active. As imprinted X-inactivation is found both in marsupial and eutherian mammals, it is believed to be evolutionarily older than random X-inactivation, which is exclusive to eutherians (122, 207, 229). In contrast to eutherians, where imprinted X-inactivation is restricted to early embryogenesis and extraembryonic tissues, it is used in all tissues of marsupials. What are the potential mechanisms of imprinted X-inactivation and have these mechanisms been conserved throughout therian evolution?

Meiotic sex chromosome inactivation (MSCI). Before addressing imprinted or random X-inactivation, we first need to introduce a third form of X-inactivation, which occurs in the male germline of many organisms: meiotic sex chromosome inactivation (MSCI) (see References 100, 238 for reviews). MSCI takes place at the pachytene stage of meiosis, when the homologous chromosomes undergo synapsis (pairing). It has been shown in mice that during this time unpaired regions both on the autosomes and on the sex chromosomes are transcriptionally silenced by a process termed MSUC (meiotic silencing of unsynapsed chromatin) (5a, 241). The original function of meiotic silencing might be the triggering of meiotic checkpoints to avoid the production of gametes with chromosomal abnormalities or aneuploidies and also as a genome defense mechanism against the spreading of foreign DNAs like transposons or retroviruses (100, 238). MSCI is a sex chromosome-specific form of MSUC, caused by the fact that the X and Y chromosomes can only pair with each other along their pseudoautosomal regions, while the X- and Y-specific parts remain unpaired (240). Apart from mice (241), meiotic silencing has also been described in more distant organisms including *Neurospora crassa* (212) and *C. elegans* (10). The mechanism in *Neurospora* involves posttranscriptional silencing by the RNAi machinery and silences not only the unpaired chromosome regions but also all homologous sequences present elsewhere in the genome (212). It is unclear if this silencing is purely based on RNAi, or if chromatin regulation is involved as well (100). During X-inactivation in male meiosis of *C. elegans* the unpaired X chromosome acquires the histone H3 lysine 9 dimethyl (H3K9me2) histone mark (10). From this stage onward the X remains transcriptionally silent and is depleted of histone marks associated with transcriptional activity until it becomes reactivated in the early embryo. In *C. elegans*, as in *Neurospora*, RNAi might also play a role during meiotic silencing, possibly by establishing H3K9 methylation (100).

During mouse MSCI/MSUC, unpaired regions are initially recognized by the double-strand brake/DNA-repair machinery, resulting

in the recruitment of multiple repressive chromatin marks including histone modifications and histone variants (**Table 1**; see below) (see Reference 238 for a review). As a consequence, the sex chromosomes form a distinct structure called the XY body or sex body, which is heterochromatic and transcriptionally silent (66, 155, 240). MSCI in mammals is thought to be based on transcriptional repression, but whether RNAi is also involved as in *Neurospora* has not been resolved (238). The silent state acquired during MSCI is maintained as postmeiotic sex chromatin (PMSC) throughout spermatogenesis with the exception of genes required during spermiogenesis, which become reactivated by an unknown mechanism (155). Furthermore, a recent study has revealed that a substantial number of X-linked genes expressed in the testis in postmeiotic cells is present in multiple copies, which might help them to overcome the repressive effect of MSCI after meiosis (152). In addition to specific reactivation and multiple copy number, another backup mechanism for meiotically repressed genes on the X chromosome exists. A number of X-linked genes have additional retroposed copies on autosomes, which are specifically expressed during spermatogenesis compensating for their silent X-linked parent genes (253). In conclusion, meiotic silencing triggered by unsynapsed chromosomal regions is a common motif in many organisms and is the root of MSCI in which silencing effects are maintained to a large extent throughout spermatogenesis.

MSCI and PMSC have recently been shown to occur in the marsupial *Monodelphis domestica* (opossum) (86, 156). Marsupial sex chromosomes are lacking pseudoautosomal regions (65) and therefore cannot pair through homology at early pachytene when autosomes undergo synapsis. However, due to their unpaired status, the sex chromosomes accumulate characteristic meiotic silencing marks like γH2AX, H3K9me2, H3K9me3, HP1β, and HP1γ and exclude signs of active transcription like Cot-1 and Pol II staining (156). At mid-pachytene the sex chromosomes finally come together in the XY body and are held together by the dense plate, a proteinacious structure. Like mice, marsupials maintain their silent sex chromosome status after meiosis by PMSC, as both the repressive chromatin signature (156) and increasing repression of X-linked genes (86) indicate. In conclusion, MSCI and PMSC seem to be mechanistically very similar in marsupial and eutherian mammals.

PMSC: postmeiotic sex chromatin

MSCI: The Ancestral Force Behind Imprinted X-Inactivation? From an evolutionary perspective, it appears plausible that MSCI might be the most ancient type of X-inactivation (88, 116, 124, 133). As the sex chromosomes increasingly diverged from each other, they might have been recognized as unpaired fragments during meiosis and been silenced by MSUC, as this mechanism was already in place for other reasons such as genome defense and as a checkpoint against chromosomal abnormalities in meiosis. The silent Xp, if it were then inherited to the female embryo in an inactive state, would automatically lead to X-linked gene dosage parity between males and females. In this so-called preinactivation hypothesis of imprinted X-inactivation, which our laboratory and others have previously proposed, X chromosome imprinting could have initially developed from meiotic silencing (38, 87, 88, 124, 133). This might be still the predominant imprinting mechanism used by marsupials today (86, 156).

Indeed, a number of recent studies have demonstrated that *XIST*, the noncoding RNA gene crucial for both imprinted and random X-inactivation in eutherians, is not present in marsupials and monotremes (46, 51, 85, 209). Instead, *XIST* seems to be an eutherian invention sharing a weak homology with the protein-coding *LNX3* gene, which is found only in noneutherian vertebrates (51). Therefore, it has been proposed that *XIST* has evolved by pseudogenization of *LNX3* (51), or at least could have acquired its transcriptional potential (85). Consequently, imprinted X-inactivation in marsupials seems to be achieved by an *XIST*-independent mechanism, possibly related to meiotic inactivation during spermatogenesis.

An alternative hypothesis would be that another noncoding RNA serves an equivalent function in marsupials as *XIST* does in eutherians (209). However, no such RNA has yet been identified.

To determine the mechanism of imprinted X-inactivation in marsupials, it will be critical to assess whether the Xp inherited from sperm enters the oocyte in a preinactivated state. Is the Xp continuously maintained throughout embryogenesis in its silent state, which it initially acquired during male meiosis? In addition, nothing is currently known about the nature of the imprint. DNA methylation is unlikely to be the global X-imprint in marsupials, as gene control regions on the inactive X are hypomethylated both in sperm (86) and in female somatic tissues (86, 98, 118). This lack of DNA methylation on X-linked promoters might also explain the incompleteness and leakiness of imprinted X-inactivation in marsupials (98). Other potential imprints could be epigenetic chromatin marks established during MSCI. Although most histones are exchanged with protamines during spermiogenesis, emerging evidence indicates that some histones and their modifications are passed on from the sperm to the embryo (175, 244). The Xp-specific chromatin configuration of marsupial preimplantation embryos is still elusive and therefore leaves open whether MSCI is the cause of imprinted X-inactivation. In conclusion, the lack of *XIST*-dependent X chromosome imprinting makes marsupials the ideal subject in which to study the potential ancestral mechanism of mammalian X-inactivation.

Evolution of *Xist* as a New Player in X-inactivation. Although low levels of *Xist* RNA are expressed during spermatogenesis (132a, 195a, 200a) and associate with the XY-body in mice (5), knockout studies revealed that *Xist* is in fact neither necessary for MSCI nor spermatogenesis (130, 134, 239). Therefore a commonality between marsupial and eutherian MSCI is its independence from *XIST*. This independence, in combination with the similarities in chromatin modifications (66, 155, 156, 240), supports the model that MSCI developed before the emergence of *Xist* and that MSCI is mechanistically conserved in marsupial and eutherian mammals. On the other hand, *Xist* is essential both for imprinted X-inactivation in extraembryonic tissues and random X-inactivation in mice (130, 179). Thus in eutherians, *Xist*-dependent X-inactivation mechanisms have at least partially taken over from the proposed ancestral *Xist*-independent form, which is still used by marsupials. The purpose for which *Xist*-dependent mechanisms might have originally evolved has not been resolved. One possibility is that *Xist*-based silencing was a new means to achieve more stable imprinted X-inactivation than the leaky and incomplete form observed in marsupials (88). This hypothesis would be greatly strengthened were a "missing link," a eutherian species with only *Xist*-based imprinted X-inactivation but without random X-inactivation, to be found. Once *Xist* had been established as a regulator of imprinted X-inactivation, relaxation of the imprint during embryonic development could have opened up the possibility of reusing *Xist* for the development of random X-inactivation (106). Random X-inactivation is indeed advantageous for females compared to imprinted X-inactivation. Maternal mutations on X-linked genes show a phenotype in females with imprinted X-inactivation as the functional paternal copy is by default inactivated. In random X-inactivation, however, cells expressing the functional paternal allele by random choice can compensate for cells with the defective maternal copy active. An alternative hypothesis would be that *Xist*-dependent silencing coevolved with random X-inactivation and only after that was it applied to imprinted X-inactivation (79). The evolutionary driving force in that case would have been first the advantages of random X-inactivation and only second the improvement of fidelity of imprinted X-inactivation. Whether eutherians at first used *Xist* to control imprinted or random X-inactivation remains a topic for speculation.

IMPRINTED X-INACTIVATION IN EUTHERIAN MAMMALS

In mice as in marsupials, one critical unresolved question regarding the mechanism of imprinted X-inactivation is the nature and origin of the responsible imprint(s). Is imprinting established exclusively in the maternal or the paternal germline, or are different maternal and paternal imprints both necessary?

Evidence for Imprinting of the Paternal X Chromosome

One line of evidence for a paternal imprint of the X chromosome comes from the observation of the development of XO mouse embryos with X chromosomes of different parental origin. XpO embryos and their ectoplacental cones are developmentally retarded during early postimplantation stages when compared with XX control embryos, wheras XmO embryos are either indistinguishable from XX controls or even larger (21, 90, 235). Thus the Xp seems less capable than the Xm in providing the appropriate dosage of X-linked genes. *Xist* is initially expressed from paternally inherited X chromosomes in biparental XpO or androgenetic (zygotes with only paternal pronuclei) XpY and XpXp preimplantation embryos (132, 173). Starting at the morula stage, *Xist* is downregulated in the majority of cells from XpO and XpY embryos. In XpXp androgenones, *Xist* is expressed from a single allele in most cells, leading eventually to random X-inactivation in both embryonic and extraembryonic tissues. Thus any potential paternal imprint on the Xp promoting *Xist* expression is gradually lost after the morula stage, which is possibly followed by a counting and choice mechanism to ensure appropriate *Xist* regulation.

The degree to which imprinted X-inactivation in eutherians and in particular in mice still relies on MSCI or if the two phenomena have been completely separated over time is also subject to recent debate. Is the silent state of the Xp inherited from the paternal germline to the embryo, or does MSCI predispose the Xp to *Xist*-dependent silencing? *Xist* starts to be expressed from the Xp in mouse embryos at the 2-cell stage, when the zygotic genome becomes activated (87, 172). At this point the *Xist* RNA-territory is confined to a small region, which gains increasingly in size during the following cell divisions, thereby progressively coating the Xp. Exclusion of markers of ongoing transcription like Cot-1 RNA or Pol II staining from the *Xist*-territory indicates that it is transcriptionally repressed. Cot-1 exclusion as a first sign of repression can be observed as early as the 2-cell stage (87; S.H. Namekawa, K.D. Huynh, B. Payer, R. Jaenisch & J.T. Lee, in preparation), and the region of Cot-1 exclusion becomes more and more prominent from the 4-cell stage onward (171, 172). On a gene-by-gene basis, imprinted X-inactivation in preimplantation embryos appears to be more complete in the vicinity of the X-inactivation center (*Xic*) than further away from it (87). Our ongoing analysis indicates that different domains of the X chromosome are silenced at different times, with some already silent at the 2-cell stage and others not silenced until as late as the blastocyst stage (S.H. Namekawa, K.D. Huynh, B. Payer, R. Jaenisch & J.T. Lee, in preparation). The *Xist* RNA-coating of the Xp is followed by a series of epigenetic changes creating the characteristic chromatin signature of the transcriptionally repressed inactive X chromosome (Xi) (172). Active marks like H3K4 methylation and H3K9 acetylation are gradually lost while macroH2A is incorporated (40), and the association with the Eed/Ezh2 Polycomb group complex leads to the accumulation of repressive H3K27 trimethylation (56, 181, 214), which is later followed by H3K9 methylation (172). Autosomal *Xist*-transgenes can recapitulate several features of imprinted X-inactivation when inherited through the paternal germline without undergoing MSCI, which has been interpreted as evidence that in the mouse, *Xist*-controlled imprinted X-inactivation and MSCI have become two independent processes (171). Nevertheless, it has not yet been established if efficient silencing on the *Xist*-transgene-harboring

***Xic*:** X-inactivation center

Xi: inactive X chromosome

Xa: active X chromosome

autosome takes place, if the initial silencing is stable over time, and if other factors in addition to *Xist*-expression are needed. Indeed, animals harboring the *Xist*-transgene are viable and normal (80, 171), thus excluding the possibility that the transgene-containing autosome is stably silenced to a large extent. Therefore, the events surrounding gamete-to-embryo transition and the mechanism by which imprinted XCI occurs remain unresolved.

Potential Mechanisms of a Paternal X Chromosome Imprint

Although *Xist* regulates imprinted X-inactivation in the extraembryonic tissues, whether it is required for the preimplantation form of XCI is not known. *Xist*-independent mechanisms, possibly related to the X-inactivation mechanism employed by marsupials, might be at work in some eutherians. For example, the heterochromatic chromatin state acquired during MSCI might predispose the Xp for future *Xist*-dependent silencing in the embryo.

A crucial property of any potential paternal or maternal imprint is that they need to pass on information from the germline to the early embryo. Therefore the imprints have to be resistant to the extensive global epigenetic reprogramming events occurring after fertilization (see References 151, 225 for reviews). The paternal pronucleus in particular becomes strongly modified as protamines are exchanged for histones, new histone modifications are acquired, and paternal DNA is actively demethylated. The maternal pronucleus appears to undergo fewer visible changes and is less obviously affected by reprogramming. Global maternal DNA methylation and several autosomal maternal and paternal DNA methylation imprints are protected against active demethylation in the zygote by the maternal factor PGC7/Stella, which is required for normal preimplantation development (154, 177). What still needs to be established is whether PGC7/Stella is also involved in the protection of imprints on the X chromosome.

An indication that X-inactivation marks can indeed resist epigenetic reprogramming in the zygote comes from nuclear transfer experiments. When nuclei from female somatic cells were transferred, the extraembryonic tissues of the resulting embryos preferentially displayed inactivation of the Xi of the donor cell (52). This indicates the persistence of epigenetic memory of the Xi (and/or the Xa) after nuclear transfer, mimicking the situation of imprinted X-inactivation. In the embryo proper, on the other hand, random X-inactivation was observed due to the erasure of the imprint in the blastocyst (see below). Further analysis, however, showed that the kinetics of epigenetic events during preimplantaion development after nuclear transfer did not completely mimic the situation during normal imprinted X-inactivation (7). This could explain why the fidelity of X-inactivation in cloned embryos is frequently perturbed (164, 262), which might also contribute to the poor survival rate of cloned animals.

Although sperm DNA is packaged to a large extent with protamines instead of histones, a significant proportion of histones and their modifications are still retained and passed on from the sperm to the embryo (175, 244). The XY bivalent acquires a distinctive sex-body chromatin signature during MSCI, which is partially maintained as PMSC throughout spermiogenesis (66, 155, 240, 243). This involves histone modifications such as H3K9 di- and trimethylation and binding of HP1β and HP1γ proteins (66, 155, 240), as well as the incorporation of the specific histone variants H2A.Z (66) and H3.3 (243).

There is at least indirect evidence that histone H3.3 is inherited with sperm chromatin to the zygote and thereafter increasingly incorporated into the paternal pronucleus during the protamine-histone exchange (236, 245). H3.3 is usually associated with active chromatin and appears in combination with H2A.Z especially at promoters and enhancers of transcriptionally active genes (91). Furthermore, H3.3 has recently been reported to be a key factor necessary for epigenetic memory of active genes

(161). Therefore, it could be speculated that some genes on the Xp might become specifically poised for transcription by incorporation of H3.3 into their regulatory regions and that *Xist* might be one of them.

On the other hand, the paternal pronucleus displays a histone modification signature distinctly different from that of the maternal pronucleus (151, 245). While the maternal pronucleus is marked by mono-, di-, and trimethylation at histone H3 lysines 4, 9, and 27, the paternal pronucleus is devoid of global di- and trimethylation marks but is rather mono-methylated on H3 K9. Recently, this initial parental asymmetry in the zygote was demonstrated to result in the attraction of distinct silencing complexes, which establish different types of constitutive heterochromatin in the paternal and maternal genome (189). The maternal constitutive heterochromatin thus becomes targeted preferentially by Suv39h proteins, which establish and maintain the H3K9 trimethylation mark. In contrast, paternal constitutive heterochromatin is labeled by H3 K27 trimethylation and bound by Rnf2/Ring1B recruiting the polycomb repressive complex 1 (PRC1) independently of Ezh2.

Future studies may well establish the link between an evolutionary older silencing mechanism through paternal epigenetic inheritance and the newer *Xist*-based silencing mechanism observed in eutherians. For example, an *Xist*-independent chromatin mark may be needed in early embryos to aid efficient gene silencing by *Xist* during imprinted X-inactivation.

Evidence for *Xist*-based Imprinting of the Maternal X Chromosome

While a paternal X chromosome imprint might predispose the Xp to imprinted X-inactivation, a maternal imprint is needed to keep the Xm in an active state and suppress *Xist* expression. Parthenogenetic preimplantation embryos (oocyte-derived without paternal pronucleus) with two maternal X chromosomes have no *Xist* expression until the morula stage, i.e., a maternal imprint blocks expression until then (99, 159). However, after the morula stage, *Xist* becomes monoallelically expressed in a majority of cells, suggesting that a counting mechanism presumably either overrides or erases this initial negative imprint. This cannot be the only maternal imprint, as embryos with additional maternal X chromosomes die shortly after implantation through the failure to inactivate the Xm in the extraembryonic tissues (61, 62, 206). The time point of this imprint has been established by serial nuclear transfer experiments in which nuclei from nongrowing and fully grown oocytes were combined. The X chromosome from the nongrowing oocyte was always inactivated in the extraembryonic tissues of postimplantation embryos, whereas the X from the fully grown oocyte remained active (227). This suggests that the imprint, which inhibits X-inactivation in the extraembryonic tissues, is placed upon the maternal X chromosome during oocyte growth. Whether the imprint acting during X-inactivation in preimplantation embryos is also regulated in the same way is not yet known, however. It will be crucial to examine whether the *Xist* alleles from nongrowing oocyte nuclei show exactly the same expression kinetics as a paternally inherited *Xist* allele. This could determine whether the only imprint on *Xist* expression is a repressive maternal one and if *Xist* is expressed paternally by default in early embryos, as the Xp lacks such an imprint (79).

Evidence that the maternal imprint acts on the maternal *Xist* allele can be observed in embryos with paternal *Xist* deletion in which the maternal X is not inactivated, leading to embryonic lethality (130). As repression of the maternal *Xist* allele is a key event for imprinted X-inactivation, identification of the repressive mark is crucial to understanding the imprinting mechanism.

Potential Mechanisms of *Xist* Imprinting

One possibility for an *Xist* imprint initially proposed was differential DNA methylation of the *Xist* promoter (2, 165, 265). Indeed, some studies suggested that the region 5′ to *Xist* might be

methylated in eggs and unmethylated in sperm (2, 265), even if bisulfite sequencing in another study (135) could not confirm that observation. A crucial negative regulator of *Xist* is its antisense partner gene *Tsix* (see **Figure 3**). *Tsix* and *Xist* expression are mutually exclusive in *cis*, and *Tsix* deletion leads to inactivation of the mutant X chromosome during random X-inactivation (110). In extraembryonic tissues, *Tsix* is exclusively expressed from the Xm in both males and females. Mutation of *Tsix* on the Xm results in upregulation of *Xist* and inactivation of both X chromosomes in females and the single X chromosome in males, causing early embryonic lethality (105, 200). This illustrates that the maternal repressive imprint on *Xist* expression and on X-inactivation in the extraembryonic tissues acts through *Tsix*. A function for *Tsix* in *Xist*-imprinting during preimplantation stages still needs to be shown. *Tsix* itself is controlled by the noncoding *Xite* and *DXPas34* repeat regions (**Figure 3**), which act as enhancers on *Tsix* expression (37, 49, 166, 221, 247). Both regions are hypermethylated in sperm but hypomethylated in oocytes, suggesting that DNA methylation might be potentially involved in paternal imprinting of *Tsix* (17). However, this is contested by an earlier study (187) in which no methylation imprints on *DXPas34* at stages prior to implantation could be detected. The importance of *DXPas34* for the regulation of *Tsix* has been underscored by the analysis of its deletion, which phenocopies to a large extent the effects of *Tsix* mutations on both imprinted and random X-inactivation (37, 247).

Candidate *trans*-acting factors for *Xist* imprinting include proteins that have been shown to bind *DXPas34* during random X-inactivation. An interesting feature of the *DXPas34* region is its clustering of binding sites for the ubiquitous chromatin insulator and transcription factor protein Ctcf, which is also a common motif found in autosomal imprinted gene loci (113). Ctcf-binding to *DXPas34* is reduced when the binding site is methylated, making it a potential epigenetic switch for X-inactivation (24). Binding of Ctcf to unmethylated DNA on the active Xm could serve several purposes. First, Ctcf could block access of *Xist* to putative enhancers downstream of *DXPas34*, thereby rendering it transcriptionally inactive (24). In addition, *DXPas34* is also bound by the Ctcf cofactor Yy1, and together these proteins can act in a complex as transcriptional activators of *Tsix* (50). Indeed, *Yy1*-deficient embryos display abnormal *Tsix* and *Xist* expression and die shortly after implantation. Furthermore, Ctcf is required for X chromosome pairing at sites around *Tsix* (including *DXPas34*) and *Xite* at the onset of random X-inactivation (260).

In contrast to its potential role on the active X chromosome where Ctcf binds *DXPas34*, CTCF has also been shown to bind the human *XIST*-promoter on the inactive X chromosome (188). In this region, CTCF might mediate *XIST* expression by either blocking repressive influences on the *XIST* promoter or activating transcription of *XIST* directly (157, 188). In summary, CTCF appears to control multiple distinct aspects of X-inactivation on both the active and inactive X chromosomes and is a candidate factor for translating imprinting information into locus-specific responses by its ability to "read" DNA methylation marks.

What is the Molecular Nature of the X Chromosome Imprint?

Is DNA methylation, although indisputably an essential mark for autosomal imprints (18, 76, 96, 115), really necessary for imprinting of the X chromosome? As discussed above, *Xist* (2, 165, 265), *Tsix* (17, 41), and *Xite* (17) have potential differentially methylated elements in their control regions. On the other hand, there is no consensus on whether differential methylation is established in the gametes and indeed maintained during preimplantation development (135, 187). If so, DNA methylation would be a secondary mark rather than the initial imprint. A study of mouse mutants of the maintenance DNA methyltransferase gene *Dnmt1* showed that DNA methylation is necessary for efficiently maintaining random but not imprinted X-inactivation in the placenta (196).

Also, de novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for random X-inactivation (199). Finally, depletion of de novo methylation from the female germline seems not to disrupt imprinted X-inactivation in the placenta as indirect data suggest (97). In conclusion, DNA methylation appears to be an unlikely candidate for the imprint responsible for protecting the Xm from inactivation in the extraembryonic tissues. Nevertheless, it has not been ruled out that DNA methylation might be the imprint responsible for repression of *Xist* during preimplantation development or might act as a paternal imprint by keeping *Tsix* repressed and *Xist* expressed on the Xp. If DNA methylation is not an essential primary imprinting mark, what could it be?

Imprinted X-inactivation is associated with the gradual accumulation of a number of specific chromatin modifications during mouse preimplantation development such as histone H3 methlyation on lysines 27 and 9 and incorporation of the histone variant macroH2A (40, 172). Although the kinetics of the acquisition of these marks on a global X chromosome-wide scale has been established, no information is yet available about the chromatin configuration of potential imprinting control regions on the X chromosome such as *DXPas34* or the *Xist* promoter in gametes or early embryos, a prerequisite to identifying potential imprinting marks alternative or in addition to DNA methylation. Just as the maintenance of X-linked gene silencing is safeguarded by multiple layers of repressive marks (43, 44, 83), the same could potentially also be true for the original imprint itself. Indeed, this is the case for autosomal imprinting marks, where imprinting control regions are marked not only by differential DNA methylation but additionally by histone modifications (113). The allele with DNA methylation is thereby also marked by repressive histone modifications such as H3K9 and H3K27 methylation, whereas the allele without DNA methylation contains activating histone marks like histone H3K4 methylation and acetylation of histones H3 and H4. There is some evidence that H3K9 methylation might direct DNA methylation and vice versa (113), suggesting that these marks might potentially also be able to compensate for each other in maintaining an epigenetic memory if one of them is absent. Autosomal imprinted genes, which are exclusively imprinted in the placenta but not in the embryo proper, do not require DNA methylation to maintain monoallelic expression (see Reference 248 for a review). Mimicking the situation of imprinted X-inactivation, these imprinted paternal alleles always remain silent while the maternal alleles are expressed. Additionally, silencing also depends on paternally expressed noncoding RNAs, like imprinted X-inactivation does on *Xist*. Instead of DNA methylation, the paternal alleles are associated with Eed-Ezh2 Polycomb group proteins and marked by histone modifications such as H3K27 and H3K9 methylation (112, 242). Indeed, the histone H3K9 methyltransferase G9a is required for efficient maintenance of imprinting in the placenta (249) as is Eed for a number of imprinted genes (126). In the case of imprinted X-inactivation, G9a is dispensable for imprinting maintenance (169), whereas Eed seems to be necessary (95, 252). It will be important to delete these factors in germ cells in order to evaluate if they have any function for the establishment of the X chromosome imprint(s). In conclusion, X chromosome imprinting may depend more on chromatin modifications than on DNA methylation. As also autosomal imprinting in the placenta seems to rely more on histone modifications than DNA methylation, this lead to the proposal that autosomal and X chromosome imprinting coevolved in the placenta (106, 194, 248). In evolutionary terms, differential DNA methylation at the X chromosome (86, 98, 118) or at autosomal imprinted genes may also play a lesser role (248). However, more recent studies found differential DNA methylation at several autosomal imprinted loci in marsupials, suggesting the possibility of regulation by DNA methylation (51a, 218a, 225a). The question therefore remains open if DNA methylation was the primary mark or was rather later

recruited as a safeguard mechanism to ensure the fidelity of silencing during embryonic autosomal imprinting and random X-inactivation.

Conclusion: Evidence Favors a Biparental Model for Imprinted X-Inactivation in Mice

In summary, mounting evidence exists for both paternal and maternal imprints for X-inactivation in mice. We therefore propose a biparental model of imprinted X-inactivation. Both inheritance of epigenetic marks on the paternal X chromosome and maternal imprinting of *Xist* on the maternal X chromsome interplay to ensure faithful imprinted X-inactivation (**Figure 2**).

Mixed Evidence for Imprinted X-Inactivation in Humans: Are We Different After All?

Imprinted X-inactivation is the exclusive mechanism applied by marsupials, whereas in mice it is used during preimplantation development and in extraembryonic tissues, but not in the embryo proper, where X-inactivation is random. Is this pattern representative for all eutherians and in particular for humans, or might differences exist within the eutherian lineage?

Imprinted X-inactivation in the placenta is not exclusive to mice, but has also been described in cows (262). During bovine preimplantation development, *Xist* RNA was detected by reverse transcriptase PCR (RT-PCR) mainly in female but to a lesser extent also in male embryos (178), which could be interpreted as evidence for *Xist* expression from the Xm in male embryos. However, as the assay was not strand-specific, potential antisense *Tsix*-transcripts could have also been detected using the *Xist*-primers. The data to date on human imprinted X-inactivation are inconclusive. Similarly, human *XIST* expression has been detected by RT-PCR both in male and female preimplantation embryos (45, 191). As in cattle, the analysis was not strand-specific; however, detection of the spliced product of *XIST* indicates expression from the Xm in males. Nevertheless, quantitative allele- and strand-specific RT-PCR and/or *XIST* RNA-fluorescence in situ hybridization analysis of human preimplantation embryos are needed. If *XIST* expression were indeed detected at significant levels from the Xm in both male and female embryos, it would resolve unequivocally whether there is *XIST*-imprinting during human preimplantation development or not. Also, still controversial is whether X-inactivation in the human placenta is random or imprinted. Some evidence supports preferential inactivation of the Xp (60, 74), but other studies report only slightly skewed or rather random X-inactivation patterns (119, 144, 263). These discrepancies between different studies might be rooted in the analysis of different cells types, maternal contamination and the restricton to a small number of analyzed genes. An X chromosome-wide allele-specific expression assessment including the status of *XIST* in the human placenta is needed for any firm conclusions.

Furthermore, it has been postulated that human *XIST* is not negatively regulated by *TSIX* as it is in the mouse, because *TSIX* transcripts apparently do not fully extend across the *XIST*-locus and were found to be coexpressed with *XIST* from the same X chromosome in certain cell types (141–143). This finding has been interpreted as additional evidence that imprinted X-inactivation, which relies in mice on *Tsix*, does not exist in humans. Yet it is still possible that *XIST* is regulated in humans by *TSIX*, because reports to date have not addressed the expression status at the crucial time period during X-inactivation in human embryos (19, 107).

In mice, random X-inactivation is a feature of ES cell differentiation, which makes them an important model system to study the underlying mechanisms in vitro. A number of studies have also examined human ES cells for their X-inactivation behavior (72, 84, 208, 215). In contrast to mouse ES cells, human ES cells did not display a consistent pattern but rather showed a high degree of variation between cell lines and even between sublines of the same parental cell line. Although some lines recapitulated

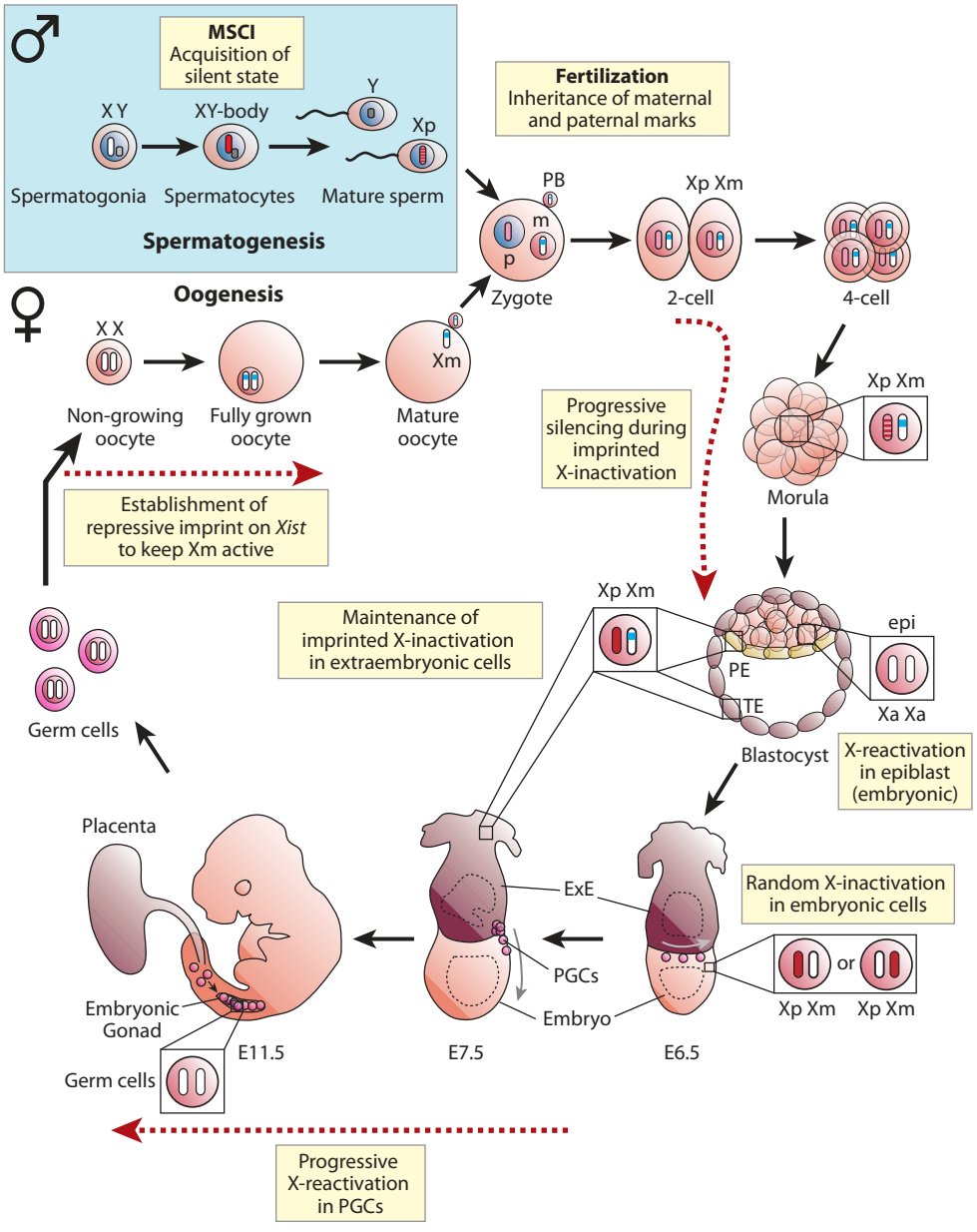


Figure 2

The X-inactivation and X-reactivation cycle during mouse development. The diagram shows primarily the critical events occurring in female mice with the exception of MSCI, which takes place in the male germline (*blue shaded area*). X-chromosome activity is depicted in white (mostly active), pink (partially active/inactive) and red (inactive). The blue stripe on active X chromosomes symbolizes a repressive imprint on *Xist*, to keep the Xm active. The pink shading of the Xp symbolizes the inheritance of epigenetic information from the male germline. Epi: epiblast; ExE: extraembryonic ectoderm; m: maternal pronucleus; p: paternal pronucleus; PE: primitive endoderm; PGCs: primordial germ cells; PB: polar body; TE: trophectoderm; Xa: active X; Xm: maternal X; Xp: paternal X.

ES cells: embryonic stem cells

X-inactivation during differentiation, others underwent X-inactivation in the undifferentiated state and sometimes lost *XIST* expression and H3K27me3 during culture. Even in lines without detectable *XIST* expression and H3K27me3 mark on the Xi, silencing was frequently maintained and only sporadically lost on a gene-by-gene basis. This variability and epigenetic instability might reflect that derivation and culture conditions of human ES cells are still suboptimal and need to be improved if cells are to be kept in a pristine undifferentiated state that retains full pluripotency. Furthermore, the long-term maintenance of cell lines with two active X chromosomes is in general a difficult feat, even in female mouse ES cells, where frequently X chromosome loss is observed (266). The genetic and epigenetic instability of mouse XX ES cells has been attributed to their global DNA hypomethylation, which these cells display in contrast to XO or XY ES cells. In human ES cells, X-inactivation in undifferentiated cells instead of X chromosome loss seems to be the more common mechanism to avoid the presence of two active X chromosomes (72, 208, 215). In conclusion, X-inactivation capability might be a useful epigenetic marker to assess the quality of human ES cell lines before they can be considered for any kind of therapeutic application, even though this will be limited to female cells.

In general, our understanding of X-inactivation in humans is less developed when compared to the information gathered from the mouse model system. The basic question regarding the degree to which imprinted X-inactivation exists in humans awaits conclusive investigation. Resolution of this question will be of particular interest from both a medical as well as a basic science perspective. Clues might emerge on how imprinted X-inactivation developed in eutherians generally, how it has been mechanistically preserved, and if ancestral *Xist*-dependent X-inactivation was initially developed in the random or imprinted form. In general, investigation of X-inactivation in a wider range of eutherian species should shed light on how much of the knowledge about murine X-inactivation can be extrapolated onto other mammals and, in particular, onto humans.

X CHROMOSOME REACTIVATION: RESETTING THE SILENT STATE BY EPIGENETIC REPROGRAMMING

In female somatic cells, the inactive X chromosome is in a very stable epigenetic state, maintained by multiple silencing marks including *Xist* RNA expression, DNA methylation, histone variants, and histone modifications (43, 44). However, there are instances during normal development (**Figure 2**) and in artificial experimental settings where the inactive state is reset and both X chromosomes become active in females. This includes the epiblast lineage in the inner cell mass of the late blastocyst, germ cells, and a number of pluripotent stem cell types. Recently more details about the X-reactivation process have emerged.

X-Reactivation in the Blastocyst

Since the discovery that mouse preimplantation embryos undergo imprinted X-inactivation (87, 128, 172), it has become clear that this inactive state had to be erased in the embryo proper before random X-inactivation could take place. Indeed, X-reactivation occurs in the inner cell mass of blastocysts between E3.5 and E4.5 around the time point of implantation into the uterus (128, 172). This reprogramming event is restricted to epiblast cells, which are positive for the pluripotency marker Nanog (128; B.P., unpublished). On the other hand, primitive endoderm (PE) and trophectoderm (TE) cells, which will form the extraembryonic tissues like the placenta, retain imprinted inactivation of the Xp. The initial sign of X-reactivation is the downregulation of *Xist* expression on the Xp, which goes hand in hand with the loss of Xp-localization of the Ezh2-Eed polycomb complex. Subsequently, the typical foci of H3K27 and H3K9 methylation on the Xp also

disappear and paternal X-linked genes become reactivated.

X-Reactivation in Primordial Germ Cells

The second X-reactivation event during embryonic development occurs during germ cell development. It has long been known that both X chromosomes in mammalian oocytes are transcriptionally active (54). Furthermore, it was shown that female PGCs (primordial germ cells) display random X-inactivation (137), which was thought to be reversed after the colonization of the genital ridges around the onset of meiosis (104, 148, 232). This is also the same time point at which global DNA-demethylation and erasure of autosomal imprints take place (71). However, more recent studies have revealed that a series of progressive chromatin changes begin much earlier during PGC migration with DNA-demethylation and exchange of histone variants in the genital ridges being the final reprogramming steps (70, 204, 205). Indeed, this early, albeit slowly advancing, reprogramming process is also reflected in the kinetics of X-reactivation in PGCs, which begins almost as soon as PGCs are specified, but is not completed until much later when oocytes undergo meiosis (223). The first sign is the downregulation of *Xist* expression, which can be observed in some PGCs as early as E7.0 (around specification) and is complete about E10.5 (after entering the genital ridges). Although the overall nuclear H3K27me3 levels increase during PGC development, the distinct foci on the Xi disappear following *Xist* downregulation (31, 48). Then, the X-linked genes furthest away from the *Xic* start to become reactivated around E8.75 during PGC migration, followed by the genes in the *Xic* vicinity, which only reactivate once germ cells start to undergo meiosis or even later (31, 223). Although X-reactivation starts early, it becomes most complete within the environment of the genital ridge where diffusible factors secreted from XX gonadal somatic cells stimulate the X-reactivation process (31). This suggests that not only an intrinsic program within the PGCs but also XX-specific extrinsic gonadal signals induce the final steps of X chromosome reprogramming. In conclusion, X-reactivation in PGCs appears to be a slow multistep process lasting over several days that initiates much earlier then previously thought right after PGC specification.

X-Reactivation in Vitro

An interesting feature of X-inactivation is its correlation with the differentiated cell state and the presence of two active X chromosomes in pluripotent stem cells and their embryonic ancestor cells (epiblast, PGCs), which all express characteristic pluripotency markers like Oct4 and Nanog. Epigenetic reprogramming and X-reactivation occur not only in vivo but also in vitro. For example, cell fusion between pluripotent stem cells and somatic cells results in reprogramming of the somatic nucleus including X-reactivation (226, 230). Remarkably, this reprogramming activity is not restricted only to female stem cells; X-reactivation has also been demonstrated in fusions between male ES cells and female somatic cells (226). Furthermore, Nanog overexpression seems to increase overall reprogramming efficiency during cell fusion (213). This, together with the expression of Nanog during X-reactivation in vivo, suggests that it might play a direct or indirect role in the process.

Another method of in vitro reprogramming is the induction of pluripotency by defined transcription factors (231; reviewed in Reference 114). Retroviral transfection with expression constructs for *Oct4*, *Sox2*, *Klf4* and *c-Myc* can revert the differentiated state of somatic cells and convert them into induced pluripotent stem (iPS) cells. These cells share a number of properties with embryo-derived pluripotent stem cells; for example, contribution to all tissues including the germline when injected into host blastocysts or gene expression and chromatin modification profiles almost indistinguishable from ES cells (127, 174, 256). The epigenetic reprogramming during iPS cell generation also involves gradual X-reactivation in female cells,

Numerator: X-linked factors (X) in the X:A ratio during X chromosome counting

Denominator: autosomal factors (A), which are used during X chromosome counting to assess the X chromosome to autosome (X:A) ratio

Blocking factor: a hypothetical complex of autosomal and X-linked factors, which protects one X chromosome (Xa) per diploid genome from inactivation

Competence factor: a hypothetical X-linked factor, which binds the future Xi and induces X-inactivation

which occurs with similar kinetics as in the induction of endogenous pluripotency genes *Oct4* and *Sox2* (127, 219). X chromosome reprogramming seems to be complete in iPS cells because after their differentiation, random X-inactivation takes place again, showing that the memory of the previous inactivation state has been erased (127).

In conclusion, X-reactivation is a hallmark of epigenetic reprogramming in diverse systems both in vivo and in vitro and is associated with the state of pluripotency. Uncovering the exact mechanisms should provide further insights into how the epigenome can be reset from a differentiated to a pluripotent state, with implications extending far beyond the field of X-inactivation research.

RANDOM X-INACTIVATION: PUZZLING THE MOSAIC TOGETHER

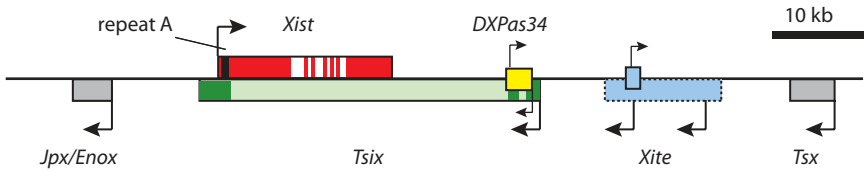
After X-reactivation the pluripotent epiblast cells harbor two active X chromosomes. Following implantation when epiblast cells start undergoing lineage-specific differentiation, random X-inactivation takes place (**Figure 2**) (147, 233). This also happens in vitro during the differentiation of such pluripotent cell lines as embryonic carcinoma and ES cells (131, 190), making them useful tools to dissect the genetic and molecular bases of the random X-inactivation process. Random X-inactivation occurs in a genetically separable stepwise manner and is controlled by DNA elements and noncoding RNAs at the *Xic* (**Figure 3**), the most prominent of which are *Xist* and *Tsix* (see References 4, 16, 182, 257 for reviews). In the initial "counting" step, cells measure the X chromosome: autosome ratio in order to ensure that the appropriate number of X chromosomes, one per diploid cell, is inactivated. After that the "choice" step occurs: one X chromosome is randomly designated to become the active X chromosome (Xa), and the other (or others if there are more than two X chromosomes) to become the Xi. In the following "silencing" period, the Xi is coated by *Xist* RNA and transformed into a transcriptionally inert state by recruitment of repressive chromatin modification complexes. During the final "maintenance" phase, the silent state of the Xi is propagated over the following cell divisions and throughout the lifetime of the organism, unless it is reverted by reprogramming in the germline (see above) or if the Xi is lost during pathological situations, e.g., cancer.

Counting and Choice, Multiple Mechanisms for One Purpose

X chromosome counting involves the assessment of X chromosome number in relation to autosome number. During this process, X-linked "numerator" and autosomal "denominator" elements are somehow titrated against each other to calculate the X chromosome: autosome ratio (4, 108, 110). According to the "blocking factor" model, a blocking factor complex, which is made out of both X chromosomal and autosomal components, breaks the symmetry between X chromosomes by binding preferentially to the *Xic* of the future Xa, thereby inhibiting *Xist* upregulation and X-inactivation (163). Knockout and transgenic analyses have identified DNA elements within the *DXPas34*, *Tsix*, and *Xite* as numerators on the X chromosome (34, 108, 110, 150, 247). When these regions are deleted, aberrant numbers of inactive X chromosomes appear. In addition to a blocking factor, an X-linked "competence factor" might also exist (108, 110). When the competence factor is produced from more than one X chromosome per diploid cell, *Xist* is activated. Although the numerator regions on the X chromosomes have been at least partially identified, the autosomal denominators are unknown. The molecular nature of blocking and competence factors remains elusive; future investigations are needed to grasp the detailed mechanism of X chromosome counting.

The question of how the active and inactive X chromosomes are chosen is tightly linked to the counting process and has long been a major focus of attention of X-inactivation research. Ultimately, the "choice" depends on

a



b

Element	Type	Functions	Transgenic phenotype	Mutant phenotype	References
Xist	Non-coding RNA	Silencing of Xi, xiRNAs	Ectopic silencing if expression induced	X-inactivation disrupted, paternal transmission embryonic lethal in females (imprinting)	130, 179, 258
Repeat A	RNA-Domain of *Xist*	Silencing domain of *Xist*, translocation of X-linked genes into silencing compartment	Not sufficient for coating	*Xist* silencing function disrupted, X-linked genes not relocated into silencing compartment	27, 259
Tsix	Non-coding RNA	Antisense regulator of *Xist*, recruitment of chromatin modifiers to *Xist*-promoter, xiRNAs	X-inactivation block → cell death (counting), ectopic pairing, choice of X without *Tsix* expression	*Xist* partially de-repressed, maternal transmission embryonic lethal in both sexes (imprinting), choice skewed toward *Tsix*-mutant Xi	105, 108, 110, 121, 158, 167, 197, 200, 224, 261
DXPas34	Multifunctional DNA-element, bidirectional promoter	Enhancer + regulator of *Tsix*, pairing, counting, insulator, Ctcf + Yy1 binding sites	X-inactivation block → cell death (counting), ectopic pairing	Down-/upregulation of *Tsix* during/after X-inactivation, *Xist* partially de-repressed, maternal transmission embryonic lethal in both sexes (imprinting), choice skewed toward mutant Xi	24 ,37, 49, 50, 108, 221, 247, 260
Xite	Non-coding RNA, enhancer	Enhancer of *Tsix*, counting, pairing	X-inactivation block → cell death (counting), ectopic pairing	Down regulation of *Tsix*, choice skewed toward mutantX	108, 166, 221, 260
Jpx/Enox	Non-coding RNA	*Xist* regulator?	ND	ND	30, 93, 237

Figure 3

Elements of the mouse X-inactivation center (*Xic*) and their functions. (*a*) Diagram depicting the location and transcriptional direction of *Xic*-elements. Elements of unknown size have a dotted outline. (*b*) Table summarizing the functions of *Xic*-elements. Transgenic phenotype refers to phenotypes observed for either autosomal transgenes or forced expression of an endogenous allele of an element on one X chromosome.

what regulates *Xist* expression in an allele-specific manner. A crucial repressor of *Xist* is its overlapping antisense gene, *Tsix* (110). The *Tsix*-expressing X chromosome becomes the Xa, whereas the *Xist*-expressing chromosome is designated to become the Xi. *Tsix* transcription itself is activated by *DXPas34* and *Xite* (37, 49, 166, 221, 247), shifting the solution to what makes the "choice" further upstream to the question of what regulates the regulators. As noted above in the context of imprinted X-inactivation, *DXPas34* is bound by

the chromatin insulator protein, Ctcf, and its binding partner and transcriptional activator, Yy1 (24, 50). This complex induces transcription of *Tsix* and also might block access of *Xist* to downstream enhancers, thereby contributing to the choice of the Xa. CTCF and YY1 also bind to the human *XIST* promoter, and families with point mutations in the CTCF binding site display a skewing of X-inactivation choice toward preferential inactivation of the mutant X chromosome (82, 184, 188).

The regulatory crosstalk between these elements is also reflected by their intrachromosomal interactions, which have been observed to occur during X-inactivation (237). Using chromosome confirmation capture (3C), it was shown that *Xite* and *Tsix* physically interact with each other whenever *Tsix* is expressed, underscoring the proposal that *Xite* is an enhancer of *Tsix* (166, 221). Another interaction that has been observed occurs between *Xist* and its neighboring noncoding gene *Jpx* (30, 93), in particular at stages when *Xist* is upregulated or poised for transcription (237). Thus *Jpx* might be the first known positive regulator of *Xist* located at the *Xic*, which stands in opposition to the other noncoding elements at the *Xic*, known for their repressive influence on *Xist*.

Although proposed some time ago (105a, 129), only over the past few years has a new mechanism involved in counting and choice been uncovered: X chromosome pairing (6, 261; reviewed in Reference 1). Before the onset of X-inactivation by *Xist* upregulation, the *Xics* briefly colocalize within the nucleus, which possibly facilitates the exchange of information between the X chromosomes to determine their future inactive or active state, respectively. This crosstalk is facilitated by the *Xite* and *Tsix* regions, necessary not only for pairing but also sufficient for ectopic pairing of autosomal transgenes with the X chromosomes (261). Ectopic pairing between X chromosomes and multicopy transgenes on autosomes outcompetes endogenous X-X pairing. This results in failure to upregulate *Xist* and undergo X-inactivation (105a). Cell differentiation is also inhibited, suggesting that X-X pairing is required for faithful counting and choice and initiation of X-inactivation (108, 261). In particular, the 1.6 kb *DXPas34* region was shown able to mediate pairing by itself, and this is thought to be accomplished, at least in part, by its binding factor Ctcf, as Ctcf knockdown abolishes the pairing process (260). Furthermore, transcription seems to be necessary, suggesting that *Tsix* and *Xite* transcription in combination with binding of Ctcf and other pairing factors is crucial for the X-X pairing mechanism. Another "X-pairing region" (*Xpr*) has been reported to lie 200 kb upstream of *Xist* and cause pairing even in undifferentiated ES cells prior to X-inactivation (3). This interaction has been postulated to occur before the pairing in the *Tsix/Xite* regions. Whether *Xpr* is crucial for X-inactivation remains to be investigated by deletion analysis.

Apart from *trans*-interaction, another type of interaction related to choice is proposed to occur in undifferentiated ES cells (146). When detecting the *Xics* by fluorescence in situ hybridization, either X chromosome can give a one- or two-pinpoint signal. In *Xist* or *Tsix* heterozygous cells, the allele destined to become inactivated mostly shows one dot and the future active X has two dots. In wild-type cells also a single dot might mark the future Xi and a double dot the future Xa. These states can switch in undifferentiated cells and become fixed only once X-inactivation occurs. This phenomenon might be explained by different strengths in sister chromatid cohesion after DNA-replication between the future Xi and Xa. Whether this is indeed the case and what it means functionally still need to be examined.

A recent alternative hypothesis for counting and choice claims that the processes occur completely stochastically (149). Analysis of *Xist* upregulation in diploid and tetraploid ES cells during differentiation revealed that each X chromosome in a cell has a certain likelihood of initiating X-inactivation depending on the X chromosome:autosome ratio. This results in a proportion of cells showing abnormally high or low numbers of *Xist* clouds during differentiation. However, the number of these

cells decreases over time, by either cell selection against them or readjustment of the number of inactive and active Xs per cell. Deletion of a large *Xic* region comprising *Xist*, *DXPas34*, *Tsix*, and *Xite* on one X chromosome in diploid female ES cells and mice did not affect inactivation of the wild-type X chromosome, suggesting that the whole region and its binding to the proposed blocking factor might be dispensable for counting. On the contrary, an X-encoded competence factor outside the deleted region might regulate the promotion of X-inactivation in a dosage-sensitive manner.

Clearly, the complexity of X chromosome counting and choice is still only poorly understood despite substantial recent advances. X-inactivation researchers now face the puzzling challenge of incorporating the multiple concepts and models into one unifying theory that will explain how the initiation of X-inactivation is precisely controlled.

How *Tsix* Regulates *Xist*

A key event in the choice of an X chromosome to become active or inactive is the repression of *Xist* by its antisense gene *Tsix*. Knockout experiments have shown that *Tsix* is instrumental in repressing *Xist*, as in heterozygous *Tsix* deletions the mutant X chromosome always becomes the *Xist*-expressing Xi (110, 121, 210). During X-inactivation in ES cells, *Tsix* is expressed on the Xa but is downregulated on the Xi causing *Xist* to be upregulated (110). While *Xist* remains expressed on the Xi during X-inactivation maintenance, *Tsix* expression ceases on the Xa and is therefore not required to keep *Xist* shut off (109).

Multiple studies have described how *Tsix* might regulate *Xist*. First, *Tsix* does not only work as a silencing DNA-element, as truncation mutants of *Tsix* transcription result in derepression of *Xist* without removal of any DNA sequences (121, 210). In addition, forced expression of *Tsix* blocks the upregulation of *Xist* from the modified X chromosome (121, 220), but splicing of *Tsix* is not necessary for blocking *Xist* in *cis* (198). Therefore, in order to repress *Xist*, *Tsix* either needs to be transcribed through the *Xist* promoter in the antisense direction and/or is required as a full-length unspliced RNA.

Greater insight into *Tsix*'s mode of action has come from studies addressing *Xist* chromatin status during X-inactivation in the presence or absence of *Tsix* (158, 197, 224). In *Tsix* mutant embryos the *Xist* promoter appears to be in a more open and transcriptionally permissive state that allows ectopic *Xist* expression from the mutant chromosome (197). Therefore it was concluded that the role of *Tsix* is to create a heterochromatic state at the *Xist* promoter on the Xa in order to keep the *Xist* gene switched off. Similar observations were made in two other related studies where *Tsix* truncation resulted in accumulation of active histone marks such as H3K4 di- and trimethylation and H3K9 acetylation and the downregulation of H3K9me3 and DNA methylation at the *Xist* promoter (157, 158). However, *Tsix* deletion does not only cause an upregulation of active marks but causes also elevated levels of H3K27me3, a mark usually associated with repressive chromatin (224). This finding, although initially appearing to be contradictory, can be explained in two different ways. First, these observations were made at partially different time points using different experimental systems. The *Xist* promoter on the Xa displays a more euchromatic histone mark configuration after differentiation, when the critical X-inactivation events have already happened, but not during X-inactivation, when the presence of the H3K27me3 mark suggests a more heterochromatic state (224). Indeed, this transient heterochromatic state might even contribute to *Xist* upregulation, a phenomenon, postulated for heterochromatin genes in *Drosophila* (250). A second explanation would be that the *Xist* promoter is an example for a so-called bivalent chromatin domain (12). Bivalent domains are characteristic for regulatory elements of developmental genes in ES cells and are marked both by H3K27 and H3K4 methylation. These genes are then expressed at low levels and poised for subsequent activation after differentiation.

As in the case of the *Xist* promoter, the bivalent domains resolve after differentiation into exclusive H3K4 methylation if the genes are to be expressed, or they remain exclusively methylated at H3K27 if they are repressed. In conclusion, it is now clear that *Tsix* regulates *Xist* by affecting its chromatin configuration. However, it still needs to be tested whether this is done purely by antisense-transcription and/or by *Tsix* full-length RNA, and if *Xist* upregulation is initiated while being in a bivalent or heterochromatic histone mark configuration.

Curiously, during X-inactivation maintenance, the chromatin marks switch between the Xa and Xi. The active *Xist* promoter on Xi is thus marked by H3K4 methylation while the inactive *Xist* promoter on Xa is marked by H3K27me3, as it also is in male cells (197, 224). Deletion of *Tsix* in male cells is not sufficient to fully derepress the *Xist* promoter (168, 247), but it is sufficient in combination with a *Eed* mutation (211). Therefore the *Xist* promoter in male cells is repressed in a synergistic manner by *Tsix* and Eed, which is necessary as part of the PRC2 complex to establish the H3K27me3 mark.

Intersection of the X-Inactivation and Short RNA Pathways

Evidence for an involvement of *Tsix* RNA into *Xist* regulation comes from the recent observation that *Xist* and *Tsix* form duplexes in vivo (167). Double-stranded *Xist/Tsix* duplexes are detectable in both male and female undifferentiated ES cells before the onset of X-inactivation and decrease in levels during differentiation. In an almost inverse correlation, small RNAs from the *Xist/Tsix* locus termed xiRNAs were found during differentiation but were not detectable before or after. As the xiRNAs are present in both male and female cells, it is suspected that they are generated specifically from *Xist/Tsix* duplexes from the Xa. The production of these xiRNAs is either directly or indirectly dependent on Dicer, a key ribonuclease in the RNAi pathway cleaving long double-stranded precursors. In *Dicer*-mutant ES cells, xiRNAs are strongly reduced, and *Xist* becomes derepressed on the Xa. On the other hand, *Xist*-coating of the Xi and recruitment of the H3K27me3 mark is also disrupted in *Dicer*-mutant cells, which can be partially rescued by *Tsix* mutation in *Dicer/Tsix* double mutant ES cells. Consequently, despite being dispensable for X-inactivation maintenance (36), Dicer appears to have a dual role in X-inactivation, both for repressing of *Xist* on the Xa and its spreading and silencing on the Xi. Furthermore, *Tsix* RNA might potentially regulate *Xist* by formation of xiRNAs. However, it is unclear how this is mechanistically achieved and especially whether xiRNAs influence the chromatin status of the *Xist* promoter.

X Chromosome-Wide Silencing and the Escape from It

After the choice has been made on which X chromosome is going to be the Xi and Xa, the recruitment of repressive complexes to the Xi initiates the silencing process (see References 77, 160, 182 for reviews). *Xist* thereby plays a critical role, although the factors that directly bind to *Xist* RNA, and how this controls the recruitment process, are poorly understood. Analysis of inducible *Xist* transgenes in ES cells revealed distinct functional domains of mouse *Xist* RNA (259). At the 5′ end of *Xist* lies the so-called repeat A sequence, which is responsible for the silencing function of *Xist*, while the coating of the X chromosome is mediated by other regions distributed over the rest of the RNA. For human *XIST*, however, the A-repeats are required both for the silencing and coating function (29). Silencing cannot be induced by *Xist* at any arbitrary time but rather is restricted to specific developmental time windows during ES cell differentiation, early embryonic development, during differentiation of the blood cell lineage, and in human cancer cell lines (29, 73, 201, 258). Therefore *Xist* expression is clearly not sufficient to cause X-inactivation on its own but needs the appropriate cellular context in which the critical epigenetic regulators are present and the X chromosome chromatin is susceptible to silencing.

A key group of players for X chromosome silencing are the polycomb repressive complexes PRC1 and PRC2. Early on during silencing, PRC2, consisting of the histone methyltransferase Ezh2 and its cofactors Eed and Suz12, establishes the characteristic H3K27me3 mark on the Xi (181, 214). The PRC1 complex with its catalytic subunit Ring1B is also recruited to the Xi during establishment of silencing and is responsible for monoubiquitination of H2AK119 (47, 58, 183). Despite their specific localization to the Xi during silencing, both Ring1B and Eed and therefore both PRC1 and PRC2 and their consequential chromatin marks H2AK119ub1 and H3K27me3 are not essential for random X-inactivation (94, 111). Both complexes and modifications are also recruited to the Xi without the crucial repeat A silencing domain of *Xist*, however, the recruitment efficiency for H3K27me3 is markedly reduced when repeat A is deleted (103, 181, 203). Nevertheless, as both marks are recruited at least to some extent even by the silencing-deficient form of *Xist*, H3K27me3 and H2AK119ub1 are clearly not sufficient to initiate silencing. PRC1 is recruited to the Xi in a PRC2-dependent and by a PRC2-independent mode (203). These observations speak for multiple levels of redundancy, both in the recruitment of silencing complexes and in their silencing function, in which PRC1 and PRC2 might be able to compensate for each other. It will be interesting to see if X-inactivation can still take place in cells defective for both PRC1 and PRC2 function.

Apart from the PRC complexes and their associated histone modifications, a number of additional characteristics are associated with the Xi during silencing. Examples are late replication timing (228), the establishment of H3K9me2 (15, 81, 139, 180) and H4K20me (103) marks, and the exclusion of active chromatin marks like H3K4me (81) and histone H4 acetylation (101). Later on, the histone variant macroH2A1 is incorporated (39) and DNA methylation is placed upon promoters of X-linked genes, which is seen as a stabilizing permanent mark important for long-term maintenance of the silent state (see Reference 78 for a review). The human inactive X chromosome appears to be organized in two distinct types of facultative heterochromatin: one characterized by *XIST* RNA association, the H3K27me3 mark, and macroH2A incorporation; and the second one defined by HP1 association and H3K9me3 and H4K20me3 histone marks (23). What the different effects of the two chromatin types on silencing of X-linked genes are and whether one domain is more efficiently or more stably silenced than the other remain to be determined.

The establishment of repression across the Xi during X-inactivation goes hand in hand with the formation of a silencing compartment set up by the *Xist* RNA, from which the transcriptional machinery is excluded (27). This compartment is first established by an A-repeat-independent mechanism and consists of a core of predominantly nongenic repetitive DNA sequences, whereas expressed X-linked genes are localized outside the compartment (27, 33). During silencing X-linked genes are recruited into the *Xist* RNA compartment in an A-repeat-dependent manner. A candidate factor involved in formation or maintenance of the silencing compartment might be the DNA-, RNA-, and nuclear matrix-binding factor SAF-A, which is enriched at the Xi (57).

Genes that do not become repressed and escape X-inactivation remain outside the *Xist* silencing compartment. Escaping genes can be subdivided into two groups: genes within the pseudoautosomal region (PAR) and genes outside of it. Genes within the PAR do not need to be dosage compensated, as they have their equivalent on the Y chromosome as well and are therefore present in equal copy numbers between males and females. On the other hand, some genes outside the PAR are present twice in females and only once in males but still escape X-inactivation. Although only few genes escape X-inactivation in mice, between 15%-25% of human X-linked genes were reported to escape (22); however, a more recent report claims that only about 5% escape (92). The majority of the escapees outside the PAR are localized to regions, which are evolutionarily younger

unconserved parts of the human X chromosome (22). This is in agreement with the hypothesis that X-linked genes became recruited gradually to the X-inactivation machinery, once during sex chromosome evolution their homologues on the Y chromosome had disappeared (see above; 153, 170; reviewed in 25, 63). Furthermore, the LINE-1 element density is low in regions where escape from X-inactivation is frequent and high in regions without many escaping genes (6a, 22, 195b). This inverse correlation supports the LINE hypothesis, in which LINE-1 repeats boost the spreading of *Xist* RNA along the Xi and thereby help the recruitment of genes to the silencing compartment (see References 123, 125 for reviews). Indeed, the spreading of *Xist* RNA into autosomes on X chromosome/autosome translocations is particularly inefficient, possibly because autosomes have a lower LINE-1 density than the X chromosome (185).

Escaping genes are frequently organized in clusters and are therefore likely separated from adjacent inactivated genes by chromatin boundaries. In one study the insulator protein Ctcf has been postulated as an instrumental factor in shielding escaping from inactivated genes (59). Ctcf thereby might separate these domains and block the spreading of CpG methylation into the escaping domain. Consequently, genes that have been initially silenced could be reactivated and thus escape the silencing while the inactivated genes are kept inactive by DNA methylation. However, Ctcf insulators on their own appear not to be sufficient to protect genes from X-inactivation, as an X-linked transgenic GFP-reporter gene flanked by chicken beta-globin insulator sequences was silenced by both random and imprinted X-inactivation (32). As a result, additional sequences besides Ctcf insulators are needed for efficient separation of inactivated from escaping X chromosome domains.

SILENCING MAINTENANCE

Once X chromosome silencing has been established, it is stably maintained over subsequent cell divisions for the entire lifetime of the organism except in the germline, where X-reactivation occurs (see above). Although *Xist* remains expressed on the Xi it is apparently not absolutely required for maintenance of X chromosome silencing, as *Xist* deletion after X-inactivation does not automatically result in global X-reactivation (20, 44). Nevertheless, both macroH2A localization (44) and H3K27me3 enrichment (264) disappear from the Xi after *Xist* deletion, which is in agreement with the observation that these three marks (*XIST* RNA, macroH2A and H3K27me3) normally co-occur on the human Xi (23). Furthermore, some X-linked genes do become reactivated after *Xist* deletion (43, 264), and additionally blocking DNA methylation or histone deacetylation greatly increases reactivation frequency (43). For random X-inactivation maintenance, DNA methylation is a key stabilizing factor, as deletion of the maintenance DNA methyltransferase gene *Dnmt1* results in X-reactivation in the embryo proper, whereas imprinted X-inactivation maintenance in the placenta is independent of DNA methylation (196). DNA methylation is not only important for maintaining gene silencing on the Xi, but is also necessary for the maintenance of the repressed state of *Xist* on the Xa. Lack of DNA methylation at the *Xist* promoter leads to frequent derepression of *Xist* resulting in inappropriate silencing of the Xa (11, 176). Recently it was shown that the DNA methylation-dependent repression of *Xist* is at least partially mediated through the DNA methylation binding protein Mbd2, which acts by the recruitment of histone deacetylases (8).

In contrast to DNA methylation, the PRC2 component Eed and as a consequence H3K27me3 may be dispensable for maintenance of random but important for maintaining imprinted X-inactivation (94, 252). This might be explained by the redundancy between the PRC2 and PRC1 silencing complexes during random X-inactivation (203). Thus it seems that multiple repressive marks on the Xi, like DNA methylation and hypoacetylated histones on one hand and *Xist* and its associated chromatin marks on the other hand, act in

complementary pathways to safeguard proper maintenance of random X-inactivation (43).

Using a random mutagenesis screen, a novel player in X-inactivation maintenance has been recently identified in SmcHD1, a protein containing a structural maintenance of chromosomes hinge domain (14). Despite normal *Xist* RNA, Eed, and H3K27me3 localization to the Xi in *Smchd1* mutant embryos, DNA methylation of X-linked CpG islands and gene repression is perturbed. The maintenance of both random and imprinted X-inactivation appears to be affected, which indicates that SmcHD1 must act also through a DNA methylation-independent mechanism, as DNA methylation is not required for maintenance of imprinted X-inactivation (196). Therefore the exact role of SmcHD1 still needs to be determined.

Nuclear compartmentalization also plays an important role. As early as 1949, Barr & Bertram noticed a distinct structure, named thereafter "Barr body" in female but not male cat neurons near the nucleolus, which they postulated to be heterochromatin related to the two X chromosomes in females (9). Indeed, the Xi localizes to the perinucleolar region within an Snf2h-enriched ring during mid-to-late S phase in an *Xist*-dependent manner (264). In *Xist*-mutant cells the perinucleolar association of the Xi is lost, leading to the disappearance of repressive chromatin marks and partial reactivation of X-linked genes. Consequently, the heterochromatic state of the Xi seems to be replicated in the perinucleolar region during S phase, which is a requirement for the faithful maintenance of X-inactivation.

XA UPREGULATION: DOSAGE COMPENSATION BETWEEN X-LINKED AND AUTOSOMAL GENES

While the main focus of dosage compensation research has previously been on the need to balance gene dosage between the sexes with unequal number of X chromosomes, another aspect of the story has long been proposed (170), but only recently has it begun to be addressed: the potential imbalance between X-linked and autosomal gene expression (see References 28, 79 for reviews). As the sex chromosomes themselves have initially evolved from autosomes (see above), X-linked genes, which were previously present in two copies, were reduced in males to only a single copy. Therefore, in order to keep X-linked gene expression at its ancestral diploid level, mechanisms similar to that in *Drosophila* were developed to boost X-dosage also in other species including *C. elegans* and mammals (69, 92, 117, 162). The upregulation of gene expression on the mammalian X chromosome then in turn might have caused the need for X-inactivation in females to avoid a gene dosage of X-linked genes twice as high as that of autosomal genes (25).

Support for this hypothesis comes from global expression comparisons between X chromosomal and autosomal gene dosage, which have now been performed in a number of organisms (69, 92, 117, 162). Hyperactivity of the Xa in both male and female mice and human males can be inferred from the observation that the X:autosome expression ratio in somatic tissues is close to 1. In the germline on the other hand, X chromosome activity is not upregulated in order to keep the gene dosage in balance with the haploid autosome set (162). The upregulation of the Xa in mammalian somatic cells resembles the dosage compensation mechanism in *Drosophila* (see References 120, 222 for reviews). However, nothing is presently known of how this is mechanistically achieved in mammals. It will be interesting to clarify how Xa upregulation is controlled—to date a neglected but potentially ancestral aspect of mammalian dosage compensation.

CONCLUDING REMARKS

Mammalian dosage compensation research has come a long way since the first description of the "Barr body" in female cat neurons (9) and Mary Lyon's visionary proposals about the random inactivation of a single X chromosome in every female cell during embryogenesis (122). Decades after these initial findings, the

Table 1 Summary of factors involved in different aspects of X chromosome inactivation (XCI)

Factor	Type	XCI Functions	Mutant XCI Phenotype	References
Polycomb group proteins				
PRC2 (Eed, Ezh2, Suz12):				
Ezh2/ Enx1	HMTase	H3K27me3 mark on Xi during imprinted + random XCI	Loss of H3K27me3 and Eed from Xi	56, 181, 214
Eed	PRC2 component	Ezh2 cofactor	Loss of H3K27me3 from Xi, some disruption of imprinted XCI, dispensable for random XCI initiation + maintenance	94, 95, 203, 214, 252
PRC1 (Bmi1, Cbx2, Cbx7, Phc1, Phc2, Ring1A, Ring1B):				
Ring1A	E3 Ubiquitin ligase	H2AK119ub1 mark on Xi during random XCI maintenance	H2AK119ub1 reduced on Xi, dispensable for random XCI maintenance	47, 58, 183
Ring1B/ Rnf2	E3 Ubiquitin ligase	H2AK119ub1 mark on Xi during imprinted + random XCI	H2AK119ub1 reduced on Xi, dispensable for random XCI initiation + maintenance	47, 58, 111, 203
DNA methylation				
Dnmt1	Maintenance DNA MTase	Maintenance of CpG methylation on Xi and at *Xist* promoter on Xa	Hypomethylation of Xi, loss of random but not imprinted XCI maintenance, *Xist* de-repression on Xa causing ectopic silencing	11, 176, 196
Dnmt3a+ Dnmt3b	De novo DNA MTases	Establishment of CpG methylation on Xi and at *Xist* promoter on Xa	Dispensable for initiation + maintenance of random and maternally imprinted XCI, *Xist* de-repression on Xa but no ectopic XCI	97, 199
Mbd2	Methylated DNA binding protein	DNA methylation-dependent recruitment of HDACs to *Xist* promoter on Xa	*Xist* derepression especially if also Dnmt1 or HDAC deficient	8
Histone variants				
macroH2A	H2A variant	Incorporation in XY body at MSCI, late repressive mark in imprinted and random XCI	X-reactivation only, if macroH2A1 RNAi in combination with HDAC and DNA methylation inhibitors	39, 40, 66, 83
γH2AX	Phosphorylated H2A variant	Incorporation in XY body at MSCI	Meiotic arrest, H2AX essential for MSCI	238*
H2A.Z	H2A variant	Replacement of macroH2A at XY body post MSCI, MSCI maintenance?	ND	66
H3.3	H3 variant	Incorporation in XY body at MSCI, predominant in paternal pronucleus	ND	236, 243, 245

(*Continued*)

Table 1 (*Continued*)

Factor	Type	XCI Functions	Mutant XCI Phenotype	References
Others				
Atr	PI3-like kinase	Phosphorylation of Ser139 of H2AX → γH2AX during MSCI	ND	238*
Brca1	Tumor suppressor	Recruitment of Atr to XY body during MSCI	Perturbed recruitment of Atr to XY body → MSCI failure	238*
Ctcf	Chromatin Insulator	Separation of XCI-escaping from inactivated genes, DNA-methylation-dependent switch and enhancer blocker within *Xic*, transcriptional activator of *Tsix*, *Xic* pairing, *Xist* activator on Xi (?)	Disruption of *Xic* pairing after Ctcf RNAi, skewed XCI-choice if *CTCF* binding site mutated in human *XIST* promoter	24, 50, 59, 82, 157, 184, 188, 260
Dicer	RNase for RNAi + miRNAs	xiRNA generation, *Xist* repression, *Xist* coating	xiRNAs reduced, *Xist* de-repressed on Xa, *Xist* coating of Xi lost, dispensable for XCI maintenance in T-cells	36, 167
Cullin3/ Spop	E3 Ubiquitin ligase	Ubiquitination of PRC1 protein Bmi1 and macroH2A	Cullin3/Spop RNAi leads to loss of macroH2A from Xi → X-reactivation if in combination with HDAC and DNA methylation inhibitors	83
G9a	HMTase	H3K9me2 mark on Xi (?) during XCI	Dispensable for XCI maintenance	169
HP1	Heterochro matin protein	Heterochromatinization of XY body during MSCI and of distinct domains on human Xi during random XCI	ND	23, 66, 155, 240
Pr-Set7	HMTase	H4K20me1 mark on Xi during random XCI	ND	103
SAF-A	Scaffold attachment factor	Immobilization of *XIST* RNA (?), stabilize XCI (?), compartment formation (?)	ND	57
SmcHD1	Structural maintenance of chromosomes	Recruitment of DNA methylation to Xi, maintenance of random and imprinted XCI	Loss of maintenance of imprinted and random XCI, loss of X-linked CpG DNA methylation	14
Yy1	Transcriptional regulator	Ctcf cofactor, binds to *XIST* promoter and regions in *Tsix*, transcriptional activator of *Tsix*	*Xist* and *Tsix* misregulated, early embryonic lethal in males and females	50, 82

Question marks symbolize proposed functions still requiring experimental verification. Abbreviations: HDAC, histone deacetylase; HMTase, histone methyltransferase; MTase, methyltransferase; ND, not determined; XCI, X chromosome inactivation. * Detailed referencing on MSCI is available in the excellent review by J. Turner (238).

discovery of the *Xic* and its defining elements finally allowed genetic analysis of their functional importance. All X-linked determinants of X-inactivation identified so far are either noncoding RNAs or DNA elements (**Figure 3**). In addition, the elucidation of Xi- and Xa-specific histone modifications, histone variants, and DNA-methylation marks added to our understanding of X-inactivation as a classic epigenetic phenomenon. Only very recently are autosomal *trans*-acting protein factors also being characterized for their roles in various aspects of X-inactivation (**Table 1**). Nuclear compartmentalization, X chromosome pairing, involvement of chromatin regulation, or small RNAs and Xa upregulation are only a few of the novel features of mammalian dosage compensation that have unfolded over the past few years and are pointing at new avenues of investigation. Despite this recent progress, many important questions remain to be answered (see Future Issues below). Mammalian dosage compensation is far from being solved and promises to remain a fruitful area of research at the intersection of epigenetics, pluripotency, and development.

SUMMARY POINTS

1. Genetic sex determination and the ensuing divergence of sex chromosomes results in potential gene dosage imbalances. Mammals developed imprinted and random X-inactivation and the upregulation of gene expression on the Xa to overcome this problem.
2. Evidence speaks for both paternal and maternal X chromosome imprinting marks, which interplay to ensure faithful imprinted X-inactivation.
3. *Xist* is a key factor for both imprinted and random X-inactivation and is controlled by multiple elements at the *Xic* and most prominently by *Tsix*. *Tsix* regulates *Xist* expression through modification of the chromatin status around the *Xist* locus.
4. X chromosome counting and choice during random X-inactivation are regulated by autosomal and X-linked factors. The pairing between the X chromosomes, which occurs during the onset of X-inactivation, appears to be instrumental.
5. The silencing of the X chromosome is achieved by the recruitment of multiple chromatin-modifying complexes and is dependent on a silencing compartment established by *Xist*-RNA. During X-inactivation maintenance, several layers of redundancy ensure faithful long-term silencing.

FUTURE ISSUES

1. How is X-inactivation achieved in marsupials without an *Xic*? Is this mode of imprinted X-inactivation based on preinactivation and inheritance of Xi through the male germline? To what extent are marsupial X-inactivation and imprinted eutherian X-inactivation conserved at the mechanistic level?
2. What is/are the X chromosome imprint(s) in mice, how is its/their erasure accomplished in the blastocyst, and how is random X-inactivation reprogrammed in the germline?
3. How many findings from model organisms can be extrapolated to humans and what is the evolutionary relationship between different modes of X-inactivation?

4. Regarding random X-inactivation, counting and choice of X chromosomes for inactivation remain largely enigmatic and will be one of the hardest puzzles to solve. The degree to which X-inactivation choice is already predetermined in the undifferentiated state or how much it is differentiation-related is unclear, as is the question how X chromosome number is measured against autosome number.
5. What regulates Xa hypertranscription?
6. What is the interplay and functional hierarchy between different epigenetic marks on the Xi?
7. How does silencing spread along the Xi and what roles do repetitive elements play in this process?

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Errata

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