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## Competing interests statement

The authors declare no competing financial interests.

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DATABASES

The following terms in this article are linked online to:

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OPINION

# X-chromosome inactivation: a hypothesis linking ontogeny and phylogeny

*Khanh D. Huynh and Jeannie T. Lee*

Abstract | In mammals, sex is determined by differential inheritance of a pair of dimorphic chromosomes: the gene-rich X chromosome and the gene-poor Y chromosome. To balance the unequal X-chromosome dosage between the XX female and XY male, mammals have adopted a unique form of dosage compensation in which one of the two X chromosomes is inactivated in the female. This mechanism involves a complex, highly coordinated sequence of events and is a very different strategy from those used by other organisms, such as the fruitfly and the worm. Why did mammals choose an inactivation mechanism when other, perhaps simpler, means could have been used? Recent data offer a compelling link between ontogeny and phylogeny. Here, we propose that X-chromosome inactivation and imprinting might have evolved from an ancient genome-defence mechanism that silences unpaired DNA.

In most sexually dimorphic animals in which sex is genetically determined, the basis for sex lies in the differential constitution of the sex chromosomes. In mammals, males have one X and one Y chromosome (XY), whereas females carry two X chromosomes (XX) (reviewed in REF. 1), which results in an imbalance of sex-linked genes between the two sexes. The Y chromosome contains only a small number of functional genes, almost all of which can be classified into two groups: the Y-chromosome-specific genes with male-specific roles (such as testis determination) and genes that have homologues on the

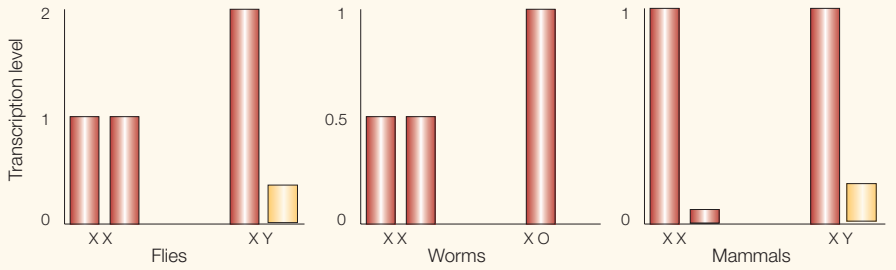


Figure 1 | **Strategies of dosage compensation.** In *Drosophila melanogaster*, male (XY) X-linked expression increases twofold. In *Caenorhabditis elegans*, hermaphrodite (XX) transcription from each of the two X chromosomes decreases twofold. In mammals, one of the two X chromosomes in females is inactivated.

X chromosome and are involved in 'housekeeping' functions[2] (also reviewed in REFS 3,4). These characteristics of Y-linked genes preclude a need for reconciling Y-linked dosage disparity between males and females. On the other hand, the X chromosome contains approximately 1,500 genes[4,5] that have a diverse range of functions. Most of these genes have no Y-chromosome homologue and so are present at twice the dosage in females than in males. This imbalance has led to the evolution of X-linked dosage compensation.

In mammals, dosage compensation occurs by X-chromosome inactivation, whereby one of the two X chromosomes is transcriptionally silenced in the female[6]. X-chromosome inactivation occurs in two forms, both of which are intricate epigenetic processes that are tightly controlled at many levels. In the 'random' form of X-chromsome inactivation, the two X chromosomes in each female cell have an equal chance of being inactivated[6]. This form of X-chromosome inactivation is a multi-step process and takes place in somatic lineages of EUTHERIAN mammals (reviewed in REF. 7). As a first step, a counting mechanism in the zygote determines whether one or two X chromosomes are present in each cell. In the presence of two or more X chromosomes, the X-chromosome inactivation pathway is set in motion. The cell then randomly chooses between its X chromosomes for silencing. Silencing is strictly *cis*-limited, involving only those genes that lie on the same X chromosome. The choice of chromosome and the silencing mechanism require competing interactions between three non-coding loci: X (inactive)-specific transcript (*Xist*)[8,9], its antisense partner *Tsix*[10], and the intergenic locus *Xite*[11]. Whereas *Xite* and *Tsix* preserve the transcriptional competence of the future active X chromosome (Xa)[10–13], *Xist* initiates silencing on the future inactive X chromosome (Xi) through propagation of its RNA along the X chromosome[14,15]. How X-chromosome counting and choice are regulated and how silencing is propagated exclusively in *cis* remain unsolved mysteries in epigenetics.

The imprinted form of X-chromosome inactivation is equally complex. In imprinted X-chromosome inactivation, the paternal X chromosome ($X_p$) is inactivated[16]. Imprinted X-chromosome inactivation is found in cells that comprise the extra-embryonic tissues of certain eutherians, notably the rodent[17] and the cow[18], but remains controversial in humans[19–21]. Interestingly, this form of X-chromosome inactivation is also found in some of the earliest mammals, including the kangaroos and opossums (metatherians), a fact that has led to the popular view that imprinted X-chromosome inactivation evolved first and was transformed into random X-chromosome inactivation in eutherians[22]. Although the rules of counting and choice do not seem to apply, imprinted X-chromosome inactivation also requires *Tsix*[12,13] and *Xist*[23]. However, their expression patterns are pre-determined in the parental germline, rather than by the zygote during early development.

Why do mammals carry out dosage compensation through such complicated means? *Drosophila melanogaster* and *Caenorhabditis elegans* have evolved an ostensibly more straightforward approach to dosage compensation (FIG. 1). As in THERIAN mammals, fruitfly sex is also determined by X and Y chromosomes: XX individuals are female and XY individuals are male (reviewed in REFS 24,25). However, unlike mammals, equalization of X-linked gene expression between the sexes is achieved by global upregulation of the single X chromosome in males[24,25]. In the worm, sex is determined by X-chromosome number (worms do not have a male-specific chromosome): XX individuals are hermaphrodites and XO individuals are males[24]. In this system, dosage compensation occurs by a twofold reduction of activity on each X chromosome in the XX hermaphrodite[24]. As in mammals, dosage compensation in the fruitfly and the worm is initiated by a counting mechanism; but unlike mammals, there is no epigenetic choice, as all X chromosomes in the nucleus are treated equally and changes in transcriptional levels are not *cis*-limited along one X chromosome. So, the fly and worm mechanisms are (at least at a superficial level) easier to imagine and execute.

Recent findings provide hints as to why mammals might have favoured a *cis*-limited silencing mechanism for dosage compensation or, more intriguingly, might even have been forced into adopting this mechanism . Here, we discuss recent insights into the ontogeny of X-chromosome inactivation and argue that they provide a glimpse into its phylogeny.

## The ontogeny of X inactivation

It has long been known that X-chromosome inactivation in mice and humans is completed during embryogenesis, so that in females dosage compensation has taken place in all cells at birth (reviewed in REFS 26–28). But when is dosage compensation necessary and when does X-chromosome inactivation take place? After Mary Lyon's first description of X-chromosome inactivation in the mouse, some debate ensued over the transcriptional status of the X chromosomes in the early embryo and the exact timing of inactivation[29]. One view held that both X chromosomes are active in the XX zygote and silencing of one X chromosome occurs at a later stage. Another view held that one X chromosome might be silenced very early on, so that XX and XY embryos would be dosage compensated in the pre-implantation stage. The possibility was also raised that both X chromosomes might be inactive in the zygote and dosage compensation could involve reactivation of one of them.

A series of elegant experiments in the 1970s and 1980s solidified the first view — specifically, that the XX zygote begins development with two active X chromosomes and X-chromosome inactivation does not take place until implantation (around day 3.5–5.5 of embryonic development in the mouse)[30–36]. For example, a late-replicating X chromosome does not appear until the BLASTOCYST stage, implying that the two X chromosomes are transcriptionally equal in the pre-implantation embryo[36]. Furthermore, expression analysis of the X-encoded hypoxanthine guanine phosphoribosyl transferase (HPRT)[32,33] and α-galactosidase (GLA)[30] proteins indicated that XX embryos have twice the activity level of these proteins than XY embryos have. So, in the consensus view, the differentiating TROPHECTODERM of the blastocyst goes through imprinted X-chromosome inactivation for the first time at the implantation stage, and the EPIBLAST lineage undergoes random X-chromosome

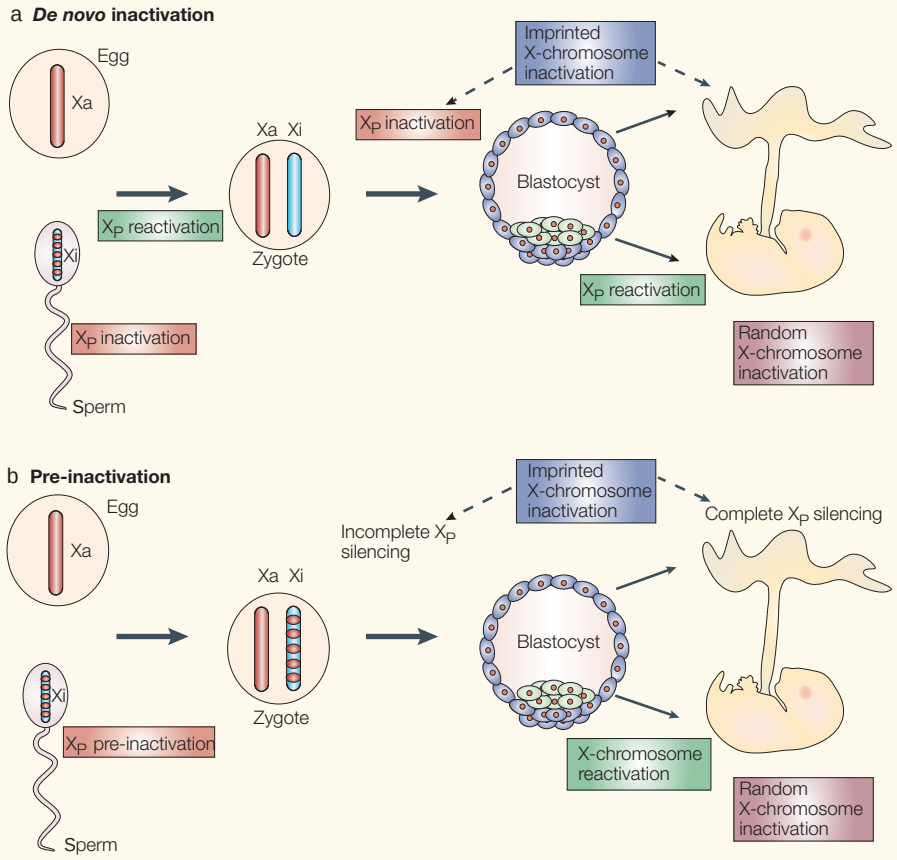


Figure 2 | **The ontogeny of X-chromosome inactivation in the mouse: two current views. a** | The *de novo* inactivation model requires many rounds of inactivation and reactivation: the paternal germline initiates meiotic sex-chromosome inactivation, but the X chromosome is completely reactivated after meiosis. The zygote inherits two fully active X chromosomes and begins re-inactivation of the paternal X chromosome ($X_p$) at the 4- to 8-cell stage. In the trophectoderm (extra-embryonic cells, shown in blue), $X_p$ silencing is maintained, therefore accounting for the imprinted form of X-chromosome inactivation. By contrast, in the epiblast (green cells), yet another round of reactivation takes place in preparation for a final round of inactivation in the form of random X-chromosome inactivation. **b** | In the pre-inactivation model, the female zygote inherits a partially silent $X_p$ and maintains the silent state throughout pre-implantation development. Silencing becomes globalized and complete in extra-embryonic tissues. This accounts for the imprinted form of X-chromosome inactivation. By contrast, the epiblast cells of the inner cell mass (ICM) undergo a single round of reactivation followed by a random form of X-chromosome inactivation.

inactivation as it differentiates into various germ lineages in the post-implantation period[34,36]. Supported by a wealth of evidence, this was the accepted model for almost 30 years.

With the advantage of new technology, recent studies have inspired the need to revise the original model. For example, it has been known for some time that *Xist* RNA, the agent of silencing, is expressed in pre-implantation XX embryos from the 2-cell stage onwards[37–40]. It is also known that some X-linked genes, such as phosphoglycerate kinase 1 (*Pgk1*), seem to be silent on the $X_p$ until after implantation[37,41,42]. Two recent papers provide evidence to indicate that X-chromosome inactivation takes place much earlier than has been conventionally accepted[43,44].

By combining FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) and immunofluoresence on pre-implantation embryos, Okamoto *et al.*[44] showed that the X chromosome that expresses a high level of *Xist* RNA carries chromatin modifications that are typically found on heterochromatin. Histone H3 is hypermethylated at lysine 9 and 27 (H3-K9; H3-K27), histone H3 is hypomethylated at lysine 4 (H3-K4), and polycomb group proteins such as embryonic ectoderm development (EED) and enhancer of zeste homologue 2 (EZH2) localize to that X chromosome. These changes begin at the 4- to 8-cell stages and accumulate as pre-implantation development proceeds. These results indicate that one X chromosome is silenced as early as the 4-cell stage. Using reverse-transcriptase PCR (RT-PCR) and FISH analyses to directly assess X-linked expression, our laboratory showed that one X chromosome is also silenced early, and that it is the $X_p$ that is affected[43]. Allele-specific RT-PCR showed that silencing does not occur uniformly along the chromosome[43]. Genes that are close to the X-inactivation centre (*Xic*) [45], from which *Xist* RNA originates, are more completely silenced than genes that are farther away, indicating that silencing occurs in a graded fashion from the *Xic*.

> "With MSCI also being present in the marsupial germline, the idea that MSUD might be the original imprinting mechanism for X-chromosome inactivation becomes rather attractive."

The degree to which genes can escape silencing and the variability between embryos shows that the pre-implantation form of X-chromosome inactivation is leaky and imperfect in many respects. Interestingly, X-chromosome inactivation becomes more complete after implantation in both the placental and somatic lineages[43]. Consistent with this, *Xist* RNA seems to spread less extensively along the X chromosome in the pre-implantation embryo than in placental and somatic cells.

Although both studies are largely in agreement that X-chromosome inactivation takes place much earlier than the conventionally held view, they differ significantly on the issue of the exact timing of X-chromosome inactivation and, therefore, how it is initiated from a mechanistic standpoint. Okamoto *et al.* reported that RNA polymerase II localizes to the $X_p$ at the 2-cell stage and that transcripts from the cysteine-rich hydrophobic domain 1 (*Chic1*) locus can be detected at this stage, implying that at least some domains of the $X_p$ are actively transcribed at this stage[44]. So, the authors conclude that X-chromosome inactivation initiates at the 4- to 8-cell stages in some cells and is generalized to all cells by the time of implantation (FIG 2a). Direct analysis of gene expression has led us to propose a different model in which dosage compensation has already occurred at the time of conception[43] (FIG. 2b). COT1 FISH, a technique that allows the visualization of nascent transcription, shows that the *Xist* RNA domain is poorly transcribed in XX embryos. This is observed first at the 2-cell stage (when general zygotic transcription occurs for the first

time[46]) and persists throughout the pre-implantation stage. Our results lead us to conclude that the mouse embryo is dosage compensated from the beginning, using a primitive form of X-chromosome inactivation that is leaky and incomplete. With the further recruitment of heterochromatic factors during pre-implantation development, X-chromosome inactivation is gradually improved and culminates in the 'complete' forms that are found in the placenta (imprinted X-chromosome inactivation) and the soma (random X-chromosome inactivation).

Although the exact timing of X-chromosome inactivation is subject to further investigation, the model above is consistent with what is currently known about the evolution of mammals, their sex chromosomes and X-chromosome inactivation. Assuming the above model is correct, what are the implications for the origin and mechanism of X-chromosome inactivation?

## The importance of the father

How is the $X_p$ silenced at conception? One possibility is that it is silenced *de novo* at the 2-cell stage when zygotic *Xist* RNA is expressed for the first time[43]. The 1- to 2-cell period represents a crucial step in the transition from maternal to zygotic gene expression[46]. At this time, the *Xist* RNA domain is small, but continues to grow as the 2-cell embryo divides to produce a 4-cell embryo[43,44]. The initially small *Xist* RNA domain indicates that silencing at the 2-cell stage is not as effective as it is later on. If correct, this could account for the reported leakiness of *Chic1* expression in the 2-cell embryo and explain how immunostaining for chromatin changes might give the impression that X-chromosome inactivation is not initiated until the 4- to 8-cell stage[44]. How the zygote would establish X-chromosome inactivation at the 2-cell stage is currently unknown; including whether the mechanism is *Xist*-dependent. Perhaps the mechanism of silencing might be likened to other imprinted chromosomal domains (review in REF. 47), whereby the $X_p$ is 'marked' during spermatogenesis and preferential silencing is subsequently executed in the zygote from the 2-cell stage onwards.

Can $X_p$ silencing take place even earlier than the 2-cell stage? Indeed, a more parsimonious solution is that the $X_p$ might arrive in the zygote in a 'pre-inactivated' state[43]. This view fits elegantly with X-chromosome dynamics in the male germline. Unique to the male germline, the process of meiotic sex-chromosome inactivation (MSCI) silences the X and Y chromosomes during the first meiotic prophase[48]. The *raison d'être* of MSCI represents yet another mystery in the field of sex-chromosome biology, although compelling rationales have been proposed. These include the need to silence X-linked genes that inhibit spermatogenesis[49] (also reviewed in REF. 50), to suppress recombination between non-homologous portions of the X and the Y chromosomes[51], or to prevent ASYNAPSED XY domains from triggering the MEIOTIC CHECKPOINT and apoptosis[52]. MSCI might also have been usurped by mammals for dosage compensation[43,53], indicating a continuity of X-chromosome silence from paternal PACHYTENE to the early embryo.

The transcriptional fate of the X and Y chromosomes after pachytene has remained highly debated, with some studies arguing for a reactivation of X- and Y-linked genes[54,55] and other genes that demonstrate persistent silence[56–60]. An analysis of the sparse literature on this subject indicates that 6 of the 9 genes that have been examined are not expressed after MSCI (FIG. 3). This supports the idea that silencing might extend beyond meiosis. Further support comes from the existence of 'retrogenes', a growing class of spermatogenesis genes that transposed from an X-linked to an autosomal location during the course of mammalian evolution[60–66]. Retrogenes include such spermatogenesis-specific genes as phosphoglycerate kinase 2 (*Pgk2*)[64], pyruvate dehydrogenase E1-α-2 (*Pdha2*)[63], glucose-6-phosphate dehydrogenase 2 (*G6pd2*)[60], and zinc-finger autosomal protein (*Zfa*)[61]. The extensive gene trafficking from the X chromosome to autosomes might have been in response to MSCI, which would have otherwise silenced those genes required for spermatogenesis[62,64]. Although a more comprehensive analysis of post-MSCI gene expression is required, the available data raise an interesting point: X-linked genes that remain silent tend to be centrally located, whereas those that become reactivated are farther from the *Xic* (FIG. 3). This pattern is strikingly similar to what is observed in the pre-implantation embryo[43]. We therefore propose that MSCI and zygotic X-chromosome inactivation form a continuum. The silencing that is initiated at MSCI in the father is propagated as an inactive $X_p$ in the daughter[43,67]. In this way, imprinted X-chromosome inactivation in the zygote might trace its origins to MSCI in the paternal germline.

In this hypothesis, fathers have a significant role in X-chromosome inactivation — a departure from the dogma that dosage compensation is restricted to females in mammals. Furthermore, the 'pre-inactivation hypothesis' greatly simplifies the

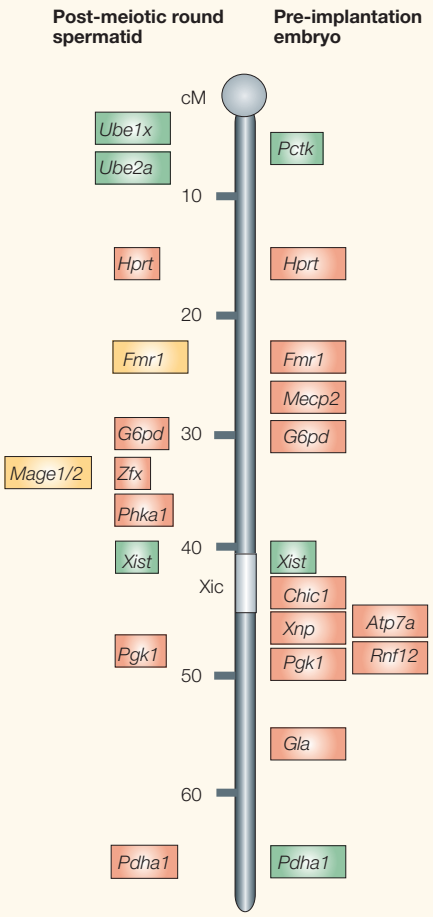


Figure 3 | **Paternal X-chromosome silencing from sperm to zygote.** The diagram shows an expression profile of the paternal X chromosome ($X_p$) in post-meiotic round spermatids and pre-implantation embryos[37,41–43,91,92]. Genes labelled in green are expressed; those labelled in red are either not expressed or show very low levels of expression; the expression state of those labelled in yellow is not conclusively known (interpretation is complicated by the fact that fragile X mental retardation 1 (*Fmr1*) is not inactivated during pachytene and the melanoma antigen gene *Mage1/2* is part of a whole family of homologous genes that might have resulted in non-specific PCR detection[54,58]). *Atp7a*, ATPase, $Cu^{2+}$ transporting, α-polypeptide; *Chic1*, cysteine-rich hydrophobic domain 1; *Gla*, α-galactosidase; *G6pd*, glucose-6-phosphate dehydrogenase X-linked[60]; *Hprt*, hypoxanthine guanine phosphoribosyl transferase[57–59]; *Mecp2*, methyl CpG binding protein 2; *Pctk*, PCTAIRE-motif protein kinase 1; *Pdha1*, pyruvate dehydrogenase E1-α-1 (REFS 54,57,58); *Pgk1*, phosphoglycerate kinase 1 (REFS 57–59); *Phka1*, phosphorylase kinase α-1 (REF. 57); *Rnf12*, ring finger protein 12; *Ube1x*, ubiquitin-activating enzyme E1, Chr X (REF. 54); *Ube2a*, ubiquitin-conjugating enzyme E2A, *RAD6* homologue[54]; *Xist*, X (inactive)-specific transcript[88–90]; *Xnp*, X-linked nuclear protein; *Zfx*, zinc finger protein X-linked[57].

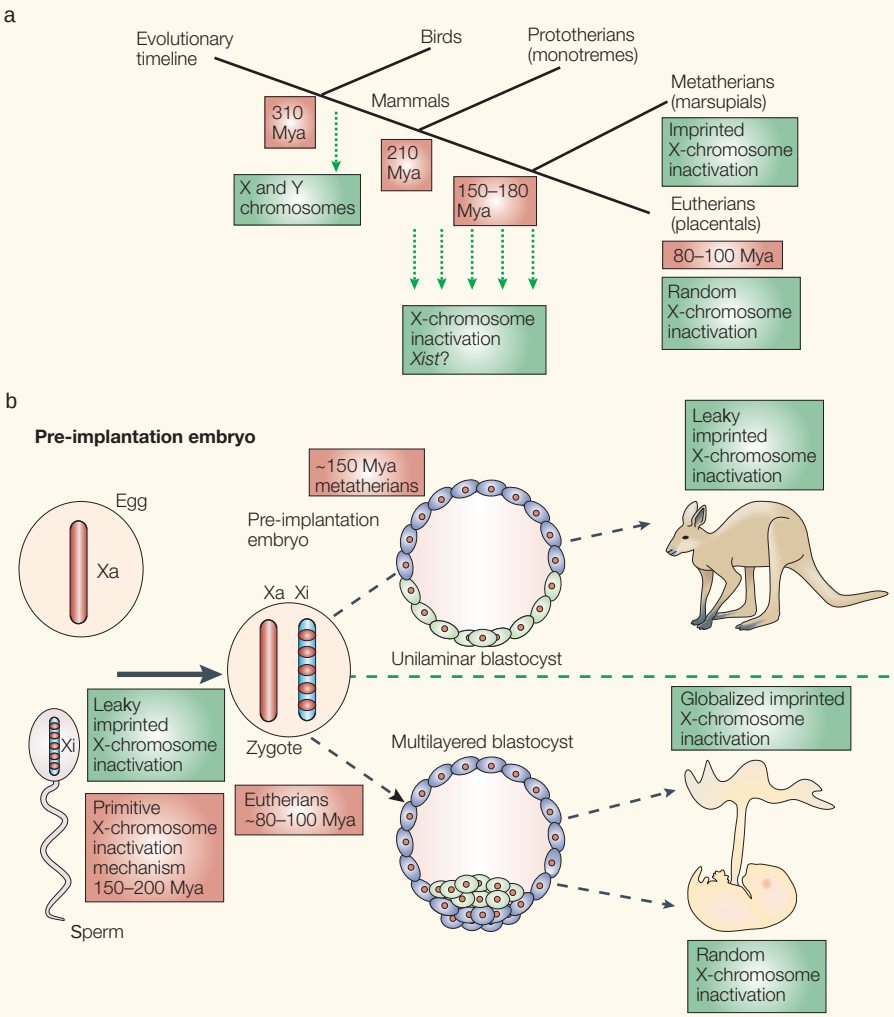


Figure 4 | **Parallels through ontogeny and phylogeny. a** | The time-frame of mammalian evolution. Dimorphic X and Y chromosomes evolved after mammals split off from the avian lineage. X-chromosome inactivation might have evolved concurrently. The timing of the appearance of *Xist* is currently unknown. **b** | A form of meiotic sex-chromosome inactivation-driven imprinted X-chromosome inactivation might have been the original mechanism of dosage compensation in mammals, evolving some 150–200 Mya (million years ago). This imprinted form is present in somatic cells of metatherians, which are some of the earliest mammals to have evolved (~150 Mya), and is mirrored in the pre-implantation embryo of the mouse, a eutherian mammal. The extra-embryonic tissues of some eutherians maintain the original imprinted mechanism of X-chromosome inactivation but show a more complete form of silencing. Random X-chromosome inactivation subsequently evolved in eutherian mammals, such as the mouse, and we can therefore trace its time of origin to 80–100 Mya.

ontogeny of X-chromosome inactivation. It posits a single round of inactivation in the paternal germline, followed by a single round of reactivation in the epiblast in preparation for random inactivation in the soma (FIG. 2b). By contrast, the classical model advocates many rounds of inactivation and reactivation from the paternal germline to the zygote and beyond (FIG. 2a). We therefore suggest that a pre-inactivation mechanism is not only consistent with the available evidence but is also the most parsimonious solution for the ontogeny of X-chromosome inactivation.

## Ontogeny recapitulates phylogeny?

Ernest Haeckel's original nineteenth century aphorism invokes the idea that the embryonic development of a species mirrors the stages of its evolutionary past. The ontogeny and phylogeny of X-chromosome inactivation might be an excellent example of this idea (FIG. 4). Imprinted X-chromosome inactivation is thought to have arisen either in metatherians (marsupials; ~150 million years ago (Mya)) or even earlier in prototherians (monotremes; ~200 Mya)[68,69]. As is evident from extant marsupials, this form of imprinted X-chromosome inactivation bears a striking resemblance to the 'primitive' form of X-chromosome inactivation in the pre-implantation mouse embryo[43,44]. Specifically, marsupial X-chromosome inactivation is incomplete in the sense that many genes escape inactivation and the extent of inactivation varies widely between cells and tissues (reviewed in REFS 1,70). So, the early mammal and the early mouse embryo share an imperfect mechanism of dosage compensation.

Between 150 and 80 Mya, X-chromosome inactivation evolved from an imperfect imprinted form, as seen in marsupials, to coexist with a more complete random form that is seen in eutherians today. As if mirroring its evolutionary history, the early mouse embryo begins development with an imperfect imprinted form that is later replaced with a more complete random form after implantation[43]. This parallel progression through evolution and development captures the idea that ontogeny recapitulates phylogeny. The recapitulation indicates that the pre-implantation mouse embryo might in fact be an excellent retrospective model for studying primordial forms of X-chromosome inactivation[43].

But are the molecular details of imprinted X-chromosome inactivation similar in marsupials and in the early mouse embryo? One important question is whether inheritance of a pre-inactivated $X_p$ might also underlie marsupial X-chromosome inactivation. Variations on such a possibility have been entertained[1,22,71]. The hypothesis predicts that MSCI would also take place in the metatherian clade. In fact, there is compelling evidence to indicate that MSCI occurs in the marsupial male germline (reviewed in REF. 50).

How can MSCI be a source of imprinting in the germline? This type of imprinting mechanism might have ancient origins in the form of meiotic silencing by unpaired DNA (MSUD). In the filamentous fungus, *Neurospora crassa*, non-homologous DNA fails to pair at meiosis and the lack of pairing results in silencing of the unpaired region[72]. MSUD is believed to have evolved originally as a defence mechanism against invading foreign DNA and transposons, but the phenomenon has now been observed more generally. In *C. elegans*, males have an XO constitution, leaving the X chromosome without a pairing partner during meiosis. The absence of pairing is associated with silencing, a result that is reminiscent of MSCI in the mammalian germline[73]. Intriguingly, lack of pairing and silencing are coincident with accumulation of chromatin imprints that include methylation of Lys9 on histone H3 (REF. 73). So, the original imprinting mechanism might have involved MSUD-induced chromatin modifications.

Might a similar imprinting mechanism be used by mammals during MSCI? Recent studies show that the state of asynapsis in the mammalian germline directly results in silencing[74–76] (see also REF. 53). This unpaired silencing depends on the coordinated actions of the breast cancer predisposition gene, *Brca1*, various modifications of the histone variant H2AX (including phosphorylation and ubiquitylation), and the ATR kinase, all of which localize to the asynapsed sex chromosomes[74–76] (FIG. 5). Taken together, these findings potentially provide a logical mechanism by which MSCI might lead to X-chromosome imprinting in the mammalian germline. With MSCI also being present in the marsupial germline, the idea that MSUD might be the original imprinting mechanism for X-chromosome inactivation becomes rather attractive. This idea also fits nicely with a recent hypothesis that genomic imprinting evolved first on the X chromosome for the purposes of X-linked dosage compensation[77]. Once fixed and further adapted on the X chromosome, a modified imprinting mechanism could have easily spread to autosomal locations by transposition of the X-linked imprinting cassette.

The role of *Xist* in the ontogeny and phylogeny of X-chromosome inactivation is of particular interest (FIG. 4a). Although *Xist* is clearly required for somatic and placental X-chromosome inactivation, MSCI does not seem to require an intact *Xist* gene[23,58,78]. Whether the pre-implantation form of imprinted X-chromosome inactivation involves *Xist* is currently not known. Because *Xist* is expressed in early embryos and the spread of *Xist* RNA loosely correlates with the gradient of silencing on the $X_p$[43], it seems likely that *Xist* will be necessary. However, the role of *Xist* in pre-implantation silencing in the mouse might strictly be to maintain $X_p$ inactivation. In this model, $X_p$ inactivation is initiated by the paternal germline through an *Xist*-independent MSUD mechanism, but silencing is maintained thereafter in the zygote in an *Xist*-dependent fashion.

The idea of heterochromatin that is induced by different mechanisms in two stages is interesting in light of a recent report on spatially distinct classes of heterochromatin on the Xi[79]. These classes of heterochromatin have distinct epigenetic marks. Class I is enriched for heterochromatin protein 1 (HP1), trimethylation at H3-K9 and trimethylation at H4-K20, but is not associated with *Xist* RNA. By contrast, class II is enriched for *Xist* RNA, trimethylation at H3-K27 and the histone variant macroH2A. We could speculate that the inherited $X_p$ resembles the class I heterochromatin of the somatic Xi and that the expression of *Xist* RNA at the 2-cell stage leads to the transition to class II heterochromatin, which would gradually provide a more complete silencing, as is observed in the trophectoderm. In this sense, X-chromosome inactivation in pre-implantation embryos can be viewed as proceeding in a progressive manner.

Is there a primordial *Xist* orthologue in marsupials? So far, such a gene has not been described. Although it might just be a matter of time before an *Xist* equivalent is found, it is formally possible that marsupial X-chromosome inactivation might be *Xist*-independent. Consistent with the idea that MSUD might have been the ancestral mechanism of imprinting, it is possible that the earliest form of X-chromosome inactivation was entirely MSCI-driven (FIG. 4b). In this model, *Xist* could have been acquired subsequently by eutherians to carry out a more global and complete form of inactivation, as observed in the post-implantation tissues (placenta and soma)[43]. On the other hand, given the obvious similarities between eutherian and metatherian X-chromosome inactivation, the ancestral form of X-chromosome inactivation might have also required *Xist* RNA, but perhaps in a form that was more localized and more variable in its degree of silencing — similar to the form that is seen in the pre-implantation mouse embryo[43].

## Why X inactivation in mammals?

Mammalian sex chromosomes are believed to have descended from a pair of identical autosomes (reviewed in REFS 80,81). Approximately 200 Mya, the X and the Y chromosomes began to differentiate into the dimorphic chromosomes that they are in mammals today, with the Y chromosome losing almost all of its genetic material (except what is required for sex determination) owing to suppression of recombination between the X and Y chromosomes during male meiosis (for reviews of Y-chromosome degeneration see REFS 3,80,81). The progressive loss of genetic material from the Y chromosome would have generated an increasing dosage imbalance between XX and XY individuals. This imbalance is thought to have given rise to the current mechanism of dosage compensation in mammals (reviewed in REF. 81). But why did mammals evolve an inactivation mechanism?

Sex chromosomes in *D. melanogaster* are also thought to have evolved from a pair of autosomes, and to have been subject to the forces that shaped the dimorphic features of the X and Y chromosomes (reviewed in REF. 81). In the interest of maintaining a genome-wide balance of gene dosage, it is easy to imagine that the activation of the single X chromosome in the male would be turned up twofold, as this would ensure that the X-to-autosome gene dosage remained the same[81]. This is a relatively frugal approach to dosage compensation because, conceptually, this mechanism requires only one significant step in evolution. By contrast, dosage compensation in mammals is an inactivation mechanism that seems complicated from two perspectives.

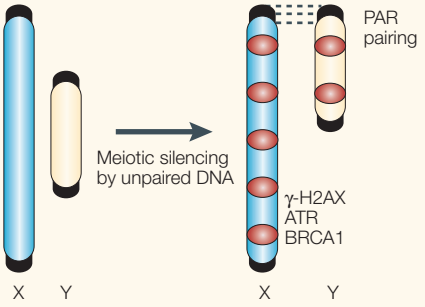


Figure 5 | **Meiotic sex-chromosome inactivation by unpaired silencing in mammals.** Unpaired regions of the X (blue) and Y (yellow) chromosomes are transcriptionally silenced at the pachytene stage of spermatogenesis. This silencing is dependent on the localization of BRCA1 (breast cancer 1, early onset), γ-H2AX (the phosphorylated form of H2A histone family, member X), and ATR (ataxia telangiectasia and rad3 related) proteins (red circles) to the unpaired regions. Dotted lines indicate limited pairing at the pseudoautosomal regions (PARs).

First, as argued above, X-chromosome inactivation is a multi-step process that involves counting, epigenetic choice and *cis*-limited silencing — a system that seems difficult to evolve simultaneously. The *D. melanogaster* system is also regulated by a chromosome-counting mechanism, but this mechanism was presumably already in place for the determination of sex[24,25]. Epigenetic choice does not take place in *D. melanogaster*, nor is dosage compensation *cis*-limited.

Second, X-chromosome inactivation is complicated from the perspective that if female X-chromosome inactivation was concurrent with male Y-chromosome degeneration it would have led to a temporary imbalance between X-chromosome and autosomal gene dosages, owing to a sudden halving of X-linked gene transcription. This is very different from fruitflies, in which concurrent male X-chromosome upregulation and male Y-chromosome degeneration would have preserved the overall X-to-autosome gene balance. According to one model, early mammals responded to this problem by a general increase in X-linked transcription as the Y chromosome decayed[81]. But because

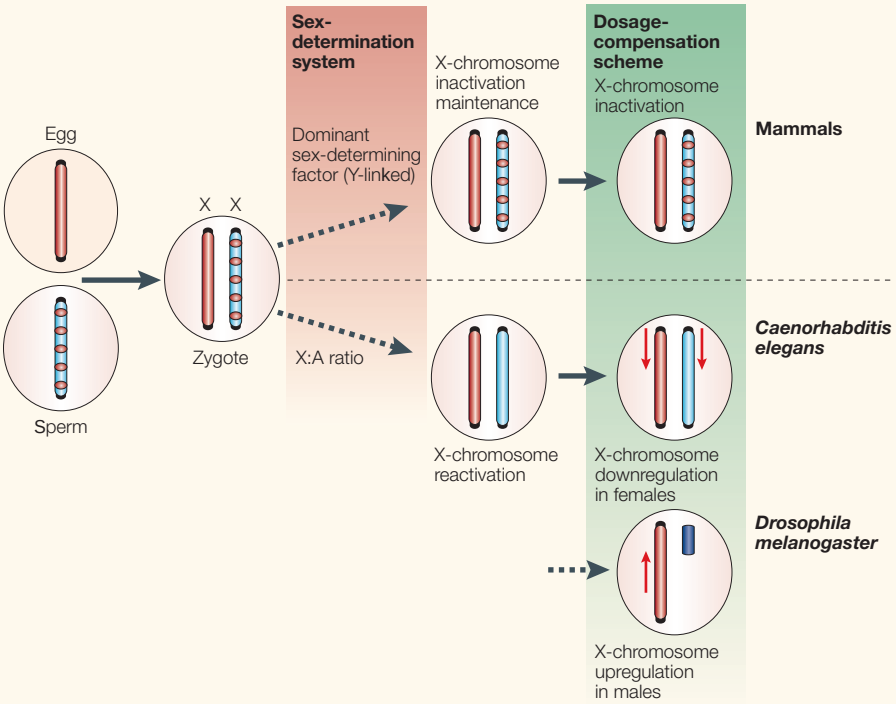


Figure 6 | **A generalized model for how the nature of the dosage-compensation mechanism depends on the method of sex determination.** In this model, heteromorphic sex chromosomes are subjected to meiotic silencing by unpaired DNA during gametogenesis, and the X chromosome is transmitted to the zygote as an inactive chromosome. Dosage compensation in the zygote will then depend on an organism's method of sex determination: sex determination that is based on a dominant sex-determining factor(s) (for example, the Y-linked *Sry*) is conducive to preserving the inherited inactive X chromosome. But sex-determining mechanisms that depend on the X:A ratio (the ratio of X chromosomes to autosomes) must reactivate the X chromosome to obtain an accurate X-chromosome number measurement. So, a non-X-chromosome inactivation type of dosage compensation can evolve in this system (for example, downregulation of the female X chromosomes in *Caenorhabditis elegans* or upregulation of the male X chromosome in *Drosophila melanogaster*). Red circles, heterochromatin.

X-chromosome upregulation occurred in both XY and XX individuals, females faced the problem of double X-chromosome dosage and were therefore subject to considerable pressure to downregulate their X chromosomes. So, in mammals, the responsibility for dosage compensation ultimately rested with the female. As argued above, MSUD-mediated silencing in the male germline would have been a simple first solution to ensure that XX zygotes started out without unwanted X-linked gene dosage.

In summary, the evolution of X-chromosome inactivation is thought to have involved a series of significant evolutionary steps in response to Y-chromosome degeneration, including generalized X-chromosome upregulation in both sexes, followed by female-specific X-chromosome inactivation[81]. This seems more complicated than the process that is thought to have occurred in the fruitfly. Using this type of logic, it might be difficult to imagine why mammals did not simply adopt the male-specific upregulation of the X chromosome *à la D. melanogaster*.

Although the mammalian system might seem unnecessarily complex, we suggest that it might in fact have been the easiest solution available. First, complete silencing might be safer than a mechanism that requires very fine tuning to allow for the precise doubling of gene expression. Second, as the Y chromosome began to lose genetic material, a simple solution for dosage compensation would have been to silence the regions of the X chromosome that no longer had pairing partners on the Y chromosome — and what better way to achieve this than through an already available, ancient mechanism of MSUD? As the Y chromosome continued to degenerate, MSUD would progressively affect more and more of the X chromosome, culminating in what we see in rodents today, where nearly all of the X chromosome is subject to silencing. Because only female offspring can inherit the X chromosome from the father, an MSUD-driven imprinted process would have immediately solved the problem of dosage compensation in the early mammal, without the need to evolve counting or choice mechanisms at the same time.

This logic also naturally raises the opposite question: why do we not see a mechanism similar to X-chromosome inactivation in the fruitfly, the worm and other organisms with heterogametic sex determination? Indeed, meiotic sex-chromosome silencing is well described in both flies[48] and worms[82]. In worms, MSUD has also recently been shown to be the mechanism that is responsible for male germline silencing[73]. We argue that these other organisms were locked into disparate mechanisms of dosage compensation because genetic circumstances limited their evolutionary choices. Specifically, in the fruitfly and the worm, sex is determined by the X-to-autosome ratio that is measured during the first few cell divisions (reviewed in REF. 24). The X-to-autosome ratio is quantified by relative expression of specific X-linked and autosomal genes. So, although MSUD might occur in the male germline, dosage compensation cannot occur by maintenance of a pre-inactivated $X_p$ in the zygote, as this would interfere with measurement of the number of X chromosomes. In mammals, sex is not influenced by the X-to-autosome ratio, but is determined by the presence or absence of the male-determining Y chromosome. So, although X-chromosome inactivation might have been the natural choice for mammals, it would not have been a logical choice for *D. melanogaster* or *C. elegans*. It seems that the strategy of dosage compensation for a given organism is directly affected by two factors — first, how sex is determined, and second, how their sex chromosomes evolved (FIG. 6).

A corollary to this hypothesis is that X-chromosome inactivation need not be unique to mammals and might arise independently in other organisms with a compatible sex-determination system. Indeed, an epigenetic phenomenon that is reminiscent of mammalian X-chromosome inactivation has been described in a dioecious plant species, *Silene latifolia* (also known as *Melandrium album*) (reviewed in REF. 83). Similar to mammals, *S. latifolia* has an XX–XY sex-determination system with the Y chromosome carrying a dominant male-determining gene. One of two X chromosomes in the female seems to be hypermethylated and late-replicating[84,85], two tell-tale signs of inert transcription. Furthermore, as in mammals, the X and Y chromosomes synapse only at their ends during meiosis in *S. latifolia*[86]. These observations indicate that MSUD might also operate in plant meiosis and that MSCI might be more widespread than is currently appreciated.

## Glossary

ASYNAPSIS
Failure of chromosomes to pair during meiosis.

BLASTOCYST
An early stage of mammalian embryonic development at which the first cell lineages become established.

COT1 FISH
A technique to visualize nascent transcription that uses the Cot1 fraction of DNA that is rich in repetitive elements that often occur in introns and 3′ untranslated regions.

EPIBLAST
An embryonic lineage that is derived from the inner cell mass of the blastocyst, which gives rise to the body of the fetus.

EUTHERIANS
Mammals that give birth to live offspring (that is, they are viviparous) and possess an allantoic placenta — the allantois is the fetal membrane that facilitates nutrient and waste exchange between the fetus and the mother.

FLUORESCENCE *IN SITU* HYBRIDIZATION
A technique in which a fluorescently labelled DNA probe is used to detect a particular chromosome or gene with the help of fluorescence microscopy.

MEIOTIC CHECKPOINT
A surveillance mechanism specific to meiosis that ensures proper chromosome segregation.

PACHYTENE
The third phase of prophase I in meiosis.

THERIAN MAMMALS
A group of mammals that includes the eutherians and marsupials.

TROPHECTODERM
The outer layer of the blastocyst-stage embryo; the precursor to the bulk of the embryonic part of the placenta.

## Conclusions

We propose that there is a link between the ontogeny and phylogeny of X-chromosome inactivation. We have suggested that our recent finding and those of others indicate that X-chromosome inactivation in mammals evolved from an ancient silencing mechanism (MSUD) that was first described in *N. crassa*. As MSUD is conserved throughout evolution, from *N. crassa*[72] to *C. elegans*[73] to mammals[75], it will be interesting to learn how widespread the MSUD-driven X-chromosome inactivation type of mechanism might be. In fact, the spectre of X-chromosome inactivation in the plant species *S. latifolia*[84,85] indicates that it might be more widespread than is currently appreciated.

There are several important questions that remain to be answered. To what extent is the $X_p$-expression pattern heritable from father to zygote in mice? How well is this mirrored by X-chromosome inactivation in marsupials and other organisms that undergo X-chromosome inactivation? Does *Xist* have a role? It will also be crucial to determine to what extent MSUD and dosage compensation can be applied to vertebrates, particularly the earliest mammals, the prototherians, and their close cousins, the birds. Preliminary evidence seems to favour dosage-compensation mechanisms in prototherians and birds[69,87]. If present, is it an inactivation type of mechanism and is it imprinted? The answers to these questions might further support the link between ontogeny and phylogeny.

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## Competing interests statement

The authors declare no competing financial interests.

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