X-CHROMOSOME INACTIVATION: COUNTING, CHOICE AND INITIATION

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In many sexually dimorphic species, a mechanism is required to ensure equivalent levels of gene expression from the sex chromosomes. In mammals, such dosage compensation is achieved by X-chromosome inactivation, a process that presents a unique medley of biological puzzles: how to silence one but not the other X chromosome in the same nucleus; how to count the number of X's and keep only one active; how to choose which X chromosome is inactivated; and how to establish this silent state rapidly and efficiently during early development. The key to most of these puzzles lies in a unique locus, the X-inactivation centre and a remarkable RNA — *Xist* — that it encodes.

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In mammals, dosage compensation of X-linked genes is achieved by the transcriptional silencing of one of the two X chromosomes in the female during early development — a process known as X inactivation. The early events in X inactivation are under the control of a key regulator, the X-chromosome-inactivation centre or *Xic*. Initiation of X inactivation involves a recognition step in which the number of X chromosomes in the cell is counted relative to cell ploidy so that only a single X chromosome is functional per diploid adult cell. One hypothesis postulates the existence of a blocking factor that is synthesized in limiting quantities sufficient for the binding of a single *Xic* per diploid cell. Initiation is also thought to include a process of choosing, whereby one of the two X chromosomes in the female cell might be preferentially selected for inactivation. Examples include the imprinted inactivation of the paternal X chromosome in extra-embryonic tissues and the biased inactivation that results from allelic differences at the X-chromosome-controlling element (*Xce*) locus in embryonic tissues.

As defined cytologically, the *Xic* is a roughly 1 Mb region that contains several elements thought to have a role in X inactivation (BOX 1) and at least four genes[1] (FIG. 1). One of these, the X (inactive)-specific transcript (*Xist*) gene, encodes a large non-coding RNA that is relatively poorly conserved. *Xist* has been shown to contribute to *Xic* function and is required for X inactivation. Other elements that lie within the *Xic* are candidates for involvement in the control of *Xist* expression, or for the mechanisms of counting and choice. One is the *DXPas34* locus, which was originally identified as a result of its unique methylation profile on the active X chromosome[2]. Another is the *Tsix* transcript, a non-coding transcript that is synthesized from the strand opposite to *Xist* and has been hypothesized to regulate the activity of *Xist* at the onset of X inactivation[3].

During random X inactivation, counting and choice must either precede, or be concomitant with, the onset of initiation and its earliest manifestation, the coating of the presumptive inactive X by *Xist*. Silencing of X-linked genes and replication asynchrony follow rapidly. Both seem to precede global histone hypoacetylation, the accumulation of a novel histone variant (macroH2A) and methylation of the inactive X, which seem to function as maintenance mechanisms for X inactivation[4–6]. However, imprinted X inactivation, which occurs in certain mammals and by which the paternal X is preferentially inactivated, might differ in some respects from random inactivation.

In this article, we review recent results concerning the events that surround the initiation of X inactivation.

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Box 1 | *Xic* and the elements of X inactivation

Xist
The X inactive-specific transcript (*Xist*) gene is expressed exclusively from the inactive X chromosome, producing a 17-kb spliced, polyadenylated transcript that is retained in the nucleus. The *Xist* transcript seems to be the primary signal for spreading the inactive state along the chromosome. But *Xist* itself does not seem to be involved in counting. Some of the elements lying outside *Xist* that influence counting and choice in X inactivation (see *Xic*) might be involved in regulating its expression.

Xic
The X-chromosome-inactivation centre (*Xic*) was originally defined through studies on structurally abnormal X chromosomes as a 'master control region', the presence of which is essential for X inactivation to occur. It is responsible for initiating X inactivation *in cis*: an X-chromosome fragment that carries a *Xic* can become inactivated, whereas one in which the *Xic* is missing cannot. The *Xic* is also involved in 'counting', whereby only a single X is kept active per two sets of autosomes in a cell, and all other *Xic*-carrying chromosomes are inactivated.

Xce
The X-chromosome-controlling element (*Xce*) affects the choice of X to be inactivated (or to remain active). In females heterozygous for different *Xce* alleles, an X chromosome that carries a strong *Xce* allele is more likely to remain active than one that carries a weak *Xce* allele, thereby leading to skewed X inactivation. The degree of skewing is rarely more than 70:30%. Refined genetic mapping using microsatellite markers indicate that the *Xce* locus might be distinct from *Xist*, lying distal and 3′ to *Xist*[38].

TsiX
TsiX is an element transcribed from the antisense strand relative to *Xist*. *Tsix* is expressed in undifferentiated ES cells and early embryos, and has been proposed to control *Xist* expression *in cis* at the onset of X inactivation[3,35]. *TsiX* antisense transcription spans the whole of the *Xist* gene, extending well over 40 kb. The 5′ end and promoter of the *TsiX* gene has been proposed to be closely associated with the *DXPas34* locus, although other (weaker) promoters might be scattered across a large region 3′ to *Xist*[34]. Targeted deletion of the 5′ end of *TsiX/DXPas34* leads to non-random inactivation of the deleted X chromosome[35] and a failure of imprinted X inactivation[52]. This might indicate that this locus and/or the transcript that it produces influences X-chromosome choice and imprinting of the X chromosome.

DXPas34
The *DXPas34* locus is a 3 kb CpG-rich region, containing a 34-mer minisatellite repeat lying roughly 15 kb downstream of the 3′ end of *Xist*. *DXPas34* is hypermethylated on the active X chromosome in somatic cells. The degree of hypermethylation was thought to correlate with allelism at the *Xce* locus, although *Xce* lies outside the *DXPas34* region[2]. The prinicipal initiation site for *TsiX* transcription has been reported to lie within *DXPas34* (REF. 3).

We place an emphasis on the role of *Xist* and the events that occur upstream and downstream of *Xist* expression. For a comprehensive review of the older literature, readers are referred to REFS 1, 7.

Xist — regulation and function

Before random X inactivation initiates in the developing embryo, *Xist* appears to be expressed at low levels from every *Xic* in the cell. Using fluorescence *in situ* hybridization (FISH), *Xist* RNA can be detected in embryonic stem (ES) cells as two small, punctate signals in female (XX) ES cells and a single punctate signal in male (XY) ES cells (FIG. 2). Female ES cells undergo X inactivation when stimulated to differentiate, and represent a useful model system for the study of X inactivation. *Xist* RNA first accumulates on and coats the future inactive X chromosome, while the *Xist* gene on the presumptive active X chromosome in male and female cells becomes silenced. The onset of X inactivation therefore seems to be intimately linked with the accumulation of *Xist* RNA in the cell.

Xist RNA stability. Monoallelic *Xist* upregulation has been associated with an increase in the half-life of the *Xist* transcript[8,9]. Johnston *et al.*[10] proposed that this change in *Xist* transcript stability is brought about by a developmentally regulated switch in *Xist* promoter use. Two promoters (P1 and P2) were shown to be used for transcription of the stable *Xist* transcript that accumulates on the inactive X chromosome in somatic cells. A putative third promoter (P0) located 6.6 kb further upstream, was hypothesized to generate the unstable *Xist* transcripts in cells that have not yet undergone X inactivation. Recent findings have raised several questions concerning the existence and role of the P0 promoter. An ES cell line carrying an *Xist* transgene that was deleted for the P0 promoter region still gave the punctate RNA FISH signal that is normally associated with unstable *Xist* transcripts in undifferentiated ES cells. This indicated that P0 might not be required for the production of unstable *Xist* transcripts in ES cells[11]. The highly repetitive and poorly conserved nature of the P0 region in both voles and humans provides a further argument against this region having an important regulatory role (Tatyana Nesterova *et al.*, personal communication).

The original observations regarding the P0 promoter were probably complicated by the presence, at that time unsuspected, of antisense transcripts running from the 3′ end of *Xist* through to, and beyond, the P1 promoter[3], and by the presence of several ribosomal protein pseudogenes lying upstream of P1, in the proposed P0 region[11,12] (FIG. 1). Nevertheless, it cannot be ruled out that a series of alternative promoters exists in the region upstream of P1/P2 and even P0, because the more recent experiments of Warshawsky and colleagues[11] were based on transgenes truncated just upstream of *Xist* that might have been influenced by position effects. The issue will only be definitively resolved by either targeted deletion of the region or its functional inhibition.

Another interesting candidate region to be considered in the context of possible regulatory elements upstream of *Xist* is the *2.1(2)P* region, which shows histone H4 hyperacetylation in undifferentiated female ES cells but not in male ES cells[13]. This H4 hyperacetylation disappears upon differentiation, suggesting that it might well be involved in *Xist* regulation before, or during, the initiation of X inactivation. Hyperacetylation of this region is substantially reduced in female ES cells carrying a mutated *Xic* (a partially deleted *Xist* gene)[16], which might reflect involvement of *2.1(2)P* in counting and/or choice, both of which involve sensing the presence of two or more *Xic*s in the nucleus[1].

Important information concerning the regulation of *Xist* has also come from experiments involving a full-length *Xist* cDNA transgene that contains the P1 and P2 promoters, but under the control of a strong inducible promoter[14]. High levels of *Xist* RNA derived from the

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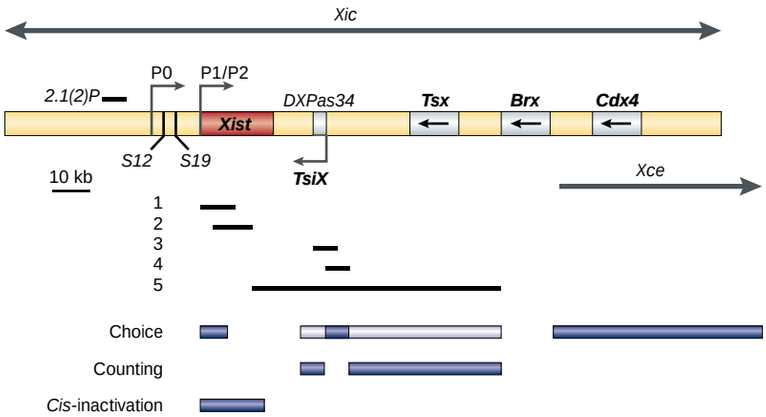

Figure 1 | **The X-inactivation centre.** A summary of the known elements and regions in the X-inactivation centre (*Xic*) thought to affect choice, counting and *cis*-inactivation during the initiation of X inactivation. (See BOX 1 for more information on *Xist*, *DXPas34*, *Tsix* and *Xce*.) Genes are shown in bold, with the direction of transcription indicated by the arrow. *Brx* (brain X-linked)[67], *Tsx* (testis X-linked)[68] and *Cdx4* (Caudal-4)[69] are genes that lie within the *Xic* that do not have, at least for the moment, any defined X-inactivation function. The *2.1(2)P* region shows differential histone H4 hyperacetylation in undifferentiated female and male embryonic stem (ES) cells and has been suggested as a possible regulatory element in X inactivation. P1 and P2 are the somatic *Xist* promoters, and P0 is a postulated *Xist* promoter in undifferentiated ES cells and early embryos. *S12* and *S19* are ribosomal protein pseudogenes found 5′ to *Xist* in the mouse *Xic*[11,12,70]. The deletions used to dissect the function of different elements in the *Xic* are shown as black lines and are described in REF. 16 (1) REF. 17 (2), REF. 34, (3) REFS 35,52 (4) and REF. 33 (5). Blue bars indicate the regions that have been implicated in specific functions. Effects on choice and counting have not so far been distinguished in the regions indicated by light bars. The terminal two *Xist* exons, lying within the 65-kb deletion, have no effect on counting or choice (C. Morey, P. A. and P. Clerc unpublished observations).

cDNA transgene were stable in undifferentiated ES cells, with a similar half-life to the *Xist* transcript in female somatic cells. This indicates that the short RNA half-life associated with low-level endogenous *Xist* expression cannot be due to an absence of *Xist* RNA stabilizing factors in undifferentiated ES cells, a result supported by studies involving human *XIST* transgenes[15]. One possibility raised by the study of Wutz and Jaenisch[14] is that stabilization of *Xist* RNA might depend on the levels of *Xist* RNA present, with low expression being associated with instability. Alternatively, the stability of the transgenic *Xist* transcript could be due to the absence of the 3′ or 5′ sequences and intronic genomic sequences that might be involved in destabilization of the endogenous *Xist* transcript in ES cells.

Xist *function*. The importance of *Xist* in the X-inactivation process has been shown by both loss- and gain-of-function experiments. Two targeted deletions of the *Xist* gene showed that *Xist* is essential for inactivation *in cis*[16,17]. In gain-of-function experiments, extra copies of *Xist*, often with considerable amounts of flanking sequence, have been introduced as transgenes either into ES cells or, by pronuclear injection, into the mouse oocyte[18–21]. Studies involving the inducible *Xist* cDNA transgene[14] have been particularly insightful. In undifferentiated or early differentiating ES cells, inducing the expression of the *Xist* cDNA transgene leads rapidly (within 24 hours or about one cell cycle) to long-range transcriptional repression *in cis*. This inactivation is dependent on continued *Xist* expression and can be reversed. It is not accompanied by any of the later characteristics of the inactive X, such as histone hypoacetylation and late replication timing. In cells that have been induced to differentiate, *Xist* must be expressed during the first 48 hours of differentiation to initiate ectopic silencing. Once 72 hours of differentiation have elapsed, continued silencing is no longer dependent on *Xist* expression and the full range of secondary X inactivation characteristics is acquired. Irrespective of whether the initial reversible repression of transcription by *Xist* in undifferentiated ES cells is a normal step in the initiation of X inactivation in female cells (as suggested by Wutz and Jaenisch[14] and possibly by recent results with androgenetic embryos), or is an artefact of the high levels of *Xist* produced by the inducible promoter in the transgene, the results are consistent with the idea that *Xist* RNA is the key factor that triggers X inactivation *in cis*.

Clues from Drosophila. Insights into the function of the *Xist* transcript have come from recent studies in *Drosophila melanogaster*. Dosage compensation in *Drosophila* is ensured through the hypertranscription of the single X chromosome present in the male, and this is associated with the hyperacetylated state of histone H4. Despite this fundamental difference with mammals in terms of strategy, certain unifying epigenetic regulatory principles might be common to both systems of dosage compensation. In each case, for instance, it seems that one, or several, molecules bind specifically to an X chromosome and are critical in remodelling the structure of the dosage-compensated chromosome. Once remodelled, the chromatin then maintains the specific transcriptional state associated with the dosage compensated X chromosome.

Dosage compensation in *Drosophila* depends on the presence of two small non-coding RNAs, *roX2* (1.1 kb) and *roX1* (3.5 kb), and the five male-specific lethal proteins (MSLs): maleless (MLE), MSL-1, MSL-2, MSL-3 and MOF (Males absent on the first)[22]. The MOF protein, which is known to have histone acetyltransferase activity, is present in both sexes but is associated with the X chromosome only in males, in a complex composed of the MSL proteins. The MSL proteins localize together, presumably as a complex, at several hundred X-chromosome sites along the entire length of the male X. In mutants lacking MOF, MLE or MSL function, binding is restricted to about 30 well-distributed sites. These 'core sites' have been proposed to represent assembly sites from which the MSL complexes spread to the other sites on the X chromosome. The association of MOF with the male X chromosome has now been shown to be RNase sensitive and to depend on interaction with the *roX2* RNA transcript[23]. *In vivo* binding of MOF to *roX2* is through its CHROMODOMAIN. Whereas the various MSL proteins in the dosage compensation complex seem to be held together through protein–protein interactions, two other MSL proteins, MLE and MSL-3, (the latter is also a chromodomain protein) might also be RNA-binding factors[23], indicating that RNA interaction might be a property of many chromatin regulatory molecules.

CHROMODOMAIN
A highly conserved sequence motif that has been identified in various animal and plant species. Chromodomain proteins seem to be either structural components of large macromolecular chromatin complexes or involved in remodelling chromatin structure.

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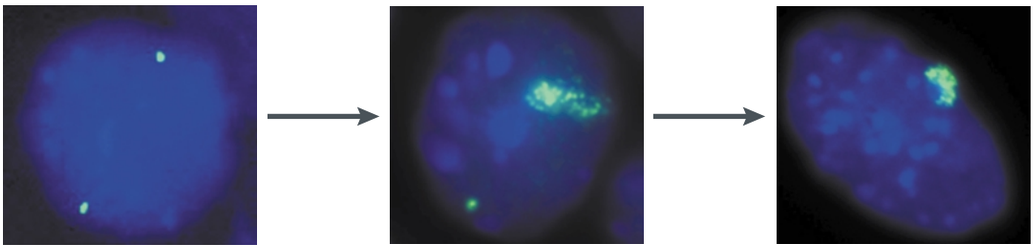

Figure 2 | ***Xist* transcription in embryonic stem cells.** Patterns of *Xist* RNA expression in female ES cells undergoing differentiation using RNA fluorescence *in situ* hybridization. The left panel shows that undifferentiated ES cells have two punctate *Xist* RNA signals, representing the presence of unstable *Xist* transcripts at the site of transcription on both (active) X chromosomes. The middle panel shows that, upon differentiation, *Xist* RNA from one of the two alleles becomes stabilized and coats the X chromosome that is to be inactivated *in cis*. The X chromosome that remains active continues to express *Xist* in its unstable form. The right panel shows that, in fully differentiated cells, *Xist* RNA coats the inactive X chromosome and the *Xist* gene on the active X has been silenced.

Recruitment of chromatin proteins by Xist. Similarly to the roX non-coding RNAs, *Xist* RNA might recruit regulatory and chromatin proteins to the mammalian X chromosome undergoing inactivation. Recent chromatin immunoprecipitation experiments indicate that *Xist* RNA might associate with chromatin that is enriched in the hypoacetylated isoforms of histones H3 and H4 (REF. 24). This is in keeping with the global hypoacetylation of the inactive X observed using immunofluorescence[25,26] and with the finding that the promoters of most genes on the inactive X chromosome are enriched for hypoacetylated histone H4 (REF. 27), as well as being hypermethylated[1]. This is suggestive of a role for the *Xist* transcript in targeting factors that are involved in transcriptional repression to the inactive X. However, the much later appearance of a globally hypoacetylated state and of hypermethylated promoters during ES cell differentiation indicates that *Xist* might not be directly involved in inducing such methylation and histone acetylation modifications. Furthermore, deletion of *Xist* in somatic cells does not seem to perturb the maintenance of the inactive state, implying that any direct role for *Xist* in recruiting chromatin modifiers might be limited to the initiation phase of X inactivation. In agreement with this, the results of Wutz and Jaenisch[14] indicate that coating of the X chromosome by *Xist* RNA rapidly induces transcriptional silencing, whether by recruiting chromatin-modifying factors or simply by changing the environment of the X chromosome, only during the first 48 hours after ES cell differentiation. The *Xist*-RNA-associated factors that are involved in this initial silencing must later be replaced or reinforced by other epigenetic silencing marks, such as deacetylation and methylation, that can be stably propagated and can maintain the inactive state through mitosis.

An association between *Xist* RNA and another chromatin protein, the histone variant macroH2A1.2 has been shown using chromatin immunoprecipitation and reverse-transcriptase polymerase chain reaction (RT-PCR) on extracts of adult female cells[24]. Immunofluorescence studies on such cells had previously revealed that macroH2A is enriched on the inactive X chromosome and forms a structure known as the MACROCHROMATIN BODY, or MCB[28]. By contrast, in undifferentiated ES cells, macroH2A is clustered away from the X chromosome[5] and seems to associate with CENTROSOMES[29]. MCB formation in differentiating female (XX) ES cells is progressive, occurring only after several days of differentiation, well after *Xist* RNA coating and inactivation have occurred[5,29]. These kinetics might indicate that macroH2A is unlikely to be involved in the initiation of random X inactivation and that its recruitment to the X chromosome must depend on factors other than *Xist* RNA alone. In fact, a recent study has shown that the association of macroH2A with the inactive X, which seems to depend on the histone part of the macroH2A molecule rather than the non-histone tail, might partly reflect the higher density of several histones on the inactive X[30]. As the association of macroH2A with the inactive X is disrupted upon loss of *Xist* RNA expression without any apparent destabilization of the inactive state[31], it does not seem to be necessary either for maintenance of the inactive state or for the initiation of random X inactivation. However, a more important role for macroH2A in imprinted X inactivation is indicated by studies in preimplantation embryos[32].

Counting and choice

Because the *Xist* transcript can induce gene silencing *in cis*, independently of flanking and intronic DNA sequences, but cannot induce inactivation of the endogenous X chromosome in the cell[14], the counting function of the *Xic* must be encoded by elements situated outside of *Xist*. Given the central role of the *Xist* transcript, such elements are likely to regulate *Xist* expression, whether directly or indirectly. Deletion analysis is being used to define such elements.

Clerc and Avner[33] created a 65-kb deletion of a region lying 3′ to the main part of the *Xist* gene (FIG. 1). The deletion ($X^{\Delta 65}$) included the terminal two *Xist* exons, the postulated principal initiation site for the *Tsix* antisense transcript and the *DXPas34* locus (BOX 1) on one of the X chromosomes in a female (XX) ES cell line. In undifferentiated mutant ES cells, *Xist* transcription from the mutated X, as visualized by RNA FISH, is markedly reduced. Upon differentiation, the deletion

MACROCHROMATIN BODY
(MCB). Discrete accumulation of the histone variant, macroH2A, on the inactive X chromosome.

CENTROSOME
The microtubule organizing centre that divides to organize the two poles of the mitotic spindle and directs assembly of the cytoskeleton, thus controlling cell division, motility and shape.

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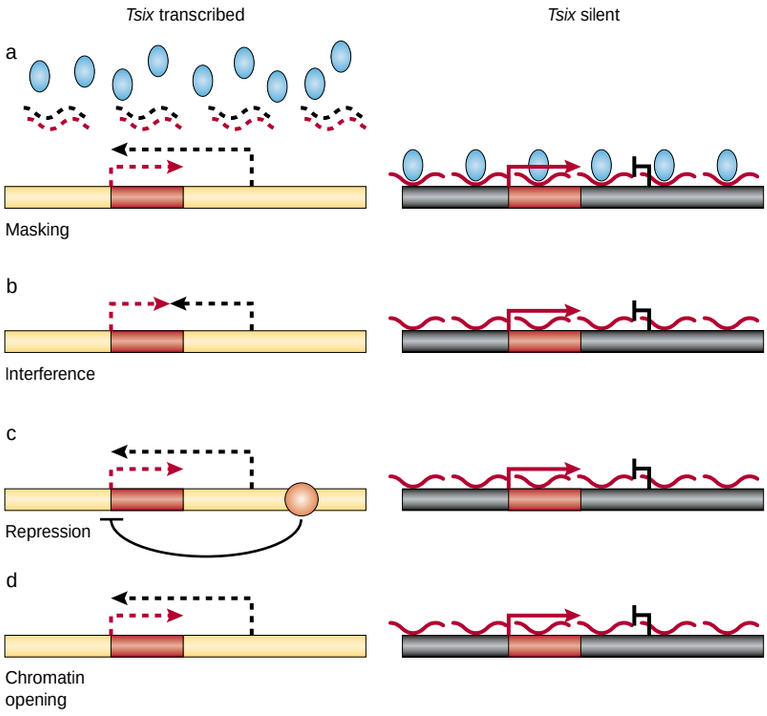

Figure 3 | **Potential roles for *Xist* antisense transcription. a** | Masking of the *Xist* transcript by the antisense transcript *TsiX* (black dashed lines) prevents binding of factors that might be involved in the stabilization of *Xist* RNA or in allowing it to associate *in cis* with chromatin. **b** | Transcriptional interference between *Xist* and *TsiX* might prevent the efficient transcription across the *Xist* gene and thereby prevent the accumulation of *Xist* transcript. **c** | Antisense transcription might be involved in the repression of high level *Xist* transcription. This might be through the binding of the putative 'blocking factor' somewhere in the *Xist 3′* region. **d** | Antisense transcription might be either involved in, or symptomatic of, 'chromatin opening', which might be required to enable the binding of developmentally regulated factors to the *Xic* region.

results in complete skewing of X inactivation, with only the mutated X chromosome being inactivated. Surprisingly, ES clones (derived from the original $XX^{\Delta 65}$ line) in which only the $X^{\Delta 65}$ chromosome is present, still initiated X inactivation upon differentiation, despite the absence of a second *Xic*. At present, this is the only example of a cell line that contains a single *Xic* and that undergoes X inactivation.

The blocking hypothesis of X-chromosome counting proposes that limited quantities of a blocking factor bind to the *Xic* of a single chromosome per diploid cell, protecting it from inactivation. The second *Xic* present in a female cell nucleus remains unprotected and is therefore inactivated. This hypothesis predicts that a single *Xic* in a diploid cell will always be blocked and therefore will always be protected from inactivation. The blocking factor could either be a diffusible molecule or, equally, a unique nuclear compartment or attachment site such as the nuclear membrane. The results from the $X^{\Delta 65}$ chromosome can be explained if a binding element for the blocking factor lies within the deleted region. In the absence of this element, the blocking factor cannot bind and the deleted X chromosome is automatically chosen for initiation of inactivation.

Clerc and co-workers have refined the analysis of the 65-kb deletion by successively adding back parts of the deleted region. Adding back the terminal two *Xist* exons did not change the behaviour of the deleted $X^{\Delta 65}$ chromosome, confirming that elements outside of the *Xist* gene itself are responsible for the phenotype (C. Morey, P. Clerc and P. A., unpublished observations).

It has become increasingly clear that the 65-kb deletion covers a complex series of regulatory loci that control counting, chromosome choice, the levels of *Xist* transcription and antisense transcriptional activity across the *Xist* gene in undifferentiated ES cells. Deletion of the *DXPas34* locus, which lies within the 65-kb deletion and is associated with the principal initiation site for the *Tsix* antisense transcript, leads to the abolition of both antisense activity and either an absence of, or a marked reduction in, *Xist* transcription from the mutated *Xic* in undifferentiated ES cells, as visualized by RNA FISH[33,34]. Upon differentiation, the deletion results in complete skewing of X inactivation, with only the mutated *Xic* inducing X inactivation[34,35]. However, the counting function of the *Xic* locus is unaffected by deletion of *DXPas34* (REF. 35). Although counting and choice are therefore distinct functions, it is possible that control of *Xist* and *Tsix* antisense transcriptional activity could be mechanistically linked to chromosome choice through a pivotal mechanism present in ES cells both before, and at the onset of, inactivation. The situation is becoming more complicated because there are good reasons to think that antisense transcription in the *Xic* could be both more widespread and complex than initially thought[34]. Furthermore, the antisense RNA itself might not have a direct role. Instead, the act of transcription might be critical in regulating the state of a large chromatin domain around *Xist*, as proposed for other loci, including the human β-globin locus[36,37] (FIG. 3).

The importance of chromatin domain formation might explain why single-copy yeast artificial chromosome transgenes for the *Xic*, which are capable of synthesizing *Xist* in a correctly regulated manner in undifferentiated cells, cannot initiate X inactivation, unlike multicopy arrays of the same transgene[18]. Multicopy arrays are thought to create a chromatin domain that provides counting and/or choice functions, albeit to variable extents[18]. By contrast, single-copy transgenes would not be able to form such a chromatin domain.

Whereas only a single region of the *Xic* might be involved in counting, several regions influence the choice of X chromosome that will be inactivated (FIG. 1). Apart from the *DXPas34*/*TsiX* region and the *Xce* locus, which lies beyond the 65 kb deletion[38], data from the human *XIC* indicate that mutation of the *XIST* promoter itself can lead to skewed X inactivation[39]. The phenotype of the *Xist* knockout described by Marahrens *et al.*[40] suggests another element involved in this choice lies within the *Xist* gene itself.

Imprinted X inactivation

X inactivation is initially subject to imprinting during the early development of some EUTHERIAN (placental) mammals — the paternally inherited X chromosome is preferentially inactivated in the first cells to differentiate,

EUTHERIANS
Mammals that give birth to live offspring (viviparous) and possess an allantoic placenta.

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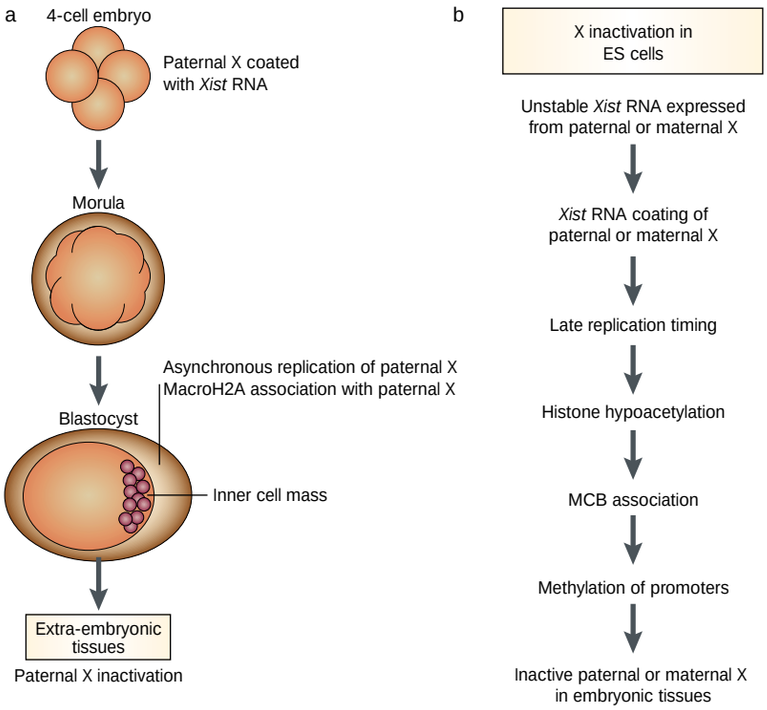

Figure 4 | **Imprinted versus random X inactivation.** Kinetics of the events underlying X inactivation in imprinted versus random X inactivation. The different epigenetic changes associated with the X-inactivation process are shown. **a** | During pre-implantation mouse development, *Xist* RNA coating of the paternal X occurs well before (at least 4 cell divisions) the first signs of inactivation in the trophectoderm of the early blastocyst, and before the novel histone variant macroH2A accumulates and the macrochromatin body forms. **b** | By contrast, during random X inactivation in embryonic stem (ES) cells, *Xist* RNA coating of the X chromosome is rapidly followed by transcriptional silencing (within roughly 24 hours, or 1 cell cycle) and MCB formation is a much later event.

which later give rise to extra-embryonic tissues (FIG. 4). Such imprinted, paternal X inactivation is observed in all tissues of marsupials. It has been hypothesized[41] that this might have been the ancient form of X inactivation, with random X inactivation evolving only later in eutherian mammals.

Recent studies in the mouse have revealed some important differences in the kinetics of the events that lead up to the establishment of the inactive state between the imprinted, extra-embryonic and the random, embryonic forms of X inactivation (FIG. 4). For example, *Xist* RNA is found to coat the paternal X from an early stage (as early as 2–4 cells), well before any sign of cellular differentiation. This coating precedes the first signs of imprinted inactivation in the TROPHECTODERM of the blastocyst by several cell divisions. This differs from the rapidity with which *Xist* RNA coating is followed by transcriptional inactivation in differentiating ES cells (within about one cell division[14]), where random X inactivation takes place.

Studies on the timing of appearance of macroH2A association with an X chromosome (MCB formation) during early embryogenesis have also pointed to a fundamental difference between imprinted and non-imprinted X inactivation (FIG. 4). In ES cells, localization of macroH2A to the inactive X chromosome occurs only late in differentiation (see above), whereas in early embryos (8–16-cell stages) and in extra-embryonic cells of the blastocyst, association of macroH2A with the paternal X chromosome undergoing imprinted X inactivation occurs rapidly[32] (FIG. 4). The early association of the paternally derived X chromosome with *Xist* RNA and, soon after this, with macroH2A might reflect a particular chromatin state that is acquired during male gametogenesis of the paternally derived X. The observation that macroH2A is associated with the SEX VESICLE in male meiosis could be compatible with such an interpretation[42,43]. In fact, the paternal genome as a whole is markedly different to the maternal genome in its chromatin state, its global methylation[44] and its replication[45] during the cleavage stages of embryogenesis. Indeed, the two genomes remain compartmentalized up to, and beyond, the 4-cell stage[46]. The arrival of the paternal X chromosome in the fertilized zygote, in a partially condensed state as a result of its passage through the male germ line, has been suggested as one way in which the X-inactivation process may initially have evolved (reviewed in REF. 1). Aspects of this state might be maintained during the initial cleavage divisions of the embryo through the conservation of chromatin structure during replication, and this might have provided the basis for selection during evolution to establish dosage compensation in the female.

The idea that the arrival of the paternal X in a partially condensed state is one of the keys to imprinted inactivation has received indirect support from the cloning experiments of Eggan and colleagues[47]. The X chromosome selected for inactivation in extra-embryonic tissues derived from a cloned somatic nucleus is always the one that had initially been inactive and which carried a mature *Xist* RNA domain and other characteristics of the inactive X at the moment of transfer. By contrast, embryonic tissues derived from the cloned adult somatic nucleus showed random inactivation. This emphasizes the specificity of the observations made in the extra-embryonic tissues and also shows the capacity of the early embryo to reverse *Xist* RNA coating and erase other epigenetic marks on the inactive X.

Although the paternal X chromosome seems to be predisposed to X inactivation, this paternal imprint can be overridden to allow dosage compensation when two paternal X chromosomes are present. Whether the counting process can occur in extra-embryonic tissues is not totally clear, although the recent finding, that cloned nuclei from ES cells (in which both X chromosomes are active) can give rise to extra-embryonic tissues showing random X inactivation[47], might indicate that counting does occur. However, other observations are less clear-cut. For instance, Okamato and colleagues recently produced female ANDROGENONES which survived to embryonic day 7.5 (E7.5) — longer than in previous experiments. These androgenetic embryos showed inactivation (late replication) of only one of the two paternal X chromosomes in all tissues, including extra-embryonic tissues, again suggestive of counting[48]. However, in these experiments, both paternal X chromosomes were in fact initially coated by *Xist* RNA, indicating that X

TROPHECTODERM
The precursor to the bulk of the embryonic part of the placenta.

SEX VESICLE OR XY BODY
Pairing of sex chromosomes during meiosis in male mammals is associated with heterochromatinization and occurs in the sex vesicle or XY-body, a specific nuclear structure that can be discerned morphologically.

ANDROGENONE
Embryo with two paternal sets of chromosomes.

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inactivation might have actually initiated on both X chromosomes, but by the late blastocyst stage one of the two paternal Xs had apparently lost this *Xist* RNA association in most cells. This might indicate that X inactivation in early preimplantation embryos is reversible and indeed relatively unstable, (as in REF. 14, discussed above), and it is this instability that provides the possibility to 'override' the imprint in the event of defective dosage compensation, rather than a counting process *per se*.

Unlike this apparent reversibility of the imprint on the paternal X, the maternal X chromosome initially carries a stable imprint to resist inactivation and remain active in the first cell lineages that differentiate — the extra-embryonic tissues. Strong evidence for this resistance to inactivation of the maternal X has been obtained by Marahrens and colleagues[17]. Female embryos that carry a mutated *Xist* allele on the paternal X, and which therefore cannot inactivate this chromosome die soon after implantation, whereas those carrying the *Xist* deletion on the maternal X grow normally, inactivating the paternal X selectively. Recent studies on embryos that are DISOMIC for the maternal X also support the idea that the maternal X is resistant to inactivation in extra-embryonic tissues, with premature embryonic death resulting from a failure to undergo X inactivation in these tissues[49]. This maternal imprint, of unknown nature, seems to be acquired relatively late during oocyte growth, as it is absent in the non-growing oocyte[50].

The actual epigenetic modifications underlying both the paternal and maternal imprint remain enigmatic. Differential methylation of the *Xist* promoter was one obvious candidate for such an imprint, but detailed bisulphite sequencing has failed to support early reports based on PCR–restriction enzyme analysis of methylation differences[51]. Given the importance of the 3′ region of *Xist* in counting and choice, the imprint might instead lie here. Support for this comes from the recently reported partial disruption of the maternal imprint in mice carrying a knockout of the region containing the putative *Tsix* promoter[52]. When maternally inherited, this deletion leads to aberrant expression of the associated *Xist* allele in a proportion of cells in early embryos and abnormal inactivation of the maternal X chromosome in extra-embryonic tissues, resulting in post-implantation lethality in 90% of cases. This might indicate that the *Tsix*/*DXPas34* region 3′ to *Xist* carries at least part of the imprint responsible for resistance to inactivation on the maternally inherited X chromosome. However, bisulphite analysis of sequences in the region 3′ to *Xist*, particularly around the *DXPas34* locus and the putative promoter of *Tsix*, have failed to reveal differential methylation in extra-embryonic tissues[53], perhaps indicating that the imprinting mark is of another nature.

A role for repeats in X inactivation

X inactivation initiates from the *Xic* and then spreads across the entire X chromosome. Inactivation can also spread into an autosomal segment when this is attached to a *Xic* by translocation. Such spreading can occur over long distances — easily 100 Mb or more[54]. Inactivation can also spread into an autosome from *Xist*-containing transgenes. Silencing of autosomal material therefore differs from that of the X only in degree: it is usually both less effective and less extensive, and this is associated with a correspondingly limited spread of *Xist* RNA into the autosome[55].

Recently, Lyon[71] suggested that LINES might function to promote spreading along the X chromosome, acting as the 'way stations' or 'booster elements' originally hypothesized by Riggs[56]. LINEs were regarded as interesting candidates for this function in view of the LINE-1-rich nature of the human and mouse X chromo-

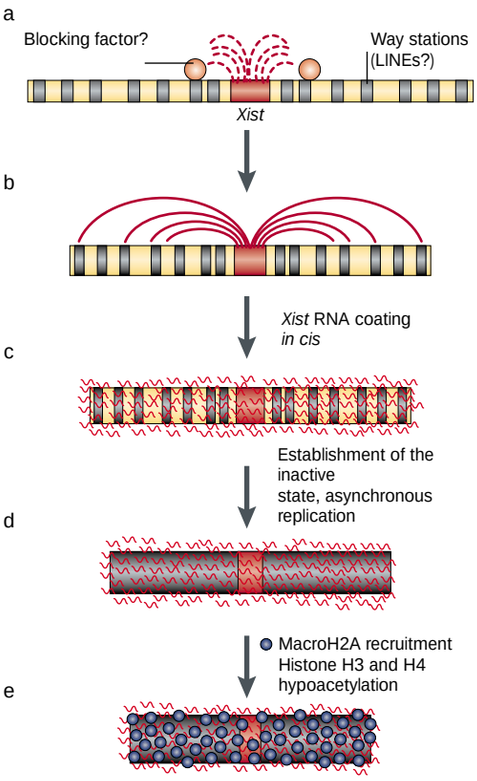

Figure 5 | **A model for X inactivation.** The model indicates speculative roles of some of the proposed players in the initiation of X inactivation. **a** | Before inactivation, *Xist* RNA is expressed in an unstable form (dotted red lines) and the postulated blocking factor(s) (red) prevents *Xist* upregulation and/or its association with the chromosome *in cis*. **b** | *Xist* RNA becomes upregulated through stabilization, transcriptional upregulation or release of the blocking factor. LINEs might participate in the spreading process in some way — either through association with nucleoprotein complexes including *Xist* or by a mechanism such as REPEAT-INDUCED GENE SILENCING (RIGS). **c** | Stabilized *Xist* RNA coats the X chromosome before its inactivation. **d** | Transcriptional silencing of genes on the X chromosome occurs as a result of *Xist* RNA coating using an unknown mechanism and is rapidly followed by a shift to asynchronous replication timing of the X chromosome. **e** | Chromatin modifications, such as the histone deacetylation and methylation of promoters of X-linked genes, as well as the recruitment of the histone variant macroH2A, presumably transform the *Xist* RNA-coated chromosome into a stably inactive and condensed chromatin state.

Animated online

UNIPARENTAL DISOMIC
An individual or embryo carrying two chromosomes inherited from the same parent

LINES
Long interspersed nuclear elements (such as L1 repeats) are retroelements present in over 100,000 copies in the mammalian genome.

REPEAT-INDUCED GENE SILENCING
(RIGS). Transgene expression in several organisms may be silenced epigenetically when repeated sequences are present. It has been proposed that interactions between homologous sequences (repeats) might lead to the formation of folded chromatin structures that attract heterochromatin-specific macromolecules

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somes. In addition, in mice there seems to be a correlation between the efficiency of the spread of inactivation into autosomes in X:autosome translocations and their LINE content[71]. Sequence analysis of the human X chromosome has now shown it to be twofold enriched for LINE-1 repetitive elements compared with the autosomes, with the greatest enrichment being in a subset of younger LINE-1 elements that were active some 60–100 million years ago[57]. LINEs therefore clearly satisfy at least one of the basic criteria for boosters — a ubiquitous distribution throughout the genome coupled to an increased frequency on the X chromosome.

A recent global analysis of the 200 or more X-linked transcribed sequences available showed that about 15% of genes on the human X chromosome escape X inactivation[58]. Most of these are located on the short arm (Xp) of the human X. Indeed, the frequency of 'escapees' on Xp is similar to that observed for autosomal genes in X:autosome translocations, a reflection of the evolutionarily recent autosomal origin of Xp[58]. Interestingly, significantly fewer LINE-1s are present in X-chromosome segments, particularly Xp22, which contains most genes that escape X inactivation. Conversely, the density of LINEs is highest in the Xq13–Xq21 region, which contains the human *XIC*. More detailed analysis of the *XIC* has shown a non-uniform LINE distribution, with a lower density of LINEs associated with the early replicating region 5′ to *XIST* and *XIST* itself than the region 3′ to *XIST*, which shows late replication[59]. The *XIST* transcript seems to associate preferentially with gene-rich (Giemsa-light) rather than LINE-rich (Giemsa-dark) chromosome bands[55,60], indicating that *XIST* and LINEs might not interact directly. One possibility is that *Xist* transcripts could mask gene-rich sequences thereby increasing the propensity of LINEs towards mediating repeat-induced gene silencing (RIGS) (Mary Lyon, personal communication).

Mice are thought to have a much smaller number of genes that escape X inactivation than humans[61], and there is evidence that the human and mouse genomes differ considerably in the number of retrotransposition-competent LINE-1 elements that they contain[62]. The mouse X chromosome might therefore have accumulated a greater and more uniform distribution of LINEs than the human X. However, escape from inactivation might not be due to inefficient spreading in some cases, but rather to inadequate maintenance of the inactive state, once established[63]. LINEs, with their tendency to be methylated,[64] could also contribute to this maintenance function.

Perspectives

The key characteristics of the X-inactivation process — its extent, remarkable stability and precise developmental regulation — suggest that it involves the sophisticated cooperation between several interacting molecules and factors, similar to many other epigenetic processes. This complexity is required to establish and then maintain a clonally heritable state of transcriptional silence (FIG. 5). The redundancy between different epigenetic modifications in the X-inactivation process presumably ensures stability both during evolution and within the individual. A particularly flagrant example of this is the increased relative stability of the inactive X in placental mammals, compared with that of marsupials. In placental mammals, the inactive X is characterized by hypoacetylation and hypermethylation of gene promoters whereas only hypoacetylation is present in the marsupial inactive X.

The superficial nature of our understanding of the role and redundancy of epigenetic mechanisms in gene regulation and higher order cellular control is emphasized by the recent discovery that several genes that are involved in a wide variety of epigenetic processes are responsible for well-defined monogenic human diseases. Examples include the *DNMT3B* gene (encoding a DNA methyltransferase) in immunodeficiency-centromeric instability-facial anomalies syndrome (ICF syndrome)[65] and the *MECP2* gene (encoding a methyl-CpG-binding protein) in Rett syndrome[66].

RNAs, rather than proteins, are particularly appealing as primary epigenetic signals in processes that require *cis*-limited gene regulation. Transcripts such as *Xist* might function by allowing the deposition of repressor complexes on the inactive X in mammals or, in the case of the *roX* RNAs, by facilitating and guiding the deposition of activator complexes onto the hypertranscribed X chromosome in *Drosophila*. The proteins that interact with these RNAs to form chromatin-associated nucleoprotein complexes are only just being deciphered in the *Drosophila* dosage-compensation system. These complexes are likely to represent an area of intense future research in mammalian X-chromosome inactivation. Other transcripts, exemplified by the widespread low-level transcription both in the antisense and sense directions, from the *Xic* region, are also likely to be an integral feature of many chromatin control mechanisms, although their mode of action is less clear.

In this review, we have indicated the many areas of uncertainty concerning X inactivation. The pivotal position for *Xist* is not in doubt, but there is much to discover about how *Xist* is controlled, and the precise sequence of events that takes place after *Xist* is expressed. Hints are emerging, and there will undoubtedly be parallels with other cellular processes. So X inactivation, along with other epigenetic processes, is likely to prove no more of a stranger than the rest of biology to evolutionary tinkering, incest, mugging and bag snatching.

Links

DATABASE LINKS *Xce* | *Xist* | *Tsix* | *roX1* | *roX2* | *MLE* | *MSL-1* | *MSL-2* | *MSL-3* | *MOF* | *DNMT3B* | *ICF* syndrome | *MECP2* | Rett syndrome

ENCYCLOPEDIA OF LIFE SCIENCES X-chromosome inactivation | X-inactivation mechanisms

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