Imprinted X inactivation and reprogramming in the preimplantation mouse embryo

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X chromosome inactivation is a developmentally regulated process that causes one of the two X chromosomes in normal female mammals to become transcriptionally silenced, thus equalizing the expression of X-linked genes between the sexes. Such dosage compensation depends upon dynamic genetic and epigenetic events occurring very early in development. X inactivation is controlled by an X inactivation centre that is associated with the expression of non-coding RNAs required for the silencing. Also associated with the inactive X are repressive histone modifications and polycomb protein-mediated states, which are progressively acquired during the inactivation process. In mouse, two forms of X inactivation have been described. Random X inactivation happens in the derivatives of the inner cell mass (ICM) giving rise to embryos where the maternally inherited X(Xm) is inactive in some cells and the paternally derived X (Xp) is inactive in others. Random X inactivation occurs around the time of implantation. Imprinted X inactivation, the preferential inactivation of the Xp chromosome, occurs earlier and, although there has been some debate as to the precise timing of initiation of this event, is apparent in all cells early in preimplantation development, then is subsequently confined to the cells of the extraembryonic lineages. A picture is emerging whereby initial epigenetic asymmetry between the two parental X chromosomes is reprogrammed in a lineage specific manner resulting in a switch from imprinted to random inactivation in embryonic derivatives. Neither the underlying reason nor the full extent of these early lineage specific epigenetic changes is known, but they may be correlated with more genome-wide reprogramming events essential for normal development.

X INACTIVATION AND ROLES FOR NON-CODING RNAs

During early development of the normal female mammal, one of the two X chromosomes becomes transcriptionally silenced (1). The choice of which X chromosome to become inactivated in embryonic lineages is random; however, in the extraembryonic lineages in the mouse, the paternally derived X is chosen to be inactivated (2). This parental origin-specific inactivation is an example of genomic imprinting; a process that causes genes to be expressed from one of the two parental chromosome homologues (3). The reason for this lineage specific difference in mouse X inactivation is not known. Interestingly, in marsupial mammals, the Xp is preferentially inactivated in all lineages (4), whereas data in humans suggest that only random X inactivation occurs, although

this remains debatable (5). It is possible, therefore, that imprinted X inactivation in the mouse extraembryonic derivatives represents the more ancestral mechanism. The dynamic epigenetic events that regulate X inactivation involve a number of steps including counting the X chromosomes such that all but one become repressed, choosing which X chromosome has to be inactivated, initiating the inactivation process, spreading the inactive state along the length of the chromosome and maintaining this repressive epigenetic state throughout the lifetime of the individual (6).

It has been shown that X inactivation is controlled by a cytologically identified chromosomal region called the X inactivation centre (Xic). Although the molecular basis of Xic remains obscure, two non-coding genes, *Xist* and its antisense transcript *Tsix*, mapped in Xic, play critical roles in X inactivation (Fig. 1). *Tsix* is expressed on the X that escapes

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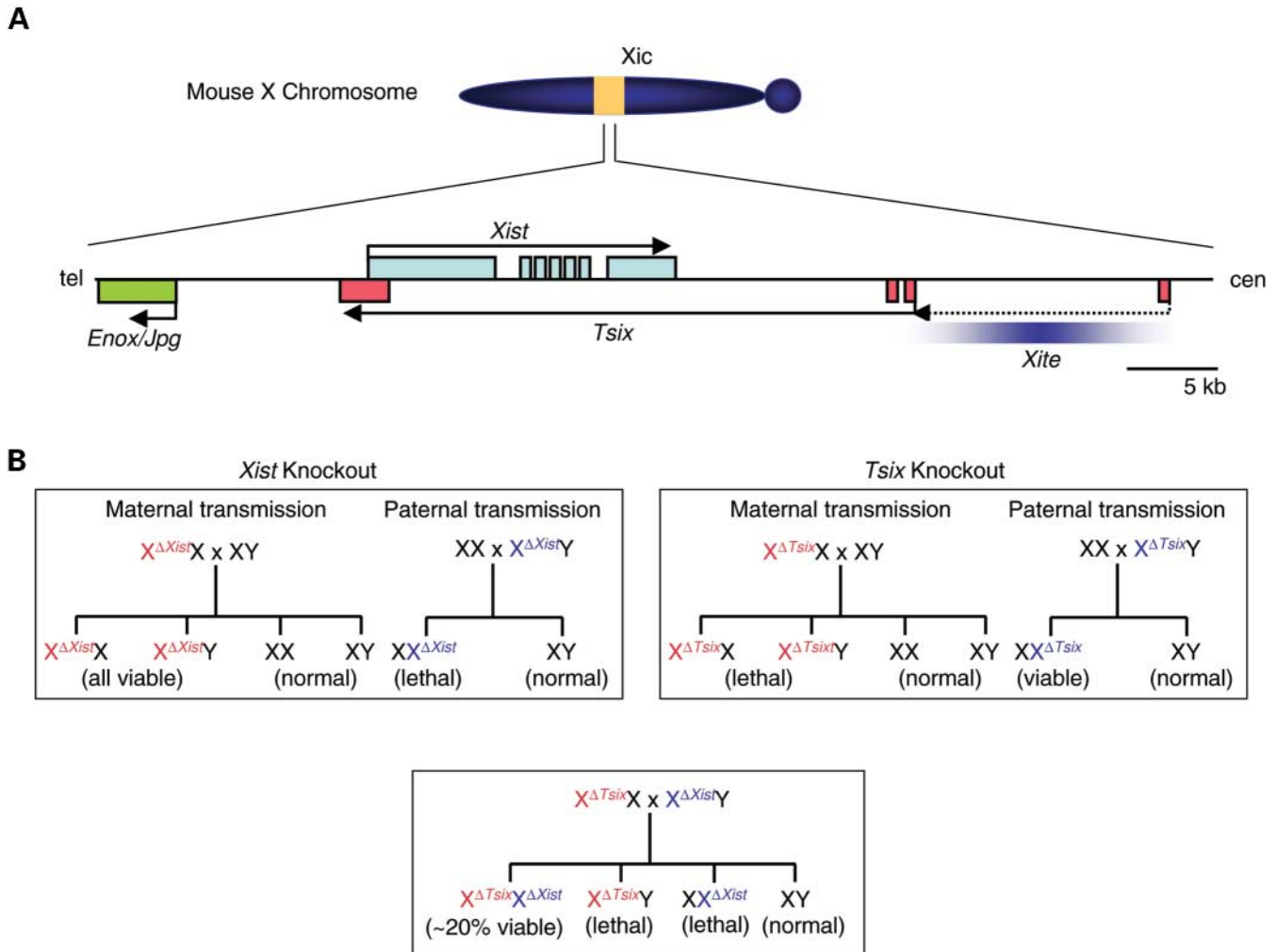
Figure 1. Genomic structures of the *Xist/Tsix* loci in the Xic and the effects of paternal and maternal transmission of the *Xist*- and *Tsix*-deficient allele. (**A**) The gene structures of *Xist* and *Tsix* and relative orientations of their transcription on the mouse X chromosome are shown. Boxes and arrows delineate exons and transcriptional orientations of each gene, respectively. Although exon1 of *Tsix* has been found about 30 kb downstream of the *Xist* gene (17), the major transcription of *Tsix* appears to occur in the vicinity of exon2 (17,18,42). *Xite* is a non-coding transcript occurring in the same direction as *Tsix* in the upstream region of exon2 of *Tsix* (43). *Enox/Jpg* located 10 kb upstream of *Xist* is a non-coding gene transcribed in an opposite direction to *Xist* (44). (**B**) Although the *Xist*-deficient allele is transmitted to both male and female pups from the mothers, only its paternal transmission causes embryonic lethality in females soon after implantation due to the failure of imprinted inactivation of the paternal X in the extraembryonic tissues (upper left) (16). In contrast, although the *Tsix*-deficient allele is transmitted to viable female pups from the fathers, its maternal transmission results in early postimplantation lethality of both male and female embryos due to functional nullisomy of the X in the extraembryonic tissues caused by ectopic inactivation of the maternal X (upper right) (17,19). Intercrosses between $X^{\Delta Tsix}X$ and $X^{\Delta Xist}Y$ reveal that a subset of females carrying the paternal $X^{\Delta Xist}$ and the maternal $X^{\Delta Tsix}$ survive to term and thereafter (bottom) (17).

inactivation and *Xist* is expressed on the X that becomes inactivated. (7–9).

The *Xist* allele becomes upregulated from the X chromosome that is to be inactivated as cells differentiate. *Xist* RNA, thus expressed, coats the X chromosome in *cis* (10,11) and induces chromosome-wide inactivation by recruiting proteins involved in heterochromatin formation, such as the polycomb group proteins (12–15). Targeted disruption of the *Xist* gene reveals that *Xist* is essential for X-inactivation to occur in *cis* (16). Paternal transmission of the *Xist*-deficient X ($X^{\Delta Xist}$) results in embryonic lethality in female embryos at the early postimplantation stage due to the failure of paternal X inactivation and the subsequent presence of two active Xs in the extraembryonic tissues (Figs 1 and 2). It is noteworthy that

complete lethality of these female embryos indicates that the imprint laid on the Xm to resist inactivation is very rigid in the extraembryonic tissues, as Xm is unable to activate *Xist*.

Transcription of *Tsix* covers a 65 kb genomic region including the entire transcription unit of *Xist* in an antisense orientation (17,18). Targeted disruption of *Tsix* reveals that *Tsix* has a repressive role in the transcriptional regulation of *Xist* in *cis* (8,17,19). A *Tsix*-deficient X chromosome ($X^{\Delta Tsix}$) is therefore able to express *Xist* and subsequently undergoes inactivation. Maternal transmission of *Tsix* deficiency causes ectopic expression of *Xist* from the maternal $X^{\Delta Tsix}$ in the extraembryonic tissues of both male and female embryos, resulting in functional nullisomy for the X chromosome and eventual death of the embryos at the early postimplantation
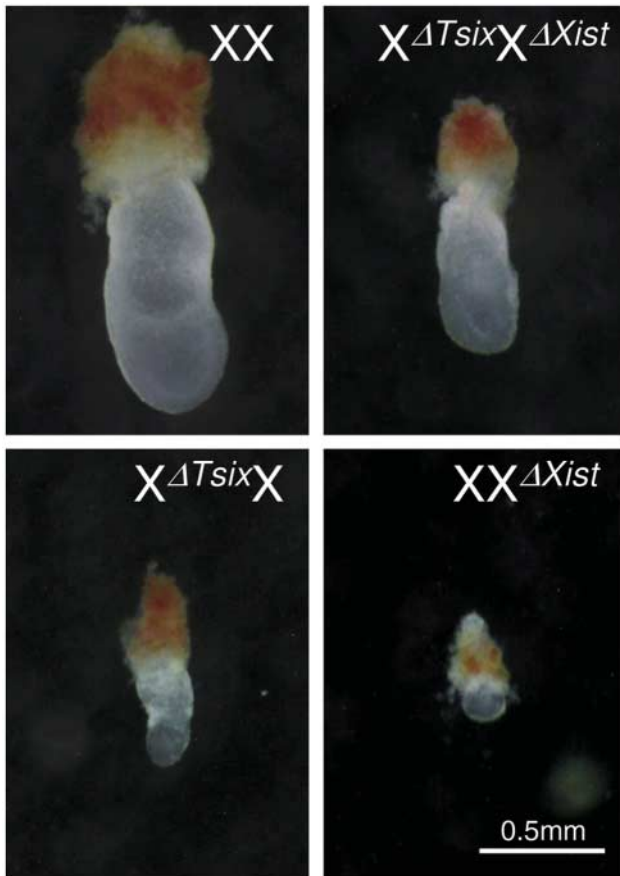
Figure 2. Comparative early postimplantation development of e7.5 normal female mouse embryos and embryos deficient for *Xist* and *Tsix* on paternally and maternally inherited X chromosomes as indicated. Heterozygotes deficient for the paternally inherited *Xist* gene fail to inactivate the paternally inherited X and die at early postimplantation stages. In contrast, heterozygotes deficient for *Tsix* on the maternally inherited X chromosome express *Xist* on that chromosome and inactivate both X chromosomes, also resulting in early post-implantation lethality. Double heterozygous females deficient in *Xist* and *Tsix* show markedly improved development and the lethality observed in the single heterozygotes is rescued at a frequency of 20% of expected females.

stage (Fig. 1). Normally, *Tsix* does not appear to be expressed until the blastocyst stage. *Tsix* transcripts have not been noted during earlier stages, and therefore, although *Tsix* is required for imprinted Xm activity in the extraembryonic lineages, it is unlikely to be responsible for the initial imprinted *Xist* repression on Xm at the earlier stages. For this, some other, currently unknown mechanism must be inferred.

It has been demonstrated that the lethality of female embryos caused by the paternally derived $X^{\Delta Xist}$ is rescued if combined with the maternally derived $X^{\Delta Tsix}$ (Fig. 1B), although such $X^{\Delta Tsix}X^{\Delta Xist}$ survivors are found only in 20% of the expected females (17). Interestingly, ectopic expression of maternal *Xist* seems to occur only after implantation in $X^{\Delta Tsix}X^{\Delta Xist}$ females (unpublished data), further supporting a role for *Tsix*-independent imprinting of Xm activity at the earlier stages. It is tempting to speculate that the 80% lethality of the double heterozygous females might be associated with the early presence of two active Xs (due to lack of *Xist*

expression from either X chromosome). However, despite the high loss, the development of $X^{\Delta Tsix}X^{\Delta Xist}$ females was greatly improved when compared with either $X^{\Delta Tsix}X$ or $XX^{\Delta Xist}$ (Fig. 2). Nonetheless, in the surviving females, the Xm becomes inactivated instead of the Xp, suggesting that *Tsix* is, at least in part, playing a role in the imprint normally laid on the Xm to resist inactivation. Furthermore, the successful 20% rescue seen in these females also raises an interesting question about the biological significance of the inactivation of Xp during normal preimplantation development. This is because in the absence of paternally expressed *Xist*, both X chromosomes might be expected to stay active in $Xm^{\Delta Tsix}Xp^{\Delta Xist}$ females until the blastocyst stage and the onset of random inactivation. Thus, in contrast to cells that have differentiated into the embryonic and extraembryonic lineages, dosage compensation of the X-linked genes may not be an absolute requirement during early preimplantation development. This is consistent with incompleteness of the inactivation status of the Xp at that time (described subsequently) (20–22).

IMPRINTED X INACTIVATION AND SUBSEQUENT REPROGRAMMING

The difference in X inactivation between the embryonic and extraembryonic lineages in the mouse is quite remarkable. Although either one of the two X chromosomes undergoes random inactivation in the embryo, the Xp is preferentially inactivated in the extraembryonic lineages, and therefore, X inactivation is imprinted (reviewed in 4). Recently, there has been some debate over when imprinted X inactivation begins in the mouse. This is a relevant issue because understanding this mechanism will provide insight into germline inherited silencing mechanisms in general, will facilitate mechanistic comparisons with autosomal imprinting and will allow consideration of the relationship between X chromosome reprogramming and genome-wide reprogramming events—issues important for our knowledge of the epigenetic control of genome function during mammalian development.

The prevailing view, held over the past 30 years, had stated that X inactivation occurs in mouse and humans after the blastocyst has implanted into the uterine wall (23). This is consistent with the first cytological evidence of a visible inactive X and the expression of X-linked gene products from both the maternally and paternally inherited X at earlier stages (20–22). Inactivation of the paternally inherited X was believed to occur for the first time in the trophectoderm and primitive endoderm (giving rise to the placenta and part of the extraembryonic membranes), leaving both Xs active in the undifferentiated inner cell mass (ICM) cells. Subsequently, in the ICM cells, either X became inactivated randomly regardless of parental origin. Three different recently published papers convincingly argue against this conventional view of the X inactivation process in the preimplantation embryos and shed light on the mechanisms of X inactivation during preimplantation development (24–26). These studies indicate that X inactivation is governed by imprinting in all cells during the preimplantation stages. Thereafter,

reprogramming occurs in the embryonic derivatives and X inactivation becomes random.

Using a range of different techniques, the three studies show that Xp has already been inactivated at the preimplantation stages in a manner that is dependent on the expression and accumulation of *Xist* RNA on the X chromosome. The transcriptional repression is progressive and is associated with the accumulation of repressive histone modifications and other proteins known to be enriched on the inactive X. The precise timing of the onset of Xp inactivation is the subject of debate and has been outlined elsewhere (27), and more experiments are required to settle the issue. Two models have been proposed. In the first model, the paternal X enters the egg during fertilization in a preinactive state (24). Upon activation of the zygotic genome at the two-cell stage, *Xist* transcripts accumulate, but in all other respects, the paternal X, in general, remains transcriptionally inactive. Transcriptional silencing of the XY bivalent during male meiosis is a well-established phenomenon consistent with the possibility that the paternal X may be inherited by the zygote in this repressed state (28). The second model suggests that the paternal X is transcriptionally active at the two to four cell stage accumulating *Xist* transcripts and, thereafter, becomes progressively transcriptionally silent (25). Regardless of whether either of these models is correct, it is clear that imprinted X inactivation takes place several cell divisions earlier than the blastocyst stage, in contrast to the original belief.

Okamoto *et al.* (25) and Mak *et al.* (26) also showed that *Xist* RNA disappears from the Xp in the epiblast lineage at the blastocyst stage with a concomitant loss of inactive X-specific histone modifications or association of polycomb group proteins members, suggesting reactivation of the hitherto inactive Xp. Intriguingly, such an event apparently takes place in the cells that become positive for Nanog, a marker for pluripotent undifferentiated cells (29,30), as early as the morula stage (26). It is likely that this population of cells is the precursor of ICM cells. During this time, it is well established that genome-wide DNA methylation reprogramming is occurring, contributing to pluripotency. It appears, therefore, that in this lineage, the X chromosome also undergoes reprogramming, causing reactivation of the Xp. This coincident reprogramming of autosomal and X chromosomes may have removed parental imprints on the X, resulting in the second wave of X inactivation being random. During this preimplantation period, and in contrast to what happens to Xp in embryonic progenitors, all autosomal germline methylation imprints tested to date seem to retain the memory of their parental origin and appear resistant to aspects of the reprogramming process (see Morgan *et al.*, this issue).

A similar X chromosome reprogramming event also happens in primordial germ cells (PGCs) that have completed migration into the genital ridge (31). It seems likely that reactivation of the inactivated X in PGCs is an essential process for the further development of the germ cells. Genome-wide reprogramming also occurs in PGCs and at this time, autosomal imprints also get reprogrammed. Therefore, X chromosome reprogramming appears at least temporally coordinated with two other genome-wide reprogramming events integral to normal development—in PGCs and in embryonic progenitors at preimplantation stages. Therefore, it seems reasonable to speculate that the regulation of autosomal and X chromosome reprogramming might be related. However, because autosomal imprints are resistant to this reprogramming in preimplantation embryos (though not in PGCs), this implicates a difference in the mechanism of imprinted X inactivation and autosomal imprinting in the embryonic progenitors. Furthermore, on the basis of non-reprogrammable inactivation of the paternal X in the extraembryonic derivatives, one might extrapolate that genome-wide reprogramming may not occur to the same extent in cells giving rise to trophoblast derivatives compared with the epiblast.

Another key question regarding imprinted X inactivation is what prevents the maternally inherited X chromosome from accumulating *Xist* RNA and becoming inactivated. Although a role for *Tsix* is implicated in random inactivation, its absence in preimplantation embryos and the failure to inactivate the Xm in Δ*Tsix* mutants suggest that the maternally inherited X carries an imprint preventing *Xist* expression on that chromosome. The nature of this imprint is not known (as described subsequently).

X INACTIVATION, DNA METHYLATION AND AUTOSOMAL IMPRINTING

In female somatic cells, the promoter of the transcriptionally repressed *Xist* allele on the active X is highly methylated, whereas the transcriptionally active one on the inactive X is not (32). Similarly, a region composed of some repetitive sequences 15 kb downstream of the *Xist* gene, which is called DXPas34, is methylated on the active X but not on the inactive X in somatic cells (33). DXPas34 is located in the vicinity of the major transcription start site of *Tsix*. These results suggest that differential methylation may play some role in transcriptional repression of the *Xist* gene on the active X and the *Tsix* gene on the inactive X.

The methylation status of these two differentially methylated regions has been studied in preimplantation mouse embryos. Earlier studies by PCR-based analyses suggested that the 5′ region of *Xist* is maternally methylated in the early cleavage stage embryos, and this differential methylation underlies imprinted expression of the paternal *Xist* (34,35). A later study using bisulphite genomic sequencing showed that this region stays unmethylated until the blastocyst stage (36). The reason for this discrepancy is unknown at the moment. Recent work by Sado *et al.* (37) in mouse embryos deficient for *de novo* DNA methyltransferases shows that differential methylation at the 5′ region of *Xist* is not the primary mechanism for the differential induction of *Xist* at the onset of random X inactivation because *Xist* remains monoallelic despite its promoter being unmethylated on both alleles. This suggests that the parental imprint for paternal expression of *Xist* does not necessarily rely on DNA methylation. For *Tsix*, the methylation profile of DXPas34 analysed by bisulphite genomic sequencing indicates that this region is not methylated on both parental alleles during preimplantation development (38), suggesting that imprinted expression of *Tsix* in the extraembryonic tissues does not depend on DNA methylation at the DXPas34 region. It has been shown that, whatever its nature, an imprint is placed on the Xm during

oocyte maturation in the prophase of meiosis I (39). Most recently, Kaneda *et al.* (40) found that DNA methylation mediated by the *de novo* methyltransferase Dnmt3a in both parental germlines can confer parental marks required for appropriate expression of the autosomal imprinted genes. It is therefore particularly interesting to consider whether Dnmt3a is also involved in the resistance of the Xm to inactivation. If the imprint on the Xm is not established in maternal germline mutants for Dnmt3a, then one would expect to see random X inactivation in the extraembryonic tissues of female embryos. However, results indicate that the paternal X is always inactive and the Xm stays active in the extraembryonic tissues of those female embryos derived from oocytes null for Dnmt3a (41), indicating that Dnmt3a-mediated DNA methylation does not confer an imprint on the Xm in the maternal germline to resist inactivation. This difference between imprinted X inactivation and autosomal imprinting suggests that there may be significant mechanistic differences between the two processes.

In conclusion, the assessment of the dynamic epigenetic changes occurring on the two X chromosomes before fertilization and in the early embryo provides valuable insight into heritable silencing mechanisms involved in this important dosage compensation mechanism. The role of genomic imprinting in the early part of this process and, subsequently, in the mouse extraembryonic derivatives is intriguing and may provide a link with the more ancestral mechanism occurring in all somatic cells of marsupial mammals. This has important implications for our understanding of the evolution of the imprinting mechanism in general. A resolution of the debate surrounding the precise timing of the onset of imprinted paternal X inactivation is also important from a mechanistic perspective. The early reprogramming events that result in a switch from imprinted to random X inactivation in embryonic progenitors and their derivatives provide a useful comparison with other reprogramming events. For example, erasure of the inactive X and autosomal imprints in PGCs might be regulated concurrently with the more genome-wide reprogramming events occurring during germ cell development. Similarly, we have suggested that genome-wide and X chromosome reprogramming might be part of the same process in ICM progenitors. However, although many investigators have, over the years, focused on similarities between autosomal imprinting and X inactivation and made mechanistic comparisons, here we draw attention to some striking differences. First, the X chromosome imprint is not resistant to genome-wide reprogramming at preimplantation stages like the autosomal imprints are. And secondly, at least one part of the machinery that confers maternal germline methylation imprints on autosomes does not contribute to imprinting on the maternally inherited X chromosome. Therefore, although germline reprogramming events that serve to erase epigenetic marks may not discriminate between X chromosomes and autosomes, evidence suggests that significant aspects of the re-establishment and maintenance of X-linked and autosomal imprints are different.

REFERENCES

1. Lyon, M. (1961) Gene action in the X chromosome of the mouse (*Mus musculus*). *Nature*, **190**, 372–373.

2. Takagi, N. and Sasaki, M. (1975) Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. *Nature*, **256**, 640–642.

3. Ferguson-Smith, A.C. and Surani, M.A. (2001) Imprinting and the epigenetic asymmetry between parental genomes. *Science*, **293**, 1086–1098.

4. Cooper, D.W., Vandeberg, J.L., Sharman, G.B. and Poole, W.E. (1983) Phosphoglycerate kinase polymorphism in kangaroos provides further evidence for paternal X inactivation. *Nat. New Biol.*, **230**, 155–157.

5. Migeon, B. and Lee, C. (2002) Species specific differences in TSIX/Tsix reveal the roles of these genes in X chromosome inactivation. *Am. J. Hum. Genet.*, **71**, 286–293.

6. Avner, P. and Heard, E. (2001) X-chromosome inactivation: counting, choice and initiation. *Nat. Rev. Genet.*, **2**, 59–67.

7. Kay, G.F., Penny, G.D., Patel, D., Ashworth, A., Brockdorff, N. and Rastan, S. (1993) Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell*, **72**, 171–182.

8. Lee, J.T. (2000) Disruption of imprinted X inactivation by parent-of-origin effects at Tsix. *Cell*, **103**, 17–27.

9. Sado, T., Wang, Z., Sasaki, H. and Li, E. (2001) Regulation of imprinted X-chromosome inactivation in mice by Tsix. *Development*, **128**, 1275–1286.

10. Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafreniere, R.G., Xing, Y., Lawrence, J. and Willard, H.F. (1992) The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell*, **71**, 527–542.

11. Clemson, C.M., McNeil, J.A., Willard, H.F. and Lawrence, J.B. (1996) XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. *J. Cell Biol.*, **132**, 259–275.

12. Brockdorff, N. (2002) X-chromosome inactivation: closing in on proteins that bind Xist RNA. *Trends Genet.*, **18**, 352–358.

13. Mak, W., Baxter, J., Silva, J., Newall, A.E., Otte, A.P. and Brockdorff, N. (2002) Mitotically stable association of polycomb group proteins eed and enx1 with the inactive x chromosome in trophoblast stem cells. *Curr. Biol.*, **12**, 1016–1020.

14. Fang, J., Chen, T., Chadwick, B., Li, E. and Zhang, Y. (2004) Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X-inactivation. *J. Biol. Chem.*, **279**, 52812–52815.

15. de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M. *et al.* (2004) Polycomb group proteins ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev. Cell*, **7**, 663–676.

16. Marahrens, Y., Panning, B., Dausman, J., Strauss, W. and Jaenisch, R. (1997) Xist-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes Dev.*, **11**, 156–166.

17. Sado, T., Wang, Z., Sasaki, H. and Li, E. (2001) Regulation of imprinted X-chromosome inactivation in mice by Tsix. *Development*, **128**, 1275–1286.

18. Lee, J.T., Davidow, L.S. and Warshawsky, D. (1999) *Tsix*, a gene antisense to Xist at the X-inactivation centre. *Nat. Genet.*, **21**, 400–404.

19. Lee, J.T. and Lu, N. (1999) Targeted mutagenesis of Tsix leads to nonrandom X inactivation. *Cell*, **99**, 47–57.

20. Sugawara, O, Takagi, N. and Sasaki, M. (1985) Correlation between X chromosome inactivation and cell differentiation in female preimplantation embryos. *Cytogenet.Cell Genet.*, **39**, 210–219.

21. Kratzer, P. and Gartler, S. (1978) HGPRT activity changes in preimplantation mouse embryos. *Nature*, **274**, 503–504.

22. Latham, K. and Rhambhatla, L. (1995) Expression of X linked genes in androgenetic, gynogenetic and normal mouse preimplantation embryos. *Dev. Genet.*, **17**, 212–222.

23. Wolpert, L. (1998) In *Principles of Development*. Oxford University Press, pp. 379–380.

24. Huynh, K.D. and Lee, J.T. (2003) Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos. *Nature*, **426**, 857–862.

25. Okamoto, I., Otte, A.P., Allis, C.D., Reinberg, D. and Heard, E. (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science*, **303**, 644–649.

26. Mak, W., Nesterova, T.B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A.P. and Brockdorff, N. (2004) Reactivation of the paternal X chromosome in early mouse embryos. *Science*, **303**, 666–669.

27. Ferguson-Smith, A.C. (2004) X inactivation: a pre or post-fertilisation turn-off? *Curr. Biol.*, **14**, R324–R325.
28. Solari, A. (1974) The behaviour of the XY pair in mammals. *Rev. Cytol.*, **38**, 273–317.
29. Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S. and Smith, A. (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*, **113**, 643–655.
30. Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, **113**, 631–642.
31. Gartler, S.M., Rivest, M. and Cole, R.E. (1980) Cytological evidence for an inactive X chromosome in murine oogonia. *Cytogenet. Cell Genet.*, **28**, 203–207.
32. Norris, D.P., Patel, D., Kay, G.F., Penny, G.D., Brockdorff, N., Sheardown, S.A. and Rastan, S. (1994) Evidence that random and imprinted Xist expression is controlled by preemptive methylation. *Cell*, **77**, 41–51.
33. Courtier, B., Heard, E. and Avner, P. (1995) Xce haplotypes show modified methylation in a region of the active X chromosome lying 3′ to Xist. *Proc. Natl Acad. Sci. USA*, **92**, 3531–3535.
34. Ariel, M., Robinson, E., McCarrey, J.R. and Cedar, H. (1995) Gamete-specific methylation correlates with imprinting of the murine *Xist* gene. *Nat. Genet.*, **9**, 312–315.
35. Zuccotti, M. and Monk, M. (1995) Methylation of the mouse *Xist* gene in sperm and eggs correlates with imprinted Xist expression and paternal X-inactivation. *Nat. Genet.*, **9**, 316–320.
36. McDonald, L.E. and Kay, G.F. (1997) Methylation analysis using bisulfite genomic sequencing: application to small numbers of intact cells. *Biotechniques*, **22**, 272–274.
37. Sado, T., Okano, M., Li, E. and Sasaki, H. (2004) *De novo* DNA methylation is dispensable for the initiation and propagation of X chromosome inactivation. *Development*, **131**, 975–982.
38. Prissette, M., El-Maarri, O., Arnaud, D., Walter, J. and Avner, P. (2001) Methylation profiles of DXPas34 during the onset of X-inactivation. *Hum. Mol. Genet.*, **10**, 31–38.
39. Tada, T., Obata, Y., Tada, M., Goto, Y., Nakatsuji, N., Tan, S., Kono, T. and Takagi, N. (2000) Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth. *Development*, **127**, 3101–3105.
40. Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E. and Sasaki, H. (2004) Essential role for *de novo* DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*, **429**, 900–903.
41. Kaneda, M., Sado, T., Okano, M., Hata, K., Tsujimoto, N., Li, E. and Sasaki, H. Role of *de novo* DNA methyltransferases in initiation of genomic imprinting and X-chromosome inactivation. *Cold Spring Harb. Symp. Quant. Biol.*, in press.
42. Shibata, S. and Lee, J.T. (2003) Characterization and quantitation of differential Tsix transcripts: implications for Tsix function. *Hum. Mol. Genet.*, **12**, 125–136.
43. Ogawa, Y. and Lee, J.T. (2003) Xite, X-inactivation intergenic transcription elements that regulate the probability of choice. *Mol. Cell*, **11**, 731–743.
44. Nesterova, T.B., Slobodyanyuk, S.Y., Elisaphenko, E.A., Shevchenko, A.I., Johnston, C., Pavlova, M.E., Rogozin, I.B., Kolesnikov, N.N., Brockdorff, N. and Zakian, S.M. (2001) Characterization of the genomic Xist locus in rodents reveals conservation of overall gene structure and tandem repeats but rapid evolution of unique sequence. *Genome Res.*, **11**, 833–849.