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Review

X-tra! X-tra! News from the Mouse X Chromosome

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Abstract

X chromosome inactivation (XCI) is the phenomenon through which one of the two X chromosomes in female mammals is silenced to achieve dosage compensation with males. XCI is a highly complex, tightly controlled and developmentally regulated process. The mouse undergoes two forms of XCI: imprinted, which occurs in all cells of the preimplantation embryo and in the extraembryonic lineage, and random, which occurs in somatic cells after implantation. This review presents results and hypotheses that have recently been proposed concerning important aspects of both imprinted and random XCI in mice. We focus on how imprinted XCI occurs during preimplantation development, including a brief discussion of the debate as to when silencing initiates. We also discuss regulation of random XCI, focusing on the requirement for *Tsix* antisense transcription through the *Xist* locus, on the regulation of *Xist* chromatin structure by Tsix and on the effect of *Tsix* regulatory elements on choice and counting. Finally, we review exciting new data revealing that X chromosomes co-localize during random XCI. To conclude, we highlight other aspects of X-linked gene regulation that make it a suitable model for epigenetics at work.
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Introduction

X chromosome inactivation (XCI) is the silencing mechanism used by eutherian mammals to equalize the expression of X-linked genes between males and females (Lyon, 1961). The process results in the silencing of a majority of genes on one of the two X chromosomes in females. To achieve transcriptional silencing of genes on one X chromosome, each cell of a female mammal must undergo a highly orchestrated set of events consisting of four stages: counting, choice, initiation and maintenance. First a cell must count the number of X chromosomes it contains. If more than one X is counted, the cell must then choose to inactive a specific X to ensure that only one remains active. The cell then initiates and propagates chromosome-wide silencing of the chosen X, and finally maintains this inactive state throughout subsequent cell divisions (Avner and Heard, 2001; Boumil and Lee, 2001).

The study of XCI in mice has been critical for understanding how this process unravels during the development of the organism and has revealed important details about its mechanism. The mouse undergoes two forms of XCI; random, which occurs in somatic cells, and imprinted, which occurs in the extraembryonic lineage (trophectoderm and primitive endoderm). In addition, recent work has shown that all cells of the preimplantation embryo exhibit the imprinted form of XCI, with the inner cell mass (ICM) of the late blastocyst reactivating the inactive X (Chaumeil et al., 2004; Huynh and Lee, 2003; Mak et al., 2004). The random form of X inactivation subsequently initiates in the epiblast and is thought to be complete by ~5.5–6.5 dpc (Gardner and Lyon, 1971; Hoppe and Whitten, 1972; Nesbitt, 1971; Rastan, 1982; Rastan et al., 1980; Takagi, 1974; Takagi et al., 1982; Tam et al., 1994; Tan et al., 1993). Both imprinted and random XCI require a specific region of the X chromosome, designated the *X inactivation center* (*Xic*). The *Xic* harbors distinct genetic elements that function in the silencing pathway, including the X inactive-specific transcript (*Xist*) and *Tsix* genes and the genetically defined *X-controlling element* (*Xce*). In particular, the *Xist* gene plays a vital role in inactivation (Borsani et al., 1991; Brown et al., 1991). *Xist* is expressed from the inactive X (Xi) and the RNA gradually spreads to coat the entire chromosome. Subsequent to this step, the Xi acquires distinctive chromatin features that are believed to be important for transcriptional silencing.

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Here we review the mechanism of XCI in the mouse by examining how imprinted XCI occurs during preimplantation development and how random XCI is initiated following implantation (Fig. 1). We also briefly recap other local and chromosome-wide aspects of X-linked gene regulation. Additional reviews are recommended for a discussion of X chromosome regulation in other mammals (Chow et al., 2005; Grutzner and Graves, 2004).

X inactivation during preimplantation development

During preimplantation development, cells of female embryos undergo imprinted XCI, in which the paternal X (Xp) chromosome is always inactivated. This silencing is dependent upon the Xist non-coding RNA and the subsequent recruitment of an ordered set of chromatin modifications.

When does imprinted XCI begin?

Until recently, the prevailing view was that XCI initiates upon embryo implantation. Yet, recent studies demonstrated that imprinted XCI occurs much earlier in the preimplantation embryo. The question of when the Xp is silenced during preimplantation has become a controversial issue, with two competing models attempting to resolve the debate (Fig. 1). Although these hypotheses are based largely on similar experimental approaches, their conclusions are conflicting. The "Pre-inactivation" hypothesis proposes that the Xp is inherited from the father in an already silent state, whereas the "de novo" model advocates that silencing is not initiated until after fertilization.

According to the "Pre-inactivation" hypothesis, the predisposition of the Xp to be inactivated during preimplantation originates from passage of this chromosome through the male germline (Huynh and Lee, 2001). In meiotic spermatocytes, the sex chromosomes form the XY body and are silenced through a process known as meiotic sex chromosome inactivation (MSCI) (Handel, 2004). This mechanism of silencing is not fully understood, but differs from XCI in that it is Xist-independent and is characterized by several distinct chromatin modifications including the histone variant H2AX (Fernandez-Capetillo et al., 2003; Turner et al., 2002). The Xp has been proposed to persist in a silent state through the end of spermatogenesis and enter the egg in a "pre-inactivated" state (Huynh and Lee, 2001, 2005). Huynh and Lee have employed an RNA fluorescence in situ hybridization (FISH) approach using a Cot-1 probe that detects nascent RNA, thus marking regions of active transcription. In support of the "Pre-inactivation" model, they reported that at the 2-cell stage one X chromosome does not stain with a FISH Cot-1 probe, suggesting that it lacks active transcription (Huynh and Lee, 2003). As MSCI and XCI differ in many respects, the "pre-

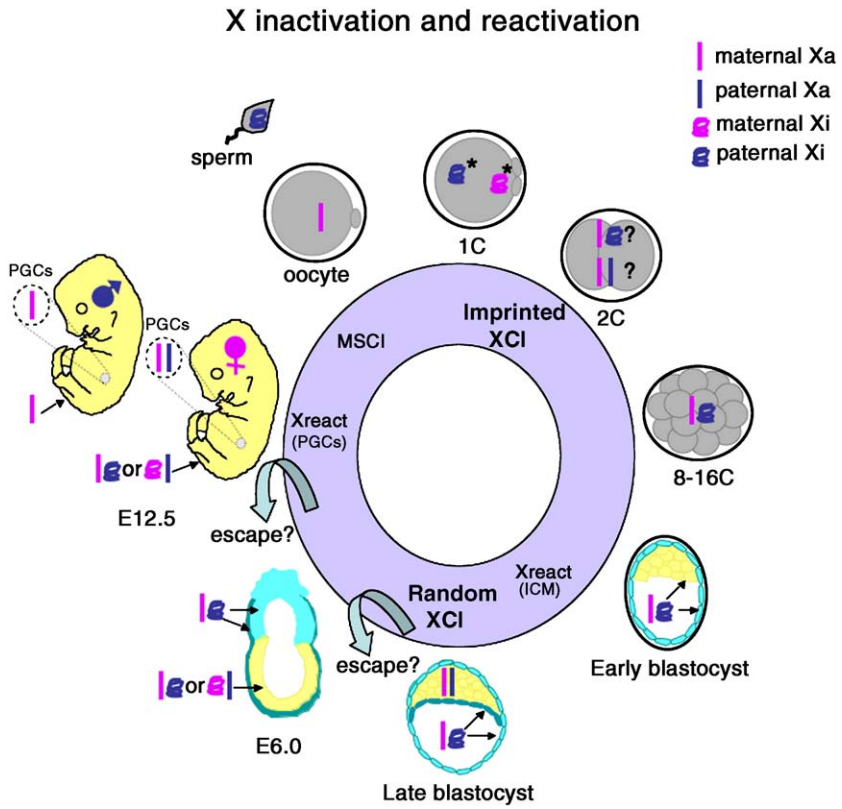

Fig. 1. Life cycle of X chromosome inactivation and reactivation in mice. The state of an X chromosome (i.e. whether X-linked genes are actively transcribed) is depicted throughout development. The asterisk in the 1 cell embryo shows that the inactivation is reflective of the silent state of the entire genome and is not X chromosome specific. In the 2-cell embryo the conflict in the literature as to whether or not the paternal X remains inactive or is reactivated is illustrated. As indicated by the curved arrows, escape from XCI could occur before random XCI (and possibly imprinted XCI) is established or be a consequence of the failure to maintain XCI post-implantation.

inactivated" Xp would have to convert to Xist-dependent silencing and acquire the correct chromatin modifications following fertilization. The "Pre-inactivation" model is attractive in that it eliminates the need for multiple rounds of inactivation and reactivation in the germline and zygote (Huynh and Lee, 2001, 2005). Two recent studies have shown that some transcriptional repression of the X chromosome persists after completion of meiosis in the male germline (Namekawa et al., 2006; Turner et al., 2006). However, other data support the idea that silencing does not persist after meiosis. First, some germ cell specific genes are reactivated in postmeiotic spermatocytes (Wang et al., 2005). Second, the presence of specific euchromatic histone modifications as well as the association of phosphorylated RNA Polymerase II with the X chromosome during these stages suggest the X may have reverted to an active state (Khalil et al., 2004).

An alternative, "de novo" model proposes that the Xp is active at fertilization and is silenced during subsequent stages of development. In support of this hypothesis, Okamoto et al. report that the Xp is active in 2-cell embryos, using RNA Polymerase II (Pol II) and Cot-1 staining as markers of transcriptional activity. In addition, assays using transcript-specific RNA FISH showed signals from both chromosomes, in accordance with the hypothesis that the paternal X is active at this stage (Okamoto et al., 2004). Furthermore, inactivation of autosomal *Xist* transgenes transmitted through the male can occur independently of MSCI, suggesting that silencing during male meiosis is not a prerequisite for XCI (Okamoto et al., 2005). However, as this latter study is based on transgenes, it remains to be determined whether this holds true for genes on the X chromosome.

It is largely unclear if experimental conditions (such embryo fixation) or other factors account for the conflicting results on the transcriptional status of the Xp in the zygote. Perhaps the key to reconciling these two models lies in a more detailed examination of the transcriptional activity of genes along the X chromosome early after fertilization using transcript-specific FISH rather than the more global Cot-1 and Pol II probes.

The paternal X becomes inactivated

Xist expression initiates at the 2-cell stage, when the zygotic genome is activated. *Xist* is imprinted in the early embryo, with exclusive expression from the Xp. It remains unclear how *Xist* is initially silenced on the maternal X (Xm) chromosome and how its imprinting is controlled. However, at later stages of development, maternal *Xist* expression is negatively regulated by the overlapping gene, *Tsix*. Expression of the Tsix non-coding RNA is detected after the 8-cell stage and is oppositely imprinted to *Xist*, with exclusive expression from the maternal allele (Sado et al., 2001). Using FISH analysis, Xist RNA can be detected in 2-cell embryos as a small punctate signal. Starting at the 4-cell stage, Xist transcripts begin to accumulate on the Xp and appear as a larger RNA domain. The accumulation of Xist RNA is the initiating event in the silencing process. Subsequently, the Xist RNA domain spreads in *cis*, eventually coating the X chromosome.

An ordered set of chromatin modifications follows Xist accumulation during pre-implantation, reflecting the progressive silencing of the X chromosome. The initial chromatin changes, which occur between the 8- and 32-cell stages, are defined by the loss of euchromatin marks: the X exhibits hypoacetylation of histone H3-Lysine 9 (H3K9) and hypomethylation of histone H3-Lysine 4 (Mak et al., 2004; Okamoto et al., 2004). Subsequently, enrichment of the EED/EZH2 Polycomb Group complex has been shown to mediate the early epigenetic mark of histone H3-Lysine 27 (H3K27) methylation on the Xi chromosome from the 16-cell stage to the early blastocyst. Finally, H3K9 methylation follows on the Xi (Heard, 2004; Mak et al., 2004; Okamoto et al., 2004). These epigenetic modifications are thought to act cooperatively on the Xi to mediate transcriptional silencing. However, although the kinetics of these chromatin changes are becoming well characterized, it remains to be determined which alterations function in establishing the inactivated state and which play a role in its maintenance. For example, chromatin changes that appear towards the end of preimplantation (after transcriptional silencing has occurred) are unlikely to function in establishment of silencing. Conversely, the H3K27 methylation epigenetic mark mediated by the Polycomb Group proteins is evident at an earlier stage, but it is still unclear whether it is required for the establishment of XCI.

X-linked gene expression

Analysis of the transcriptional status of X-linked genes in 8–16-cell embryos revealed a gradient of silencing along the chromosome, with an inverse correlation between the degree of silencing and distance from the *Xic* (Huynh and Lee, 2003). Thus, most genes within 10 cM of the *Xic* were predominantly silent on the Xp, whereas those further away were more likely to be expressed. These data suggest that silencing is progressive and reflects the degree of spreading of Xist RNA in these embryos. These results support a model in which silencing is first established at the *Xic* and then proceeds to spread bidirectionally toward the ends of the chromosomes.

Reactivation of the X chromosome in the ICM

In early blastocysts, all cells exhibit the hallmark signs of XCI on the Xp: a large domain of Xist RNA, indicating its coating of the chromosome, association of the Polycomb Group proteins, H3K27 methylation and transcriptional silencing (Mak et al., 2004; Okamoto et al., 2004; Plath et al., 2003). However, at this point, cells of the ICM undergo a reversal of XCI. This reversal is characterized by loss of the factors that associated with the X chromosome earlier during development. Thus, in the ICM of expanding or hatching blastocysts, Xist RNA is dispersed or absent, no EED/EZH2 domain is present, and some cells also lack H3K27 methylation, presumably as a result of dissociation of the EED/EZH2 complex from the chromosome. These results highlight once again that during preimplantation, X-inactivation is strictly Xist-dependent, such that when *Xist* is downregulated, the silencing modifications are also lost.

In contrast to the ICM, cells of the trophectoderm maintain their imprinted XCI and the Xp remains silent following implantation. Recently, transient association of EED with the X chromosome in the trophectoderm progenitor cells was proposed to play a role in the maintenance of transcriptional silencing during subsequent differentiation (Kalantry et al., 2006). Thus, differentiated *eed*−/− cells exhibit reactivation of the X chromosome (Wang et al., 2001). However, in undifferentiated *eed*−/− mutant trophectoderm stem (TS) cells, the Xp is not reactivated despite the absence of Xist RNA coating, H3K27 methylation, macroH2A and H4-1mK20 (Kalantry et al., 2006). These apparently conflicting results suggest that our knowledge of how X chromosome silencing occurs in the trophectoderm lineage is incomplete.

Distinct sequence requirements for imprinted versus random XCI?

Transgenic approaches have been used to study which DNA sequences are important for XCI. *Xist/Tsix* spanning transgenes present at more than one copy at autosomal loci can induce Xist-dependent silencing in *cis* at ectopic loci when the cell undergoes random XCI; these transgenes range in size from 80 kb in line πJL1.4.1 (Lee et al., 1999b) to 460 kb in line L412 (Heard et al., 1999) (Fig. 2B). Recently, transgenic lines containing a single copy insertion of X sequences onto autosomes were examined for their ability to undergo Xist-induced silencing in *cis* during preimplantation, i.e. imprinted XCI (Okamoto et al., 2005). Specifically, in transgenic line 53BL (Fig. 2B), a 460 kb transgenic fragment containing *Xist* and *Tsix* conferred silencing in *cis* upon paternal transmission and exhibited the characteristic chromatin modifications normally seen on the Xi during this time. Thus, this transgene contains sufficient X chromosome sequence for Xist-induced silencing in *cis* and therefore provides a useful system to identify minimal sequences required for imprinted XCI. Conversely, transgenic line L412, which has 2 copies of the same transgene and was earlier shown to exhibit random XCI, did not confer silencing during preimplantation. While transgenic studies initially suggested that distinct elements may be required for imprinted versus random XCI, the answer may be slightly more complex since these studies may be confounded by position effects, transgene copy number, introduction of foreign sequence and integrity of transgenic DNA.

Random XCI

As previously introduced, random XCI is a multi-step process that initiates in the embryo-proper upon implantation and is considered to be complete in the 6.5 dpc post-implantation embryo (Rastan, 1982; Takagi et al., 1982). First, X chromosome counting and choice ensure that only one X remains active. In males, a defect in counting would result in the inactivation of the single X. In females a defect in choice is evident by loss of random XCI, while a defect in counting might result in the inactivation of both or none of the X chromosomes. Once choice is made, initiation is then evident by the downregulation of *Tsix* and upregulation of the *Xist* transcription on the future Xi (Lee et al., 1999a). In contrast to earlier reports, it is unlikely that this latter step involves the stabilization of Xist RNA (Panning and Jaenisch, 1998; Sheardown et al., 1997; Sun et al., 2006). The Xist RNA coats the Xi in cis followed by a similar sequence of chromatin modifications observed during preimplantation. In addition, DNA hypermethylation is a late step in this process. These epigenetic modifications eventually serve to maintain Xi repression in a Xist-independent manner, although *Xist* continues to be highly expressed and coat the Xi even after XCI is fully established. Since random XCI occurs upon differentiation of female embryonic stem (ES) cells, which are derived from the blastocyst ICM, ES cells have served as a system to elucidate molecular mechanisms of random XCI that are difficult to access in vivo. Other reviews nicely present the specific chromatin modifications leading to the establishment of random XCI in both ES cells and embryos (Heard, 2005; Plath et al., 2002). Here we focus on recent developments in deducing how *Tsix* regulates *Xist* during early events of random XCI, and then we discuss some exciting news on nuclear organization of the X chromosomes during this process, as well as the identification of elements essential for XCI.

The complex dependence of Xist on Tsix

Within the *Xic* chromosomal region, *Xist* and *Tsix* are essential to mediate the early steps in XCI. *Xist* deletions confirm that Xist is necessary for silencing of the X chromosome in *cis* (Penny et al., 1996; Marahrens et al., 1997). The choice of which X to inactivate, however, appears to involve a complex interplay between *Xist* and *Tsix*. While the analyses of *Tsix* (major promoter) deletion alleles $Tsix^{\Delta CpG}$ (Fig. 2B) (Lee and Lu, 1999) and $Tsix^{AA2\Delta 1.7}$ (Sado et al., 2001) show that *Tsix* is critical for repressing *Xist* on the active X (Xa), it is not known how *Tsix* exerts its effects. Here we highlight three developments in our understanding of how *Tsix* mediates the control of XCI: the requirement for *Tsix* antisense transcription through *Xist*, the regulation of *Xist* chromatin structure by *Tsix* and the effect of *Tsix* regulatory elements on choice and counting.

To discriminate between a role for the Tsix RNA itself or antisense transcription through *Xist*, two groups independently generated *Tsix* mutant alleles that express a truncated Tsix RNA that terminates before it overlaps with *Xist*-coding sequences (Luikenhuis et al., 2001; Shibata and Lee, 2004). The resulting mutant alleles are always chosen as the Xi, implying a role for antisense transcription through *Xist*. Furthermore, knocking in the Tsix cDNA in *cis* cannot complement this phenotype (Shibata and Lee, 2004), emphasizing that transcription through the overlapping region is required for the ability of Tsix to repress Xist (Shibata and Lee, 2003). While analyses of *Tsix* mutant alleles have demonstrated that Tsix is essential for regulating choice of XCI in females, a Tsix-independent mechanism appears to regulate counting of random XCI in males, since viable males can inherit a *Tsix* deletion on their single X chromosome (Lee and Lu, 1999; Sado et al., 2001; Ohhata et al., 2006).

While the interplay between *Tsix* and *Xist* remains incompletely understood, recent studies suggest that *Tsix* regulates

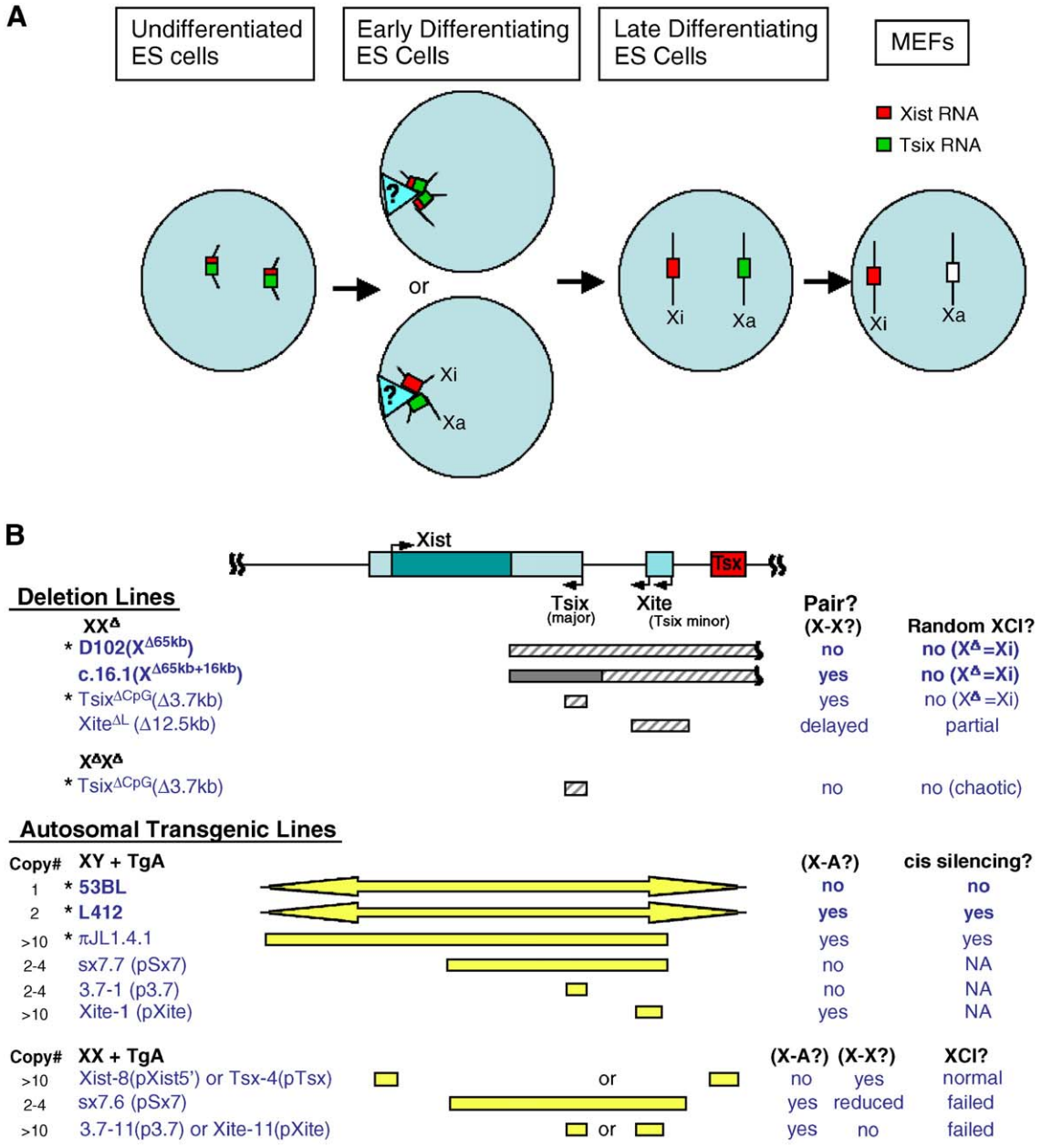

Fig. 2. Initiation of random XCI. (A) Co-localization of X chromosomes occurs early in differentiating ES cells. The X chromosomes are shown in the nucleus of differentiating ES cells and terminally differentiated MEFs. The *Xics* are depicted as rectangles. Red and green rectangles indicate *Xist* and *Tsix* expression, respectively, and the white box represents the absence of *Tsix* expression from the established Xa in MEFs. The blue triangle denotes a hypothetical anchor point for the co-localization of the X chromosomes. (B) Deletion and transgenic ES cell lines suggest sequence requirements for Xic–Xic pairing. Schematic of the *Xist*/*Tsix* locus is shown at the top, with arrows denoting the start and direction of transcription. Transcripts that initiate from the *Xite* region also coincide with minor Tsix transcripts (Ogawa and Lee, 2003; Sado et al., 2001). For summary of transcripts at this locus see Rougeulle and Avner (2004). *Tsx*, the closest protein-coding gene, is indicated by a red box. ES cell lines analyzed by Bacher et al. (2006) are in bold; the other lines were assayed by Xu et al. (2006). The asterisk designates lines from which mice have been generated. For deletion lines, cross-hatched boxes indicated deleted sequence with size of deletions denoted in parentheses next to the line name. For line c.16.1 the gray box indicates the 16 kb of reinserted sequence. For autosomal transgenic lines (names in parentheses) yellow boxes designate the region of *Xist*/*Tsix* sequence that was used to generate the transgene. Estimated transgene copy numbers are designated. Pairing between two Xs (in female lines) or an X and A (in transgenic lines) is indicated to the right. Delayed shows that pairing occurred later than normal during differentiation of ES cells while reduced indicates pairing occurred less often than expected. Whether or not random XCI occurred is noted for deletion lines (random XCI). Partial corresponds to skewing of XCI for the deletion allele. For male transgenic lines (XY+TgA) the ability of the transgene to induce Xist-dependent silencing was noted (cis-silencing). For female transgenic lines the ability of cell to undergo XCI was noted.

Xist transcription, at least in part, through chromatin remodeling at the *Xist* locus. Sado et al. modified the *Xist* locus in the presence or absence of a *Tsix* deletion in *cis* such that a truncated nonfunctional *Xist* was under the influence of the endogenous *Xist* promoter and determined the consequence in E13.5 tissues and mouse embryonic fibroblasts (MEFs) (Sado et al., 2005).

Here, the nonfunctional Xist RNA was expressed in the absence of *Tsix* transcription in *cis*. In addition, the active chromatin modification H3-2meK4, was observed on the *Xist* promoter of the nonfunctional *Xist* allele in the absence but not in the presence of *Tsix* expression in *cis*, suggesting that *Tsix* functions to prevent the recruitment of activating marks. In another study, Navarro et al. profiled the chromatin at the *Xist* and *Tsix* loci in TS cells that undergo imprinted XCI, in MEFs where random XCI is well established and in undifferentiated ES cells, where random XCI has not yet occurred (Navarro et al., 2005). Active *Xist* loci were enriched for H3-2meK4. Comparison of wild-type and *Tsix* mutant ES cells indicated that recruitment of H3-2meK4 throughout the *Xist* transcription unit (but not the promoter) is dependent upon *Tsix* expression. Sun et al. have performed a similar analysis in undifferentiated and differentiated ES cells, supporting the idea that *Tsix*-dependent chromatin modifications are important for controlling random XCI (Sun et al., 2006). These studies suggest that *Tsix* is affecting *Xist* local chromatin structure during random and imprinted XCI. While Sado et al propose from their analyses of cells in which random XCI is complete that Tsix represses *Xist* transcription by recruiting repressive chromatin modifications at the *Xist* promoter on the Xa, other groups suggest that Tsix causes the *Xist* gene body to become euchromatic in ES cells, which have not undergone XCI (Navarro et al., 2005; Sun et al., 2006). This latter activity may reflect an additional role for Tsix in priming the *Xist* locus for activation or inactivation (Navarro et al., 2005).

Deletion and transgenic analyses of *Tsix* regulatory elements indicate that they are key to determining both the future Xa and Xi and may control choice and counting during random XCI (Fig. 2B). Initially, a 65-kb deletion of sequence distal to *Xist* ($X^{\Delta 65\ kb}$, Fig. 2B), which removed all identified antisense transcripts to *Xist* as well as other genes, perturbed choice in XX female cells and counting in XY and XO cells (Clerc and Avner, 1998; Rougeulle and Avner, 2004). In contrast, genetic manipulations that perturb *Tsix* expression from its major promoter lead to nonrandom XCI but counting in XY cells appeared unaffected (Lee, 2000; Lee and Lu, 1999; Luikenhuis et al., 2001; Morey et al., 2001; Sado et al., 2001). Subsequently, the X-intergenic transcription element, *Xite*, was characterized upstream of the major *Tsix* promoter and was shown to be the region from which multiple transcripts were initiating (Ogawa and Lee, 2003) [partly coincident with minor *Tsix* promoters (Sado et al., 2001)]. Deletion of the *Xite* region in ES cells ($Xite^{\Delta L}$, Fig. 2B) skewed random XCI, leading to the idea that *Xite* is affecting choice and may be the elusive *Xce*. While XX cells heterozygous for deletions of sequence 3′ of *Xist* have identified sequences that regulate XCI choice, homozygous deletion of the *Tsix* major promoter in ES cells and mice ($Tsix^{\Delta CpG}$, Fig. 2B) led to failure of random XCI, indicating that the deleted sequence is also required for counting in females. The observed failure in XCI was termed "chaotic choice" since three possible outcomes were observed: two Xa's, two Xi's or, the most rare event, one Xi and one Xa. This indicates that the Tsix deleted sequence is also important for counting in female ES cells as well as choosing both the future Xi and Xa (Lee, 2002, 2005). In addition, when XX ES cells with high copy number transgenes of the *Tsix* major promoter (p3.7) or the *Xite* region (pXite), were differentiated, the cells exhibited a defect in growth and there was no evidence of XCI, suggesting that an excess of these elements was inhibiting counting and choice (Fig. 2B) (Lee, 2005). While this outcome could be due to the transgenic sequences sequestering *trans*-acting factors essential for *Tsix* expression away from the endogenous X chromosome, leading to its inactivation, Lee proposes that random XCI requires two factors, a "blocking factor" for the future Xa and a "competence factor" to enable inactivation of the future Xi (Lee and Lu, 1999). This model contrasts with an earlier proposal that the Xa requires a "blocking factor" to protect it from being inactivated and the Xi arises by default (Rastan, 1983).

Before departing from a discussion of *Tsix* mediated control of XCI, it should be noted that while many of the experiments cited here rely on the assumption that the ES cell system recapitulates random X inactivation in the mouse embryo, there may be some potential problems with this assumption. For example, a recent study reported that XX female ES cells were globally hypomethylated relative to XY and XO ES cells (Zvetkova et al., 2005). The authors propose that the hypomethylated state is a disadvantage to XX ES cells and therefore the reason why these cells tend to be unstable. Consequently, it is critical to determine whether the ICM of female blastocysts is also hypomethylated. Although female ES cells represent a powerful tool to study random XCI, these results are a reminder that, when possible, findings should be verified in the embryo.

Initiating random XCI

Whereas sequences conferring choice and initiation of XCI are being delimited, less is known about the mechanism through which these elements may function. Recent evidence suggests that these sequences may be important to bring the X chromosomes together. The *Xics* of two X chromosomes were shown to transiently co-localize within interphase nuclei during the early stages of ES cell differentiation (Fig. 2) and this transient pairing appears to precede the upregulation of *Xist* expression from one of the X chromosomes and the subsequent accumulation of characteristic repressive chromatin modifications (Bacher et al., 2006; Xu et al., 2006). [For reviews see (Carrel, 2006; Morey and Bickmore, 2006).] Thus, X chromosome pairing appears to be important for choice and initiation of XCI. Interestingly, Bacher et al. also demonstrate that the Xs co-localize at the nuclear periphery, suggesting that pairing may also be due to localization within a common nuclear sub-compartment. Deletion of 65 kb sequence 3′ to *Xist* that is required for counting abolished X chromosome pairing ($X^{\Delta 65\ kb}$, Fig. 2B). However, reintroducing sequence 16 kb of spanning the *Tsix* regulatory elements into the 65 kb deletion restores co-localization but does not restore random XCI ($X^{\Delta 65\ kb+16\ kb}$, Fig. 2B), indicating that X chromosome pairing is necessary but not sufficient for choice/counting (Clerc and Avner, 1998; Morey et al., 2001). The deletion and transgenic analyses in

both studies, as summarized in Fig. 2B, suggest that elements regulating choice are required for X–X pairing, and, when present at autosomal loci, can interfere with this process (Bacher et al., 2006; Xu et al., 2006). Additional proof that this process has a role in counting will come from X co-localization tests in cells with more than two X chromosomes or with increased autosomal ploidy (e.g. >2N) (Morey and Bickmore, 2006). Finally, the co-localization of Xs reported in these studies is consistent with the proposal that choice/counting/initiation involves transvection of X chromosomes (Marahrens, 1999). At this time, transvection (defined as the pairing of homologous chromosomes) has been only shown to regulate transcription in *Drosophila* (Duncan, 2002). In addition, observations of Bacher et al. may also be consistent with an early hypothesis that XCI involves co-localization of Xs to the nuclear periphery (Comings, 1968).

When is choice of the future Xa and Xi determined? Contrary to the established view, Williams and Wu have hypothesized that XCI does not even involve choice between two X chromosomes (Williams and Wu, 2004) and suggest that the future Xa and Xi are determined prior to developmental events that trigger the initiation of XCI (such as differentiation of ES cells). In general, they propose that only one of the two X chromosomes is susceptible to epigenetic modification designating the future Xa or Xi, while the other X is destined for the opposite outcome, apparently by default. While allele-specific chromatin and DNA modification are apparent after XCI initiates, they have not been reported in undifferentiated ES cells. However, using a DNA FISH procedure that preserves nuclear architecture in ES cells Panning and colleagues observe differences in the two X chromosomes prior to XCI (Mlynarczyk-Evans et al., 2006), thus supporting the hypothesis by Williams and Wu. These data suggest that the X chromosomes in ES cells have two distinct states, one more accessible to the X chromosome DNA probes than the other, perhaps correlating with yet-to-be defined epigenetic differences between the future Xa or Xi.

Defining elements required for random XCI

It is a challenge to define the minimal *cis* sequences and *trans* elements required for the initial XCI events of choice and counting. With regards to choice, Cattanach and colleagues characterized a putative X-linked element in the mouse, designated the *Xce*. Different *Xce* alleles skew X chromosome inactivation, resulting in a nonrandom X-linked phenotype (Cattanach and Isaacson, 1965, 1967). Though *Xce* has been mapped to the distal region of the mouse *Xic* (Chadwick et al., 2006; Simmler et al., 1993), it has eluded molecular identification and its mode of action remains only phenotypically defined. It is not known how the distinction of *Xce* alleles occurs in an *Xce* heterozygous animal, although characteristic epigenetic modifications and chromatin structure may play a role (Avner et al., 1998; Chao et al., 2002; Percec and Bartolomei, 2002; Prissette et al., 2001; Simmler et al., 1993). Notably, the *Xce* effect appears to be limited to tissues of the embryo proper, as the paternally imprinted inactivation characteristic of mouse extraembryonic tissues is not perturbed in *Xce* heterozygous females (Rastan, 1983). Lee and colleagues proposed that the regulatory element defined as *Xite* functions as the *Xce* (Ogawa and Lee, 2003). Ultimately, genetically engineered swapping of candidate *Xce* elements will define the *Xce*.

Two approaches have been used to identify trans factors involved in random XCI. First, an ENU mutagenesis screen has identified multiple autosomal mutant loci that perturbed the *Xce* effect (Percec et al., 2002, 2003) and may harbor candidates for the "blocking factor," a hypothetical complex proposed to function on the Xa (Lyon, 1971; Rastan, 1983). Fine mapping of the autosomal loci are required to elucidate whether or not these regions harbor elements that affect *Xce*, although preliminary data have indicated that the effect is more complex and not defined by a single locus [personal observation, (Chadwick and Willard, 2005)]. Second, a recent SAGE screen identified multiple genes that are differentially expressed in males and female 6.5 dpc embryos and, consequently, may include candidate genes for regulating XCI (Bourdet et al., 2006). For now, the minimal *cis* and *trans* elements required for XCI remain elusive, especially since no single copy *Xist*/*Tsix* containing transgene can recapitulate random XCI.

Other processes of X-linked gene regulation

X chromosome reactivation in PGCs and cloned embryos

Once random XCI is established in somatic cells, it is maintained in subsequent cell divisions. At a specific time in developing primordial germ cells (PGCs) both X chromosomes are active once again. This could occur either if PGCs develop from a population of cells in which X reactivation occurs, or if they arise by expansion of a stem cell population in which XCI has never occurred. Studies favor the former hypothesis and indicate that the reactivation of the Xi occurs through the erasure of the marks that distinguish the Xi and Xa (McLaren, 2003). A similar process occurs at autosomal imprinting loci at this time, where the somatic imprinted marks are also being erased (Hajkova et al., 2002).

Not much is known about the mechanism of X chromosome reactivation in PGCs. For example, is this process dependent upon X-linked genes? Is it independent of the *Xic*? To some extent, X chromosome reactivation in the PGCs is mimicked when somatic nuclei are transferred into oocytes during mouse cloning procedures (Bao et al., 2005; Eggan et al., 2000; Nolen et al., 2005). Marks associated with the Xi are erased, although incompletely, and XCI becomes randomized again in cloned embryos. Undoubtedly, this will provide a useful system to begin understanding the mechanism through which the X chromosome is reactivated.

Exceptions in X-linked gene regulation

We end this review with three additional anomalies of X-linked gene regulation for which the mechanisms remain to be resolved. First, a few genes escape XCI in mice, as opposed to

Table 1
Questions addressing inactivation and reactivation of the X chromosome

	Question
1	When is imprinted XCI established?
2	What are the *cis* elements required for imprinted XCI?
3	When is *Tsix* first required for imprinted XCI?
4	How does *Tsix* affect *Xist* chromatin structure during imprinted and random XCI?
5	Are *Tsix* and *Xite* regulatory sequences exclusively responsible for choice (and counting) in random XCI?
6	How is the *Xce* affecting choice in skewed/nonrandom XCI?
7	What are the *trans* factors involved in choice during random XCI?
8	What are the *cis* elements required for X chromosome pairing during early steps of random X inactivation?
9	Is X chromosome pairing during early steps of random XCI occurring at a fixed location in the nuclear periphery?
10	Is the hypomethylated state reported in female ES cells also present in the ICM of female blastocysts?
11	Are the steps of random XCI observed in differentiating ES cells recapitulating the steps of random XCI that occur in the embryo?
12	How do X chromosomal imprinted genes escape random XCI? (Is "escape" a consequence of the inability to maintain random XCI?)
13	Do genes that escape random XCI also escape imprinted XCI?
14	How are X-linked imprinted genes regulated in the midst of imprinted and random XCI?

the 15–20% that escape XCI in humans (Carrel and Willard, 2005; Disteche et al., 2002). While escape has been proposed to be dependent upon the vertebrate insulator factor CTCF controlling boundary elements present between escapees and non-escapees (Filippova et al., 2005), many questions remain. For example, it is still unclear whether the genes that escape XCI do so from the very beginning of the X inactivation process or if they are initially inactivated and then become reactivated (Fig. 1). The latter mechanism was reported for mouse escapee *Smcx* (Lingenfelter et al., 1998). Furthermore, it also remains to be determined if genes can escape imprinted XCI. Secondly, in addition to the genes that escape XCI, there are new reports of imprinted X-linked genes, adding even greater complexity to X-linked gene expression (Davies et al., 2005; Kobayashi et al., 2006; Raefski and O'Neill, 2005). The neuronal specific expression of imprinted genes from the Xm may serve as a mouse model for uncovering complexities of Turner Syndrome. The *Rhox5*/*Pem* gene was recently reported to be imprinted, with expression from the Xp during implantation and from the Xm during postimplantation. As hinted by Eakin and Hadjantonakis, paternal-specific expression detected in preimplantation embryo may be a consequence of failure to completely inactivate the distally located X-linked *Rhox5* that is expressed in sperm (Eakin and Hadjantonakis, 2006). Finally, X-linked gene expression levels on Xa are reported to be elevated relative to autosomal gene expression levels, such that expression levels from a single X corresponds to expression levels from two autosomes. Thus, not only is the Xi unique in its silencing, but the Xa is also unique in its elevated gene expression levels (Nguyen and Disteche, 2006). Elucidating the mechanisms behind these X-linked anomalies will further our understanding of the requirements for establishing and maintaining random XCI.

Conclusion

We have presented a brief overview of the complex stages of X chromosome inactivation and reactivation, focusing on recent exciting studies that will encourage investigators to reevaluate and broaden approaches to study these dynamic processes. We conclude in Table 1 with questions that undoubtedly will be tackled with the many pioneering and creative approaches that have and will continue to unravel the complexities of X chromosome regulation throughout development.

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