X-Chromosome Kiss and Tell: How the Xs Go Their Separate Ways

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Loci associated with noncoding RNAs have important roles in X-chromosome inactivation (XCI), the dosage compensation mechanism by which one of two X chromosomes in female cells becomes transcriptionally silenced. The Xs start out as epigenetically equivalent chromosomes, but XCI requires a cell to treat two identical X chromosomes in completely different ways: One X chromosome must remain transcriptionally active while the other becomes repressed. In the embryo of eutherian mammals, the choice to inactivate the maternal or paternal X chromosome is random. The fact that the Xs always adopt opposite fates hints at the existence of a *trans*-sensing mechanism to ensure the mutually exclusive silencing of one of the two Xs. This paper highlights recent evidence supporting a model for mutually exclusive choice that involves homologous chromosome pairing and the placement of asymmetric chromatin marks on the two Xs.

NONCODING RNAs OF THE X-INACTIVATION CENTER

XCI is the transcriptional silencing of one X chromosome in female cells in order to equalize the dosage of X-linked genes between males (XY) and females (XX) (Lyon 1961). There are two lineage-specific forms of XCI, referred to as "imprinted" and "random" XCI (Boumil and Lee 2001; Heard 2005; Lucchesi et al. 2005). The imprinted form of XCI occurs in extraembryonic tissues of eutherians and is characterized by exclusive silencing of the paternal X (Huynh and Lee 2001; Takagi and Sasaki 1975). Random XCI—where both Xs have an equal chance of undergoing inactivation—is a multistep process that occurs in the embryo proper (Avner and Heard 2001; Cohen and Lee 2002; Clerc and Avner 2003; Heard 2004). These phases have been defined genetically and consist of counting, choice, establishment of silencing, and maintenance of silencing. The counting mechanism determines the X-to-autosome ratio and inactivates one X chromosome per diploid nucleus. This is followed by a choice step where the Xs are designated to become active and inactive Xs (Xa and Xi, respectively). Transcriptional silencing of Xi begins during the establishment phase and is propagated along the chromosome. Finally, the silent Xi is preserved in new cell populations during the maintenance phase.

Both random and imprinted XCI require the X-inactivation center (*Xic*), an X-linked domain that contains a number of noncoding RNA (ncRNA) genes important for XCI (shown in Fig. 1) (Plath et al. 2002; Willard and Carrel 2001). The *Xist* gene (X-inactive specific transcript) encodes a 17-kb alternatively spliced ncRNA that accumulates *in cis* along the X chromosome designated for silencing (Borsani et al. 1991; Brockdorff et al. 1991, 1992; Brown et al. 1991, 1992; Clemson et al. 1996). This noncoding locus is essential for the silencing step (Penny et al. 1996; Marahrens et al. 1997). *Xist* expression is regulated with the help of its noncoding antisense partner, *Tsix* (Lee et al. 1999a; Lee and Lu 1999; Sado et al. 2001). The transcription of *Tsix* inhibits *Xist* expression *in cis*, effectively blocking silencing on the future Xa (Luikenhuis et al. 2001; Morey et al. 2001; Sado et al. 2001; Stavropoulos et al. 2001; Lee 2002a). *Tsix* expression is regulated by another locus that makes the ncRNA called *Xite*, located upstream of the major *Tsix* transcriptional start site. *Xite* functions in part as an enhancer of *Tsix* to ensure the persistence of *Tsix* expression during cellular differentiation (Ogawa and Lee 2003; Stavropoulos et al. 2005). In short, *Xist* silences the future Xi, whereas *Tsix* and *Xite* together designate the future Xa.

X-CHROMOSOME INACTIVATION: TWO IDENTICAL SUBSTRATES, TWO OPPOSITE OUTCOMES

Recent models suggest that these noncoding genes work together to mediate counting and choice and determine the pattern of X-inactivation in a cell-autonomous fashion. The process of XCI requires a cell to act oppositely upon two epigenetically equivalent chromosomes: As one X persists as a transcriptionally active chromosome, the other becomes globally silent. In the embryo of eutherian mammals, the choice to inactivate the maternal or paternal X is random and invariably takes place in a mutually exclusive manner. The precision with which choice is determined implies the existence of a cross-talking process or a feedback mechanism to guarantee the distinct fates of the two Xs. The loss of mutual exclusion in homozygous *Tsix* knockout mice has provided the first experimental evidence for the idea of *trans*-sensing (Lee 2002a, 2005).

Conceptually, mutually exclusive fates of the X chromosomes could be achieved in several ways (Fig. 2). One possibility is that the two X chromosomes are not really equivalent at the beginning of XCI (Fig. 2A), a possibility congruent with the imprinted status of the Xs in extraembryonic cells in which the stereotypical paternal

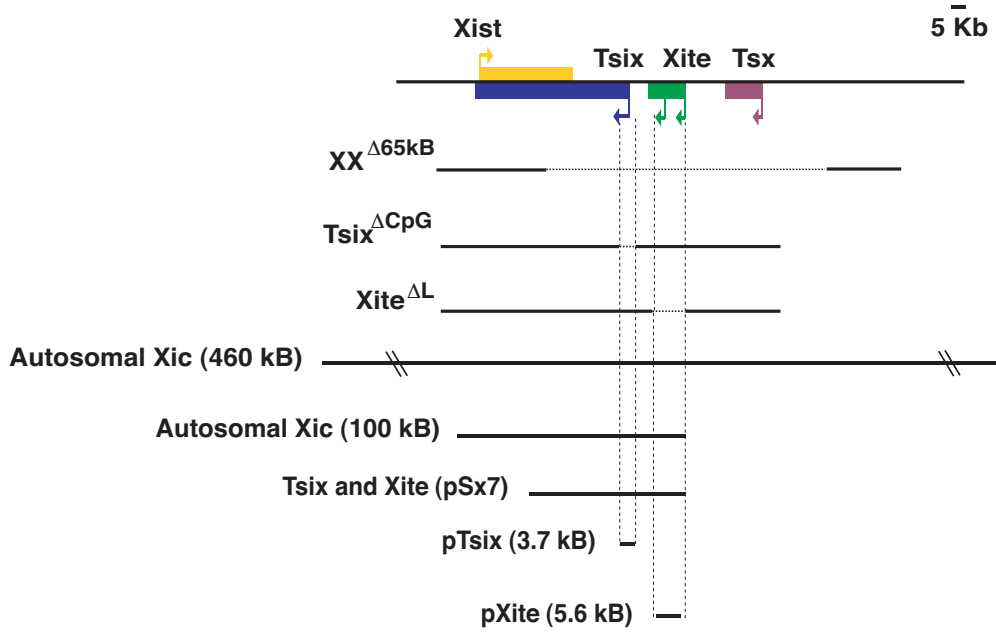

Figure 1. Map of the X-inactivation center (*Xic*) and locations of existing genetic deletions and transgene insertions. (*Dashed lines*) Genetic deletions (designated by Δ); (*solid lines*) autosomal insertions of various regions of the *Xic.* The 460-kb *Xic* autosomal transgene, encompassing 130 kb 5′ and 310 kb 3′ of *Xist*, is not drawn to scale (indicated by the diagonal cross bars).

X silencing is the rule. Although the field has yet to reach a consensus on the nature of the "imprint," differential CpG methylation within the *Xist-Tsix-Xite* regions has been implicated (Norris et al. 1994; Ariel et al. 1995; Courtier et al. 1995; Zuccotti and Monk 1995; McDonald et al. 1998; Prissette et al. 2001; Boumil et al. 2006). We note that slight differences in the methylation pattern and the lack of functional evidence thus far leave open the question of which, if any, of these marks constitute the primary imprint. Because the *Tsix* domains of differential methylation coincide with binding sites for the chromatin insulator and transcription factor, CTCF (Chao et al. 2002; Boumil et al. 2006), parallels to genomic imprinting at the *H19/Igf2* locus and *Rasgrf1* locus have frequently been drawn (Bell and Felsenfeld 2000; Hark et al. 2000; Holmgren et al. 2001; Yoon et al. 2002, 2005). At these autosomal imprinted loci, the differential binding of CTCF to differentially methylated imprinting centers appears to be of primary importance in setting up the mutually exclusive fates of the maternal and paternal chromosomes.

In a similar vein, what has been considered "random" X-inactivation could employ such a chromosome-specific mark, but that mark would be imposed zygotically rather than gametically. In a departure from conventional thought, two recent models suggest that "differential states" are already present prior to the onset of XCI (Williams and Wu 2004; Mlynarczyk-Evans et al. 2006). Although the nature of the "states" is unclear, the states are proposed to result in a situation in which both X chromosomes are not inactivated in a completely stochastic sense, but exist in predeterministic states that can alternate between the two Xs before XCI that predisposes one X to be silent at the onset of XCI. In one case, it is argued that the two active Xs of female embryonic stem (ES) cells switch between states in which the sister chromatids are in close apposition and another in which they are farther apart (Mlynarczyk-Evans et al. 2006). The model further proposes that the configuration in which the *Xic*s are farther apart "predetermines" the future Xa. Additional characterization will be required to demonstrate if and how they are involved in X-inactivation choice.

A second possibility—one generally preferred by the field—is the concept of a limiting factor that is present in quantities sufficient for only one X chromosome (Fig. 2B) (Lyon 1972; Rastan 1983), and it is the stochastic binding of this factor, or factors to the Xs, that determines the random pattern of silencing. This putative factor could be a "negative" factor (acting to repress the *Xic*) or a "positive" factor (acting to induce it), depending on its mechanism of action. For instance, in the classic one-factor hypothesis, the X chromosomes are predisposed to inactivate by default, and the binding of the so-called "blocking factor" (BF) to one X is specifically required to block its *Xic* from initiating silencing. Conversely, if X-inactivation is actively triggered and does not occur by default, the interaction of the single positive factor—so-called competence factor (CF)—would be required to initiate inactivation. An alternative to the one-factor hypothesis proposes that two factors (one BF and one CF) are required for XCI (Lee and Lu 1999; Lee 2005). The nature of the negative/positive factor could be a unique factor, a complex of factors, or a unique privileged site within the nucleus. In any case, the limited quantity of the factors and their sole action on one or the other X are directly responsible for the "asymmetric" action on two otherwise epigenetically equivalent Xs.

A third possibility invokes direct contact between the Xs as the basis of communication and determination of

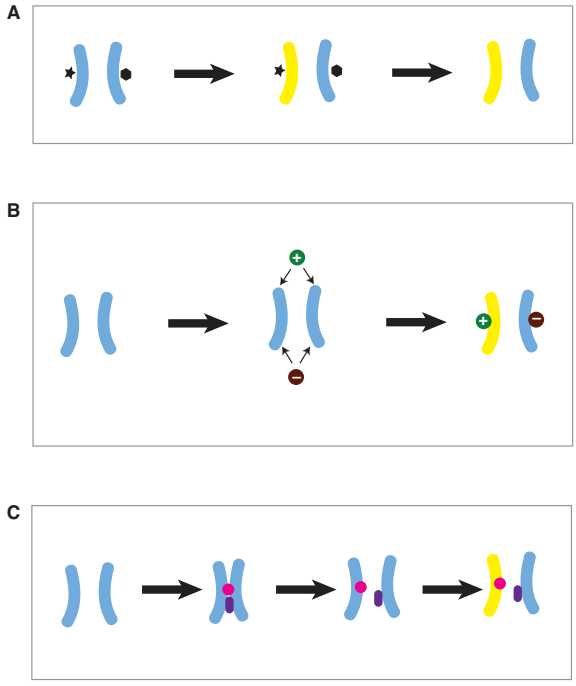

Figure 2. Conceptual models of mutually exclusive X-chromosome inactivation. (*Light blue* chromosomes) Active Xs; (*yellow* chromosomes) X chromosomes that are inactivated. X-inactivation proceeds from left to right. (*Panel A*) X chromosomes are not equivalent before the onset of XCI. Even before X-inactivation is initiated, the presence of marks or different "states" (represented by the star and the hexagon) distinguishes the two Xs. At the onset of X-inactivation, the X chromosome containing the mutually exclusive mark is silenced. This mark may or may not persist into the differentiated state. (*Panel B*) Limited positive and negative factors interact with one of the two X chromosomes. Before XCI begins, both X chromosomes are competent to become inactivated. The presence of a single positive/competence factor (*green*) or negative/blocking factor (*red*) can associate stochastically with either X. Binding of the positive factor triggers inactivation, whereas the negative factor protects the X from inactivation. (*Panel C*) X chromosomes interact with one another to coordinate mutually exclusive choice. Before XCI initiation, both X chromosomes are epigenetically equivalent. Pairing of the two Xs facilitates cross-talk for the formation of asymmetrical chromosomes, with the association of two factors (represented by a *pink circle* and *purple oval*). The asymmetry allows the cell to act uniquely upon one X, but not the other. Although schematized here as soluble factors, this asymmetry may also be chromosomally based (e.g., a different chromatin state, histone, and/or DNA modifications).

their distinct fates (Fig. 2C). *Trans*-chromosomal interaction occurs in phenomena such as transvection in *Drosophila* and *Neurospora* (Aramayo and Metzenberg 1996; Wu and Morris 1999; Chen et al. 2002; Duncan 2002; Coulthard et al. 2005; Vazquez et al. 2006) and in mammalian autosomal imprinting (LaSalle and Lalande 1995, 1996). Such *trans*-sensing mechanisms have also been proposed for X-inactivation (Marahrens 1999; Lee 2002b). In principle, physical contact between the two Xs could coordinate the silencing process and ensure that one and only one X becomes the future Xi, thereby providing a mechanism for establishing distinct fates for the two Xs.

We now highlight advances within the past year that shed light on the nature of mutually exclusive choice and the mechanism by which asymmetric marks are placed on the Xs. Intriguingly, homologous Xs do appear to come in physical contact just prior to the onset of XCI. Furthermore, the initiation of XCI is preceded by chromatin modifications unique to the *Xic* on the future Xi. Here, we propose a speculative model of early events in X-inactivation linking the physical pairing between the two X chromosomes to the establishment of *Xic* asymmetry.

THE EPHEMERAL ACT OF PAIRING

Although *trans*-sensing has long been suspected to occur at the *Xic*, experimental evidence for such interactions has, until recently, been completely lacking. The "chaotic choice" phenotype in female cells lacking *Tsix* provided the first experimental evidence for the idea of necessary cross-talk (Lee 2005). In homozygous *Tsix*$^{-/-}$ ES cells, cell differentiation results in aberrant XCI patterns in which female nuclei exhibit two Xi, one Xi, or no Xi. In recent papers, two groups independently examined whether homologous pairing of the X chromosomes might mediate *trans*-sensing. They used fluorescence in situ hybridization (FISH) to monitor the X–X distances during the various phases of XCI in female mouse ES cells, a model system that faithfully recapitulates the steps of XCI upon cell differentiation in culture (Lee et al. 1996; Panning et al. 1997; Clerc and Avner 1998; Marahrens et al. 1998). They found that the two X chromosomes transiently pair with each other during the onset of XCI, most likely just prior to *Xist* up-regulation (Bacher et al. 2006; Xu et al. 2006). Curiously, it appears that the majority of X–X pairs occur very close to the periphery of the nuclear envelope (Bacher et al. 2006), although the significance of this is presently not known, as no specific nuclear compartment has been identified.

Both groups found that the X–X associations are transient, as X–X pairing disappears during later stages of cellular differentiation and in fully differentiated somatic cells (Bacher et al. 2006; Xu et al. 2006). By asking whether pairing coincides with several chromatin changes that occur in sequence during XCI, Xu et al. (2006) observed that the association takes place in the Xist+ fraction but not in the Ezh2+ or the H3-3meK27+ subpopulation, suggesting that pairing occurs specifically in the fraction of cells that has entered the XCI pathway, but has not yet recruited the full silencing machinery. The brevity and timing of the X–X association are intriguing, as indeed the time window coincides with X chromosome counting and choice—the point at which the future Xi and Xa are designated.

How much of the X chromosome is engaged in pairing interactions and how close do the Xs actually get? By testing probes along the length of the X, from centromere to telomere, Xu et al. (2006) found that X–X pairing occurs specifically between the *Xic* regulatory regions and not between any other regions of the X chromosome. Then, biochemical analysis using the "chromosome conformation capture" (3C) technique (Dekker et al. 2002) determined with greater precision that the two *Xic* regions are in

direct physical contact with each other and that this contact takes place when pairing is seen to occur by FISH analysis.

WHY DO X-CHROMOSOMES PAIR?

The *Xic* region contains genes for the noncoding *Xite*, *Tsix*, and *Xist* RNAs, the major players involved in regulating counting, choice, and silencing during XCI. Could these regulatory sequences facilitate the transient X–X interactions necessary for mutually exclusive choice? Genetic analysis shows that, interestingly, the *Xic* domains required for pairing map precisely to genes that regulate counting and choice—*Tsix* and *Xite*. An X chromosome carrying a 65-kb deletion downstream from *Xist* loses the ability to pair with its wild-type homolog (Fig. 1) (Clerc and Avner 1998; Bacher et al. 2006). Within this 65-kb region, subdeletions involving either 12.5 kb of *Xite* (Ogawa and Lee 2003) or 3.7 kb of *Tsix* (Lee and Lu 1999) are sufficient to perturb the pairing process (Xu et al. 2006). Notably, the loss of pairing in the homozygous deletion of *Tsix* specifically correlates with aberrant XCI patterns in the differentiating female ES cells: The occurrence of cells with two Xi, one Xi, and no Xi in any differentiating population implies a disruption in both counting (i.e., aberrant numbers of Xis) and mutually exclusive choice (i.e., the two- and no-Xi phenotype) (Lee 2002a, 2005; Ogawa and Lee 2003). Thus, deletions of elements necessary for X–X pairing compromise the regulation of counting and choice.

The connection between counting/choice and X–X pairing is further supported by analysis of transgenic cell lines containing multiple copies of *Tsix* or *Xite* transgenes inserted into autosomes (summarized in Fig. 3). Male ES cells carrying full-length *Xic* transgenes (100–460 kb) display novel interchromosomal association between the X and the transgene-bearing autosome (Bacher et al. 2006; Xu et al. 2006). Significantly, these male cells also display ectopic XCI (Lee et al. 1996, 1999b; Heard et al. 1999), further correlating pairing with the ability to undergo XCI. In female ES cells, the same transgenes also induce de novo X–autosome (X–A) pairing, and regions as small as 3.7 kb of *Tsix* and 5.6 kb of *Xite*—at least when multimerized—are sufficient to create new X–A pairs (Xu et al. 2006). A direct physical interaction between the X and the autosome can also be visualized by 3C analysis (Xu et al. 2006). Thus, sequences within *Tsix* and *Xite* not only are necessary, but are also sufficient to form new pairs.

The analysis of *Tsix/Xite* transgenes in a female context revealed an intriguing difference from that in a male. The presence of extra copies of *Tsix* and *Xite* in an XX context disrupts cell differentiation, whereas it has no measurable effect in XY cells (Lee 2005). The aberrant cell differentiation apparently results from the absence of *Xist* upregulation and XCI in the transgenic female cells. It was then observed that the occurrence of ectopic X–A pairs competitively inhibits the formation of endogenous X–X pairs (Xu et al. 2006). Taken together, these results suggest that X–X pairing is a prerequisite for the initiation of XCI, which in turn is required for proper cell differentiation. It is therefore hypothesized that homologous chromosome pairing is one of the earliest events of XCI and is specifically required for counting and choice, without which the silencing mechanism of XCI cannot be called upon in female cells undergoing differentiation.

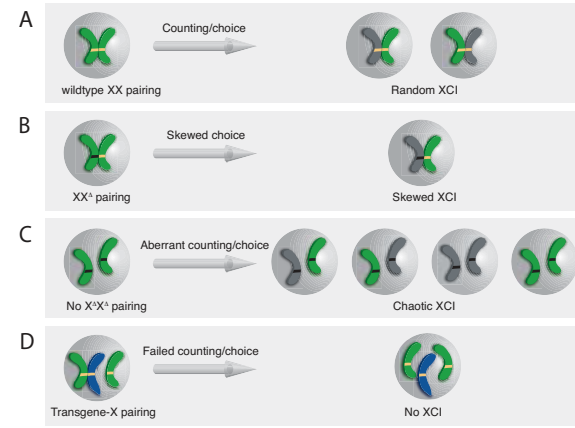

Figure 3. Summary of the effects of *Xic*, *Tsix*, and *Xite* genetic deletions and autosomal transgenes on the X–X pairing event and XCI. (*Panel A*) In wild-type XX ES cells, the homologous pairing between two *Xic* regions (designated by the *yellow regions*) on two active X chromosomes (*green* chromosomes) generates cross-talk resulting in asymmetrically "marked" chromosomes (future Xi shown in *gray*) for mutually exclusive choice. (*Panel B*) Cell lines containing either a single-copy deletion of *Xic* or *Tsix* (designated as XX^{Δ} cells, with the deletion in *black*) exhibit normal X–X pairing with skewed choice and inactivation on the mutated X. The single-copy deletion of *Xite* disrupts the dynamics of the X–X pairing event, also resulting in skewed inactivation of the mutated X. (*Panel C*) Homozygous deletion of *Tsix* (designated as $X^{\Delta}X^{\Delta}$ cells) disrupts X–X pairing and results in chaotic choice, with cells containing either 0, 1 Xi, or 2 Xi, and abnormal XCI. (*Panel D*) Cell lines containing multiple copies of either the *Xic*, *Tsix*, or *Xite* transgenes (*yellow regions*) on autosomes (*blue*) exhibit de novo X–autosome pairing and disruptions in X–X pairing. These abnormal *trans*-interactions may out-compete the normal X–X association, resulting in the presence of two Xa per cell and the failure of counting/choice and XCI.

The discovery of pairing provokes many new questions regarding the mechanism of XCI. Does the pairing process involve specific DNA domains within *Tsix* and *Xite*? Does it require transcription, the ncRNA outputs of *Tsix* and *Xite*, or particular chromatin modifications to the genetic locus? Interestingly, transgenic subfragments of *Tsix* and *Xite* that are most effective at nucleating pairing carry promoter elements (Xu et al. 2006). If transcription of these ncRNAs is indeed required, then pairing, counting, and choice must be added to the list of the already diverse functions of ncRNA elements in gene regulation. Finally, what do the Xs communicate to each other while paired, and how are the distinct fates of each X decided by this mysterious act of cross-talking?

FROM THERE TO HERE: PAIRING AND ASYMMETRIC *XIC* FATES

Prior to pairing, the two X chromosomes appear to be epigenetically equivalent. When they pair and come apart again, the Xs appear to be marked for different transcriptional fates. If mutually exclusive choice arises from these *trans*-allelic interactions, the interactions must create physical differences between the two chromosomes

that signal for one X to remain active and the other X to become repressed. In theory, the asymmetry could be created by many possibilities, including differential binding of a protein factor, transient localization of the Xs to a certain nuclear region, or specific "marks" and epigenetic modifications.

Recent evidence supports the idea of allele-specific differences in chromatin states. It was shown previously that the *Xist* loci exist in different chromatin environments consistent with the expression status of *Xist*: The active *Xist* gene is associated with an "open" chromatin state with increased levels of H3-K4 dimethylation. In contrast, the silent *Xist* allele exists in a "closed" chromatin state characterized by H3-K9 dimethylation and H3-K27 trimethylation (Goto and Monk 1998; Navarro et al. 2005; Sado et al. 2005). These observations—made using cells that have already undergone XCI—do not indicate whether the chromatin modifications are a cause or consequence of the asymmetrical expression of *Xist*. Additionally, *Tsix* has been recently shown to influence the local chromatin structure of the *Xist* promoter region in post-XCI cells and embryos (Navarro et al. 2005; Sado et al. 2005). These studies suggest an interplay between *Tsix* and the local chromatin environment of *Xist* that would support transcription of the locus, where *Tsix* may function to maintain an open chromatin state at *Xist*. However, because these experiments were also carried out using cells that had already undergone XCI, they contribute to the uncertainty of whether the chromatin modifications actually correlate with the monoallelic expression of *Xist* and the role of *Tsix* in this process (Sado et al. 2005). That is, could these chromatin marks be the causal asymmetry for mutually exclusive choice and therefore predict the monoallelic expression pattern of *Xist*?

To address this question, a recent study examined the chromatin environment at *Xist* at time points before, during, and after the onset of XCI using female ES cells (Sun et al. 2006). The study used an allele-specific chromatin immunoprecipitation (ChIP) to assay the relative levels of histone modifications on the two X chromosomes of hybrid female ES cells (one X of *Mus castaneus* origin and the other of 129 origin). Three of the most studied histone modifications were examined: histone H3-K4 dimethylation, H3-K27 trimethylation, and H4 acetylation. The results are consistent with the idea that, before XCI, the two Xs are epigenetically identical because each modification was detected at relatively equal levels on both X chromosomes in wild-type female ES cells. In fibroblasts, which have already undergone XCI, the expressed *Xist* allele is enriched with H3-K4 dimethylation and H4 acetylation and reduced in H3-K27 trimethylation, consistent with the previous reports showing that the expressed *Xist* allele is in an open chromatin state (Navarro et al. 2005; Sado et al. 2005).

The situation during the onset of XCI, however, revealed an unexpected result—the presence of a transient heterochromatic environment at the *Xist* loci (Sun et al. 2006). During this phase of XCI, the authors note that *Xist* expression is up-regulated about 30-fold and that, oddly, this transcription occurs from the locus buried in "heterochromatin." This *Xist* locus contains increased H3-K27 trimethylation and a modest decrease in H4 acetylation levels at this stage of XCI, marks normally characteristic of silenced genes. The chromatin environments, summarized in Figure 4, are completely opposite

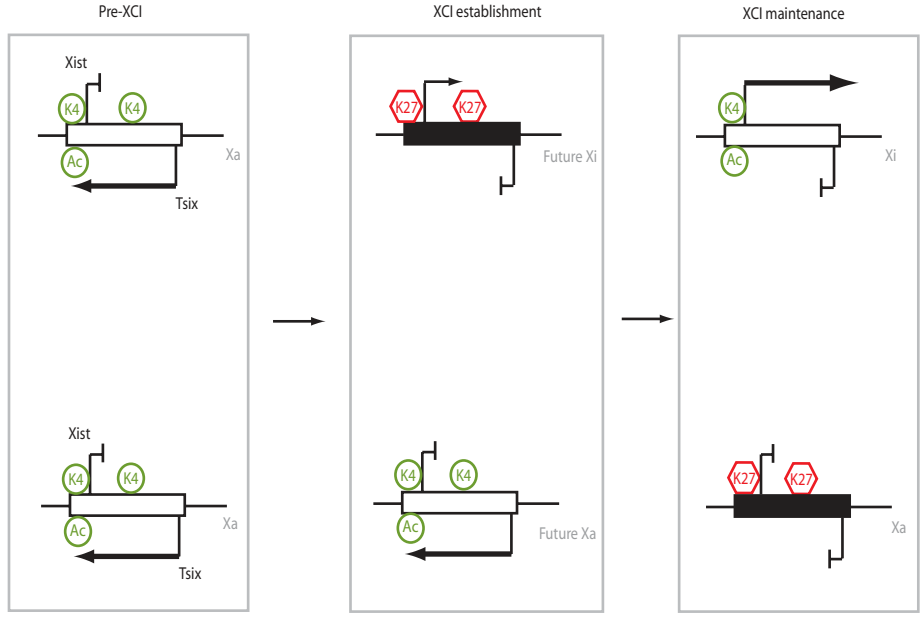

Figure 4. Different chromatin modifications within the *Xist* region (represented as a rectangle) distinguish and predict the future Xi and Xa. The *Xist* region, with modifications shown occurring at the promoter region (upstream of the start site) or within the *Xist* gene body (downstream from the start site). Before XCI, the two X chromosomes are epigenetically identical and the *Xist* region is euchromatic (*open rectangle*), with robust *Tsix* expression. (*Green*) Euchromatic modifications, with histone H3-K4 dimethylation denoted as K4 and histone H4 acetylation as Ac. The *Xist* region becomes heterochromatic (*filled rectangle*), and histone H3-K27 trimethylation is represented in red as K27. During the establishment of XCI, the two X chromosomes exhibit different chromatin modifications, with the future Xi being heterochromatic (with paradoxical *Xist* expression) and the future Xa being euchromatic. These modifications reverse during the final maintenance phase of XCI.

for the future Xa and Xi, and the marks change as XCI progresses. The *Xist* region is euchromatic on both alleles before XCI, supporting the hypothesis that the two Xs are epigenetically equivalent at the pre-XCI stage. Following counting/choice, the future Xi acquires heterochromatic features at the *Xist* region along with the onset of *Xist* expression. The future Xa allele continues to be euchromatic within the *Xist* region after the pairing and counting/choice events. During the final maintenance phase of XCI, the chromatin patterns invert, with the Xi allele exhibiting euchromatic features and increased expression of *Xist,* and the Xa allele being heterochromatic and therefore transcriptionally silent.

What is the function of *Tsix* in influencing the local chromatin environment and regulating the monoallelic expression patterns of *Xist*? The authors repeated the same allele-specific ChIP experiments using a heterozygous female mutant of *Tsix*, and found that each *Xist* allele contains different chromatin modifications corresponding to the absence of *Tsix* (Sun et al. 2006). On the *Tsix*$^{-}$ allele, the *Xist* locus is heterochromatic with decreased H3-K4 dimethylation, H4 hypoacetylation, and increased H3-K27 trimethylation. Indeed, it is this mutated X that is invariably silenced during XCI: During the onset of XCI, *Xist* becomes expressed exclusively from the *Tsix*$^{-}$ chromosome. Intriguingly, these asymmetric chromatin marks occur in the *Tsix*$^{+/-}$ ES cells even before induction of XCI by cell differentiation. These observations demonstrate that the chromatin marks preempt asymmetric *Xist* expression and thereby argue that the *Tsix*-driven chromatin changes are causally linked to the establishment of unequal and mutually exclusive X-chromosome fates.

Taken together, these experiments delineate a series of preemptive asymmetrical chromatin changes at *Xist* that differentiate the two X chromosomes and predict the monoallelic expression pattern of *Xist* (summarized in Fig. 4). Before the onset of XCI, the biallelic expression of *Tsix* on both X chromosomes keeps each *Xist* locus in an open, euchromatic state. During the onset of XCI, *Tsix* is silenced on one of the two X chromosomes. The loss of *Tsix* expression from that *Xic* allele influences the formation of the heterochromatic state in *Xist*, which leads paradoxically, in turn, to the transcriptional activation of that *Xist* allele. The euchromatic status of the second allele prevents the up-regulation of *Xist* on the future Xa. For reasons entirely unknown at the present time, the chromatin environment at *Xist* inverts to more conventional patterns after the establishment of XCI, in agreement with observations made by other studies in late-stage embryos and in differentiated cells (Navarro et al. 2005; Sado et al. 2005).

A number of interesting problems remain to be resolved. First, does *Xist* truly favor a repressive chromatin environment for transcription and is heterochromatin sufficient to induce *Xist* expression? If so, *Xist* may be classified as a heterochromatin-preferring gene, much like the *Light* gene of *Drosophila melanogaster*, which appears to require heterochromatin for transcriptional activation (Wakimoto and Hearn 1990; Yasuhara and Wakimoto 2006). Second, why do the chromatin states invert after the establishment of XCI patterns, especially when *Xist* expression continues into the maintenance phase of XCI, and become constitutive in all somatic female cells? If heterochromatin is required to initiate *Xist* expression, one might expect it to be required for maintenance of expression in somatic cells. Finally, when in the XCI pathway does the heterochromatic state arise—during the pairing process or after the pairs come apart?

MODEL: CROSS-TALKING GENERATES X-ASYMMETRY FOR MUTUALLY EXCLUSIVE CHOICE

In this review, we have presented evidence supporting a model of XCI where X–X communication results in mutually exclusive choice. Our model incorporates facets of the one-factor model for X-chromosome counting/choice, which is currently widely accepted by the field (Lyon 1972; Rastan 1983). It posits the generation of protein factors, produced primarily from autosomes (but does not rule out X-linked factors) in amounts proportional to the actual number of chromosomes present. These proteins form the complex dubbed the blocking factor (BF), which binds one *Xic* and represses the initiation of silencing on that X (Lyon 1972; Rastan 1983). All remaining *Xic*s, lacking the association with the limiting BF, are induced by default to initiate silencing on the linked chromosome.

Although the single-factor (BF) model is simple and elegant, recent observations from *Tsix* and *Xite* genetic analyses have not been easy to reconcile. The alternative "two-factor" model has been proposed based on the principal observation that *Xic* mutations behave differently in the XX and XY contexts (Lee and Lu 1999; Lee 2005). First, given that *Tsix*–/+ and *Tsix*–/– mutations in female cells lead to loss of *Xist* repression, one would expect male cells lacking *Tsix* to inappropriately up-regulate *Xist* and silence its sole X. However, *Tsix*-/Y ES cells do not undergo XCI to any significant degree (Lee and Lu 1999; Sado et al. 2001), and *Tsix*-/Y mice are perfectly normal. (Note: This applies when the *Tsix* mutation is paternally inherited to avoid imprinting effects at *Tsix* [Lee 2000; Sado et al. 2001].) Along similar lines, female ES cells carrying multicopy *Xite* and *Tsix* transgenes form no Xi at all. Thus, although one could argue that one X is protected by BF, the second X is clearly not silenced "by default." Finally, in reporter assays where the *Xist* promoter drives expression of luciferase, differentiation of female cells results in increased luciferase expression only in XX and not XY cells (Sun et al. 2006).

Clearly then, XY cells lack a factor that would ordinarily be present in XX cells. The two-factor model thus incorporates the need for BF binding on the future Xa and introduces the need for a second factor—dubbed the competence factor (CF). CF is believed to play a part in inducing the inactivation of the future Xi and would be produced only in the context of supernumerary Xs. Indeed, CF is thought to consist of X-linked factors not titrated away by the fixed quantity of autosomal factors produced during the counting process. Naturally, every X in excess of one would produce one X-linked CF, enabling the silencing of all but one X (one that binds BF) in the genome.

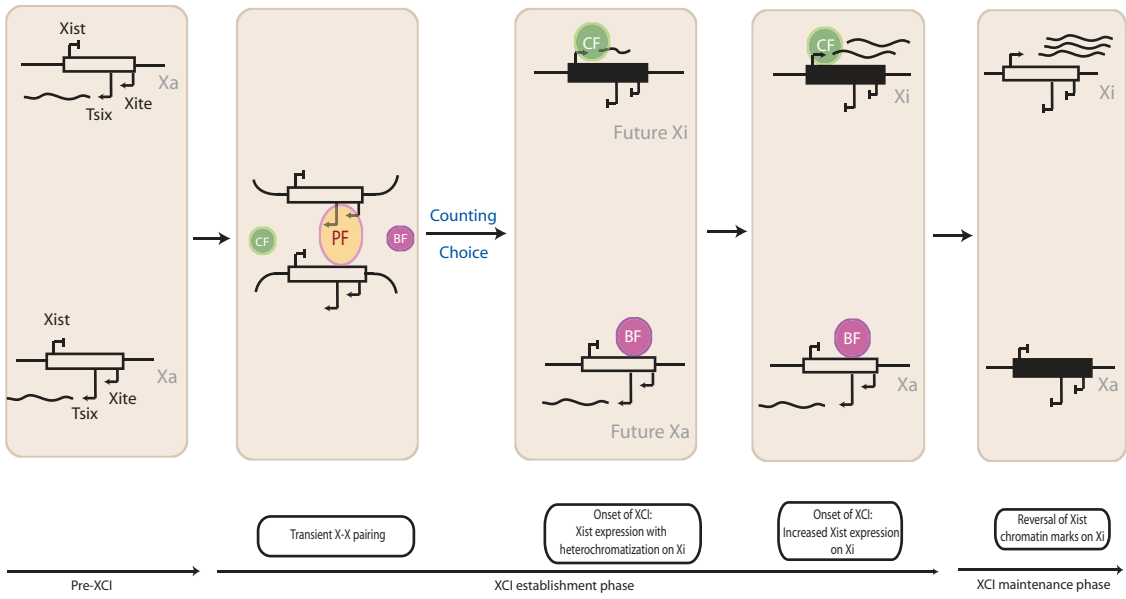

Figure 5. A model for mutually exclusive choice arising from cross-talk between paired X chromosomes, resulting in asymmetrical Xs. The two X chromosomes are both euchromatic and epigenetically identical at the pre-XCI stage. The two chromosomes are then paired together, perhaps with the help of a pairing factor (PF), to facilitate the cross-talk needed to distinguish between the chromosomes. Following the transient X–X pairing event, the future Xi and future Xa are distinguished by different chromatin modifications within the *Xist* region. Binding of a competence factor (CF) to an unknown site in this region of the future Xi assists with the transcriptional up-regulation of *Xist* despite the heterochromatic environment. The binding of a blocking factor (BF), again to an unknown site, may function to help *Tsix* keep *Xist* repressed on the euchromatic future Xa. The chromatin modifications switch for the Xi and Xa alleles during the final maintenance phase of XCI.

We hypothesize that this counting process is intimately linked to pairing, perhaps occurring simultaneously in time and space (Fig. 5). BF and CF must bind mutually exclusively. The proposed cross-talk during the paired state may provide a platform on which the asymmetric distribution of BF and CF takes place. During the pre-XCI stage, the X chromosomes are epigenetically identical and exist in random positions relative to each other in the nucleus. As XCI begins, the two Xs transiently pair via putative pairing factor(s) (PF) and they do so perhaps within a particular region in the nucleus, with closest contact points occurring at *Tsix* and *Xite*. It is envisioned that BF and CF compete to bind. Where the hypothetical BF and CF would bind is a subject of debate. Previously, it has been speculated that BF may bind *Tsix* or *Xite* of the future Xa and ensure the persistent expression of *Tsix* (Lee and Lu 1999; Lee 2005). On the other hand, CF may bind either to *Tsix/Xite* to down-regulate their Xa-promoting activities or it may bind *Xist* directly, perhaps recruited there by the newly created transient heterochromatic status of the *Tsix/Xist* domain.

By this model, cross-talking via X–X pairing eventually leads to the mutually exclusive binding of BF and CF and divergent X-fates. BF's unique binding to one *Xic* enables the monoallelic persistence of *Tsix* expression on the future Xa. The absence of BF binding and the unique binding of CF to the remaining *Xic* would then silence *Tsix* and enable the induction of *Xist*. Therefore, pairing generates the asymmetry in *Tsix* expression, which in turn dictates the asymmetric chromatin modifications and expression patterns of *Xist*.

The future promises to bring exciting new revelations pertaining to the mechanisms of counting, choice, and XCI. With no shortage of models, it seems, however, that XCI is likely to become even more complicated before the mechanisms are clarified. What we have discussed herein applies primarily to the random form of XCI. It is possible that imprinted XCI employs a significantly different mechanism of silencing, as indeed the marsupial genome appears to lack *Xist* (Duret et al. 2006; Hore et al., in prep.; L.S. Davidow et al., in prep.) . In this case, one wonders whether pairing and differential chromatin marks also direct the initiation of XCI in the marsupial. Recent studies have also introduced the contrasting viewpoint that an XX-specific CF may not be required for XCI (Morey et al. 2004; Vigneau et al. 2006). On the basis of results from a deletion of the *DXPas*34 repeat in *Tsix*, the work suggests that inhibition of *Tsix* transcription (at least in the context of this deletion) has similar effects *in cis* in male and female cells, thus questioning whether XX–XY differences exist and whether they could be used as a basis for postulating a need for a CF. Yet, as discussed above, XX–XY differences do actually exist. Clearly, many unanswered questions regarding mechanisms remain and will continue to challenge the field for years to come.

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REFERENCES

Aramayo R. and Metzenberg R.L. 1996. Meiotic transvection in fungi. *Cell* **86:** 103.

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.

Avner P. and Heard E. 2001. X-chromosome inactivation: Counting, choice and initiation. *Nat. Rev. Genet*. **2:** 59.

Bacher C.P., Guggiari M., Brors B., Augui S., Clerc P., Avner P., Eils R., and Heard E. 2006. Transient colocalization of X-inactivation centers accompanies the initiation of X inactivation. *Nat. Cell Biol*. **8:** 293.

Bell A.C. and Felsenfeld G. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* **405:** 482.

Borsani G., Tonlorenzi R., Simmler M.C., Dandolo L., Arnaud D., Capra V., Grompe M., Pizzuti A., Muzny D., and Lawrence C., et al. 1991. Characterization of a murine gene expressed from the inactive X chromosome. *Nature* **351:** 325.

Boumil R.M. and Lee J.T. 2001. Forty years of decoding the silence in X-chromosome inactivation. *Hum. Mol. Genet*. **10:** 2225.

Boumil R.M., Ogawa Y., Sun B.K., Huynh K.D., and Lee J.T. 2006. Differential methylation of Xite and CTCF sites in Tsix mirrors the pattern of X-inactivation choice in mice. *Mol. Cell. Biol*. **26:** 2109.

Brockdorff N., Ashworth A., Kay G.F., McCabe V.M., Norris D.P., Cooper P.J., Swift S., and Rastan S. 1992. The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. *Cell* **71:** 515.

Brockdorff N., Ashworth A., Kay G.F., Cooper P., Smith S., McCabe V.M., Norris D.P., Penny G.D., Patel D., and Rastan S. 1991. Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. *Nature* **351:** 329.

Brown C.J., Ballabio A., Rupert J.L., Lafreniere R.G., Grompe M., Tonlorenzi R., and Willard H. 1991. A gene from the region of the human X inactivation center is expressed exclusively from the inactive X chromosome. *Nature* **349:** 38.

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.

Chao W., Huynh K.D., Spencer R.J., Davidow L.S., and Lee J.T. 2002. CTCF, a candidate trans-acting factor for X-inactivation choice. *Science* **295:** 345.

Chen J.L., Huisinga K.L., Viering M.M., Ou S.A., Wu C.T., and Geyer P.K. 2002. Enhancer action in trans is permitted throughout the *Drosophila* genome. *Proc. Natl. Acad. Sci*. **99:** 3723.

Clemson C.M., McNeil J.A., Willard H., 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.

Clerc P. and Avner P. 1998. Role of the region 3′ to Xist exon 6 in the counting process of X-chromosome inactivation. *Nat. Genet*. **19:** 249.

———. 2003. Multiple elements within the Xic regulate random X inactivation in mice. *Semin. Cell Dev. Biol*. **14:** 85.

Cohen D.E. and Lee J.T. 2002. X-chromosome inactivation and the search for chromosome-wide silencers. *Curr. Opin. Genet. Dev*. **12:** 219.

Coulthard A.B., Nolan N., Bell J.B., and Hilliker A.J. 2005. Transvection at the vestigial locus of *Drosophila melanogaster*. *Genetics* **170:** 1711.

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*. **92:** 3531.

Dekker J., Rippe K., Dekker M., and Kleckner N. 2002. Capturing chromosome conformation. *Science* **295:** 1306.

Duncan I.W. 2002. Transvection effects in *Drosophila*. *Annu. Rev. Genet*. **36:** 521.

Duret L., Chureau C., Samain S., Weissenbach J., and Avner P. 2006. The Xist RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. *Science* **312:** 1653.

Goto T. and Monk M. 1998. Regulation of X-chromosome inactivation in development in mice and humans. *Microbiol. Mol. Biol. Rev*. **62:** 362.

Hark A.T., Schoenherr C.J., Katz D.J., Ingram R.S., Levorse J.M., and Tilghman S.M. 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* **405:** 486.

Heard E. 2004. Recent advances in X-chromosome inactivation. *Curr. Opin. Cell Biol*. **16:** 247.

———. 2005. Delving into the diversity of facultative heterochromatin: The epigenetics of the inactive X chromosome. *Curr. Opin. Genet. Dev*. **15:** 482.

Heard E., Mongelard F., Arnaud D., and Avner P. 1999. Xist yeast artificial chromosome transgenes function as X-inactivation centers only in multicopy arrays and not as single copies. *Mol. Cell. Biol*. **19:** 3156.

Holmgren C., Kanduri C., Dell G., Ward A., Mukhopadhya R., Kanduri M., Lobanenkov V., and Ohlsson R. 2001. CpG methylation regulates the Igf2/H19 insulator. *Curr. Biol*. **11:** 1128.

Huynh K.D. and Lee J.T. 2001. Imprinted X inactivation in eutherians: A model of gametic execution and zygotic relaxation. *Curr. Opin. Cell Biol*. **13:** 690.

LaSalle J.M. and Lalande M. 1995. Domain organization of allele-specific replication within the GABRB3 gene cluster requires a biparental 15q11-13 contribution. *Nat. Genet*. **9:** 386.

———. 1996. Homologous association of oppositely imprinted chromosomal domains. *Science* **272:** 725.

Lee J.T. 2000. Disruption of imprinted X inactivation by parent-of-origin effects at Tsix. *Cell* **103:** 17.

———. 2002a. Homozygous Tsix mutant mice reveal a sex-ratio distortion and revert to random X-inactivation. *Nat. Genet*. **32:** 195.

———. 2002b. Is X-chromosome inactivation a homology effect? *Adv. Genet*. **46:** 25.

———. 2005. Regulation of X-chromosome counting by Tsix and Xite sequences. *Science* **309:** 768.

Lee J.T. and Lu N. 1999. Targeted mutagenesis of Tsix leads to nonrandom X inactivation. *Cell* **99:** 47.

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

Lee J.T., Lu N., and Han Y. 1999b. Genetic analysis of the mouse X inactivation center defines an 80-kb multifunction domain. *Proc. Natl. Acad. Sci*. **96:** 3836.

Lee J.T., Strauss W.M., Dausman J.A., and Jaenisch R. 1996. A 450 kb transgene displays properties of the mammalian X-inactivation center. *Cell* **86:** 83.

Lucchesi J.C., Kelly W.G., and Panning B. 2005. Chromatin remodeling in dosage compensation. *Annu. Rev. Genet*. **39:** 615.

Luikenhuis S., Wutz A., and Jaenisch R. 2001. Antisense transcription through the Xist locus mediates Tsix function in embryonic stem cells. *Mol. Cell. Biol*. **21:** 8512.

Lyon M.F. 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* **190:** 372.

———. 1972. X-chromosome inactivation and developmental patterns in mammals. *Biol. Rev. Camb. Philos. Soc*. **47:** 1.

Marahrens Y. 1999. X-inactivation by chromosomal pairing events. *Genes Dev*. **13:** 2624.

Marahrens Y., Loring J., and Jaenisch R. 1998. Role of the Xist gene in X chromosome choosing. *Cell* **92:** 657.

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.

McDonald L.E., Paterson C.A., and Kay G.F. 1998. Bisulfite genomic sequencing-derived methylation profile of the xist gene throughout early mouse development. *Genomics* **54:** 379.

Mlynarczyk-Evans S., Royce-Tolland M., Alexander M.K., Andersen A.A., Kalantry S., Gribnau J., and Panning B. 2006. X chromosomes alternate between two states prior to random X-inactivation. *PLoS Biol*. **4:** e159.

Morey C., Arnaud D., Avner P., and Clerc P. 2001. Tsix-mediated repression of Xist accumulation is not sufficient for normal random X inactivation. *Hum. Mol. Genet*. **10:** 1403.

Morey C., Navarro P., Debrand E., Avner P., Rougeulle C., and Clerc P. 2004. The region 3′ to Xist mediates X chromosome counting and H3 Lys-4 dimethylation within the Xist gene. *EMBO J*. **23:** 594.

Navarro P., Pichard S., Ciaudo C., Avner P., and Rougeulle C. 2005. Tsix transcription across the Xist gene alters chromatin conformation without affecting Xist transcription: Implications for X-chromosome inactivation. *Genes Dev*. **19:** 1474.

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.

Ogawa Y. and Lee J.T. 2003. Xite, X-inactivation intergenic transcription elements that regulate the probability of choice. *Mol. Cell* **11:** 731.

Panning B., Dausman J., and Jaenisch R. 1997. X chromosome inactivation is mediated by Xist RNA stabilization. *Cell* **90:** 907.

Penny G.D., Kay G.F., Sheardown S.A., Rastan S., and Brockdorff N. 1996. Requirement for Xist in X chromosome inactivation. *Nature* **379:** 131.

Plath K., Mlynarczyk-Evans S., Nusinow D.A., and Panning B. 2002. Xist RNA and the mechanism of X chromosome inactivation. *Annu. Rev. Genet*. **36:** 233.

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.

Rastan S. 1983. Non-random X-chromosome inactivation in mouse X-autosome translocation embryos—Location of the inactivation centre. *J. Embryol. Exp. Morphol*. **78:** 1.

Sado T., Hoki Y., and Sasaki H. 2005. Tsix silences Xist through modification of chromatin structure. *Dev. Cell* **9:** 159.

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

Stavropoulos N., Lu N., and Lee J.T. 2001. A functional role for Tsix transcription in blocking Xist RNA accumulation but not in X-chromosome choice. *Proc. Natl. Acad. Sci*. **98:** 10232.

Stavropoulos N., Rowntree R.K., and Lee J.T. 2005. Identification of developmentally specific enhancers for Tsix in the regulation of X chromosome inactivation. *Mol. Cell. Biol*. **25:** 2757.

Sun B.K., Deaton A.M., and Lee J.T. 2006. A transient heterochromatic state in Xist preempts X inactivation choice without RNA stabilization. *Mol. Cell* **21:** 617.

Takagi N. and Sasaki M. 1975. Preferential inactivation of the paternally derived X-chromosome in the extraembryonic membranes of the mouse. *Nature* **256:** 640.

Vazquez J., Muller M., Pirrotta V., and Sedat J.W. 2006. The Mcp element mediates stable long-range chromosome-chromosome interactions in *Drosophila*. *Mol. Biol. Cell* **17:** 2158.

Vigneau S., Augui S., Navarro P., Avner P., and Clerc P. 2006. An essential role for the DXPas34 tandem repeat and Tsix transcription in the counting process of X chromosome inactivation. *Proc. Natl. Acad. Sci*. **103:** 7390.

Wakimoto B.T. and Hearn M.G. 1990. The effects of chromosome rearrangements on the expression of heterochromatic genes in chromosome 2L of *Drosophila melanogaster*. *Genetics* **125:** 141.

Willard H.F. and Carrel L. 2001. Making sense (and antisense) of the X inactivation center. *Proc. Natl. Acad. Sci*. **98:** 10025.

Williams B.R. and Wu C.T. 2004. Does random X-inactivation in mammals reflect a random choice between two X chromosomes? *Genetics* **167:** 1525.

Wu C.T. and Morris J.R. 1999. Transvection and other homology effects. *Curr. Opin. Genet. Dev*. **9:** 237.

Xu N., Tsai C.L., and Lee J.T. 2006. Transient homologous chromosome pairing marks the onset of X inactivation. *Science* **311:** 1149.

Yasuhara J.C. and Wakimoto B.T. 2006. Oxymoron no more: The expanding world of heterochromatic genes. *Trends Genet*. **22:** 330.

Yoon B.J., Herman H., Sikora A., Smith L.T., Plass C., and Soloway P.D. 2002. Regulation of DNA methylation of Rasgrf1. *Nat. Genet*. **30:** 92.

Yoon B., Herman H., Hu B., Park Y.J., Lindroth A., Bell A., West A.G., Chang Y., Stablewski A., Piel J.C., Loukinov D.I., Lobanenkov V.V., and Soloway P.D. 2005. Rasgrf1 imprinting is regulated by a CTCF-dependent methylation-sensitive enhancer blocker. *Mol. Cell. Biol*. **25:** 11184.

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.