X inactivation: ***Tsix* and *Xist* as yin and yang**

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A new study shows that expression of *Tsix*, an antisense *Xist* gene, can be controlled by imprinting, and that high *Tsix* activity during X inactivation can protect the future active X chromosome from silencing by *Xist*. *Tsix* and *Xist* seem to have a yin and yang relationship, with opposite effects on X inactivation.

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The evolution of sexual dimorphism and of chromosomal sex determination led to the problem of how to equalize sex-chromosome gene dosage between males and females. In mammals, where females carry two copies of the gene-rich X chromosome and males have only one, equal X-linked gene dosage is achieved by the transcriptional inactivation of one of the two X chromosomes in female cells. Two different mechanisms have evolved to inactivate a single X in females. In imprinted X inactivation, the parental origin of an X chromosome determines its fate, such that the maternally inherited X (Xm) remains active and the paternally inherited X (Xp) is silenced in every cell. In random X inactivation, there is an equal probability of the Xp or the Xm being silenced in any cell. X inactivation is imprinted in marsupials and in the extraembryonic tissues of the mouse, whereas it is random in primates and in mouse embryonic tissues.

Imprinted X inactivation results in appropriate dosage compensation because of the stereotyped genetic contributions of the parents to the embryo: mothers always contribute an X chromosome, whereas fathers determine the sex of the zygote by contributing either an X or a Y. Therefore, if every Xp is imprinted to be inactivated, normal female embryos inactivate a single X chromosome, and male embryos, which lack an Xp and have no need for dosage compensation, do not silence their single X. Imprinting is controlled by epigenetic modifications of the genome that are established in the parents' germ cells, often taking the form of differential methylation at cytosine-guanine dinucleotides (CpGs) in *cis*-regulatory DNA sequences. Imprinting of X-inactivation could consist of a spermatocyte-specific mark promoting silencing of the Xp and/or an oocyte-specific mark blocking X inactivation of the Xm.

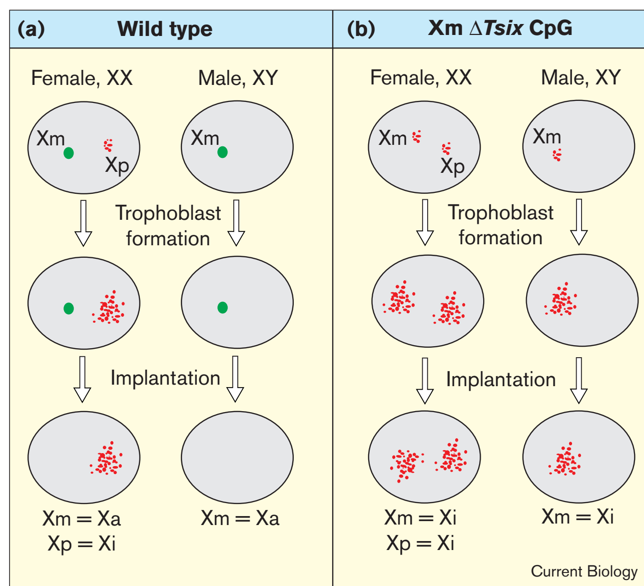
Random X inactivation is thought to require multiple functions to yield reliable dosage compensation: counting of X chromosomes, choice of an active X (Xa), initiation of silencing on the future inactive X (Xi), and maintenance of the Xi's silent state. These functions have been mapped to an 80 kilobase region of the X chromosome known as the X inactivation center (Xic) [1]. Counting assesses the need for dosage compensation by selecting only one Xa per diploid genome. In molecular terms, counting is thought to result from the binding of a limiting, autosomally encoded blocking factor complex to an Xic DNA element called the counting element. Binding of blocking factor to an X chromosome's counting element prevents the X inactivation machinery from functioning *in cis* on that chromosome, the future Xa [2]. The choice of which X will become the Xa occurs only when more than one X chromosome is present. Molecularly, it reflects the likelihood that blocking factor will assemble on the counting element of a given X chromosome, and it is affected by multiple *cis*-acting choice elements.

After counting and choice have taken place, X inactivation is initiated at all X chromosomes not chosen to become the Xa. That is, any X not bound by blocking factor is silenced by the X inactivation machinery and becomes an Xi. In males, the single Xm is always selected to remain active, and there are no additional X chromosomes on which to initiate inactivation. By contrast, in female cells, the paternal X is chosen as Xa and the maternal X is inactivated in about half of all cells, and the opposite pattern is observed in the other half. After X inactivation is initiated, the silent state of the Xi is clonally maintained through multiple silencing mechanisms, such that all females are mosaic for X-linked traits.

In placental mammals, the two mechanisms of X inactivation have co-opted a common molecular effector, the Xi-specific transcript, *Xist*. *Xist*, which resides within the Xic, is a large, processed, non-coding nuclear RNA that is required for initiation of X inactivation [3]. During onset of both imprinted and random X inactivation in female cells, *Xist* spreads from its site of transcription, coating the entire Xi; this spreading correlates with X-linked gene silencing. Though no chromatin remodeling or gene silencing activity has been proven, it is widely thought that *Xist* acts in a ribonucleoprotein complex (RNP) with such activities. Indeed, a recent study [4] using an inducible *Xist* cDNA showed that *Xist* can reversibly silence genes *in cis* outside of its normal context in X inactivation.

As both imprinted and random X inactivation mechanisms act through *Xist* to implement their respective decisions to

Figure 1



X inactivation in extraembryonic cells is controlled by the imprinted *Tsix* CpG. **(a)** Female and male nuclei of eight-cell embryos before blastocyst formation are depicted schematically as they appear by RNA FISH [16]. A pinpoint of *Tsix* RNA (green) is expressed from the Xm, which is present in both sexes, and dispersed *Xist* RNA (red) is expressed from the Xp, which is present only in females. In trophoblast cells of the blastocyst, *Xist* RNA spreads and forms a larger particulate domain surrounding the Xp, and Xp genes are silenced; *Tsix* transcription from the Xm persists. After implantation and formation of extraembryonic tissues, *Tsix* transcription from the Xm is quenched. Thus, imprinted X-inactivation results in the Xm remaining active in both female and male cells. **(b)** Female and male nuclei of cells bearing the *Tsix* CpG deletion on the Xm are depicted. *Xist* expression is observed now from both the Xp and the Xm, and no *Tsix* expression is apparent. Upon trophoblast formation, in addition to coating the Xp in female cells, *Xist* RNA coats the deletion-bearing Xm in both sexes and Xm genes are ectopically silenced. Thus, in imprinted X-inactivation, pinpoint *Tsix* expression prior to differentiation correlates with protection of an X chromosome from inactivation. The *Tsix* CpG likely acts as an imprinting center bearing an Xm imprint directing high *Tsix* expression, and/or an Xp imprint shutting off *Tsix* expression.

inactivate a given X chromosome, we can gain insight into X inactivation by studying how each mechanism regulates the function of *Xist*. Some important clues have been provided recently by Jeannie Lee's laboratory. In 1999, Lee and co-authors [5] reported the discovery of an antisense *Xist* transcript, termed *Tsix*, which initiates downstream of the *Xist* gene and is transcribed through it. In embryonic stem (ES) cells, which initiate random X inactivation upon differentiation, expression of the *Tsix* transcript is observed during the pre-inactivation period in which *Xist* is unstable and has not yet spread from its site of transcription. As soon as inactivation is initiated, visualized by spread of *Xist* RNA over the Xi, *Tsix* expression is extinguished from that chromosome. On the Xa, in contrast, low-level *Xist* and *Tsix* transcription persist for a time until both are quenched. *Tsix* is an attractive potential regulator of *Xist* function because it is located *in cis* to *Xist* and because it is expressed whenever *Xist* lacks the ability to spread and/or silence genes.

To study the function of *Tsix*, Lee's group [6] engineered a 3.7 kilobase deletion of the CpG-rich region encompassing the *Tsix* promoter and major transcriptional start site, drastically decreasing the amount of *Tsix* RNA that is produced. Male ES cells carrying the deletion properly counted the single X and did not initiate X inactivation. Female ES cells carrying the *Tsix* deletion on one X chromosome exhibited skewing of X inactivation, such that the wild-type X was always chosen as Xa, and the deleted X was inactivated [6]. These results indicate that *Tsix* regulates choice, but not counting, in random X inactivation.

Now, Lee [7] has generated mice heterozygous for the *Tsix* deletion. In contrast to its subtle effects on random X inactivation, Lee's new results indicate that the *Tsix* regulatory region plays an indispensible role in imprinted X inactivation within the developing mouse extraembryonic tissues. She observed a dramatic parent-of-origin effect in the phenotypes of progeny of deletion-carrying mice. Mutant mice inheriting the deletion from their father were born at the expected frequency, and were healthy. In contrast, mutant mice inheriting the deletion from their mother were conceived at normal frequency, but only 18% survived to term. Developmental analysis indicated a defect in post-implantation placental outgrowth, consistent with embryonic lethality as well as growth retardation and smaller adult body mass of rare survivors.

To determine whether the *Tsix* deletion affected *Xist* expression in extraembryonic tissues, Lee [7] examined the expression of *Tsix* and *Xist* in pre-implantation mouse blastocysts. Blastocysts contain the future embryonic epiblast cells, as well as the future extraembryonic trophoblast cells in which imprinted X inactivation occurs. As depicted in Figure 1, male and female trophoblast cells normally express *Tsix* from the Xm, and female cells express *Xist* from the Xp. In mutant blastocysts with a maternally inherited deletion, *Tsix* expression was abolished. Furthermore, *Xist* was now expressed from both the Xm and the Xp in females, and from the single Xm in males.

Using an *in vitro* differentiation assay, in which blastocysts normally 'implant' in a petri dish and the differentiating trophoblast grows outward, Lee [7] found that blastocysts bearing the maternally inherited deletion attached poorly and had little trophoblast outgrowth. Using *in situ* hybridization to determine the distribution of *Xist* RNA, Lee showed that *Xist* coated the single Xm in 50% of mutant male trophoblasts, and coated both the Xm and the Xp in 60% of mutant female trophoblasts. Taken together, these results suggest that extraembryonic cell death is caused by disruption of imprinted

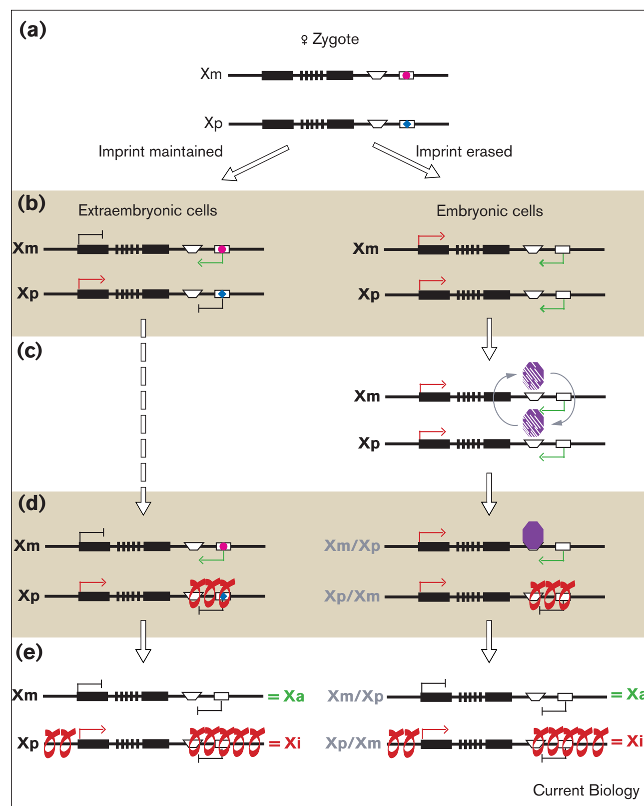


Figure 2

A model for imprinted and random X inactivation. The black line diagrams represent elements of the X inactivation center: solid rectangles represent the exons of the *Xist* gene; the open trapezoid represents the counting element; the open rectangle represents the *Tsix* CpG which includes the major transcriptional start site of *Tsix*. The diagram is not to scale and the position of the counting element has not been mapped and is tentative. **(a)** A zygote is produced by the joining of maternally and paternally inherited haploid genomes. Female zygotes receive an Xm with a maternally imprinted *Tsix* CpG (pink circle) and an Xp with a paternally imprinted *Tsix* CpG (blue diamond). In the cells that give rise to the extraembryonic tissues, these imprints are maintained, whereas in the cells that give rise to the embryo proper, these imprints are erased. **(b)** Before differentiation, future extraembryonic cells express *Tsix* only from the Xm (green arrow) and *Xist* only from the Xp (red arrow), as a result of the imprints at the *Tsix* CpG. In contrast, future embryonic cells express both *Tsix* and *Xist* from the Xm and the Xp, because imprints at the *Tsix* CpG have been erased. **(c)** In future embryonic cells, a single, autosomally encoded blocking factor (purple octagon) assembles and tests binding at the available counting elements before stably binding to one of them. **(d)** In future extraembryonic cells, the *Xist* allele which is highly expressed – the Xp – produces functional *Xist* (red loops) that can silence neighboring genes reversibly. In future embryonic cells, stable binding of the blocking factor to the counting element of either the Xm or the Xp blocks *Xist*'s ability to silence that chromosome, either by directly or indirectly affecting *Xist* transcription or activity. On the other X, low levels of functional *Xist* can silence neighboring genes reversibly, perhaps including *Tsix*. **(e)** When a spreading or stabilizing factor becomes available, the X expressing functional *Xist* is rapidly coated and silenced. In extraembryonic cells, the *Tsix*-expressing Xm is protected and becomes the Xa, whereas in embryonic cells, the X which has bound blocking factor becomes the Xa. After differentiation, expression of *Tsix* and *Xist* from the Xa is extinguished. The silent epigenotype of the Xi is locked in by multiple mechanisms. Please note that although the blocking factor does not have to be invoked in order for imprinted X inactivation to occur reliably, we believe that it probably does play a partially redundant role here, preferentially binding the counting element of the X which has lower *Xist* activity, the Xm. Also note that blocking factor binding in step (c) and reversible *Xist*-mediated silencing in step (d) are separated only for illustration of the model and may occur simultaneously or in the reverse order.

X inactivation, such that the Xm in male and female cells is ectopically inactivated.

Lee [7] has demonstrated that the *Tsix* promoter is the probable location of an imprint that sets *Tsix* expression and controls whether or not an X chromosome is inactivated in an extraembryonic cell. This *Tsix*-regulating imprint is probably established during gametogenesis. Differential methylation of the 4 kilobase CpG-rich region that includes the *Tsix* promoter is the likely way in which maternal and paternal effects are exerted, turning zygotic *Tsix* expression up or down, respectively. Future work should ascertain whether specific methylation patterns are conferred on this region in oocytes and spermatocytes. Specific mutation of sequences exhibiting differential methylation will determine whether epigenetic modification has positive or negative effects on *Tsix* transcription.

Lee's study [7] indicates that imprinted X-inactivation is effected through *Tsix*, which seems to be a negative regulator of *Xist* function. The mechanisms by which *Tsix* could regulate *Xist* fall into two general classes: first, those in which *Tsix* function is mediated by the antisense transcript; and second, those in which the antisense transcript is incidental to *Tsix* function. In the first class, an interaction between the *Tsix* and *Xist* transcripts could disrupt *Xist* RNA function post-transcriptionally. Such an interaction might prevent *Xist* folding or complex formation. Another possibility is that the formation of a *Tsix*–*Xist* duplex might result in *Xist* RNA destabilization via a double stranded RNA interference-like mechanism. RNA interference, whereby a small amount of duplex RNA stimulates RNA turnover in a sequence-specific manner, has been demonstrated recently to function in mouse embryos in the developmental window in which *Tsix* regulates imprinted X inactivation [8]. It will be important to determine whether transcription through the *Xist* locus, producing a transcript antisense to *Xist* RNA, is required to mediate *Tsix* function, as would be predicted by this class of mechanism.

Alternatively, in a second class of mechanisms, *Tsix* could regulate *Xist* function at the transcriptional level. *Xist* and *Tsix* may fit into a more general category of oppositely imprinted functional–nonfunctional gene pairs, the best-studied example of which is *Igf2* and *H19*. These two imprinted genes are transcribed in a mutually exclusive manner: *Igf2* encodes a functional mRNA, whereas *H19* encodes a nonsense RNA. Differential methylation of an imprinting center allows a transcriptional enhancer, required

by both genes, to act only on *Igf2* on the maternal chromosome, and only on *H19* on the paternal chromosome. *H19* transcription appears to be incidental, and can be seen merely as a readout of *Igf2* repression [9].

Tsix and *Xist* activity could be regulated in an analogous, mutually exclusive manner, with the *Tsix* imprint determining whether a *cis*-regulatory element acts on *Xist*, producing functional RNA, or not, resulting in incidental production of nonsense RNA from the *Tsix* promoter. In this scenario, the *Tsix* CpG deletion [6] likely removes two functional domains: an imprinting center that binds a regulatory factor in an imprint-sensitive manner, and the minimal *Tsix* promoter, which responds to the presence of the regulatory factor. If this type of mechanism is operational, reintroduction of the imprinting center without the minimal *Tsix* promoter should restore proper *Xist* regulation without restoring the *Tsix* transcript.

With Lee's results in mind, we propose a general model for X inactivation, depicted in Figure 2. Under this model, the initiation of X inactivation is a default process that must be kept in check to produce an Xa. Imprinted and random X inactivation mechanisms counter *Xist*-mediated silencing in different ways, each ensuring that one future Xa is protected in every cell. Both mechanisms block *Xist*'s early, short-range silencing activity *in cis*, such that *Xist* activity is retained only on the future Xi. Initiation of X inactivation occurs when this early activity is converted to late activity, in which *Xist* spreads rapidly, coating the entire Xi within one cell cycle [4,10]. Late activity might be contingent upon availability of a stabilizing or spreading factor, or upregulation of *Xist* transcription. After passage of a differentiation milestone, *Xist*-mediated silencing is locked in, and the Xi epigenotype is maintained. Meanwhile, Xa gene expression is ensured because this chromosome has escaped *Xist* action during the critical X inactivation window.

In imprinted X inactivation, the *Tsix* imprint prevents inactivation of the future Xa, whereas in random X inactivation, counting is used to prevent Xa silencing. Imprinting protects an X chromosome from inactivation by causing expression *in cis* of *Tsix*. Parental imprints set high-level *Tsix* expression on the Xm, protecting it from inactivation, and abolish *Tsix* expression from the Xp, allowing *Xist* action and *de facto* generation of a paternal Xi. In random X inactivation, the future Xa is protected from silencing by binding of blocking factor to the counting element of a single X chromosome. Blocking factor binding prevents *Xist* activity *in cis*, randomly selecting either Xm or Xp to become Xa. Our model allows us to propose answers to several persistent questions — and to explain some puzzling results — in the X inactivation field.

Where is the blocking factor binding site, or counting element? If a *cis*-acting counting element exists, it should be possible to isolate a DNA element that causes ectopic X inactivation in male cells by titrating blocking factor away from the endogenous X. Conversely, it should be possible to delete this element in male cells, causing ectopic X inactivation because the deleted chromosome cannot be blocked from inactivation. In fact, a 65 kilobase deletion downstream of *Xist*, including the *Tsix* promoter, not only skewed random X inactivation in XX cells, as Lee's deletion does, but caused ectopic X inactivation in a cell line with a single X chromosome [11]. A report [12] that a 35 kilobase genomic *Xist* transgene triggered counting and occasional inactivation of the single X in male cell lines argues that the 6 kilobase region downstream of *Xist* absent in the deletion and included in the transgene may contain the counting element.

How can deletions in the *Xist* gene cause primary skewing of X inactivation? Deletions in *Xist* which affect the RNA's function result in the deleted X being chosen as the Xa [13], suggesting that during random X inactivation there is discrimination between a functional and a nonfunctional *Xist* allele before the Xa is designated. Under this model, if early *Xist* activity can act on the counting element DNA, remodeling its chromatin environment, the affinity of the counting element for blocking factor might be reduced. Then, the X exhibiting weaker *Xist* activity would have relatively higher affinity for blocking factor binding and would tend to be chosen to remain active. Choosing as Xa the chromosome with the weakest *Xist* allele, which is least able to form a stable Xi, would improve female survival.

What is *Tsix*'s role in random X inactivation? Imprinted X inactivation, which is controlled by *Tsix*, can be seen as the total skewing of X chromosome choice. Perhaps it is not surprising, then, that *Tsix* influences choice in random X inactivation. This model suggests that, in Lee's ES cell lines, deletion of *Tsix* skewed X inactivation because the deleted X was less likely than the wild-type X to bind blocking factor and to be chosen as Xa. Therefore, *Tsix* and *Xist* both affect choice. We propose that *Tsix* and *Xist* have a yin–yang relationship and that the relative ratio of their activities mediates choice by determining which counting element blocking factor will assemble on. If the *Tsix*:*Xist* ratio is high, *Xist* is rendered nonfunctional and blocking factor can bind the counting element; if *Tsix*:*Xist* is low, *Xist* can become functional, act on the counting element, and reduce the likelihood of blocking factor binding. During initiation of X inactivation, *Xist* might even shut down *Tsix* expression from the future Xi, which would provide positive feedback on *Xist* activity. Positive feedback loops are often employed in nature to lock in all-or-nothing decisions such as X inactivation.

How does blocking factor prevent *Xist* activity *in cis*? We believe that blocking factor may act through *Tsix*. In fact,

the counting element may represent an alternative antisense *Xist* promoter that is functional only on blocking factor binding, allowing blocking factor to counter *Xist* function by upregulating antisense transcription. As the counting element is genetically separable from the imprinted promoter studied by Lee [7,11], antisense transcription may be turned on transiently during X inactivation even in *Tsix* CpG-deleted male cells, shielding the single X chromosome from *Xist* action. The possibility of another antisense promoter seems especially likely given Lee and Lu's [6] ability to detect low-level antisense transcription in their *Tsix* deletion-bearing cells.

Why is imprinting incomplete? The 18% survival rate of Xm *Tsix* CpG deletion-bearing pups in Lee's study [7], and rare viable XpO, XpXp, and XmXm animals in previous studies [14], demonstrates that imprinted X-inactivation is not absolute. This raises the question of whether a weak form of random X inactivation is operative in parallel during imprinted X inactivation, or whether the latter mechanism is sloppy enough that strong selection of occasional, properly dosage-compensated cells allows some survival. In our model for imprinted X inactivation, the existence of a protective blocking factor is not required to choose a single Xa; however, the system can also operate if the blocking factor is available. By ensuring *Xist* is unable to act on the counting element of the imprinted Xm, *Tsix* could constitutively direct blocking factor activity to the Xm in extraembryonic cells. Perhaps overlap between the imprinted and random mechanisms, with blocking factor occasionally able to bind in a non-imprinted manner, could explain why imprinted control of X inactivation in mouse extraembryonic tissues is incomplete.

This new understanding of *Tsix* as an imprinting center, as well as a probable effector of choice in random X inactivation, leads back to an evolutionary perspective. If imprinting was the original mechanism of X inactivation in the embryonic as well as the extraembryonic tissues of the mouse ancestor, as is generally believed, then *Tsix*'s original function may have been in imprinting, and its mechanism of countering *Xist* function may have been coopted later by a random X inactivation mechanism. So do organisms, such as humans, in which *Xist* expression is never imprinted and X inactivation is generally random [15], employ *Tsix* in the regulation of *Xist* function?

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