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Investigation of cellular mechanisms for designating one active and one inactive X chromosome in female mammals

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Investigation of Cellular Mechanisms for Designating

One Active and One Inactive X Chromosome in Female Mammals

by

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DISSERTATION

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For Barbara, who is willing to entertain an unconventional idea.

Acknowledgements

I want to acknowledge the many people who supported and inspired me during my graduate work. In the process, I will try to give a sense of what was important in each case, in the hope that readers of this thesis may find a useful lesson or two.

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Barbara has attracted to her lab a group of strong scientists with diverse backgrounds who are friendly and warm-hearted. It has been wonderful to work in the Panning lab, and I want to thank everyone for sharing their expertise and always offering their help. From the departed lab members, I learned the following lessons: Olga told me not to be offended if a hypothesis turns out to be wrong, because our job is to discover how nature really is; Peter taught me that good lab notebook skills add value to experiments; Faraz modeled a productive approach to finding the right career; Kathrin demonstrated that no task is impossible, however big it may be; and Hannah shared her insight that asking the right question, rather than being driven by a favorite experiment, is the best long-term approach in science. I admire the current Panning lab members (listed in order of their joining, from recent to ancient) for the following: Jason for staying

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I am incredibly lucky to have a wonderful, close family, whose love and encouragement have seen me though the many challenges of graduate school. I inherited much of my stubbornness and determination from my paternal grandparents, Stephanie and Duke. The legacy of my maternal grandparents, Ruth and Bert, is the value of education and lifelong learning. Fifty-two years after Grandpa Bert's death, I am incredibly proud to follow in his footsteps by becoming a Ph.D.-level biological scientist. My aunts, uncles, cousins, brother, and in-laws have expressed interest in and support for my work above and beyond all reasonable expectation. Aunts Carol and Martha have insisted on receiving copies of every article I've been an author on, and have actually attempted to read them! Carol flew to San Francisco to attend my thesis talk even though it was the weekend before her own wedding, and the only thing that could keep Martha away was having to administer her high school students' final exams. Uncle Bob has amicably grilled me on my research every time we see each other. Over the years, I've enjoyed talking shop about lab with my Aunt Glenna, a hospital microbiology technician. Cousin Holly has been my faithful email correspondent, listening to my problems and encouraging me through grad school. Though we've chosen divergent career paths, my brother Alex's insight into my professional life is

always on target and I am thankful for the deepened relationship we share in adulthood. Uncle John and Cousin Chris showed their support at my thesis talk—my third graduation they've attended! My mother-in-law, Kathy, who also attended the talk, has always probed me to articulate *why* I am pursuing this research, while my father-in-law and his wife, Bill and Vera, generously send their congratulations at each milestone without probing for details. Finally, my Aunt Anne was an inspiration in the final six months of her life; she showed me that each day holds infinite possibility.

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Investigation of Cellular Mechanisms for Designating One Active and One Inactive X Chromosome in Female Mammals

Susanna Mlynarczyk-Evans

Abstract

In mammals, where females have two copies of the X chromosome and males have only one, X-chromosomal genes are expressed at comparable levels in both sexes. This dosage compensation is achieved by X-chromosome inactivation in females. At a critical decision point early in development, one of the two X chromosomes, apparently chosen at random in each cell, is transcriptionally silenced, and then clonally maintained for a female's lifetime. How can a cell distinguish between two chromosomes of the same DNA sequence to silence only one of them? What is the source of the apparent randomness? What restricts X-inactivation to females? In this thesis, I present research pertaining to these questions. Cytological investigations in pluripotent female cells revealed that, before X-inactivation is initiated, the future Xa and the future Xi already differ from one another along their entire lengths, suggesting they adopt distinct states. Analysis in cells poised for random X-inactivation supported a mechanism in which X chromosomes alternate between states in a mutually exclusive manner, such that the future Xi state is exhibited by the maternally-inherited X chromosome in 50% of cells and by the paternally-inherited X in the remaining 50%. Thus, upon initiation of X-inactivation, distinct properties of the future Xi may direct the silencing machinery to one chromosome per cell, leading to apparently random X-inactivation in the population. Analysis in pluripotent male cells showed that the single X chromosome does not adopt

either state observed in females. The presence of a second copy of X-chromosomal sequences triggers adoption of two states, suggesting that the X-inactivation mechanism uses homology sensing to restrict the future Xi state to female cells. Taken together, this body of work reveals the parameters of the mechanism by which a mammalian cell differentiates one X chromosome for silencing in females, in a manner that appears random. Lessons from the X chromosomes may have implications for the regulation of random monoallelic genes on every autosome pair in the mammalian genome.

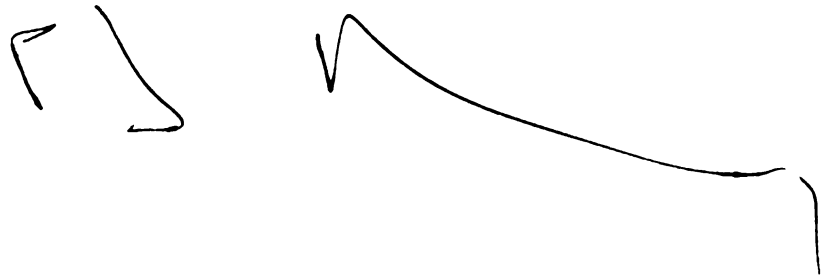

Table of Contents

List of Tables

List of Figures

Prologue.

Thinking about how to achieve random, exclusive X-inactivation

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Thinking about how to achieve random, exclusive X-inactivation

Summary

The inactivation of one entire X chromosome in female mammals is a dramatic example of epigenetic regulation, in which heritable changes in gene function are achieved without altering DNA sequence: in this case, two homologous chromosomes are differentially regulated within the same nucleus for the lifetime of a female individual. In a brief window early in development, the active and inactive X chromosomes appear to be designated at random and in a mutually exclusive manner within each female cell. What is the cellular mechanism that is responsible for this striking form of regulation? In this chapter, I will place this unanswered question in the context of classic experiments and theories in the field of X-inactivation. I begin with an overview of major developments in the field's history, touching on the discoveries of important molecular components that may play roles in designating the Xa and Xi. By considering the evolutionary process that led to the advent of X-inactivation, the parameters within which the mechanism arose are revealed. A review of the developmental regulation of X-inactivation indicates that input from the developmental program impinges on the mechanism. I take a fresh look at classic genetic observations that have formed the basis of thinking about the problem of designating one Xa and one Xi. Finally, I examine a diverse range of models that have been proposed over 45 years to account for this enigmatic process. These ideas reveal key mechanistic features that will likely turn out to be components of the process by which one Xa and one Xi are designated in each female cell.

A note on terminology

In the field of X-inactivation, the process of designating one Xa and one Xi per cell in a mutually exclusive and apparently random manner is commonly referred to as the two steps of "X chromosome counting" and "random choice of the Xa (or Xi)." I choose to avoid these terms for several interrelated reasons. First, it unclear whether this process can be divided into two distinct mechanistic steps. Second, these terms cast a particular human interpretation on the process that may differ from how the cell "thinks." Third, and most importantly, use of these terms may limit one's open-mindedness to considering a diverse range of mechanisms—an approach that may be necessary if we hope to discover the solution to this biological puzzle.

Historical Background

The proposal of X-chromosome inactivation in females as the mammalian dosage compensation mechanism represents one of the appealing hypotheses in the history of biology. As articulated by Mary F. Lyon, a British mouse geneticist at the Medical Research Council, in 1961, this hypothesis brought together findings in the disparate fields of cytology, genetics, and development. Early cytological observations formed the seed of the hypothesis. In his experiments with neurons of cats in the 1940s, a Canadian anatomist named Murray Barr had noticed that "the sex of a somatic cell... may be detected with no more elaborate equipment than a compound microscope." Barr proposed that the distinct, intensely staining body in the nuclei of female, but not male, cells was composed of "heterochromatin of the sex chromosomes" (Barr and Bertram, 1949). In the mid-20th century, the community of mouse geneticists was aware of classic

work in *Drosophila* that had elucidated the relationship between cytological observations of heterochromatin and the silencing of genes in genetic experiments. In his 1967 monograph, *Sex Chromosomes And Sex-Linked Genes*, Susumu Ohno of the City of Hope Medical Center in California wrote, "When the single X-derivation of the sex chromatin body [which was Ohno's own contribution by 1960] came to light, it occurred to many of us[1] that heterochromatinization of one of the two X's of the female might be the means for achieving dosage compensation for all the X-linked genes at once" (Ohno, 1967, p. 97).

Two mouse geneticists independently published separate formulations of the X-chromosome inactivation hypothesis at about the same time: Lyon and Liane B. Russell, of the Oak Ridge National Laboratory in Tennessee. In a succinct and penetrating letter to *Nature* that appeared in April of 1961, Lyon proposed that one X chromosome in the cells of female mammals is "genetically inactivated;" that this chromosome "can be either maternal or paternal in origin, in different cells of the same animal;" and that inactivation occurs "early in embryonic development," after which the inactive state is stably inherited by descendant cells such that female mammals exhibit mosaic expression of traits borne on the X chromosome (Lyon, 1961). Buried in a detailed review on the genetics of mammalian sex chromosomes, which appeared six weeks later in *Science*, Russell presented her own hypothesis. Like Lyon, she reviewed X;autosome translocations in

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[1] It seems that many researchers felt they had anticipated the X-inactivation hypothesis, and it is true that an extensive literature preceded it. In a June 1962 review of human X chromosome genetics, the author made the following statement before attributing the hypothesis to Lyon: "Apparently [the idea] occurred to several persons (e.g., Stewart, 1960; Ohno, 1961a; Beutler, Yeh, and Fairbanks, 1962; Grumbach and Morishima, 1962) at about the same time so that assigning priority, always a hazardous as well as a useless task, is particularly difficult" (McKusick, 1962). Though Ohno and the others (my list would include several more) had described the genetic "rules" for the occurrence of the Barr body, their early publications give no hint that they suspected X-inactivation's significance as a dosage compensation mechanism.

the mouse that caused position effect variegation, which indicated that the X has the potential to express a "heterochromatic effect." Noting the dependence of the variegation phenotype on X chromosome number, Russell wrote that the body of data "permits the hypothesis that, in mammals, genic balance requires the action of *one* X in a manner which precludes realization of its heterochromatic potentialities, so that only any *additional* X's present assume the properties characteristic of heterochromatin" (Russell, 1961). Today, Lyon alone is cited as the originator of the X-inactivation hypothesis. It seems that she owes her place in history to a confluence of chance (the earlier publication date), completeness of her hypothesis, and clarity of presentation. Her paper has a timelessness to it that makes it worth re-reading today.

Lyon's proposal was one of those appealing hypotheses that so simply explained data from so many different experimental approaches that it appears to have been rapidly accepted as the paradigm in the field. There were a few minor protests, mostly from people who felt cheated by not receiving credit themselves (Russell picked on the issue of the completeness of X-inactivation, arguing that X-inactivation spread unevenly along the chromosome (Russell, 1963)—indeed, in humans, X-inactivation is remarkably incomplete, with 15% of genes partially or completely escaping silencing (Carrel and Willard, 2005). Overall, Lyon's hypothesis provided a strong basis for the next phase of investigation, and researchers quickly got down to work on several major questions: What is the genetic basis of X-inactivation? And what is the mechanism of silencing?

What the center holds

Early genetic studies were aimed at identifying the source of the inactivating potential, which was present in some translocation products of the X chromosome, and absent from others. Initially, different investigators favored the possibility of different

numbers of sources—one (Russell, 1963) or multiple (Eicher, 1970)—but analysis eventually revealed a single "*X-inactivation center*" in both mouse and human (*Xic* and *XIC*, respectively; Rastan, 1983; Therman and Patau, 1974). In a way, this controversy over how centralized control over the X-inactivation potential of a given genomic sequence really is continues today, as different groups argue the relative importance of *cis*-elements on the X chromosome in promoting spread of silencing into surrounding genes, vs. the intrinsic ability of the X-inactivation center to silence any linked sequences, regardless of their nature (Lyon, 1998; Popova et al., 2006).

Once evidence that the *Xic* was a single locus was produced, the next logical step was to use newly developed methods to clone the *Xic*, sequence it, and see what lessons it held. This proved to take some time, as nobody knew of any sequences that were expressed from the *Xic*. As the 1990's dawned, everything changed when a young Italian researcher, Andrea Ballabio, recently hired as an assistant professor at Baylor College of Medicine in Houston, realized that a lambda clone he had isolated from a cDNA library in a screen for the X-linked human steroid sulfatase (Ballabio et al., 1987), exhibited an unusual expression profile. Ballabio collaborated with Hunt Willard, then at Stanford, whose group was studying translocation breakpoints to refine the location of the human *XIC*. Willard's group quickly determined that Ballabio's 750 bp clone corresponded to a gene that was a compelling candidate for involvement in the X-inactivation process: it was located in the minimal defined *XIC*, and contrary to the usual rule with X-linked genes, it was expressed exclusively from the inactive X chromosome (Brown et al., 1991a; Brown et al., 1991b). After discovery of the human *X-Inactive Specific Transcript* (*XIST*), it was no great trick to pull out a clone of the mouse *Xist* gene, which shared the interesting properties of its homolog (Borsani et al., 1991; Brockdorff et al., 1991).

The years since the cloning of *XIST/Xist* have been exciting ones in the field of X-inactivation. Characterization of *XIST/Xist* revealed that the gene encodes a very long, spliced RNA (up to 18 kb in mouse, 19 kb in human) that is not translated into protein, but remains in the nucleus (Brockdorff et al., 1992; Brown et al., 1992) and physically associates with the inactive X chromosome along its length (Clemson et al., 1996; Panning and Jaenisch, 1996). The developmental regulation of *Xist*, discussed in greater detail below, suggested a role in initiation of X-inactivation. Analysis of *Xist*-knockout mice demonstrated that the gene is required *in cis* for X-inactivation (Marahrens et al., 1997; Penny et al., 1996), and transgenic lines showed that ectopic *Xist* expression was sufficient to induce silencing in linked sequences (Matsuura et al., 1996; Lee et al., 1996; Herzing et al., 1997; Heard et al., 1999; Wutz and Jaenisch, 2000). Detailed sequence analysis of the *Xist* transcript has begun, in the hopes of identifying RNA elements that confer *Xist*'s silencing and cis-coating activities (Beletskii et al., 2001; Caparros et al., 2002; Wutz et al., 2002). As is so often the case, identification of the gene led to previously unimagined directions of investigation into the process.

The discovery of the *Xist* gene touched off a fast-moving era of genetic analysis of the *Xic*. Through use of *Xic* transgenes, several groups showed that the presence of a second *Xic* triggers X-inactivation on one of the two chromosomes (Heard et al., 1999; Herzing et al., 1997; Lee et al., 1999b; Lee et al., 1996), confirming earlier conclusions based on X chromosome translocations and deletions (Rastan and Robertson, 1985). A number of groups realized at nearly the same time that the *Xist* locus was transcribed from both DNA strands prior to X-inactivation in mouse ES cells, and an antisense gene that traverses the entire *Xist* transcribed region and more was mapped and dubbed *Tsix* (Lee et al., 1999a; Debrand et al., 1999; Mise et al., 1999; Sado et al., 2001). *Tsix*

clearly plays an important role in regulating the initial process of X-inactivation in mouse, although its function appears not to be conserved in humans (Chow et al., 2003; Lee et al., 1999a; Migeon et al., 2001; Migeon et al., 2002). Through targeted genetic analysis, investigators found that *Xist* and *Tsix* exerted opposing effects on the process of designating the Xa and Xi: *Xist* transcription promotes Xi fate (Marahrens et al., 1998; Newall et al., 2001; Nesterova et al., 2003; Gribnau et al., 2005), and *Tsix* promotes Xa fate (Lee and Lu, 1999; Luikenhuis et al., 2001; Sado et al., 2001; Stavropoulos et al., 2001). These experiments also confirmed that the mechanism for assigning fates to the X chromosomes ensures mutual exclusivity. For example, in the presence of an *Xist*-mutant X chromosome that always becomes the Xa, the wild-type homolog always becomes the Xi in a primary manner (Gribnau et al., 2005; Marahrens et al., 1998). A detailed discussion of the function of genetic elements in the mouse *Xic* can be found in Appendix 1. To conclude, molecular genetic analysis has identified important parameters of the cellular process that designates the Xa and the Xi, and has clearly shown that this mechanism must direct *Xist* upregulation from just one X chromosome in each female cell.

Searching for silence

From the early days of the X-inactivation field, researchers have been concerned with discovering what distinguishes the inactive X chromosome from the active X to make it silent. Even before the nature of the Barr body was understood, this structure was recognized as being "allocyclic": having a distinct timing of replication relative to the active chromosomes (reviewed in Gartler et al., 1992). The Xi was typically observed to replicate late in S phase, a property that is a common feature of silenced genes and heterochromatin (Goren and Cedar, 2003), though in very early development the Xi

replicates precociously in extra-embryonic lineages (Sugawara et al., 1983). It has also long been noted that the inactive X chromosome tends to exhibit peripheral nuclear localization, another feature of silent chromatin. In the 1980's, the significance of DNA hyper-methylation on the Xi was vigorously investigated through the use of DNA demethylating agents (Mohandas et al., 1981). Methylation of cytosine residues in genomic DNA is used in many epigenetic mechanisms to achieve stable gene repression (Bird, 2002). In the 1990's and 2000's the roles of DNA methyltransferase enzymes were directly investigated through gene knockout experiments (Beard et al., 1995; Panning and Jaenisch, 1996; Sado et al., 2000; Sado et al., 2004).

In the 1990's, the composition of chromatin proteins on the Xi and their post-translational modifications came under investigation. In general, chromatin marks that are associated with silencing in other genomic regions/developmental contexts are enriched, and those associated with gene activity are depleted, from the inactive X chromosome (Jenuwein and Allis, 2001). The pantheon of chromatin modifications observed on the Xi has exploded in recent years, now including: hypo-acetylation of histones H2A, H3 and H4; hypo-methylation of histone H3-Lys4; hyper-methylation of H3- Lys9, H3-Lys27, H4-Lys20; ubiquitination of histone H2A; and enrichment of histone variant macroH2A (reviewed in Heard, 2005). The enzymes responsible for deposition of these marks are sometimes also observed to be enriched on the Xi, often in a developmental or cell cycle-regulated manner. Finally, it is important to note that *Xist* RNA is responsible for recruiting these chromatin features, directly or indirectly, and also for retaining some of them on the Xi. The latter point is revealed when *Xist* is deleted from the Xi, revealing minor silencing defects (Csankovszki et al., 2001). It seems that the abundance of distinguishing features on the Xi collaborate to keep the chromosome silent for the lifetime of a female individual. Despite the many molecular features of the

Xi that have been discovered, not one of them has so far been found to be critical in maintaining silence, and in fact a large number of them can be lost without reactivating the chromosome, unless the differentiation state of the cell changes (Kalantry et al., 2006). Instead, it seems that the only sure-fire way to relieve silencing is to expose the Xi to the nuclear environment of a pluripotent cell (Takagi et al., 1983; Eggan et al., 2000), and even under this condition, the reactivation process is piecemeal and varies along the chromosome (Nolen et al., 2005). In considering the mechanism that initially designates the Xa and the Xi, it is a good idea to keep the silencing machinery in mind, as it may be employed in more than one stage of the X-inactivation process.

Evolution of Sex Chromosomes and Dosage Compensation

To better understand the framework of the X-inactivation mechanism, it is useful to consider the biological problem that X-inactivation solves. This problem relates to the sex determination system in mammals, and the subsequent series of selective pressures that arose in the course of evolution.

Among multicellular organisms, the existence of distinct male and female sexes is nearly universal. Although the core developmental programs for each sex appear to have been largely conserved over evolution, the gene networks that set sex-specific developmental programs in motion evolve rapidly (Hodgkin, 1992; Marshall Graves and Shetty, 2001). In some organisms, the two sexes are genetically identical, and environmental inputs such as temperature or hormone exposure tip the developmental program in the male or female direction. More commonly, however, sex is determined by genetic mechanisms, with allele content at a sex-determining locus controlling male vs. female development. The advent of genetic sex determination is thought to have

occurred many times over evolution, and each time the pair of ordinary autosomes that becomes home to the sex-determining locus has been transformed into a pair of neo-sex chromosomes, termed X and Y, or Z and W, depending on whether the heterogametic sex is male (XY, as in mammals), or female (ZW, as in birds) (Ohno, 1967). After the sex chromosomes are born, it is thought that they experience unique selective pressures initiating a process of genetic degradation on the Y or W and leading to advent of dosage compensation and fixation of gene content on the X or Z (Charlesworth et al., 2005).

The need for dosage compensation arises when gene content diverges between the two sex chromosomes. In the case of the mammalian sex chromosomes, the X is one of the largest and most gene-rich chromosomes, home to more than 1000 genes (5% of the genome) that are important in both sexes, many performing basic metabolic functions (Ross et al., 2005); in contrast, the Y is a fraction of the physical size and encodes about a tenth as many genes, many of which are expressed exclusively in the male reproductive system (Skaletsky et al., 2003). Though the mammalian X and Y chromosomes do retain a short stretch of homology—the pseudo-autosomal region—by which they pair during meiosis in males, they have become highly divergent over the approximately 300 million years since their birth as sex chromosomes (Ross et al., 2005).

The evolutionary path by which the dosage compensation mechanism is likely to have evolved in mammals is illustrated in Figure 1. The early steps in this process are shared with other XX/XY sex determining systems, and it is useful to consider other well-studied systems as counterpoints. As gene degradation begins on the neo-Y chromosome, the heterogametic (male) sex faces a reduction by half of the corresponding gene products. The resulting imbalance in X to autosomal gene products

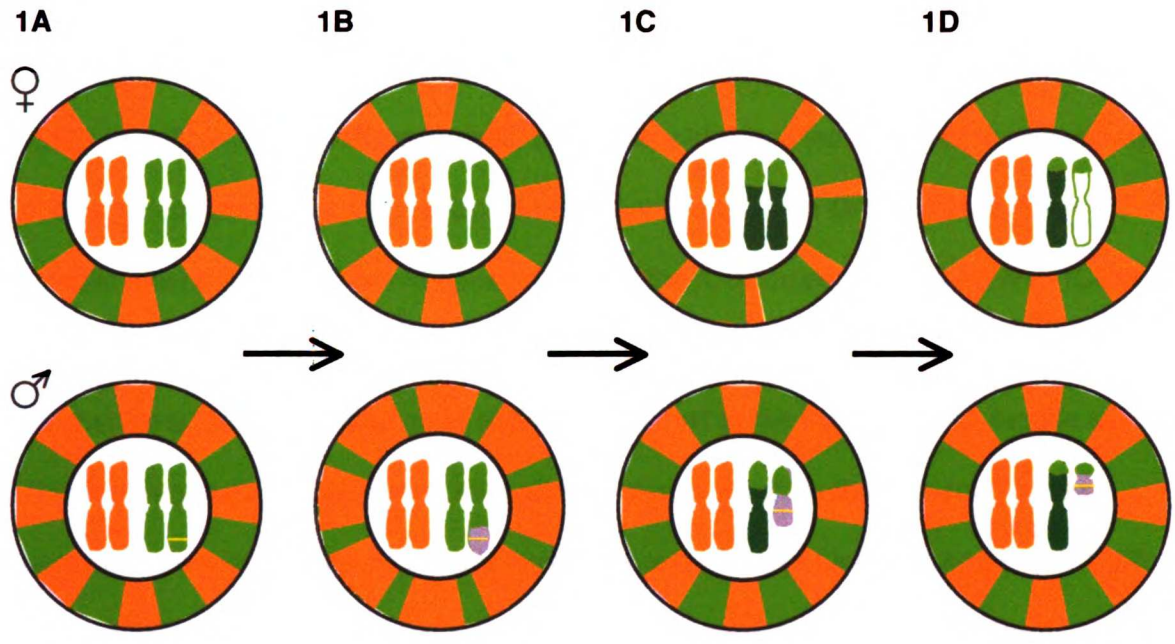

Figure 1. Evolution of sex chromosomes and dosage compensation in mammals. Female (top) and male (bottom) cells are depicted with orange chromosomes representing an autosome pair and green chromosomes representing the (neo-) sex chromosome pair. Colored outer ring represents the pool of gene products produced from the chromosomes of corresponding color.

(1A) The advent of genetic sex determination by acquisition of a master sex determining locus (yellow line) on the neo-Y chromosome leads to pressure to maintain tight linkage between alleles that reinforce genetic control over the sexual differentiation program. This leads to buildup of mutations, translocations, and loss of homology to the X in the region surrounding the sex-determining locus on the Y.

(1B) Lack of recombination around the sex-determining locus leads to decay of surrounding genes on the Y (purple region), further suppressing recombination between the X and Y. This leads to an imbalance between X and autosomal gene products in males (note shift in composition of outer ring), producing pressure to increase expression of X-linked genes that have lost homologs from the Y.

(1C) A mechanism for up-regulation of X-chromosomal genes (dark green) evolves; its action is probably restricted to genes that have lost homologs. This solves the dosage imbalance for males, but females become imbalanced due to hyper-expression of X-chromosomal genes (note shift in composition of outer ring).

(1D) Female-specific epigenetic inactivation of an entire X chromosome (white with green outline) evolves, such that the dosage balance is restored in females without disrupting the balance in males. The Y chromosome is now free to decay, with homologous X-chromosomal genes progressively co-opted into the X-upregulation and -inactivation system.

within the cell is deleterious, as exemplified by the poor ability of diploid organisms to survive loss of one copy of a single chromosome. The consequence is selective pressure for up-regulation of the corresponding region of the X chromosome (Ohno, 1967) specifically in the heterogametic, male sex, which has led to evolution of a variety of dosage compensation mechanisms in different lineages. In *Drosophila*, a male-specific mechanism for two-fold up-regulation of the X chromosome has evolved (Cline and Meyer, 1996). However, in nematodes and in mammals, the dosage compensation mechanisms reduce X-linked gene expression by half in the homogametic, female sex. This fact suggests an intervening evolutionary step in which X-chromosomal genes became upregulated two-fold in both sexes of these lineages (Jegalian and Page, 1998; Ohno, 1967), changing the selective pressure to a female-specific need to down-regulate X chromosome activity. Recent studies in mammals have supported this idea, showing that the genes on a single X chromosome are expressed, on average, at two-fold greater levels than those on each copy of an autosome (Adler et al., 1997; Nguyen and Disteche, 2006). Although nematodes and mammals are similar in that they execute their dosage compensation programs in the homogametic, female sex, these dosage compensation programs are fundamentally different. In nematodes, both X chromosomes are transcriptionally repressed by half in females/hermaphrodites (Cline and Meyer, 1996); whereas in mammals, one of the two X chromosomes is completely silenced while the other continues to be expressed. Therefore, each of these lineages has employed a unique evolutionary series of up-regulations (and down-regulations) to independently build a mechanism through which the heterogametic and homogametic sexes achieve comparable dosage of the X chromosome that remains balanced with autosome dosage (Figure 2).

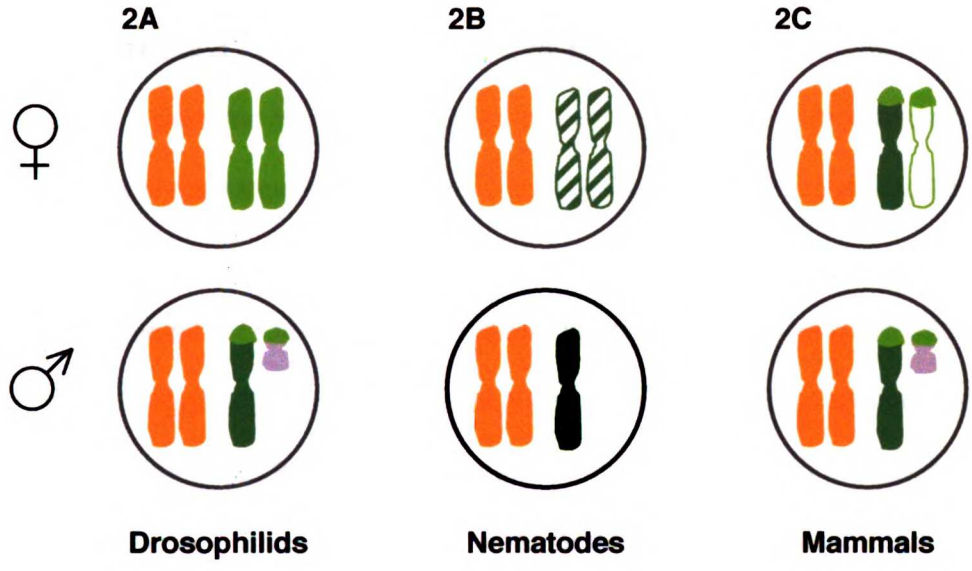

Figure 2. Solutions to the problem of dosage compensation in three animal lineages. Female (top) and male (bottom) cells are depicted with orange chromosomes representing an autosome pair and green chromosomes representing the sex chromosome pair. The gene-poor Y-specific region is represented in purple.

(2A) In drosophilids, the single X chromosome in males is upregulated two-fold (dark green), while no adjustments in X chromosome gene expression occur in females.

(2B) In nematodes, I hypothesize that the X chromosome has become intrinsically upregulated in both sexes relative to the autosomes. Then, in hermaphrodites, the two X chromosomes are epigenetically down-regulated by two-fold (cross-hatching); in males, the Y chromosome, which is not needed to determine sex in *C. elegans*, has been completely lost.

(2C) In mammals, the X chromosome has become upregulated by two-fold relative to the autosomes, and one of the two X chromosomes is silenced (white with green outline) in females.

The mammalian X and Y chromosomes are thought to have originated separately from the Z and W sex chromosome system of the bird/reptile lineage. A large block of the X chromosome is common to the mammalian lineage, being present in all placental mammals, as well as marsupials and monotremes (Graves, 1996), which diverged approximately 180 and 210 million years ago, respectively. This observation suggests that the X and Y were the sex chromosomes in the mammalian common ancestor. The X chromosome of the placental lineage also contains evolutionarily more recent strata that were translocated from autosomes (Ross et al., 2005). Recently, it has been found that a monotreme sex chromosome system also shares homology with the bird Z and W chromosomes, suggesting a complicated picture of sex chromosome evolution in the amniotes (Grutzner et al., 2004). In monotremes, where the X and Y chromosomes share much homology, it remains unclear whether dosage compensation occurs (Rens et al., 2004). The existence of chromosome-wide X-inactivation in marsupials is well established, and it is always the paternally-inherited X chromosome that is silenced (Sharman, 1971). In fact, this was one of the first examples of genomic imprinting, whereby gene expression depends upon the parental origin of the chromosome. It has been noted that although X-inactivation is random in most tissues of placental mammals, the extraembyronic tissues of rodents display imprinted silencing of the paternal X, suggesting a possible evolutionary connection. Inactivation in marsupials may be less stringent than in placental mammals—appearing incomplete, variable, and tissue-specific (Graves, 1996)—although these features have also been noted in human females (Carrel and Willard, 2005).

Investigators working in marsupial systems have been searching for an *Xist* homolog without success for 15 years. A new study employing comparative genomic analysis of the *Xic* among mammalian lineages revealed that this master regulator of X-

inactivation in placental mammals is on an autosome in monotremes, and although it is located on the X chromosome in marsupials, the *Xist* gene is not present. Instead, *Xist* seems to have evolved in the placental lineage at the location of a protein-coding gene, *Lnx3*, shared by all other vertebrates, including monotremes and marsupials (Duret et al., 2006). Thus, although marsupials and placental mammals share extensive X chromosome homology, chromosome-wide X-inactivation, and the *Xic*-homologous region in an X-chromosomal position, the evolutionary relationship between the *Xist*-dependent dosage compensation mechanism in the placental lineage and the process occurring in marsupials is unclear. It will be important to establish the characteristics of dosage compensation (if any) in monotremes to determine which characteristics of mammalian X-inactivation are ancestral, and which are inventions of the different lineages.

In thinking about the molecular mechanism of X-inactivation in placental mammals, it is valuable to bear in mind the evolutionary history of the process. For example, the fact that upregulation of X-chromosomal genes appears of have preceded cis-limited inactivation may mean that a specific set of DNA elements was already present on the X to facilitate upregulatrion, and/or that chromosome-wide epigenetic regulation was already in place, thus providing a groundwork for chromosome-wide control of gene expression level. Furthermore, it is important to keep evolutionary parsimony in mind, such that it takes the fewest number of steps to bridge the distance between the X-inactivation mechanisms in marsupials and placental mammals (Brown and Chandra, 1973).

Developmental Cycle of X-Inactivation

One of the original tenets of Lyon's hypothesis was that X-chromosome inactivation occurs in a developmentally regulated manner. At the earliest stages of mouse development, the X chromosomes inherited from the mother and the father are both transcriptionally active, at least to some extent (Epstein et al., 1978). X-inactivation occurs in three waves affecting distinct cell populations of the early embryo, each coinciding with a restriction in developmental potential (Monk and Harper, 1979; Sugawara et al., 1985). The pattern of inactivation differs between the waves: in the first two, which occur as placental tissues are determined, X-inactivation is imprinted such that the paternal X chromosome is silenced in each cell; in the third wave, which occurs in the cells that comprise the embryo proper, X-inactivation is random.

The cycle of X-inactivation and -reactivation in the mouse is illustrated in Figure 3. Zygotic transcription begins at the two-cell stage, at which time X-chromosomal genes are expressed from both the maternal and the paternal X chromosomes (Okamoto et al., 2005). Female embryos are thus X:A dosage-imbalanced, and this manifests itself as a growth disadvantage of female embryos compared to their male siblings during pre-implantation development (Burgoyne et al., 1995; Thornhill and Burgoyne, 1993). *Xist* is also expressed from the two-cell stage (Zuccotti et al., 2002) specifically from the paternal X chromosome, and as the cleavages proceed, progressive silencing of the paternal X emanating from the *Xic* in a bidirectional gradient can be observed in blastomeres of the morula (Huynh and Lee, 2003; Latham and Rambhatla, 1995). By the early blastocyst stage (day 3.5, 32 to 64 cells), the first differentiation event has occurred in the embryo: trophectoderm cells, a placental cell type, have been determined, and exhibit complete inactivation of the paternally inherited

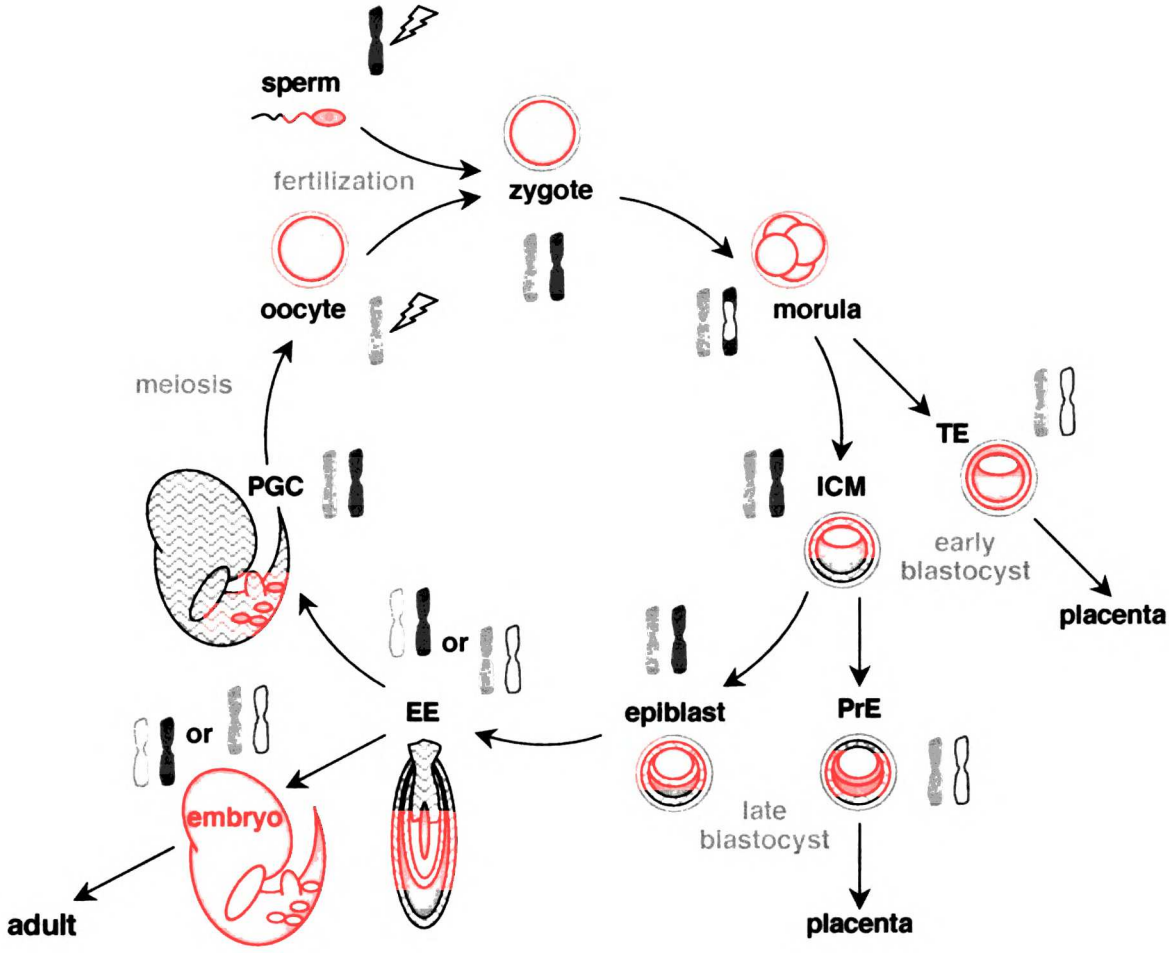

Figure 3. The X-inactivation and -reactivation cycle in mouse.
Maternal (pink) and paternal (blue) X chromosome activity status is indicated by filled (active) or empty (inactive) chromosomes. The stages of mouse development at which changes in X-inactivation status occur are schematized. Yellow color indicates the cell population whose X chromosome activity is indicated by the adjacent chromosomes. Grey hatched fill indicates tissues in which X-inactivation has already occurred. The extra-embryonic tissues are not shown in the embryo/PGC schematic. Partially empty paternal X chromosome at the morula stage indicates partial paternal X-inactivation. Lightning bolts indicate that at meiosis, the imprinting status of both X chromosomes in females and the single X chromosome in males is reset to reflect maternal and paternal origin, respectively. TE, trophectoderm; ICM, inner cell mass; PrE, primitive endoderm; EE, embryonic ectoderm; PGC, primordial germ cell. See text for more details.

X chromosome (Huynh and Lee, 2003; Mak et al., 2004; Okamoto et al., 2004; Sugawara et al., 1983). Meanwhile, paternal *Xist* expression and partial paternal X-inactivation are reversed in the pluripotent inner cell mass (ICM), which now exhibits gene expression from both X chromosomes (Mak et al., 2004). By the late blastocyst stage (day 4.5, ~128 cells), the ICM gives rise to the pluripotent epiblast and to the primitive endoderm, another restricted lineage that will contribute to the placenta. The primitive endoderm again exhibits paternal *Xist* expression (Kunath et al., 2005) and X-inactivation (Takagi and Sasaki, 1975; West et al., 1977), while both X chromosomes in the epiblast remain active. Shortly after implantation in the uterus, at the egg cylinder stage of development (day 5.5, ~500 cells), X-inactivation occurs in the embryonic ectoderm (Sugawara et al., 1983), which will give rise to the definitive germ layers of the embryo proper. This time, the maternal and paternal X chromosomes are inactivated with equal frequency (Sugawara et al., 1983), as inferred in Lyon's original hypothesis. After the initial X-inactivation event in each of these cell types, the inactive X chromosome is clonally maintained in all descendant cells for the lifetime of a female individual. The major exception to this rule is found in the germline, where X-inactivation initially occurs, but is reversed within a few cell generations in the primordial germ cells as they prepare to enter meiosis (Tam et al., 1994). Thus, the two X chromosomes in a female become equivalent for meiotic recombination and are passed on to the next generation with potential for gene activity.

The link between differentiation and X-inactivation extends *in vitro*. Pluripotent embryonic stem (ES) cells can be derived from the ICM of blastocyst embryos, and female ES cell lines exhibit activity of both X chromosomes. Again, the X:A dosage imbalance is probably deleterious, and may explain in part the relative difficulty of deriving female ES cell lines, their less vigorous growth compared to male ES cell lines,

and their karyotypic instability, especially with respect to X chromosome constitution (Kawase et al., 1994; Zvetkova et al., 2005). Differentiation can be triggered *in vitro* by several methods, and X-inactivation follows within a day or two (Martin et al., 1978; Paterno and McBurney, 1985). Cell types are produced that correspond the embryonic lineages, and these exhibit random X-inactivation as *in vivo*. Evidence that extraembryonic lineages, which are also produced during ES cell differentiation, may exhibit imprinted paternal X-inactivation under certain conditions *in vitro* is presented in Appendix 2. Whereas the mouse embryo is tiny and almost inaccessible to manipulation at the stage of development when random X-inactivation occurs, embryonic stem cells represent a convenient model system for studying this process *in vitro*. For example, use of an inducible *Xist* transgene in ES cells allowed the discovery that *Xist* expression in the absence of differentiation does not lead to heritable silencing, thus indicating that a developmentally-regulated factor controls the timing of *Xist*-mediated silencing (Wutz and Jaenisch, 2000). In conclusion, work *in vivo* and *in vitro* showing that X-inactivation is coordinated with the developmental program has implications for models of the regulatory network that controls initiation of X-inactivation.

Classical Genetics of X-Inactivation

The fact that two chromosomes of the same DNA sequence are treated differently within the same cell, and that different cells make the distinction independently such that equal numbers of cells choose the maternally or the paternally inherited chromosome, has caught the attention of scientists even before they understood the biological significance of the inactive X chromosome. After more than 45 years of study and postulation, the cellular mechanism that solves this problem is still mysterious. Below, I will review data

that are relevant to the problem and formed the basis for the earliest models seeking to account for this mechanism.

Influence of X chromosome number

A single X chromosome is typically active in each somatic cell of male and female mammals. This fact became apparent in the late 1950s and early 1960s when clinicians began applying reliable new karyotyping methods to their patients. Normal XY males and XO Turner's syndrome females have one Xa, normal XX females and XXY Klinefelter's syndrome males have one Xa, and male and female Klinefelter's patients with up to five X chromosomes all have a single Xa. Dozens of case reports, too numerous to cite, can be found in the medical journals of this era, serving as fodder for discussion of the mechanism regulating Barr body number. The ceonsensus of these reports was that if there are "n" X chromosomes in a cell, n-1 of them will be inactivated.

There is a shortcoming of the data on which the n-1 rule was based: because the patients were examined many years after the initial X-inactivation event, it is unclear whether the occurrence of a single Xa in every cell was the result of tightly regulated *primary* event, or if a mixture of X-inactivation outcomes occurred initially, followed by a *secondary* effect in which only those cells achieving a 1Xa:2A ratio survived. This issue can be addressed by examination of X-inactivation patterns in early embryos possessing supernumerary X chromosomes. There is a paucity of data from the mouse concerning the fate of supernumerary X chromosomes because of experimental challenges in carrying out such experiments—in particular, how to arrange inheritance of additional copies of a single chromosome, and how to circumvent problems associated with imprinted X-inactivation defects in the extraembryonic tissues so that the phenotype of the randomly inactivating cells can be examined. In two studies employing large

translocation products of the X chromosome that can be inherited in addition to the normal X, diploid embryos that were effectively XXX and XXY were generated provided good evidence that one Xa remains active in a primary fashion (Shao and Takagi, 1990; Goto and Takagi, 1998). In one of these studies, extraembryonic defects were rescued by aggregation with tetraploid cells (Goto and Takagi, 1998). Thus, experimental data from the mouse supports the original inference that inactivation of all but one X chromosome in each cell is a primary aspect of the X-inactivation mechanism.

Influence of ploidy

The number of active and inactive X chromosomes is also related to the number of autosomal chromosome complements in a cell. Tetraploid and octaploid cells were observed to have twice and four times as many Xi's (and Xa's) as normal diploid cells, leading to the more sophisticated formulation of the n-1 rule: Xi number equals n-p/2, where p is the ploidy of the cell (Harnden, 1961). Many of the early polyploid samples on which this conclusion was based are likely to have arisen by failure of cytokinesis after the primary X-inactivation event, and are therefore uninformative with respect to the initial regulation of X-inactivation. However, polyploidy is a fairly frequent cause of miscarriage in humans, so that it has been possible to analyze X-inactivation patterns in polyploid tissue obtained shortly after X-inactivation. In samples from XXXX and XXYY tetraploid embryos, two Xa's (and two Xi's—never three—in females) are observed (Carr, 1971). Tetraploid mice have been generated experimentally, and have confirmed the observations in humans. Among XXYY and XXXX tetraploid mouse embryos analyzed a few days after the X-inactivation window, two Xa chromosomes were observed almost exclusively in the embryo proper (Webb et al., 1992). Thus, although

early data were problematic, controlled experiments have shown that the n-p/2 rule reflects a primary effect.

Analysis of odd-numbered ploidies is also of interest to the X-inactivation mechanism. The pattern of X-inactivation in haploids has largely not been studied. One experimentally-generated single-X haploid rabbit embryo examined at the sixth day of development did not display an Xi (Hansen-Melander and Melander, 1972). The number of Xa's in samples from triploid human embryos is variable; it is clear that the ratio of 2X:3A in XXY triploids can trigger X-inactivation, as some cells containing one Xi are detected in these embryos (Carr, 1971). Cultured specimens from triploid humans have mostly been reported to have two Xa's per cell (Migeon et al., 1979; Mittwoch et al., 1963; Vogel et al., 1983). A new study suggests that primary fetal samples exhibit one Xa, and that selection for two Xa's occurs during subsequent culture (Gartler et al., 2006). Experimental manipulations in the mouse have shown that the predominant pattern of X-inactivation results in a single Xa in both male XXY and female XXX triploids, though some variability is seen (Endo et al., 1982). Taken together, the data show that the mechanism of X-inactivation designates two Xa's in nearly every tetraploid cell, and one Xa in most triploid cells; however, the aberrant patterns that arise in rare cases should also inform our thinking about the X-inactivation mechanism (Table 1).

	number of Xi					
	0	1	2	# cells	# embryos	reference
triploids						
XXX	0%	30%	70%	198	3	(Endo et al., 1982)
XXY	7%	93%	0%	193	3	(Endo et al., 1982)
tetraploids						
XXXX	7%	3%	91%	107	3	(Webb et al., 1992)
XXYY	98%	2%	0%	112	3	(Webb et al., 1992)

Table 1. Number of Xi in triploid and tetraploid mouse embryos. Embryonic tissues from triploids at E7 to E8, and tetraploids at E10, were analyzed. Note that in the tetraploid study, the Xi was not detected in 12% of control diploid female cells, so Xi number may be under-represented.

Randomness and skewing

It has long been appreciated that the maternal and paternal X chromosomes are inactivated in equal proportions of female cells (Ohno and Hauschka, 1960). This fact has been interpreted to indicate that the mechanism designating the Xa and Xi makes a random choice between the two X chromosomes in each cell. However, the example of mating type switching in fission yeast demonstrates that highly stereotyped switching patterns can also produce a population of cells exhibiting two alternative fates at equal frequency (Klar, 1987). Thus, while the X-inactivation mechanism needs to account for the observation of equal frequencies of cells bearing a maternal Xi and a paternal Xi in the population, this mechanism need not necessarily make a random choice between the two chromosomes in each cell (Williams and Wu, 2004).

The X-inactivation mechanism must also be able to account for the fact that non-random frequencies are seen in some cases. When certain divergent mouse strains are crossed, skewed ratios of X-inactivation (e.g., 70/30 and 80/20) are observed. This skewing away from randomness is mediated by the *X choosing element (Xce)* locus (Cattanach, 1975), which has been mapped to a location just proximal to the *Xic*, downstream of *Xist* (Chadwick et al., 2006; Simmler et al., 1993). In heterozygous females, the "stronger" *Xce* allele-bearing X chromosome becomes the Xa more frequently than that bearing the weaker *Xce* allele. The primary nature of the *Xce* effect on the initial X-inactivation event has been confirmed in recent studies examining X-inactivation ratios in early embryos (Chadwick and Willard, 2005). Thus, a given chromosome is not always designated as the Xa and as the Xi in equal proportions of cells, suggesting that the mechanism of X-inactivation involves some element of competition between the two X chromosomes, the outcome of which can be influenced by *cis*-elements. The *Xce* effect is mediated by autosomal loci which presumably

encode trans factors (Percec et al., 2002; Percec et al., 2003), demonstrating the complex interplay between molecular components of the X-inactivation mechanism.

Models for Random, Exclusive X-Inactivation: Themes and Variations

How does a cell determine the number and identity of X chromosomes to be inactivated? Many mechanistic models have been proposed over the years, but most have been forgotten by the field. A single idea, which I call the classic blocking factor model, predominates in modern discourse, although it is no better supported by data than many of the other ideas. I have reviewed the literature and recovered a diverse range of models, presented in Figure 4, A though K, and described in detail in the figure legends. I have tried to represent each model in terms of the modern understanding that X-inactivation is controlled by *Xist* expression *in cis*. But for a few exceptions, which are included as food for thought, each of these models satisfies the constraints of the basic rule: #Xi = n–p/2. In order to satisfy the rule, the models invoke a range of common themes, which I will now discuss.

Regulation by X and autosome number

To account for the relationship between Xa number and the number of X chromosomes and autosomes, many models employ a contribution of the autosomes to the system to "choose" one chromosome as Xa. In the classic blocking factor model, two copies of the autosomes produce a limiting quantity of a trans-acting "blocking factor" that is sufficient to bind to a site on just one copy of the X chromosome, marking it to resist *Xist* upregulation *in cis* and to remain active (Figure 4A, ii.). The core of this

Figure 4. A catalogue of models for designation of one Xa and one Xi.
Each conceptual framework has been applied to the modern understanding of X chromosome fate being controlled by *Xist* expression level *in cis*. I have applied my own standard terminology across all of the models to facilitate comparisons. For each model, the series of windows reflects the steps occurring within a single female cell. Most of the models are designed for a random outcome, but for the sake of illustration, presentation is standardized such that only the outcome in which the upper X chromosome becomes the Xa and the lower X chromosome becomes the Xi is shown, unless the two possible outcomes differ conceptually. Windows may refer to events involving autosomes, X chromosomes, or both, as indicated. In each model, the bold window indicates the step in which the two *Xist* alleles achieve differential regulation. A star to the upper left of a window indicates that the step requires input from the developmental program, for example, availability of factors that up-regulate any un-blocked *Xist* allele. Keys identify symbols used on each page. In general, repressors of X chromosome activity are depicted in pink; primary blocking factors that keep an X active in green with any secondary activators in blue; and inhibitors of the foregoing components in orange.

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4A Blocking Factor (BF)

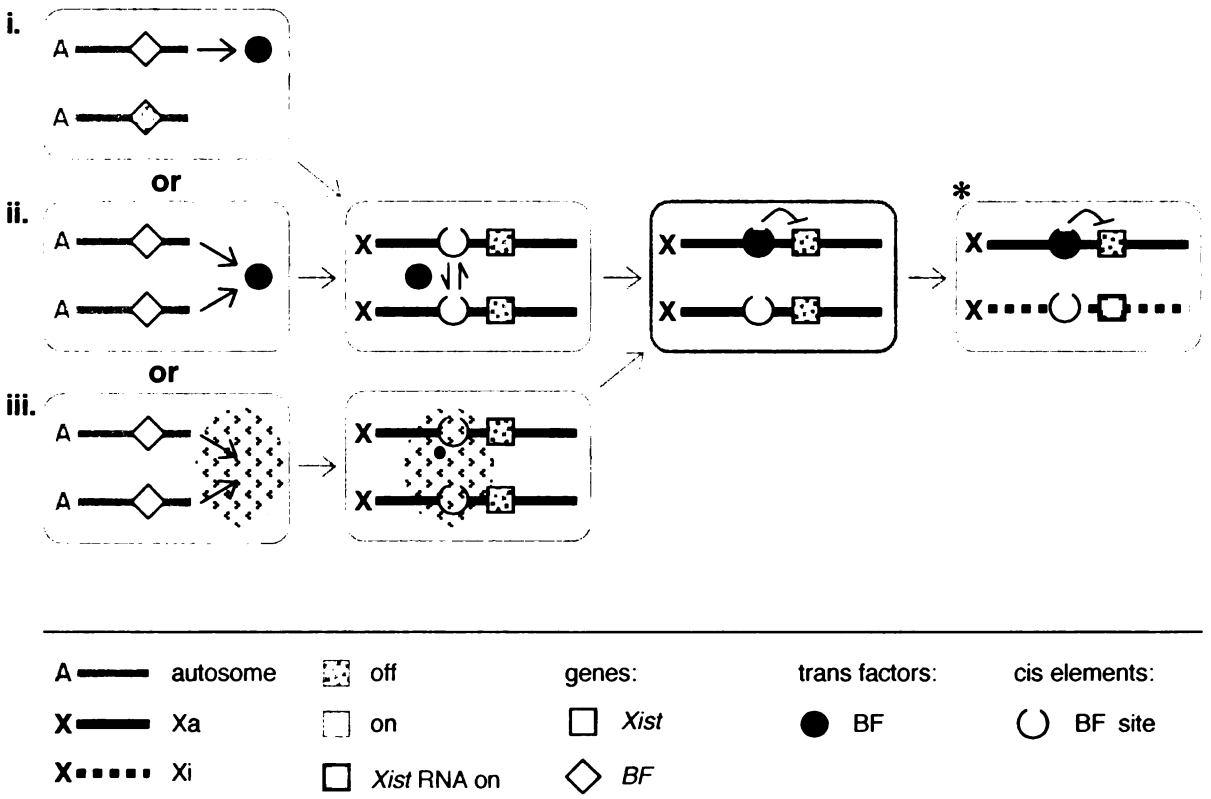

Figure 4. *(continued)*

(4A) Classic blocking factor (BF) model (based on Russell, 1964; Eicher, 1970; Rastan, 1983). Following path ii., a diploid autosomal complement produces a limiting quantity of BF that is sufficient to bind to a site on one X chromosome, repressing *Xist* and blocking that chromosome from inactivation (Panning and Jaenisch, 1998). The need for a "developmental cue" in the final step (*) to upregulate *Xist* on the future Xi has been recognized (Brown, 1991). Path i. shows an early suggestion for how to limit production to one BF per diploid cell, which stipulated inactivation (by imprinting) of one of copy of the autosomal BF locus (Brown and Chandra, 1973). Path iii. shows a modern version of the BF model in which a limiting but not necessarily small pool of BF forms a homo-multimeric complex that binds to one X chromosome through highly cooperative interactions (Nicodemi and Prisco, 2006).

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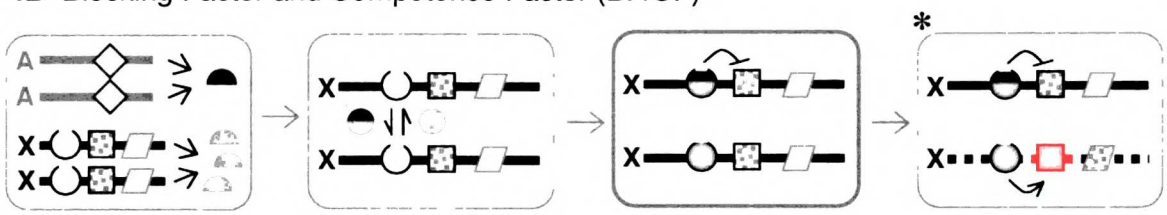
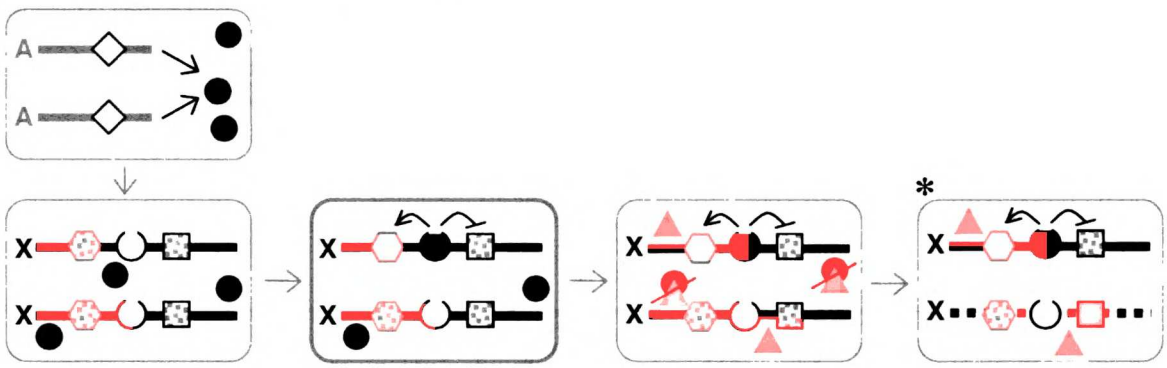
4B Blocking Factor and Competence Factor (BF/CF)



4C Blocking Factor with Feedback



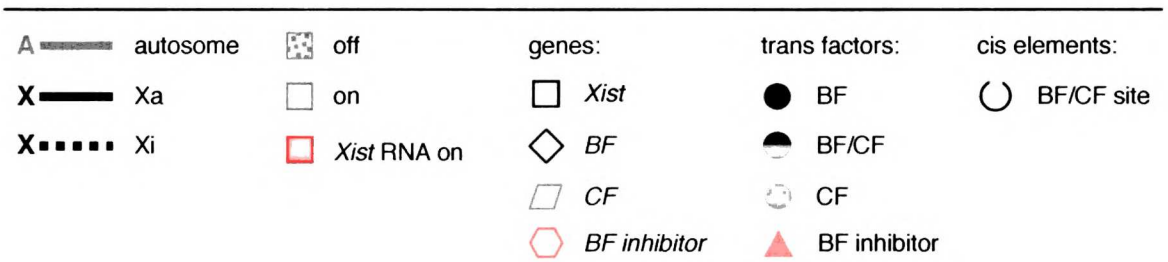


Figure 4. *(continued)*

(4B) Blocking factor and competence factor (Lee, 2005; Lee et al., 1999). Similar to (4A) but with the addition of a *trans* factor encoded by the X (competence factor, CF) that is required to assemble with BF to form an active blocking complex (BF/CF) which binds to one X chromosome to designate it as the Xa. The remaining, untitrated CF binds to the second X chromosome and is required for up-regulation of the *cis*-linked *Xist* allele.

(4C) Blocking factor with feedback (based on Grumbach et al., 1963). Similar to (4A) but including an auto-regulatory feedback loop. BF binding activates expression of an inhibitor that neutralizes any remaining unbound BF (slash mark; inactivated units not shown in following window). This model originally featured episomes in the blocking factor role; they would integrate at a site on the X and begin expression of the inhibitor. The amount of BF does not have to be as tightly regulated as under the classic model; however, the predictions of this model for polyploid cells are inconsistent with experimental observations.

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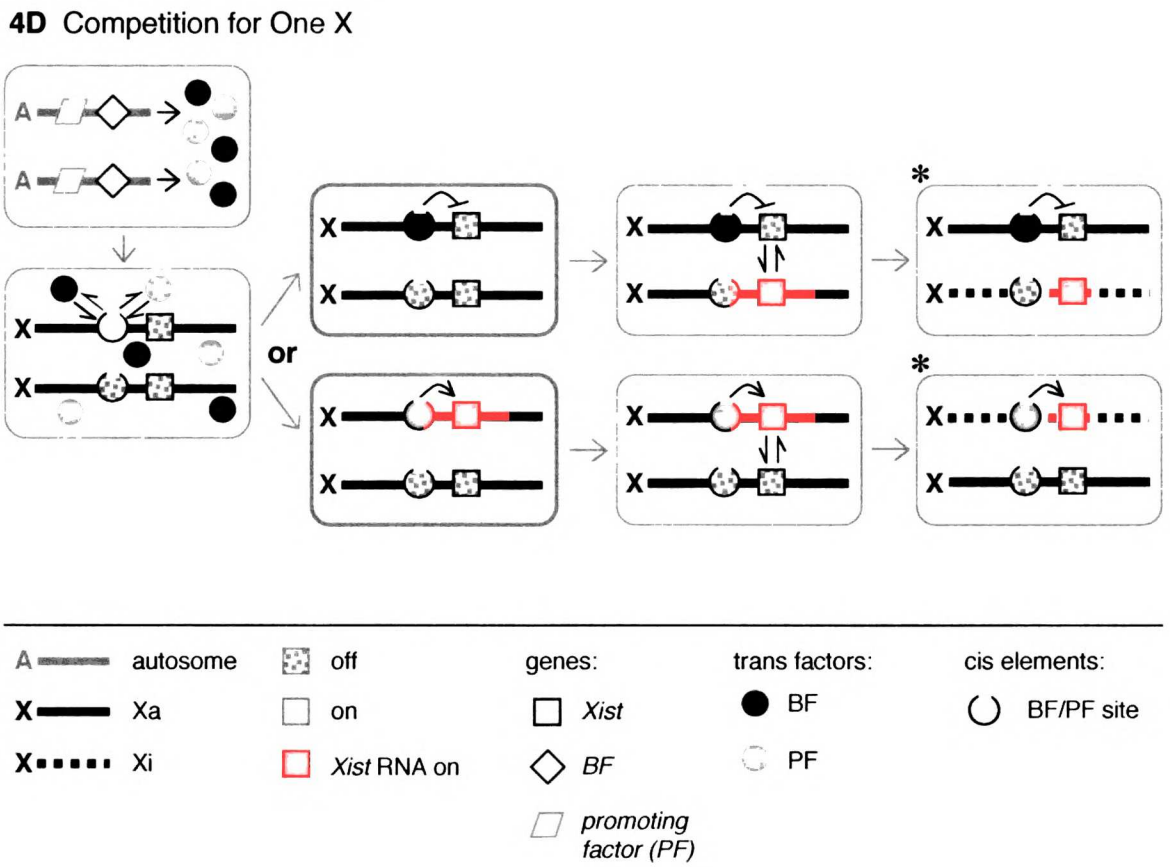

Figure 4. *(continued)*

(4D) Competition for one X chromosome (Williams and Wu, 2004). This model employs the device of rendering one X chromosome "immune" from the choice mechanism, e.g., through imprinting (which is known to occur at the *Xic*, directing X-inactivation non-randomly in the extraembryonic tissues). Blocking and promoting factors (BF and PF) then compete to occupy a binding site on the one available X. Thus, a random choice is made, but it involves only one X chromosome. BF or PF binding exerts a negative or positive influence, respectively, on the *cis*-linked *Xist* allele. A key requirement of the model is that the two *Xist* alleles communicate to ensure that the immune X chromosome assumes the opposite fate to the chosen one. There is no need for BF or PF to be limiting in quantity; the factors simply should have equal affinities at their physiological concentrations. For simplicity, unbound BF/PF units are omitted from the windows that follow the random choice.

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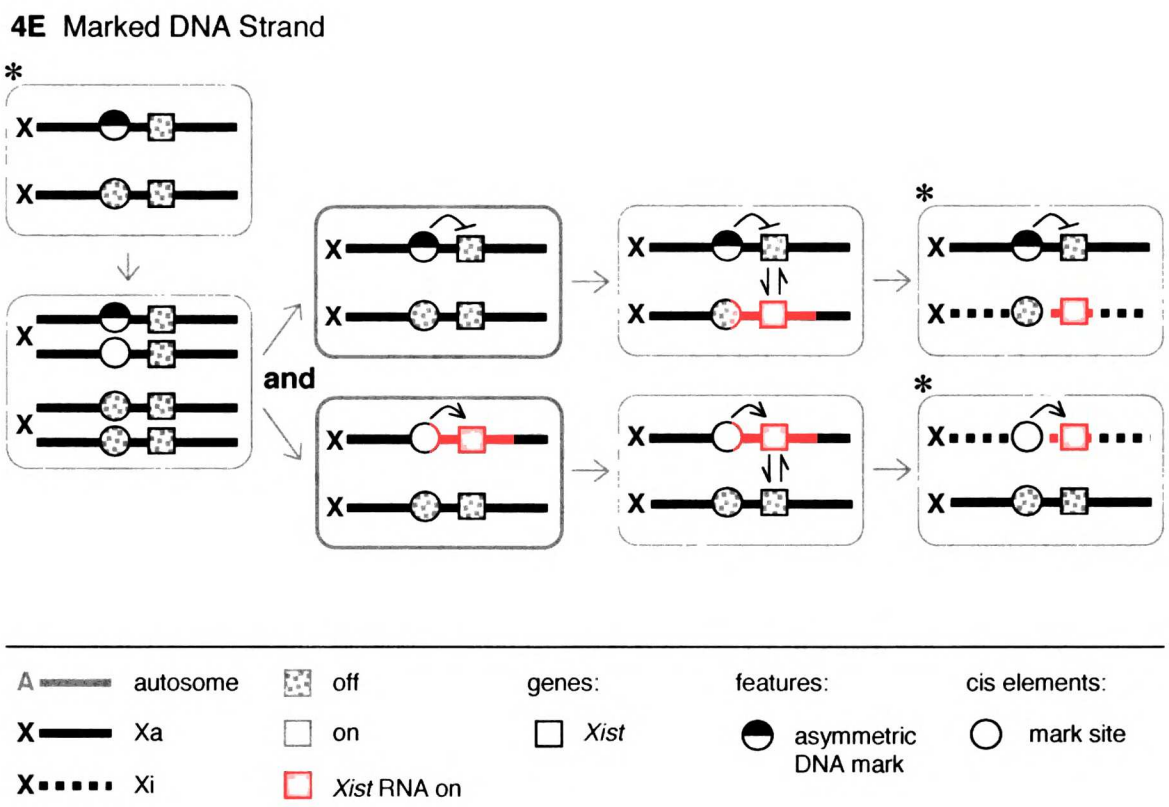

Figure 4. *(continued)*

(4E) Marked DNA strand (Williams and Wu, 2004). In this model, one X chromosome is immune as in (4D). In a parent cell, the non-immune X receives an asymmetric mark on one DNA strand (e.g., a nick). After DNA replication, this mark remains on one of the two resulting sister chromatids, and the marked X is inherited by one of the two daughter cells. Presence or absence of the mark in the daughter determines the fate of the non-immune chromosome. Communication between X chromosomes as in (4D) ensures one Xa and one Xi in each cell. This model is unique in that no randomness is involved in generating a 50/50 ratio of X-inactivation in the population; in fact, the asymmetric mark may be introduced non-randomly on the same strand each time.

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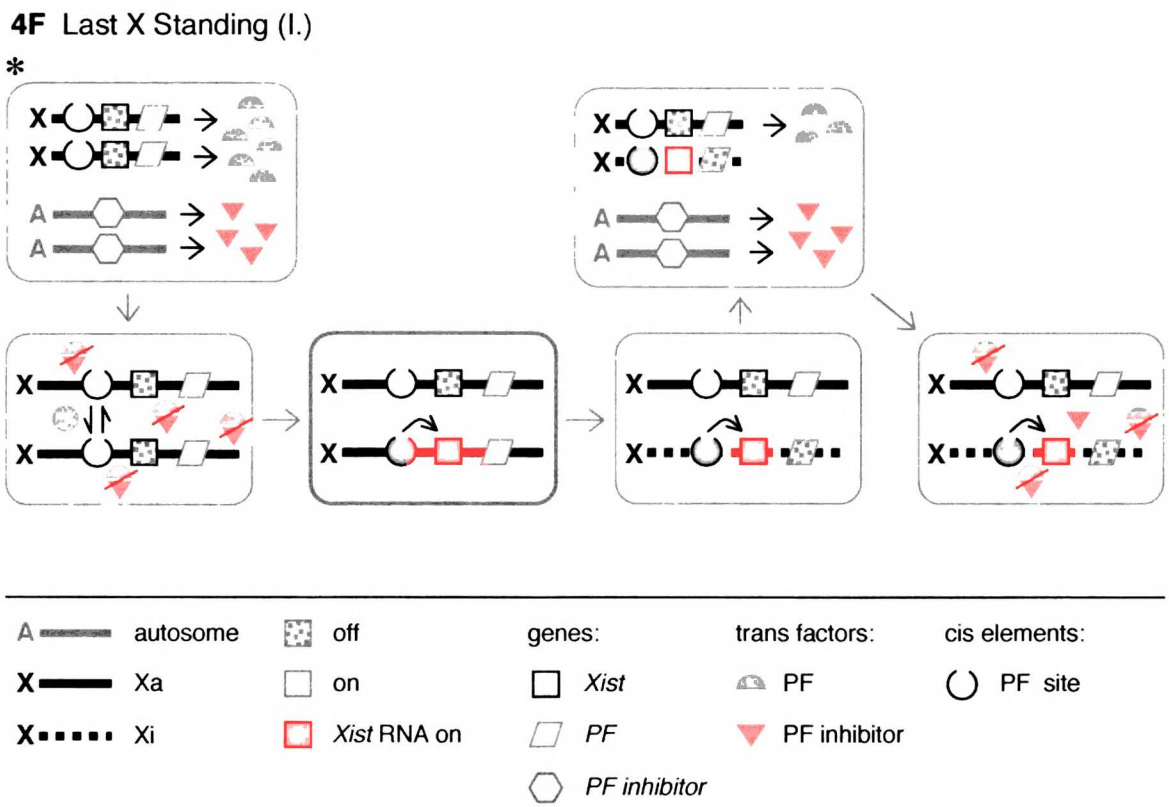

Figure 4. *(continued)*

(4F) Last X standing (I.) (based on Gartler and Riggs, 1983). This model does not employ a blocking function on the X to designate the future Xa. Instead, X chromosomes are inactivated one at a time until the ratio of Xa:A reaches 1:2. Here, the X chromosome produces a promoting factor (PF) that acts on the X chromosome. Meanwhile, an autosome produces a trans-acting inhibitor of PF that binds to and neutralizes PF (slash mark; inactivated units not shown in following window). The ratio of expression of the two factors is 3PF:2I. After PF binds to an X chromosome, X-inactivation is initiated and the cis-linked PF gene is silenced. The ratio of factors is altered to 1.5PF:2I (all PF neutralized) and no further X chromosomes can be designated as Xi.

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4G Last X Standing (II.)

*

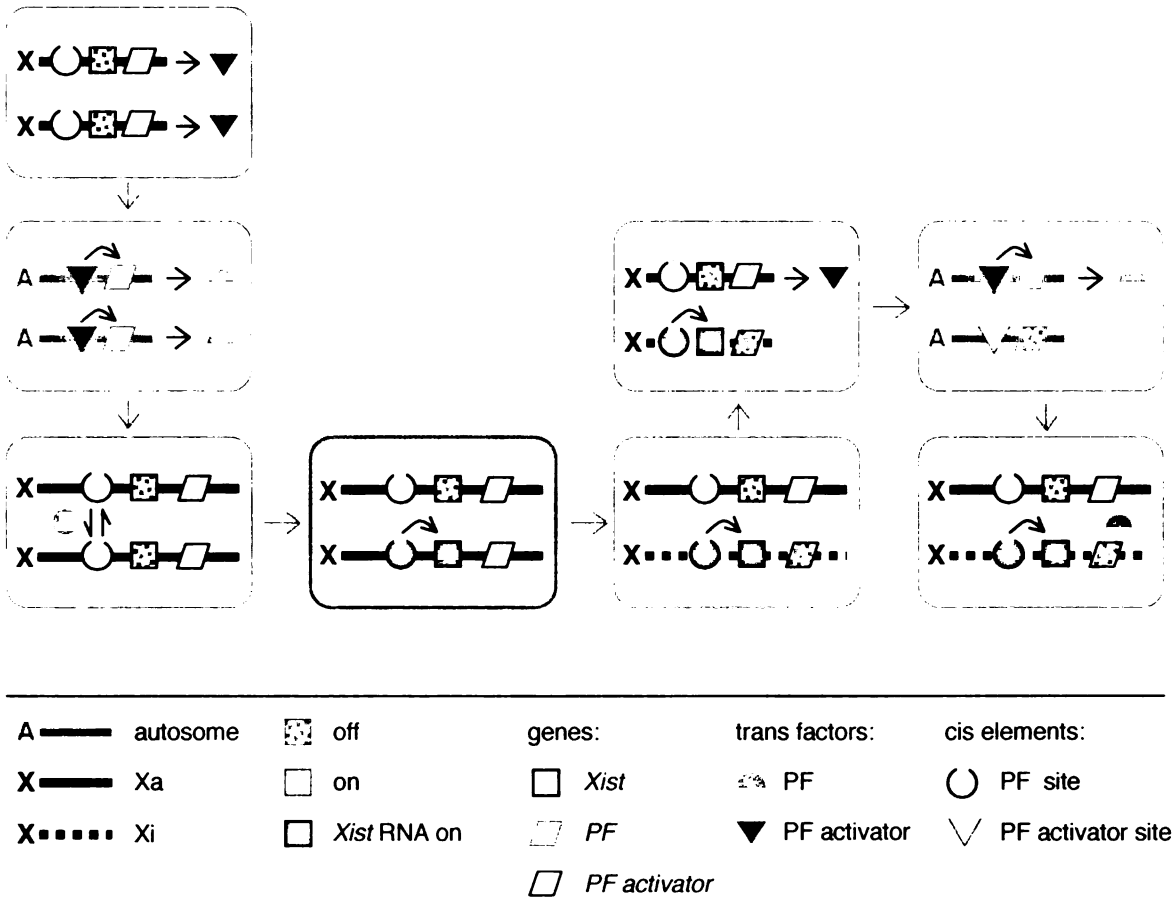

Figure 4. *(continued)*

(4G) Last X standing (II.) (based on Lyon, 1972). This model is conceptually similar to (4F). In this case, the X chromosome produces an activator of an autosomal locus that encodes the promoting factor (PF). A feedback loop ensues, with inactivation of one X chromosome leading to a drop in PF below the threshold needed to inactivate any further X chromosome. Note that once the first step of the system is triggered in the last X standin models (4F and 4G), the entire sequence can be carried out without further input from the developmental program (Lyon, 1972).

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4H Competition for Cooperative Trans Factors

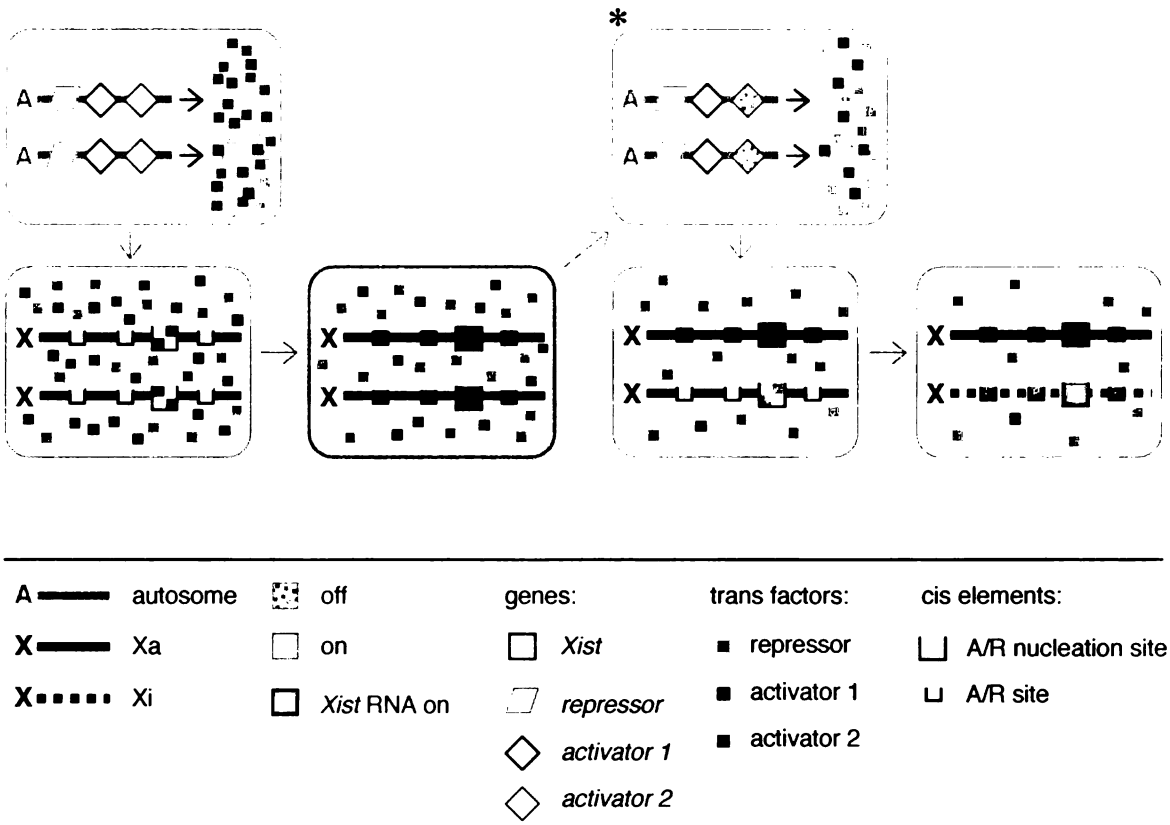
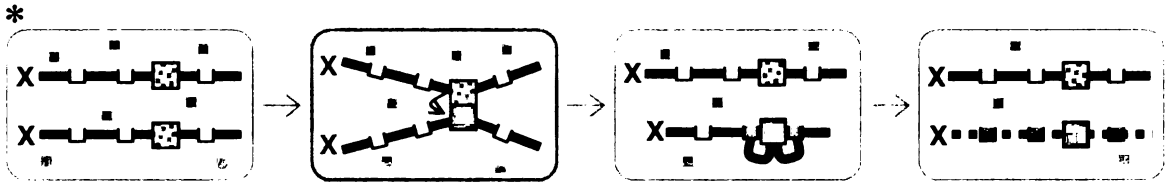


Figure 4. *(continued)*

(4H) Competition for cooperative trans factors (McBurney, 1988). This model was published three years before the discovery of *Xist* and evokes chromosome-wide binding of a trans-acting repressor to silence the Xi. The repressor must compete for binding with two activators, which have a higher affinity for the chromosome. All three factors work by nucleating at the *Xic* via cooperative interactions and spreading via *cis* elements along the X chromosome. Initially, the two activators, one of which is limiting and has the highest affinity, occupy the two X chromosomes in a mutually exclusive manner. When a developmental event leads to silencing of the non-limiting activator gene, the second X chromosome is now available for occupancy by the repressor, which nucleates, spreads, and silences the Xi. The model is already complex enough that I did not incorporate *Xist*; however, *Xist* expression could easily follow the template provided by the repressor.

(figure continues next page)

4l Transvection



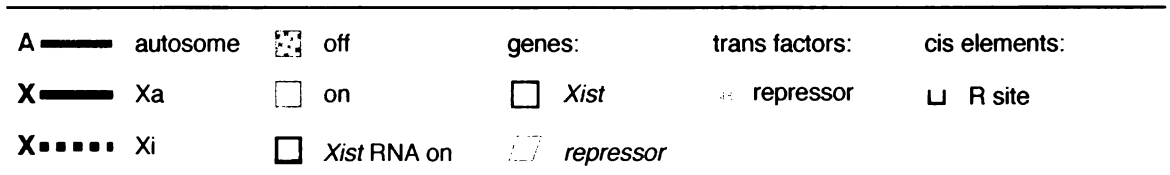
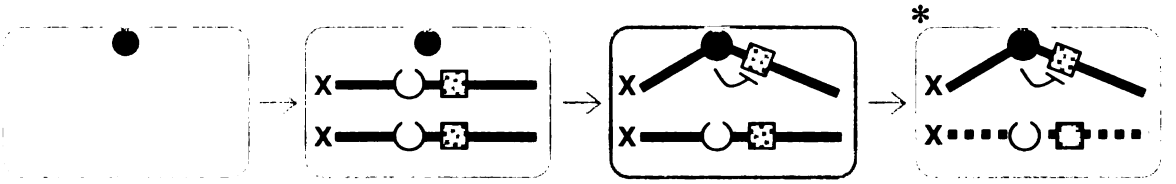


Figure 4. *(continued)*

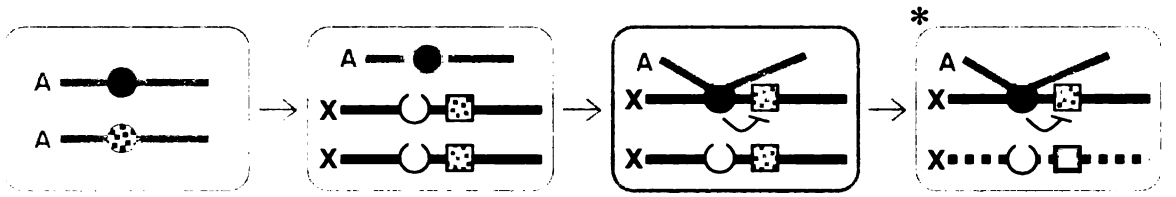
(4l) Transvection (Marahrens, 1999). (A similar model was proposed earlier by Muscatelli et al. (1992)). In response to a developmental cue, transvection (physical pairing) between the two *Xist* alleles is triggered, leading to trans-activation and heterochromatinization of one of the two alleles (symbolized by binding of repressor). This activated, heterochromatic allele then spreads inactivation along the chromosome by sequential pairing with *cis*-linked elements. Although this model is intriguing, it is unclear how it would fare in the case of aneuploidy or polyploidy.

(figure continues next page)

4J Single Blocking Location (BL)



4K One Active Blocking Element (BE)



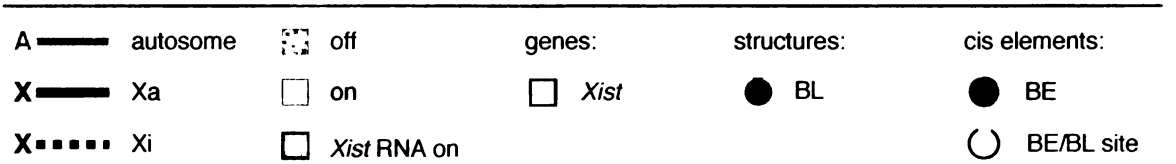


Figure 4. *(continued)*

(4J) Single blocking location (based on Comings, 1968). A diploid cell is posited to have a unique location (a "nuclear attachment site" in the original paper) that protects an X chromosome from *Xist* up-regulation and X-inactivation. After sequestration of the future Xa, this model is carried out much like the classic BF model (4A).

(4K) One active blocking element (based on Lomvardas et al., 2006). This model is based on a recent study showing physical pairing between a trans-acting enhancer element and the single expressed allele chosen from among a large number of genes in a multi-gene family. My adaptation for the case of X-inactivation employs an autosomal blocking element that is active on only one homolog (the other copy may be imprinted, for example). A *trans* interaction between one X chromosome and the blocking element protects that X from inactivation, as in (4J).

idea has early origins (Russell, 1964), and was articulated by Eicher (1970) and later Rastan (1983), the latter of whom is usually cited as the originator of the hypothesis. The idea of a limiting component (in the extreme case, one unit) has seemed problematic to many. One early model invoked a feedback loop initiated by binding of blocking factor to one X that destroys the remaining unbound blocking factor (Grumbach et al., 1963), Figure 4C), although this model does not fare well in polyploid scenarios. A common modern suggestion is to invoke a limited pool of a blocking factor with weak affinity to the X and to itself such that exclusive binding to one X chromosome may be accomplished via nucleation and highly cooperative assembly (e.g., (Nicodemi and Prisco, 2006), Figure 4A, iii.). One model explicitly takes the idea of cooperative binding to a chromosome-wide level (Figure 4H). The pool of an autosomally-encoded activating factor is proposed to be limited to an amount that can nucleate, spread, and occupy one entire X chromosome, protecting it from being occupied by a repressor that is not limiting (McBurney, 1988).

Perhaps the best solution to how to control the amount of blocking factor available is to employ something that is inherently present in a single copy. One old model invoked a "single nuclear attachment site" to block the associated X chromosome from silencing (Comings, 1968), Figure 4J); this site would, of course, have to scale with ploidy. A recent study from another field brought to mind an appealing possible solution. In this study, the choice of a single active odorant receptor gene was found to involve a single-copy enhancer element that interacts *in trans* with one of 1000 structural genes scattered across other chromosomes (Lomvardas et al., 2006). By analogy, in X-inactivation, the limiting autosomal factor could be a single copy of an autosomal "blocking element," with the second copy having been inactivated by an imprinting or random mechanism, as was the case in the odorant receptor study. Trans interaction

with one X would then designate that chromosome to become the Xa (Figure 4K). In some ways, this idea was implicit in an early version of the blocking factor model which invoked inactivation of one copy of the autosomal blocking factor locus by imprinting, so that only one active copy was available for choosing the Xa (Brown and Chandra, 1973) (Figure 4A, i.).

One class of models turns the blocking factor idea on its head. These models do not specifically designate the Xa. Rather, they invoke what I will call a "promoting factor" controlled by X chromosome dosage which designates inactive X chromosomes until the ratio of Xa:A falls to 1:2. I have dubbed these the "last X standing" models (Figure 4F and 4G). They rely upon feedback between the X chromosomes and autosomes. In one case, the X chromosome itself encodes the promoting factor (Gartler and Riggs, 1983); in the other case, the X encodes an activator of an autosomally-encoded promoting factor (Lyon, 1972). The outcome is similar in both cases; when the chromosome bound by PF is inactivated, the regulatory circuit causes a drop in PF levels such that no more X chromosomes may be bound. Again, precise regulation of the amount of PF is important, but some fudge room can be made by invoking cooperative binding as for the blocking factor.

A single model invokes simultaneous choice of the Xa and the Xi through mutually exclusive binding of a blocking complex and a competence factor (Figure 4B; (Lee, 2005; Lee et al., 1999b). This model was proposed to account for phenotypes observed in cells containing transgenic copies of certain *Xic* elements, and is discussed further in Appendix 1. Note that this model requires tight regulation of two trans factors.

While the models discussed so far do not require an X chromosome to know what its homologous partner is doing within the same nucleus, or even if it has a partner, the remaining models invoke communication between X chromosomes. Interestingly,

these models do not involve direct input from autosomal ploidy, and have the appeal that they do not require precise regulation of trans factor availability. To be accurate, one of these models actually *does* invoke a singular trans factor in a female cell, but in this case the factor is the other X chromosome. This model depends upon transvection, or direct physical pairing and trans-regulation, between *Xist* alleles on opposite chromosomes, leading to activation of one which silences the Xi (Figure 4I). The outline of this model was suggested by Muscatelli et al. (1992), and a specific version was apparently independently developed by Marahrens, 1999. A transvection model accounts for restriction of X-inactivation to females, since males do not have a second allele to facilitate *Xist* activation. However, different sets of assumptions need to be made about how the system operates to explain the results in polyploid vs. aneuploid cells. That said, this model seems less fanciful following the recent demonstration of transient colocalization of *Xic* alleles at the onset of X-inactivation (Bacher et al., 2006; Xu et al., 2006). Two other models also require communication between X chromosomes to ensure each adopts a unique fate as Xa or Xi (Figure 4D and 4E). The novel device in these models is to assign a fate to just *one* of the two X chromosomes, and then to communicate this fate to the homolog which takes on the opposite fate (Williams and Wu, 2004). The means of communication is not specified, but existence of some sort of mechanism can reasonably be assumed based on the fact that changing the frequency with which one X chromosome adopts a particular fate results in a complementary change by the other X chromosome (e.g., in the *Xce* effect, and as discussed in greater detail in Appendix 1). More problematic is how to make these models operate in an aneuploid or polyploid cell. Notably, in mechanisms that exploit communication between X chromosomes, such as the transvection model, the mechanism does not operate in male cells because of the lack of an essential

component of the system: a homolog. By contrast, the regulatory circuits of the blocking and promoting factor models are fully operational in male cells, and the output of each circuit simply does not require X-inactivation.

Breaking symmetry

Each model presents an idea for rendering two initially equivalent chromosomes non-equivalent. Once they differ from one another, it is conceptually easy to carry out different regulatory programs on each, as recognized in the following statement from the early days of the field: "Possibly one X chromosome gets the lead and through feedback mechanisms the function of the other is repressed" (McKusick, 1962). It is possible to imagine that the X-inactivation mechanism evolved to exploit stochastic differences between the X chromosomes, such as the chance interaction between a particular factor and one of the two chromosomes. Most of the models are presented as breaking symmetry at a regulatory element at the *Xic*, which then switches *Xist* expression on or off. This is usually achieved by binding of a trans factor to one chromosome, although in one case an asymmetric mark on one X chromosome, such as a nick on one strand of the DNA, provides the initial differentiation (Figure 4E; (Williams and Wu, 2004). One of the models explicitly invokes differentiation between X chromosomes along their entire lengths prior to initiation of silencing (McBurney, 1988), a feature that could be introduced into most of the other models if desired.

Switchlike behavior

In nature, X chromosome fate decisions occur in a switchlike manner: a female cell appears always to designate one Xa and one Xi. Therefore, a prominent feature of these models is the attempt to achieve switchlike behavior. The theoretical mechanisms

that are most appealing have clear on and off states, as in the single blocking element hypothesis (Figure 4K) where intermediate states are not possible. The attention in the models to precise regulation of the abundance of blocking factor is a reflection of the concern over intermediate states, in which neither or both X chromosomes are bound, and robustness of the system is an issue with several of the models. As an example, the blocking factor with competence factor model (Figure 4B) requires two separate factors to be tightly regulated in their production. It is critical to control the ratio between the two factors, which should be a simple problem, as well as the overall abundance of both factors, which is mechanistically challenging. Overproduction of both, even in the proper ratio, would lead to a chaotic situation in which each chromosome could randomly bind either complex independently, leading to lethal decisions about X chromosome fate (both Xa, or both Xi) in 50% of cases. Several of the models involve cooperative interactions, which contribute to robustness and switchlike behavior of a number of biological systems; in theory, the chromosome-wide assembly of factors by cooperative interactions should lead to very stable states (Figure 4H). The models in which X chromosomes communicate to share information about each other's fate (Figure 4D, 4E, and 4I) also have the potential to display clear states, although the molecular means of achieving this communication is unclear at present.

Apparent randomness

All of the models are able to account for the apparently random pattern of X-inactivation in a population of female cells. In most of them, randomness stems from a choice between two equivalent, alternative intermolecular interactions (e.g., blocking factor can bind equally well to its *cis*-element on either X chromosome). The asymmetric mark model points out that apparent randomness can also be achieved in the complete

absence of chance—in each parent cell an asymmetric mark is reproducibly introduced on the same DNA strand, and the two daughters always exhibit inactivation of opposite X chromosomes (Figure 4E; Williams and Wu, 2004). As already discussed, the ratio of X-inactivation can become partially or completely skewed toward one of the two X chromosomes (e.g., by the *Xce* effect). Thus, a good model should be able to accommodate partial to total non-randomness. The asymmetric deterministic mark encounters difficulty on this point, whereas models involving stochastic binding interactions easily account for skewing by alteration of the affinity for the interaction on one of the two X chromosomes.

Developmental regulation

A number of models specifically recognize the need for coordination of initiation of X-inactivation with the developmental program. One modification of the classic blocking factor model added a "developmental cue" in the form of a trans factor that becomes available after BF binding to facilitate *Xist* upregulation on the unprotected allele (Brown, 1991). One could also imagine shutoff of a factor that prevents *Xist* upregulation and/or spreading in pluripotent cells. Such a step is necessary in all of the blocking factor family of models; without it, inactivation will never be initiated, or both chromosomes will be inactivated before the protective factor can bind. In fact, designation of the Xa under these models could in theory operate continuously without initiation of X-inactivation for a number of cell generations, perhaps ever since fertilization, before the developmental cue induces a change that allows *Xist* upregulation. A less parsimonious option is for production of the blocking factor to be triggered by a first developmental cue, followed by a second cue for the initiation of X-inactivation. Such tight coordination of two developmental signals might rely on a

checkpoint or monitoring mechanism that senses blocking factor binding, adding an additional level of complexity to the models. The marked DNA strand model (Figure 4E) is especially demanding of developmental input, requiring at least two separate, carefully timed, points of regulation to initiate marking and to carry out inactivation with precise cell cycle spacing. To initiate silencing on the future Xi, the chromosome-wide trans factor model (Figure 4H) postulates shutoff of a protective mechanism specific to pluripotent cells (McBurney, 1988), and therefore requires only one point of developmental regulation. Several of the proposed mechanisms are appealing in that, once set in motion, they require no further developmental regulation. For example, triggering the first step in the "last X standing" models leads to automatic silencing of X chromosomes until the Xa:A ratio reaches the proper level (Lyon, 1972). The transvection mechanism as proposed by Marahrens is similar in that once initiated, it could run to completion (Marahrens, 1999).

Taken together, the foregoing models provide a diverse range of ideas on how each female cell may designate one active and one inactive X chromosome, such that the maternal and paternal X chromosomes are inactivated in equal numbers of cells. While none is likely to be correct in every detail, these models reveal major themes that are critical to thinking about this problem. It is these key issues that will undoubtedly be addressed in the actual mechanism when it is revealed.

Thesis Guide

The main sections of this thesis are concerned with uncovering the parameters of the "random" X-inactivation mechanism.

In Chapter 1, evidence is presented that in pluripotent female cells, which have not yet undergone X-inactivation, the two X chromosomes differ from one another along their entire lengths. These differences, revealed by a cytological assay, suggest that the two X chromosomes adopt distinct states prior to X-inactivation. Employing *Xic* mutations that are known to cause completely non-random X-inactivation upon differentiation, the two X chromosome states in pluripotent cells are shown to be predictive of the fates of the X chromosomes as Xa and Xi. In cells that are poised for random X-inactivation, the maternal and paternal X chromosomes appear to exhibit each state with equal frequency. Analysis of X chromosome behavior in clonal cell lines suggests that the two X chromosomes switch between states. These data indicate that random X-inactivation may be achieved by a system in which X chromosomes alternate between two mutually exclusive states that determine their fates in response to the developmental signal that initiates X-inactivation.

Chapter 2 presents an investigation of the sequence requirements for an X chromosome to adopt the future Xa and future Xi states. Analysis in pluripotent male cells shows that the single X chromosome does not adopt either of the distinct states observed in females, and this difference is due to absence of a second, homologous X chromosome. The ability of *Xic* mutations to control the state of an X chromosome also depends on the presence of a homolog. In male cells carrying an *Xic* transgene on an autosome, the single X chromosome adopts a female-like future Xi state, correlating with ectopic X-inactivation observed upon differentiation in this cell line. Analysis of the behavior of an X chromosome in the presence of a severely truncated homolog lacking the *Xic* shows that X-chromosomal sequences respond to the presence of homology at a local level, adopt distinct states, and switch between them without being linked to the *Xic*. Taken together, these data indicate that homology sensing is employed in the

regulation of X-inactivation. The data raise the possibility that the ability of certain X chromosomal sequences to respond to the presence of homology and adopt two states preceded the advent of the *Xic*. The *Xic* may then have co-opted this chromosome-wide differentiation system to spread silencing along a single X chromosome in females.

In the Epilogue, I discuss the implications of the research presented in Chapters 1 and 2 for the X-inactivation mechanism. I then identify several areas for future investigation, including: further genetic analysis of the determinants of X chromosome states; exploring the cell biology of chromosome dynamics suggested by my experiments; searching for the molecular factors that underlie the differences between homologs; and defining the behavior of random monoallelic loci on the autosomes, which appear also to adopt two states. I provide rationales for studying these areas, and describe unpublished data and outlines for further experiments.

The appendices present two other significant projects that I began, but was not able to complete. These projects are also related to the cellular mechanisms for differential marking of the future Xa and the future Xi—one in random and the other in imprinted X-inactivation.

In Appendix 1, I review in detail the molecular genetic analysis of mouse *Xic* function in random X-inactivation, and compare what is known about the analogous elements in the human *XIC*. Throughout this section, I point out areas where further genetic analysis would be fruitful. I then describe my rationale and strategy for a genetic targeting experiment to remove a genomic region downstream of *Xist*, and my progress toward generating and analyzing male and female ES cell lines bearing this deletion.

In Appendix 2, I review what is known about the regulation of imprinted X-inactivation in the extraembryonic tissues of the mouse. Then I present preliminary evidence that the imprint is retained in female ES cells, and can control X-inactivation in

the primitive endoderm derivatives produced by ES cell differentiation *in vitro*. I describe a series of follow-up experiments aimed at confirming these results and building a complete story for future publication. In conclusion, I discuss the implications of retention of the imprint for the X-inactivation field.

Acknowledgements

I would like to thank Barbara Panning for encouraging my interest in the old X-inactivation literature. My anecdotal account of the history of X-inactivation is based largely on a review of the field she presented to her lab in its early days. I also want to thank her for providing several of the classic X-inactivation model papers to me.

Chapter 1.

X chromosomes alternate between two states prior to random X-inactivation

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Summary

Early in the development of female mammals, one of the two X chromosomes is silenced in half of cells and the other X chromosome is silenced in the remaining half. The basis of this apparent randomness is not understood. We show that before X-inactivation, the two X chromosomes appear to exist in distinct states that correspond to their fates as the active and inactive X chromosomes. *Xist* and *Tsix*, non-coding RNAs that control X chromosome fates upon X-inactivation, also determine the states of the X chromosomes prior to X-inactivation. In wild-type ES cells, X chromosomes switch between states; among the progeny of a single cell, a given X chromosome exhibits each state with equal frequency. We propose a model in which the concerted switching of homologous X chromosomes between mutually exclusive future active and future inactive states provides the basis for the apparently random silencing of one X chromosome in female cells.

Introduction

At least ten percent of mammalian genes are transcribed from only one allele that, in most instances, is chosen at random (Singh et al., 2003). The mechanisms for achieving differential regulation of homologous alleles in a stochastic manner are poorly understood (Ohlsson et al., 1998). X-chromosome inactivation in mammals is an example of random monoallelic expression; the majority of genes on one X chromosome

in XX females are silenced to equalize expression of X-linked genes with XY males (Lyon, 1961). Understanding the process by which the two X chromosomes are assigned active and inactive fates in a stochastic manner will provide insight into how randomness can be achieved in other biological contexts.

X-inactivation is a well-defined system for studying the mechanisms that generate the randomness of monoallelic expression. In the earliest stages of female embryogenesis, X-linked genes exhibit biallelic expression in female cells. Upon receipt of a developmental cue, one X chromosome is silenced in half of the cells of the embryo and the other X chromosome is silenced in the remaining half. Once the identities of the active and inactive X chromosomes (Xa and Xi) are established, they are stably propagated throughout all subsequent cell divisions (Avner and Heard, 2001). Female mouse embryonic stem (ES) cells provide a model system to study the mechanisms that control the initial, stochastic determination of the Xa and Xi *in vitro*. As in the pluripotent cells of the early embryo, genes are expressed from both X chromosomes in female ES cells. X-inactivation can be induced *in vitro*, recapitulating the random silencing process that occurs *in vivo* during differentiation (Martin et al., 1978). In addition, genetic elements have been identified that affect the randomness of X-inactivation (Lee, 2002; Ogawa and Lee, 2003), making female ES cells a useful tool for the study of the mechanisms that control random monoallelic expression.

The *X-inactivation center* (*Xic*) is a master *cis*-regulatory element on the X chromosome that controls X-inactivation (Avner and Heard, 2001). The *Xic* contains a number of elements, including *Xist* and *Tsix*, a sense/antisense pair of non-coding RNAs that are transcribed from both X chromosomes prior to X-inactivation (Lee et al., 1999; Panning and Jaenisch, 1996; Sheardown et al., 1997). When embryonic cells differentiate and X-inactivation is initiated, *Xist* RNA spreads *in cis* from the *Xic* to coat

and silence one X chromosome (Panning and Jaenisch, 1996; Sheardown et al., 1997). *Xist* and *Tsix* play a role in the stochastic determination of which X chromosome will become the Xi and which will become the Xa. In cells heterozygous for either an *Xist* or a *Tsix* mutation, X-inactivation is non-random: an *Xist* mutant chromosome always becomes the Xa (Gribnau et al., 2005; Marahrens et al., 1998) and a *Tsix* mutant chromosome always becomes the Xi (Lee and Lu, 1999; Luikenhuis et al., 2001; Sado et al., 2001). Heterozygous mutations of *Xist* or *Tsix* also exhibit effects *in trans*: in addition to determining the fate of the mutant X chromosome, each mutation also causes the wild-type X chromosome to adopt the opposite fate. Thus, information about the fate of one X chromosome must be transmitted to the other X chromosome, ensuring that decisions about their fates are coordinated and that there is random and exclusive silencing of one X chromosome in female cells. The manner in which the opposing activities of *Xist* and *Tsix* regulate the fates of both X chromosomes in female cells remains mysterious.

In this paper, we present evidence that in individual pluripotent embryonic cells that are poised for X-inactivation, the X chromosomes exist in two mutually exclusive states. In heterozygous *Xist* and *Tsix* mutant cells, these states predict the fates of the X chromosomes, indicating that one X chromosome adopts a future Xa state and the other X chromosome adopts a future Xi state. In wild-type cells, X chromosomes switch between these states such that, among the progeny of a single cell, a given X chromosome exhibits each state with equal frequency. Thus, the concerted switching of homologous X chromosomes between mutually exclusive future Xa and future Xi states may provide the basis for the apparent randomness of X-inactivation.

Materials and Methods

Cell lines and culture

Wild-type female ES cells (Marahrens et al., 1997), Δ*Xist*/+ ES cells (Csankovszki et al., 1999; Gribnau et al., 2005), 129-tet/*cas* ES cells (Gribnau et al., 2005), *Tsix*-pA/+ ES cells (Luikenhuis et al., 2001), X.2/X^{wt} ES cells (Tada et al., 1993) and MEFs were cultured according to standard practice. Wild-type and Δ*Xist*/+ (Csankovszki et al., 1999) 3.5 days post conception blastocysts were harvested by standard procedures and cultured overnight in ES medium without LIF. To identify cells in S phase, cells were cultured with BrdU (Amersham) for 15 to 30 minutes.

Cell-cycle fractionation

Flow cytometry was performed on live ES cells labeled with BrdU (Amersham) and stained with 40 μg/mL Hoechst 33342 (Molecular Probes) for 45 minutes prior to harvesting. Cells were resuspended in ES medium plus Hoechst, 7% Cell Dissociation Buffer (Gibco), and 10 mM EDTA for sorting, and were cooled during the procedure. Cytometry was performed using a FACSDiVa Cell Sorter (Becton Dickinson). Cell cycle profiles were generated by excitation with a violet laser; Hoechst emission was measured with a HQ445/50 bandpass filter (Chroma Technology). Cells were gated to exclude debris and double cells. For microscopy, fractions were sorted into PBS in multiwell slides pre-treated with 1 mg/mL poly-L-lysine, and allowed to settle and adhere.

Sample preparation

ES cells and MEFs were fixed for FISH using PFA (Marahrens et al., 1998) or MeOH (Gribnau et al., 2003). Blastocysts were treated with acid tyrode to remove zona pellucidae, applied to 2% gelatin-coated slides using a Cytospin apparatus (Shandon),

Table 1. BACs used as templates for FISH probes. BACs covering the indicated genes were obtained from the BACPAC Resources Center at Children's Hospital Oakland Research Institute (http://bacpac.chori.org/).

probe	BAC
Hba1	RP23-71G18
Fn1 (B)	RP23-27O4
Ccnb3	RP24-270D24
Hprt (B)	RP23-412J16
Mecp2 (A)	RP23-77L16
Xic	RP23-309B17
Pgk1	RP24-90H17
Grpr (B)	RP23-231H22
2CEN (A)	RP23-382P22

PFA-fixed for 10 minutes, and permeabilized with a 5 minute incubation in PBS + 0.5% Triton X-100.

FISH

BACs used for genomic probes are listed in Table 1. All pair-wise DNA FISH was performed on loci separated by 40 Mb or less; linked sequences can be reliably scored as being on the same chromosome over distances of up to 50 Mb (Ensminger and Chess, 2004). A collection of PCR products spanning exon 1 was used to make *Xist* probes. Probes were generated using a BioPrime kit (Invitrogen), or using cy3-dCTP or FITC-dUTP (Amersham; Enzo Life Sciences) with kit reagents. Strand-specific probes to detect *Xist* and *Tsix* RNA were generated by *in vitro* transcription with FITC-UTP or bio-CTP (Enzo Life Sciences) from an *Xist* exon 7 template.

FISH for genomic DNA was performed as described (Gribnau et al., 2003). Biotinylated probes were detected with FITC-avidin (Vector) or cy3-streptavidin (Amersham). Combined DNA and RNA FISH was performed as described (van Raamsdonk and Tilghman, 2001), with the addition of a pepsin pre-treatment prior to the initial step (Gribnau et al., 2003). BrdU detection was performed as described (Gribnau

et al., 2003) using mouse monoclonal α-BrdU antibody (Becton Dickinson) and FITC-conjugated α-mouse (Vector).

Samples were scored on an Olympus BX60 microscope. Images were collected with a Hamamatsu ORCA-ER digital camera using Openlab 4.0.1 software, assembled using Adobe Photoshop 7.0, and levels adjusted to enhance contrast. ICM cells of blastocysts were scored from 3-D images collected using a DeltaVision system as described below.

FISH signal intensity quantification

Images were collected as 0.1 μm optical section stacks using an Olympus IX70 microscope with a motorized stage controlled by DeltaVision 2.10 software (Applied Precision, LLC) and a MicroMax CCD camera (Roper Scientific). SoftWorx 2.50 software was employed to deconvolve 3-D images and sum pixel intensity through relevant sections of image stack to generate 2-D projections representing total intensity of each FISH signal. The software was allowed to delineate the signal circumference and to integrate pixel intensities to generate an overall intensity value in arbitrary units.

Mimosine arrest and release replication timing assay

S-phase time-course experiments were performed using a mimosine arrest-release protocol (Gribnau et al., 2003). DNA from each time point was isolated and sonicated as described (Hansen et al., 1993). BrdU-labeled human DNA (0.5 μg) was mixed with 10 μg of DNA from each time point for normalization. Labeled DNA was immunopurified using an α-BrdU monoclonal antibody (Becton Dickinson) and Protein G Sepharose 4 Fast Flow beads (Amersham) and resuspended in 1 ml of 1mM Tris, 0.1mM EDTA pH8.0. PCR primers are listed in Table 2. For FACS analysis of mimosine arrest/release timepoints, samples were fixed overnight in 70% ethanol, treated with 0.2 μg/mL RNase,

Table 2. PCR primers used to assay replication timing. Immunopurified BrdU-containing DNA from mimosine arrest-release time course fractions was assayed using the following primers. Standard PCR conditions were used for 34 to 39 cycles, with the exception that annealing time was extended for the first seven cycles.

locus	primer sequences (5' – 3')
standard (human *ZIP1*)	ATCTCCAGTCAGTGGCTAGTCC CACGCTTGGTCCACGTTGGGATTT
Xic	AAGTCAATAAAGCACTCCCCATCTC TTGGCTCAGTGCTTATGGTG
Pgk1	TGCAACTGTTAGACCTGAGGAACCTTG TTGCCCAGCAGAGATTTGAGTTCAGC

stained with 20 μg/mL propidium iodide (Molecular Probes), and analyzed using a FACSCalibur (Becton Dickinson).

Statistics

All p-values were determined by comparing the observed distribution of signal patterns at each allele to a random, 50/50 distribution (null hypothesis) using a chi-square distribution test with one degree of freedom.

Results

X-chromosomal loci show a high frequency of singlet/doublet FISH signals in ES cells

While using fluorescence *in situ* hybridization (FISH) to visualize the *Xic* in female ES cells fixed with paraformaldehyde (PFA), we observed that a high frequency of cells displayed a single pinpoint FISH signal at one allele and a double pinpoint at the other. To determine whether this feature was unique to the *Xic*, we analyzed a number of other X-chromosomal loci. Cells were scored as showing a singlet signal for each

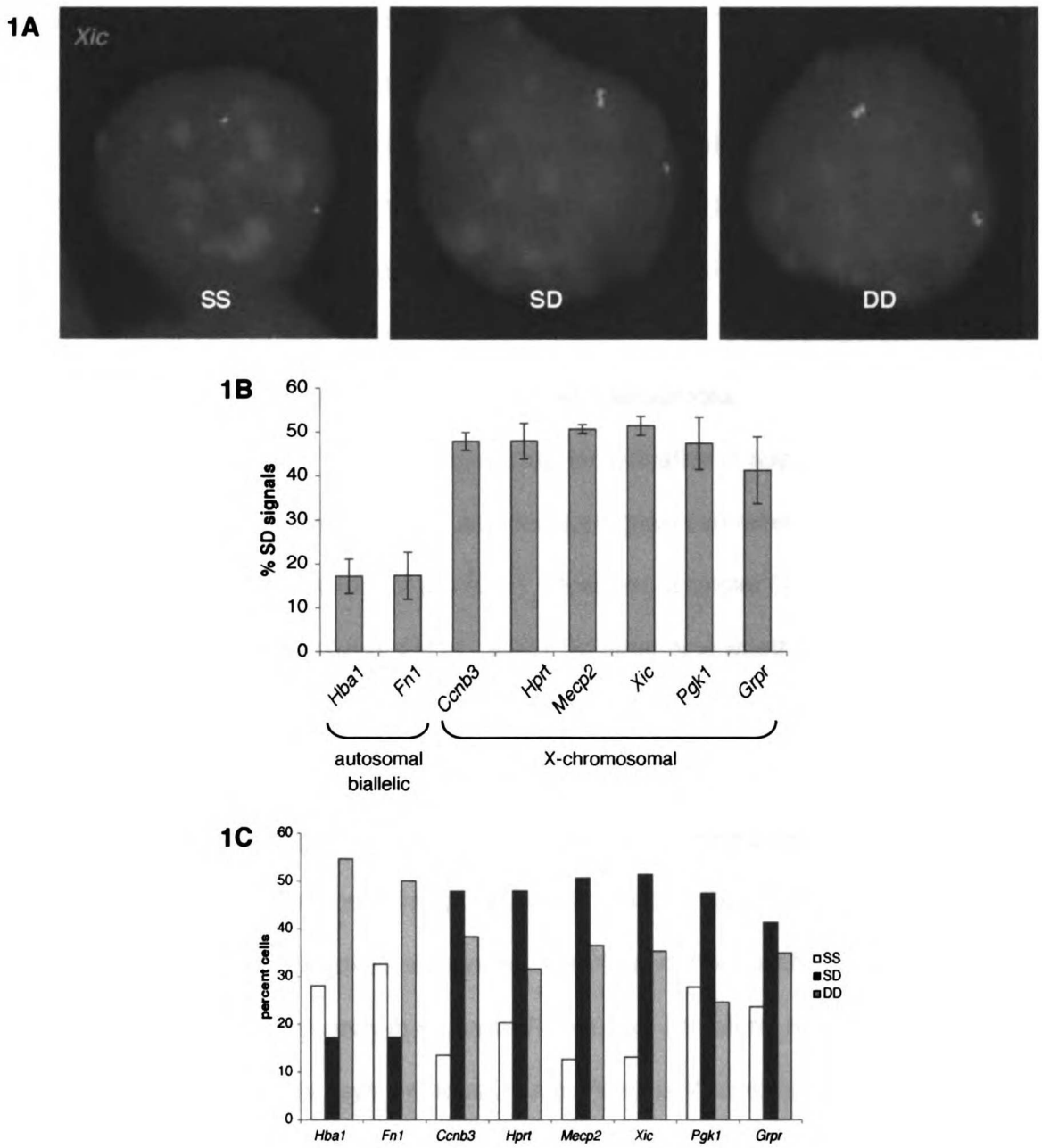


Figure 1. X-chromosomal loci display a high proportion of SD FISH signals in female ES cells.
(1A) FISH for *Xic* genomic sequences (red) demonstrates the three classes of signals in PFA-fixed female ES cells. DNA was stained with DAPI (blue).
(1B) ES cells display an elevated proportion of SD signals at X-chromosomal loci. Average data from 2-4 experiments (n>150), scored by two independent scorers, are presented. Error bars indicate one standard deviation.
(1C) Full presentation of the proportions of PFA-fixed ES cells exhibiting SS (white), SD (black), and DD (grey) signals at autosomal (*Hba1*, *Fn1*) and X-chromosomal (*Ccnb3*, *Hprt*, *Mecp2*, *Xic*, *Pgk1*, *Grpr*) loci. Average data from the samples scored in Figure 1B are shown.

allele (SS), a doublet signal for each allele (DD), or a pattern in which one allele appeared as a singlet and the other as a doublet (SD) (Figure 1A). For all X-chromosomal loci examined, the SD signal class was the most abundant, comprising 40 to 50% of the population (Figure 1B and 1C). This proportion is significantly greater than the fraction of SD signals observed for two autosomal loci, which exhibited the SD pattern in fewer than 20% of cells (Figure 1B and 1C). Thus, the high proportion of cells displaying SD signals is a unique feature of X-linked sequences.

A high frequency of SD FISH signals can be indicative of asynchronous replication of the two alleles, as singlet and doublet signals can reflect unreplicated and replicated loci respectively (Selig et al., 1992). However, a singlet FISH signal can also occur at a replicated locus (Azuara et al., 2003). To determine whether the high proportion of cells exhibiting SD signals was due to asynchronous replication, we directly measured the replication timing of X-linked sequences. ES cells were blocked in G1 and released into S phase (Figure 2A). At hourly intervals, cells were pulsed with BrdU to label replicating DNA. BrdU-containing DNA was immunopurified from each time point and assayed by PCR for X-linked sequences. The *Xic* and *Pgk1* each showed a single peak of BrdU incorporation early in S phase (Figure 2B). Therefore, neither X-linked locus was subject to highly asynchronous DNA replication. We next performed FISH for the *Xic* and *Pgk1* in female ES cells sorted by DNA content (Figure 2C). The proportion of cells exhibiting SD signals increased at the beginning of S phase, remained fairly constant throughout S phase, and decreased at the end of S phase (Figure 2D). Taken together, these data demonstrate that even though both alleles of each of these X-chromosomal loci replicated early, SD signals persisted throughout S phase. Furthermore, they suggest that the singlets in cells exhibiting SD FISH signals are replicated alleles.

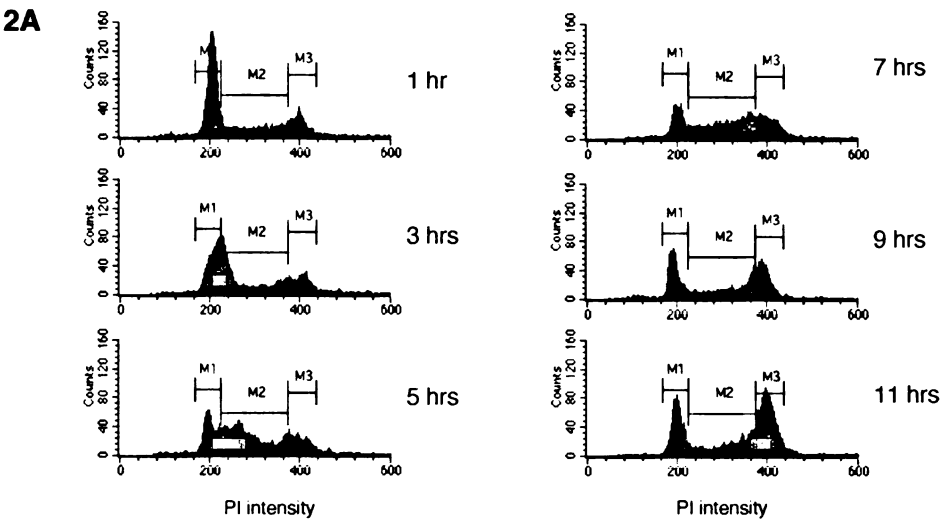


Figure 2. SD FISH signals at X-chromosomal loci are independent of asynchronous DNA replication in ES cells.

(2A) FACS analysis of female ES cell samples from a mimosine arrest/release timecourse. Samples collected at two-hour intervals, starting at 1 hour after washout of mimosine and continuing until 11 hours after washout, were analyzed by propidium iodide (PI) staining for DNA content. Histograms illustrate release of the majority of cells from G1 arrest and synchrony of progression through S phase. Co-cultured, replication-incompetent feeder cells exhibiting 2n DNA content contribute to the apparent G1 peak in each sample.

(2B) Analysis of replication timing of X-chromosomal sequences from the mimosine arrest/release timecourse monitored in 2A. Samples were pulsed with BrdU at hourly intervals after release from G1 arrest and DNA was isolated. An equal amount of bulk BrdU-labeled human DNA was added to each time point to allow assessment of immunoprecipitation efficiency across samples. BrdU-labeled DNA was immunopurified, and sequences present in each fraction were assessed by PCR. Standard shows consistent amplification of a human sequence across samples. IgG represents PCR analysis from labeled DNA purified with mouse IgG instead of anti-BrdU antiserum. –BrdU indicates analysis of an anti-BrdU immunoprecipitation from unlabeled DNA. Pre-IP depicts PCR analysis of the input DNA. Analysis of *Xic* and *Pgk1* reveals a single peak of replication for each locus in female ES cells. *(figure continues next page)*

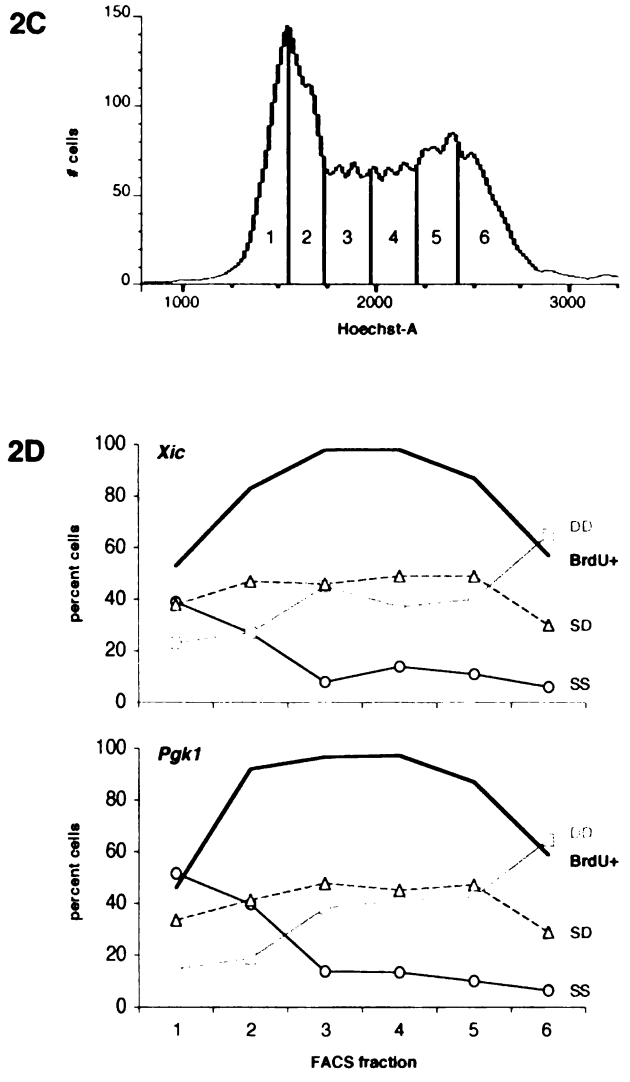


Figure 2. *(continued)*

(2C) Live female ES cells, pulse-labeled with BrdU, were sorted into six fractions by Hoechst-staining for DNA content.

(2D) FISH for the *Xic* (upper panel) and *Pgk1* (lower panel) in the fractions depicted in 2C shows constant, high proportions of SD signals (red triangles) throughout S-phase. Proportions of SS (black circles) and DD (grey squares) are also shown. The high proportion of BrdU-positive cells in all fractions (bold black line), shows that a substantial proportion of cells in all six fractions are in S phase. Over 80% of cycling ES cells are in S phase and fewer than 10% are in G1 (Savatier et al., 1994), inevitably leading to the inclusion of early S phase cells in fraction 1. Data are representative of 2-3 independent experiments.

(figure continues next page)

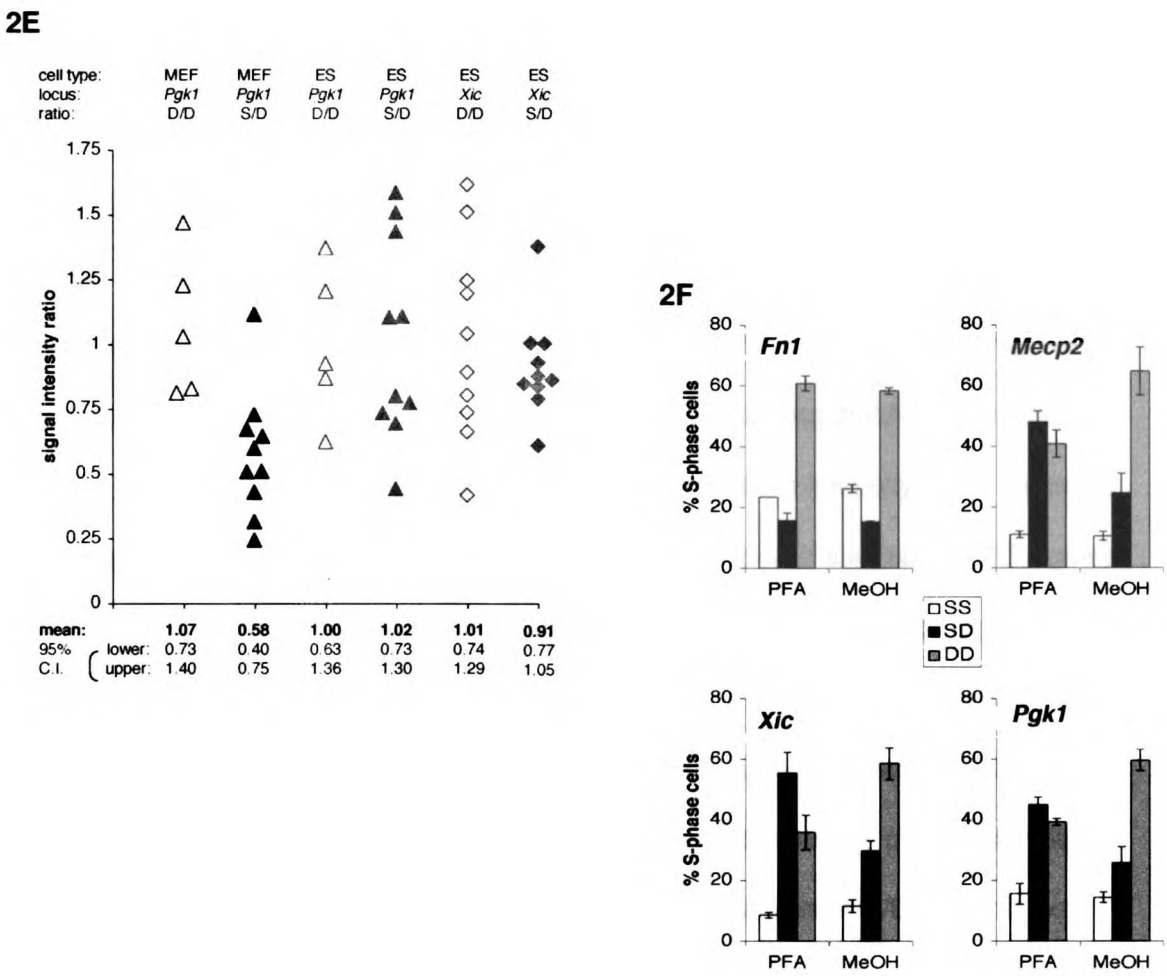


Figure 2. *(continued)*

(2E) In ES cells, singlet and doublet FISH signals for X-chromosomal loci exhibit equivalent fluorescence intensity. Plots show the ratios of S/D (solid symbols) or D/D (open symbols) FISH signal intensities in individual MEF or ES cell nuclei displaying an SD or DD pattern for *Pgk1* or the *Xic* as indicated. The intensity of both pinpoints in each doublet was summed to calculate the total intensity of doublet signals. When calculating the D/D intensity ratios the two doublets in a cell were randomly assigned to the numerator or denominator. Mean ratio values and 95% confidence intervals for the means are indicated.

(2F) Comparison of the proportions of cells displaying SS (white), SD (black), and DD (grey) signals for an autosomal locus (*Fn1*) and three X-chromosomal loci (*Mecp2*, *Xic*, and *Pgk1*) in S-phase ES cells upon PFA or MeOH fixation.

If a singlet signal in a cell showing an SD signal pattern represents a replicated allele, then it should contain the same amount of DNA as the two pinpoints comprising the doublet signal. To test this hypothesis, we determined the relative fluorescence intensity of singlet and doublet signals for the *Xic* and *Pgk1*. We validated this assay in female fibroblasts, in which singlet FISH signals correspond to unreplicated loci and doublet FISH signals correspond to replicated alleles (Gartler et al., 1999). In female mouse embryo fibroblasts (MEFs) exhibiting DD signals for *Pgk1*, the distribution of D/D intensity ratios centered around one (Figure 2E), demonstrating that two replicated alleles display equal signal intensities. We next compared the intensity of the singlet to the sum of the two pinpoints in the doublet (S/D) in female MEFs exhibiting SD signals for *Pgk1*. The S/D intensity ratios centered around 0.5 (Figure 2E), indicating that relative fluorescence intensity can be used to measure a two-fold difference in DNA content. Quantification of FISH signals can therefore be used to assay differences in DNA content at individual loci in single cells. In ES cells, the ranges of S/D intensity ratios for both the *Xic* and *Pgk1* were very similar to the ranges of D/D ratios: in both cases, the distributions centered approximately around one (Figure 2E). These results indicated that the singlet and doublet FISH signals in ES cells exhibiting an SD pattern for the *Xic* and *Pgk1* contained the same amount of DNA. In combination, these analyses demonstrate that the unusually high proportion of ES cells displaying SD signals for X-chromosomal loci reflects something other than asynchronous DNA replication. We refer to the high frequency of PFA-fixed cells exhibiting SD signals that are Independent of Asynchronous DNA Replication as SIAR.

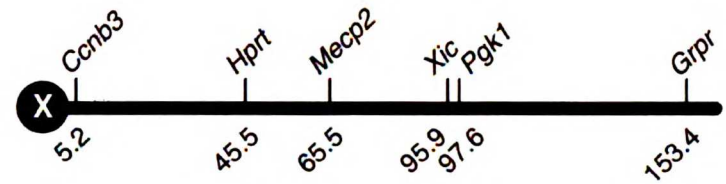
In a recent study, it was suggested that nuclear organization was important for replicated sequences to appear as singlet FISH signals (Azuara et al., 2003). To determine whether SIAR required an intact nucleus, we compared the proportion of ES

cells exhibiting SD signals upon PFA fixation to that seen upon methanol:acetic acid (MeOH) fixation. While PFA fixation preserves the three-dimensional organization of the nucleus by cross-linking nucleic acids and proteins, MeOH fixation destroys nuclear architecture by extracting the bulk of histones and other chromatin proteins (Hendzel and Bazett-Jones, 1997). The distribution of SS, SD, and DD signals for an autosomal gene, *Fn1*, did not differ significantly between fixation methods (Figure 2F). In contrast, these two fixation conditions resulted in different distributions of FISH signals for the X-chromosomal loci *Mecp2*, *Pgk1*, and the *Xic*. For these loci, the proportion of cells displaying SD signals decreased while the proportion of cells exhibiting DD signals increased in MeOH-fixed samples compared to PFA-fixed samples (Figure 2F). The changes in the relative proportions of cells exhibiting SD and DD signals indicate that when nuclear structure is disrupted, some replicated X-chromosomal loci that would appear as singlets in an intact nucleus resolve into doublets. In addition, these results suggest that native chromatin structure and/or nuclear organization is necessary for some replicated alleles of X-chromosomal loci to appear as singlets.

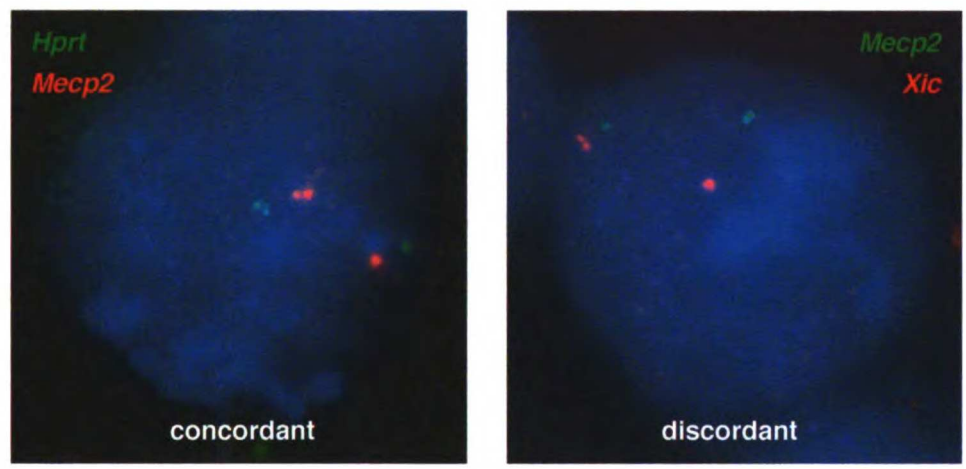
X chromosomes differ prior to X-inactivation

All X-chromosomal loci examined (Figure 3A) exhibited SIAR in female ES cells. We tested whether the singlet FISH signals for multiple loci appeared on the same chromosome or were randomly distributed between the two X chromosomes. Closely linked loci were analyzed pair-wise and, among the cells that exhibited SD signals for both probes, the proportion in which singlet signals occurred on the same chromosome (concordant signals) was determined (Figure 3B). *Ccnb3* and *Hprt*, *Hprt* and *Mecp2*, and *Mecp2* and *Pgk1* each exhibited approximately 65% concordant signals (Figure 3C). This 65% concordance is significantly higher than the 50% concordance that would be

3A



3B



3C

	SD SD cells:			
locus pair	concordant	discordant	p =	(n)
Ccnb3 & Hprt	**64%**	36%	0.009	(84)
Hprt & Mecp2	**65%**	35%	0.014	(66)
Mecp2 & Pgk1	**65%**	35%	0.001	(115)
Mecp2 & Xic	38%	**62%**	0.006	(134)
Xic & Pgk1	38%	**62%**	0.019	(104)

Figure 3. X chromosomes differ from one another in ES cells.

(3A) Map of the X-chromosome showing positions (Mb) of loci assayed for SIAR.

(3B) Left: concordant *Mecp2* (red) and *Hprt* (green), and right: discordant *Xic* (red) and *Mecp2* (green) FISH signals.

(3C) Frequencies of concordance and discordance for specified locus pairs in ES cells. p-values, determined using a chi-squared test, reflect the probability that the observed distributions are random.

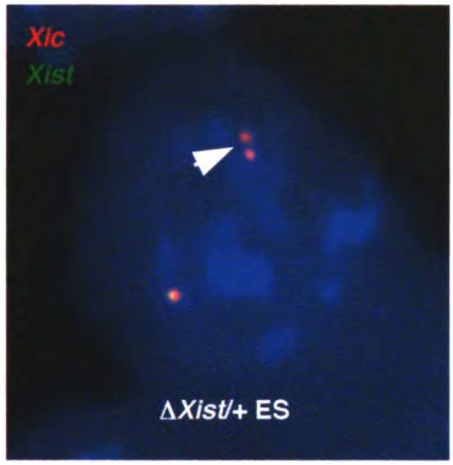
expected for a random distribution of singlet signals between chromosomes ($p < 0.02$), indicating that the behavior of loci on each X chromosome is coordinated. One X chromosome exhibits a higher frequency of singlet signals along its length, and the other X chromosome exhibits a higher frequency of doublet signals. These results suggest that, even though X-inactivation has not yet occurred and X-linked genes are biallelically expressed, the two X chromosomes in female ES cells already differ from each other.

When carrying out pair-wise analysis of X-chromosomal loci, we found that the *Xic* was unusual in that it was oppositely coordinated with adjacent genes. *Mecp2* and the *Xic*, and the *Xic* and *Pgk1* exhibited a bias against concordant signals, displaying concordance in only 38% of cells scored (Figure 3C). The *Xic* contains *Xist*, which is unusual in that it is the only gene expressed exclusively from the Xi. The opposite behavior of the *Xic* relative to other X-chromosomal loci in ES cells therefore parallels the opposite expression patterns of *Xist* and X-linked genes after X-inactivation. This parallel suggests a relationship between the appearance of the X chromosomes by FISH prior to X-inactivation and their fates as the Xa and Xi.

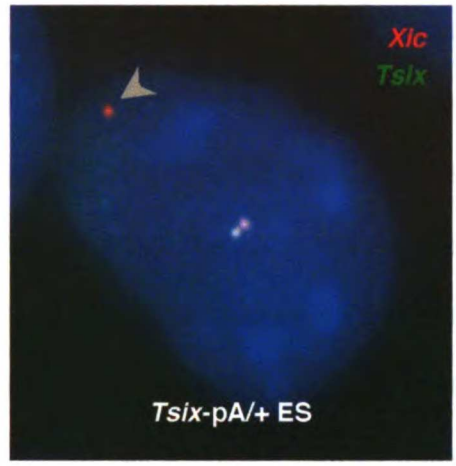
Xist and *Tsix* control SIAR and X chromosome fate

To determine if there was a correlation between the appearance of the X chromosomes by FISH prior to X-inactivation and their fates after X-inactivation, we analyzed SIAR in ES cell lines that will undergo non-random X-inactivation. If such a correlation exists, then the identities of the X chromosomes displaying singlet and doublet FISH signals should be non-random in ES cell lines that are poised for non-random X-inactivation. In ES cells heterozygous for an *Xist* mutant chromosome (Δ*Xist*/+), the wild-type X chromosome is always silenced and the mutant chromosome always remains active upon X-inactivation (Csankovszki et al., 1999; Gribnau et al.,

4A



4B



4C

		singlet in SD cells:			
locus	ES cell line	future Xa	future Xi*	p =	(n)
Xic	ΔXist/+*	31%	**69%**	<0.001	152
	TsixpA*/+	32%	**68%**	<0.001	112
Pgk1	ΔXist/+*	**65%**	35%	0.002	108
	TsixpA*/+	**71%**	29%	<0.001	73
Mecp2	ΔXist/+*	**65%**	35%	0.013	71
	TsixpA*/+	**68%**	32%	0.001	84

Figure 4. The future Xa and future Xi exhibit distinct frequencies of singlet FISH signals.
(4A) Allele-specific FISH for the *Xic* (red) in Δ*Xist*/+ ES cells. An *Xist* probe (green) identifies the wild-type allele. White arrowhead indicates the Δ*Xist* allele.
(4B) Allele-specific FISH for the *Xic* (red) in *Tsix*–pA /+ ES cells. *Tsix* RNA (green) identifies the wild-type allele. Grey arrowhead indicates the *Tsix*–pA allele.
(4C) Table summarizing scoring of allele-specific FISH in Δ*Xist*/+ and *Tsix*–pA/+ ES cells. For three X-chromosomal loci, SD cells were scored for identity of the allele displaying the singlet signal. The X chromosome indicated in black always becomes the Xa, and that in grey and marked with an asterisk always becomes the Xi. The allele indicated in green will be the expressed allele after X-inactivation and the allele indicated in red will be the silent allele. p-values reflect the probability that the observed distributions are random.

2005). In ES cells bearing a *Tsix* mutant chromosome (*Tsix*-pA/+), the wild-type X chromosome remains active and the mutant chromosome is inactivated upon X-inactivation (Luikenhuis et al., 2001).

We performed allele-specific FISH for X-chromosomal loci in Δ*Xist*/+ES cells (Figure 4A) or *Tsix*-pA/+ ES cells (Figure 4B). The *Xic*, *Pgk1*, and *Mecp2* were scored individually in both cell lines. While the overall proportions of SS, SD, and DD signal patterns for these loci did not differ between wild-type ES cells and *Xist* and *Tsix* mutant ES cells (data not shown), the identities of the alleles displaying the singlet and doublet FISH signals in cells with SD signals were non-random in the mutant cell lines. In both Δ*Xist*/+ and *Tsix*-pA/+ cell lines, the X chromosome that will become the Xi exhibited singlet signals for the *Xic* at a high frequency (approximately 70% of SD cells; Figure 4C) and singlet signals for *Pgk1* or *Mecp2* at a low frequency (approximately 30% of SD cells; Figure 4C). The future Xa showed the opposite patterns (Figure 4C). These results demonstrate that *Xist* and *Tsix* mutations affect SIAR and that, prior to non-random X-inactivation, the future Xi and future Xa show different probabilities of exhibiting a singlet signal for the *Xic* and other X-chromosomal loci.

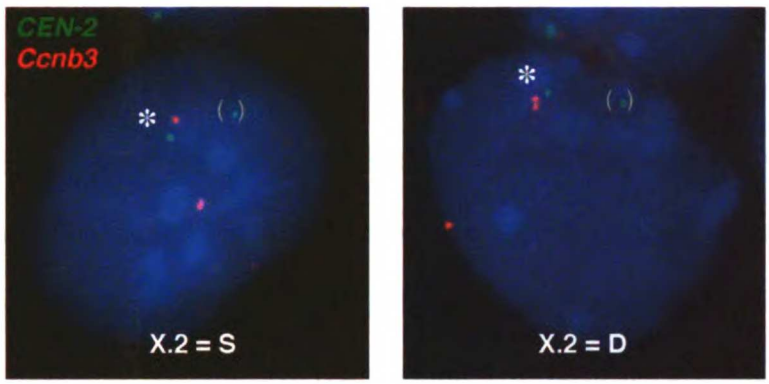
X-chromosomal loci showed distinct frequencies of FISH signal patterns on the future Xa and future Xi in *Xist* and *Tsix* mutant ES cells, supporting the idea that the two X chromosomes adopt distinct states prior to non-random X-inactivation. One X chromosome exists in a future Xi state, which causes singlet FISH signals to occur at a high frequency at the *Xic* and at a low frequency at other X-linked sequences. The second X chromosome adopts the future Xa state, which causes singlet FISH signals to occur at a low frequency at the *Xic* and at a high frequency at other X-chromosomal loci.

X chromosomes alternate between two states prior to random X-inactivation

The coordination of SIAR in wild-type ES cells (Figure 3C) indicated that the two X chromosomes existed in two distinct states similar to those observed in *Xist* and *Tsix* mutants. We hypothesized that in cells poised for random X-inactivation, these X chromosome states might also be indicative of X chromosome fates. Two predictions arise from this hypothesis. First, each X chromosome in wild-type ES cells should exist in the future Xi state in the same proportion of cells in which that chromosome will be inactivated. Second, because the fate of each X chromosome is not yet fixed in wild-type ES cells, the state of each X chromosome must not be fixed either.

In an ES cell line that will undergo random X-inactivation, one X chromosome should exist in the future Xi state in half of the cells in the population and the other X chromosome should exist in the future Xi state in the remaining half. By FISH, an X-linked gene should appear as a singlet on one X chromosome in 50% of SD cells and on the other X chromosome in the remaining 50%. To test this prediction, we performed allele-specific FISH in X.2/X^{wt} ES cells, which are poised to undergo random X-inactivation (Tada et al., 1993). In these cells, one X chromosome is marked by a centromeric fusion to chromosome 2 (Tada et al., 1993). *Ccnb3*, an X-chromosomal locus that exhibited SIAR (Figure 1B) and is closely linked to the fusion point, was analyzed in combination with a probe proximal to the centromere of chromosome 2, which identified the X.2 chromosome (Figure 5A). The X.2 chromosome and the wild-type X chromosome each exhibited a singlet signal for *Ccnb3* in approximately 50% of SD cells (Figure 5B), consistent with the marked X chromosome existing in the future Xi state in half of the cells and the wild-type X chromosome adopting this state in the remaining half.

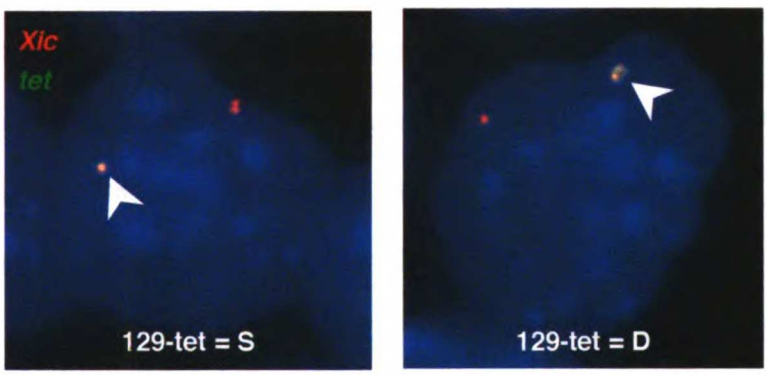
5A



5B

	Ccnb3 singlet in SD cells:			
cell line	marked	unmarked	(n)	p =
X.2/X^{wt}	47%	53%	179	0.501
X.2/X^{wt} clone 6G	46%	54%	52	0.579
X.2/X^{wt} clone 7C	50%	50%	50	1.000
X.2/X^{wt} clone 7D	47%	53%	64	0.617
X.2/X^{wt} clone 7E	52%	48%	50	0.777

5C



5D

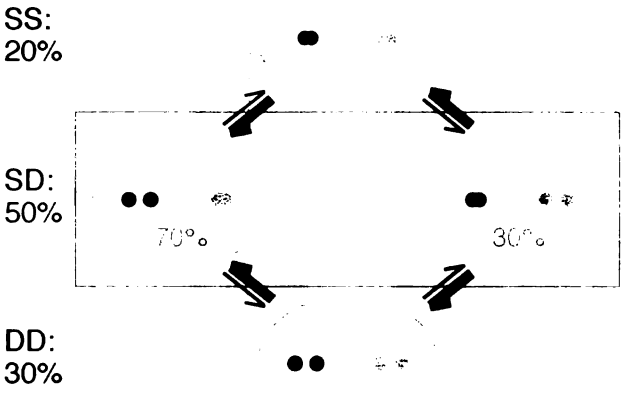
	Xic singlet in SD cells:			
	129-tet	*cas*	(n)	p =
129-tet/*cas* clone A	60%	40%	209	0.003
129-tet/*cas* clone B	60%	40%	237	0.002

Figure 5. X chromosomes appear to switch between states.
(5A) The two SD signal configurations observed for *Ccnb3* (red) by allele-specific FISH in X.2/X^{wt} ES cells. The marked allele (x.2 fusion chromosome, asterisk) is scored by its proximity to a *CEN-2* probe (green). The *CEN-2* signal on wild-type chromosome 2 is indicated by parentheses.
(5B) Allele-specific scoring of *Ccnb3* in X.2/X^{wt} ES and four single cell-derived clones. Non-significant p-values indicate a random distribution.
(5C) Allele-specific FISH for the *Xic* (red) in 129-tet/*cas* ES cells, showing the two SD signal configurations in which the 129-tet allele, identified using a tet-operator probe (green; yellow overlap, indicated by white arrowheads), appears as the singlet (left) or the doublet (right).
(5D) Allele-specific scoring of the *Xic* in two independently derived 129-tet/*cas* ES cell lines. p-values indicate that the *Xic* on the 129 chromosome exhibits a singlet signal at a higher frequency than would be expected by random chance.

X-inactivation is partially skewed in cells that are heterozygous at the *Xce*, an X-linked control element that influences randomness of X-inactivation (Cattanach, 1975). We analyzed ES cell lines containing X chromosomes that carry different *Xce* alleles to determine whether the frequency with which a chromosome adopts each state correlates with the degree of skewing observed upon X-inactivation. In ES cells heterozygous for *M. musculus* 129 and *M. castaneus* Ei X chromosomes (129/*cas*), the 129 X chromosome will be inactivated in approximately 80% of cells, and the *cas* X chromosome will be inactivated in the remaining 20% (Cattanach and Rasberry, 1994; Ogawa and Lee, 2003). Allele-specific FISH for the *Xic* (Figure 5C) was performed in two independent 129-tet/*cas* ES cell lines in which the *Xic* allele on the 129 X chromosome is marked by a tet-operator array integration that does not disrupt the *Xce* effect (Gribnau et al., 2005). Based on frequency with which the future Xi exhibited a singlet FISH signal in mutant cells destined for non-random X-inactivation, we calculated that in 129/*cas* ES cells, the *Xic* on the 129 allele should appear as a singlet in approximately 62% of SD cells (Figure 6). Consistent with this prediction, the singlet appeared on the 129 allele in 60% of SD cells (Figure 5D). Together, analysis of ES cell lines that undergo completely random, completely non-random, and skewed X-inactivation suggests that there is a relationship between the state of an X chromosome in ES cells and its fate as the Xa or Xi.

The fates of the X chromosomes are not fixed in wild-type ES cells, as a population of ES cells derived from a single progenitor cell undergoes random X-inactivation upon differentiation (Gribnau et al., 2005; Penny et al., 1996). If the states of the X chromosomes in ES cells reflect their fates upon X-inactivation, the states should not be fixed in ES cells that undergo random or skewed X-inactivation. To test this hypothesis, we first analyzed four clonal derivatives of the X.2/X^{wt} ES cell line, each of

6A



6B

	navy	aqua
pS=	0.35	0.55
pD= (1-pS)	0.65	0.45

Figure 6. A dynamic model for SIAR.

(6A) We hypothesize that after replication, both alleles of an X-chromosomal locus fluctuate between appearing as a singlet and a doublet FISH signal, and that one allele preferentially appears as a doublet (navy), while the homologous allele preferentially appears as a singlet (aqua). Schematics show the four possible FISH signal patterns for a single locus (SS, SD or DS, DD); the approximate percentages of cells displaying each pattern for the *Xic* are indicated. SD/DS FISH signals are subdivided into the approximate percentages in which the singlet is displayed by the aqua allele vs. the navy allele, based on allele-specific FISH in Δ*Xist*/+ and *Tsix*–pA /+ ES cells (Figure 4C). Several observations are consistent with this model. First, SS, SD, and DD populations coexisted at apparent equilibrium (Figure 2D, fractions 3-5) until chromosome condensation at G2/M (Figure 2D, fraction 6), suggesting that loci may interconvert between appearing as singlet and doublet FISH signals. Second, most X-linked loci on one X chromosome exhibited approximately 65% concordance when analyzed pair-wise, showing singlet signals for both loci on one chromosome and doublet signals for both loci on the other chromosome (Figure 3C). This degree of concordance can be accounted for by the independent fluctuation of linked loci, which all exhibit the same probability of appearing as a singlet along a given chromosome. Third, most loci on the future Xa in Δ*Xist*/+ and *Tsix*–pA /+ ES cells exhibited an elevated frequency of appearing as singlet signals. However, these loci did not appear as singlets in every cell, consistent with loci fluctuating between appearing as a singlet or a doublet and spending more time as a singlet on the future Xa.

(6B) Calculation of the probabilities with which alleles on each chromosome appear as singlets or doublets in the dynamic model described above. The frequencies with which the navy and aqua alleles appear as a singlet or doublet signal are described by the terms pS and pD, where pD=1-pS. The table lists pS and pD values estimated from our data. We assumed that the identities of the future Xa and future Xi are fixed in Δ*Xist*/+ and *Tsix*–pA/+ ES cells, and that for the *Xic* locus, the navy allele (low pS, mostly doublet) occurred on the future Xa and the aqua allele (high pS, mostly singlet) occurred on the future Xi. The overall frequency of SS (~20%), SD (~50%), DD (~30%) FISH signals in the wild-type population were considered. For the navy allele, the pS was calculated by adding the fraction of SS signals (where both the navy and aqua alleles appeared as singlets) to the fraction of SD signals in which the navy allele appeared as the singlet (as determined from the Δ*Xist*/+ and *Tsix*–pA/+ cell lines, in which it appeared as the singlet in approximately 30% of SD signals). A similar calculation was performed for the aqua allele.

(figure continues next page)

6C Calculation of proportion SD cells exhibiting a singlet signal on a given allele when chromosome states are fixed:

Proportion SD in which aqua allele is singlet = navyDaquaS/(navySaquaD + navyDaquaS)

Calculated = 0.69

Observed = 0.65 to 0.71 (Fig. 4C)

6D Model for coordination of the states of two adjacent loci along the chromosome:

	pS=		pD=	
	navy	aqua	navy	aqua
locus A	0.35	0.55	0.65	0.45
locus B	0.35	0.55	0.65	0.45

	formulas				calculations			
	A^SB^S	A^SB^D	A^DB^S	A^DB^D	A^SB^S	A^SB^D	A^DB^S	A^DB^D
navy	pS^A*pS^B	pS^A*pD^B	pD^A*pS^B	pD^A*pD^B	0.1225	0.2275	0.2275	0.4225
aqua	pS^A*pS^B	pS^A*pD^B	pD^A*pS^B	pD^A*pD^B	0.3025	0.2475	0.2475	0.2025

Proportion concordant = [(navy A^SB^S * aqua A^DB^D) + (navy A^DB^D * aqua A^SB^S)]/[(navy A^SB^S * aqua A^DB^D) + (navy A^DB^D * aqua A^SB^S) + (navy A^SB^D * aqua A^DB^S) + (navy A^DB^S * aqua A^SB^D)]

Predicted = 0.58

Observed = 0.64 to 0.65 (Fig. 3C)

6E Prediction of proportion SD cells exhibiting a singlet signal on a given allele in 129-tet/*cas* population subject to 80%/20% skewing:

Proportion SD in which *129-tet* allele is singlet = 0.8(proportion aqua allele singlet) + 0.2(proportion navy allele singlet)

Predicted = 0.8(0.69) + 0.2(0.31) = 0.62

Observed = 0.60 (Fig. 5D)

Figure 6. *(continued)*

(6C) Illustration of the calculation for the frequency with which an allele fixed in the aqua state (high pS) will appear as the singlet in a cell exhibiting a SD signal at a given locus. Calculations were based on the values listed in Figure 6B. The calculation shows that strict differences in the underlying states of the X chromosomes could result in the observed ~69% bias in singlet allele identity seen in heterozygous *Xist* and *Tsix* mutant ES cells (Figure 4C).

(6D) Prediction of FISH signal concordance frequencies for two linked loci that inhabit the same state (both navy on one chromosome, both aqua on the other) and that are fluctuating independently. Even though states are strictly coordinated on each homolog under the model, the predicted frequency of concordant SD SD FISH signals is 58%, comparable to the observed frequency of 62% to 65% (Figure 3C).

(6E) Use of the model to predict the degree of skewing in singlet allele identity in 129-tet/*cas* ES cells. The proportion of the time that the 129 *Xic* allele is expected to appear as the singlet in SD cells is calculated based on the results of Figure 6C. The model assumes that the 129 *Xic* allele is in the aqua state in 80% of cells, and in the navy state in the remaining 20% of cells. The model predicts 62% skewing toward the 129 allele appearing as the singlet signal, which is in close agreement to the 60% skewing observed (Figure 5D).

which is subject to random X-inactivation (data not shown). In each cell line, the X.2 chromosome exhibited a singlet signal for *Ccnb3* in approximately half of cells displaying SD signals (Figure 5B). Thus, each clone recapitulated the pattern observed in the parental cell line. In the two clonally derived 129-tet/*cas* ES cell lines, the *Xic* on the 129 chromosome also appeared as a singlet in some cells exhibiting SD signals and a doublet in others, at a frequency in agreement with the skewing of X-inactivation in both cell lines (Figure 5D and Figure 6). Thus, the marked chromosome in these clonal cell lines, which would have existed in either the future Xa or the future Xi state in the founding cell of each clone, assumed the future Xa state in some daughter cells and the future Xi state in others. Therefore, the states of the X chromosomes cannot be fixed; rather, the two X chromosomes must switch between states in cycling ES cells.

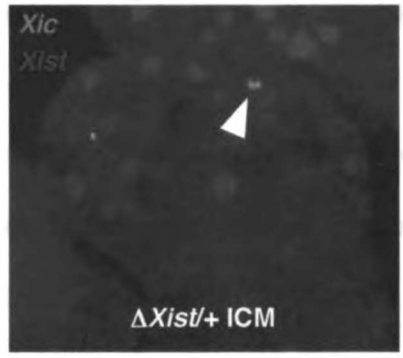
SIAR is restricted to cells poised for random X-inactivation

If future Xi and future Xa states underlie random X-inactivation, then these states should be observed *in vivo.* We performed FISH for the *Xic* in blastocyst-stage female mouse embryos. Cells from the inner cell mass (ICM) of these embryos are poised for random X-inactivation (Sugawara et al., 1983). The percentage of ICM cells exhibiting SD signals for the *Xic* was comparable to that seen in ES cells (Figure 7A), suggesting that ICM cells also display SIAR. Allele-specific FISH for the *Xic* in Δ*Xist*/+ blastocysts (Figure 7B) indicated that the wild-type X chromosome, which will become the Xi, more frequently exhibited a singlet signal, and the mutant X chromosome, which will become the Xa, more frequently appeared as a doublet (12/15 SD cells, p=0.02). These observations suggest that, as in ES cells, the two *Xic* loci in cells of early embryos adopt distinct configurations that are regulated by *Xist* and correlate with fate. Thus, the

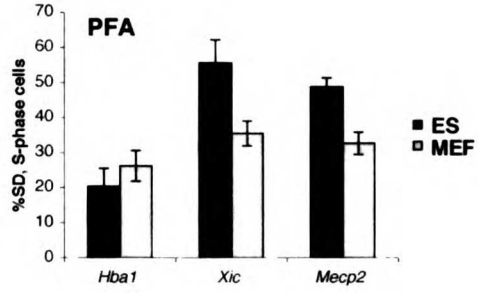
7A

	Xic	
cell type	**%SD**	**(n)**
ES	51	(182)
ICM	53	(40)
TE	31	(36)
MEF	28	(141)

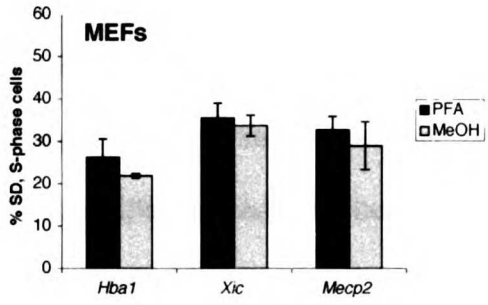
7B



7C



7D



7E

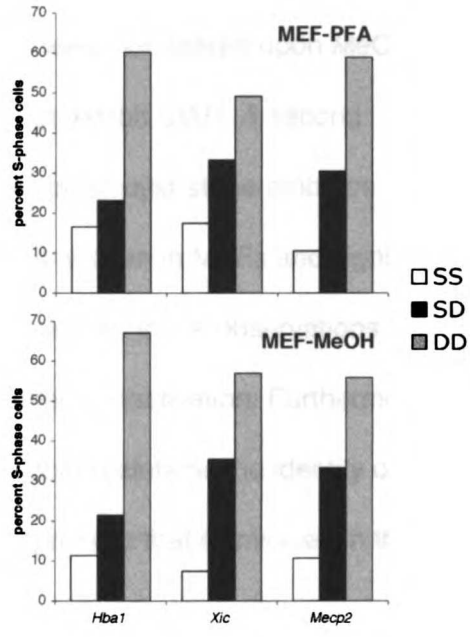


Figure 7. SIAR is specific to pluripotent cells *in vitro* and *in vivo*.

(7A) Percent SD signals observed by FISH for the *Xic* in cell types that are poised for X-inactivation (ES and ICM), and in trophectoderm (TE) cells and MEFs, which have completed X-inactivation.

(7B) Allele-specific FISH for the *Xic* (red) in Δ*Xist*/+ ICM cells. An *Xist* probe (green) identifies the wild-type allele; white arrowhead indicates the Δ*Xist* allele. In 12 /15 SD cells ($p = 0.02$), the Δ*Xist* allele exhibited the doublet signal.

(7C) Comparison of the proportion of PFA-fixed S-phase (BrdU+) ES cells (dark grey) and MEFs (light grey) exhibiting SD signals for an autosomal locus (*Hba1*) and two X-chromosomal loci (*Xic*, *Mecp2*). Data represent 2-3 independent experiments.

(7D) Percent SD signals observed for an autosomal biallelic locus (*Hba1*) and two X chromosomal loci (*Mecp2* and the *Xic*) in S-phase MEFs fixed with PFA (dark grey) or MeOH (light grey). Average data from 2 experiments ($n \geq 150$) are presented; error bars indicate one standard deviation.

(7E) Full presentation of the proportions of S-phase MEFs fixed with PFA (upper) or MeOH (lower) exhibiting SS (white), SD (black), and DD (grey) signals at *Hba1*, the *Xic*, and *Mecp2*. Representative experiments are plotted.

chromosome states reflected by FISH may play a role in the initial random designation of the Xa and Xi *in vivo*.

After one X chromosome is silenced, it remains the Xi throughout all subsequent cell divisions. We analyzed differentiated cells to determine whether SIAR persists after X-inactivation is established. In MEFs, the X-chromosomal loci *Mecp2* and the *Xic* exhibited significantly reduced proportions of SD signals when compared to ES cells (Figure 7A and 7C), and the proportions of SD signals were not altered upon MeOH fixation (Figure 7D and 7E), indicating that MEFs do not exhibit SIAR. A second differentiated cell population, trophectoderm cells from blastocyst-stage embryos, showed a proportion of SD *Xic* signals comparable to that seen in MEFs and significantly lower than that seen in ES cells (Figure 7A). Taken together, these observations indicate that SIAR occurs only in cells that are poised for X-inactivation. Furthermore, they suggest that SIAR does not reflect a mechanism that maintains the identity of the Xa and Xi in differentiated cells, but instead reflects a process that is involved in the initial designation of X chromosome fates.

Discussion

In this study, we used FISH to demonstrate that the two X chromosomes in female mouse ES cells differ prior to X-inactivation. On one X chromosome, the *Xic* tended to appear as a singlet signal when assayed by FISH and other X-linked genes more often appeared as doublets. The second X chromosome was more likely to show the opposite pattern. In ES cell lines that are destined for non-random X-inactivation, the future Xi exhibited a high frequency of singlet signals for the *Xic* and of doublet signals for other X-chromosomal loci, while the future Xa showed the opposite pattern. Taken together,

these data suggest that, prior to X-inactivation, the two X chromosomes in a female cell exist in distinct future Xi and future Xa states.

We propose that an absolute difference between the X chromosomes underlies the future Xi and future Xa states. The state of the X chromosome in turn affects the probability that a locus will appear as a singlet or doublet FISH signal. In *Tsix*-pA/+ and Δ*Xist*/+ ES cells, where a given X chromosome will become the Xi in 100% of cells, the *Xic* appeared as a singlet on that chromosome in 70% of SD cells. A simple explanation for this observation is that FISH reflects dynamic behavior of loci, and the future Xi and future Xa differ in their dynamics. For example, the *Xic* may fluctuate between appearing as a singlet or doublet on both the future Xi and the future Xa, appearing more frequently as a singlet on the future Xi, and more frequently as a doublet on the future Xa (Figure S3). In this model, the frequency with which a locus on the future Xi or Xa appears as a singlet or doublet in the population, and not its appearance in a single cell at any given point in time, reveals the underlying state of the chromosome.
In cell lines destined for random and completely non-random X-inactivation, the frequency with which a given X chromosome adopts the future Xi state correlates with the frequency with which it will be inactivated. The same relationship was observed in cell lines that will display skewed X-inactivation due to the *Xce* effect. Thus, the *Xce* effect is manifested prior to X-inactivation. This result is consistent with the suggestion that *Xce* effect influences the initial assignment of X chromosome fates (Percec et al., 2002).

Our observation that X chromosomes adopt distinct future Xi and future Xa states suggests that X chromosomes know their fates prior to silencing. These states exist even in cell lines that will undergo random X-inactivation, raising the question of how randomness is achieved. In clonal populations of ES cells that will undergo random X-

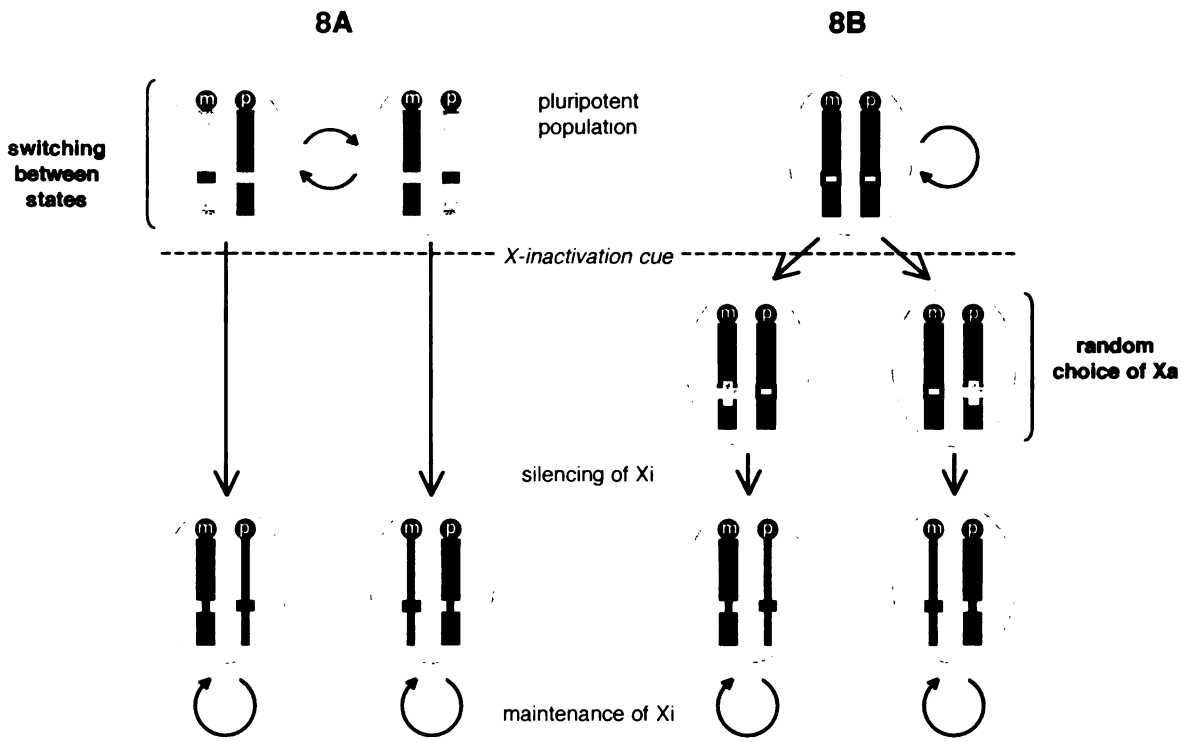


Figure 8. Models for achieving randomness in X-inactivation.

(8A) The data in the present study suggest a model in which the X chromosomes in pluripotent cells (m indicates maternal X chromosome, p indicates paternal X chromosome) coordinately switch between future Xa (light blue with dark blue *Xic*) and future Xi (dark blue with light blue *Xic*) states in cycling cells. The fates of the X chromosomes as the Xa (green with red *Xic*) or the Xi (red with green *Xic*) are determined by their states at the time that the cell receives the cue to initiate X-inactivation.

(8B) The prevailing model holds that the two X chromosomes in pluripotent cells (black, *Xic* indicated in white) are equivalent until the cue that initiates X-inactivation causes differential marking of the two X chromosomes (grey cross), thus designating the Xa and the Xi.

inactivation, a marked X chromosome showed a singlet signal for an X-linked gene in 50% of cells, suggesting that X chromosomes can switch states.

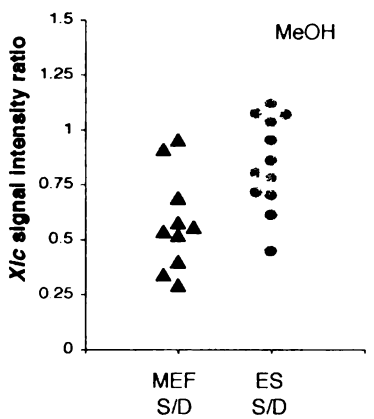
We propose a model in which switching of X chromosomes between mutually exclusive future Xi and future Xa states is the source of the apparent randomness of X-inactivation (Figure 8A). In each cell, one X chromosome adopts the future Xi state and the other X chromosome adopts the future Xa state. When a cell receives the cue to initiate X-inactivation, the chromosome that exists in the future Xi state in that cell will be silenced. However, as long as that cell remains pluripotent, the states of the chromosomes are not fixed and can switch in a concerted fashion. Because of this switching, the two X chromosomes each assume the future Xi state in half of the cells in a population. This randomization of states provides the basis for silencing to occur in an apparently random manner when X-inactivation is triggered. In this model, the mechanisms that determine the randomness of X-inactivation function prior to the receipt of the differentiation signal that initiates X-inactivation. In contrast, the prevailing model for randomness of X-inactivation posits that the two X chromosomes are equivalent in pluripotent cells, and that designation of which chromosome will be silenced occurs stochastically upon receipt of the signal that triggers X-inactivation (Figure 8B) (Avner and Heard, 2001; Rastan, 1983). Instead, our data support a model similar to the class of models proposed by Williams and Wu (2004), who speculate that a switching-based mechanism, like that regulating mating type switching in fission yeast, may underlie the randomness of X-inactivation.

X-chromosomal loci exhibited SD signals in a significant fraction of MeOH-fixed and of PFA-fixed ES cells. In a recent study, Gribnau et al. (2005) also observed a high frequency of SD FISH signals for X-chromosomal loci in MeOH-fixed ES cells. Based on the large fraction of cells exhibiting SD signals, it was suggested that X-linked genes are

subject to asynchronous replication in ES cells. We directly assayed replication timing of two X-chromosomal loci, *Pgk1* and the *Xic*, in ES cells. Each exhibited a single peak of replication. Cells showing SD FISH signals for the *Xic* or *Pgk1* occurred at a high frequency even after both alleles of these two X-chromosomal loci had replicated. In addition, a FISH signal intensity quantification assay revealed that in individual ES cells, the singlet and doublet signals for the *Xic* or for *Pgk1* contained comparable amounts of DNA (Figure 9A). Together, these data indicate that the high frequency of SD FISH signals for X-chromosomal loci in ES cells is not due to highly asynchronous DNA replication.

In PFA-fixed ES cells, the *Xic* was unusual in that it tended to exhibit a singlet FISH signal while other genes on the same X chromosome more often showed doublet signals. In contrast, the *Xic* and adjacent genes exhibited concordant FISH signals when ES cells were fixed with MeOH (Figure 9B) (Gribnau et al., 2005). Thus, the opposite behavior of the *Xic* was observed only in PFA-fixed ES cells. In addition, when ES cells were fixed with PFA, the future Xi showed a higher frequency of singlet signals for the *Xic* or of doublet signals for other X-linked genes. In contrast, when ES cells were fixed with MeOH, the future Xi and future Xa showed equal probabilities of exhibiting singlet signals for the *Xic* or other X-linked genes (Figure 9C) (Gribnau et al., 2005). PFA fixation maintains nuclear structure while MeOH extracts histones and other chromatin proteins and perturbs nuclear organization (Hendzel and Bazett-Jones, 1997). Therefore, the differences between PFA- and MeOH-fixed samples suggest that some aspect of chromatin structure or nuclear organization is required for replicated alleles on the future Xi and future Xa to appear as singlets with different probabilities. One possible explanation is that there is differential cohesion between sister chromatids on the two X chromosomes. Whatever the physical basis of the singlet and doublet FISH

9A



9B

	% SD SD cells concordant	discordant	p =	(n)
Mecp2 &Cnbp2	77	23	0.003	(30)
Mecp2 & Xic	62	38	0.275	(21)

9C

		% singlet in SD cells marked	unmarked	p =	(n)
Tsix -pA/+	*Xic*	47	53	0.475	(159)

Figure 9. Features of the mechanism governing random, exclusive X-inactivation are not revealed in MeOH-fixed samples.

(9A) *Xic* FISH signal intensity quantification as in Figure 2E for MeOH-fixed MEFs and ES cells. S/D FISH signal intensity ratios in individual nuclei displaying SD signals are plotted. The signal intensity ratio distributions are consistent with the interpretations that (1) in MEFs exhibiting a SD pattern, most singlet and doublet signals represent unreplicated and replicated alleles, respectively, and that (2) in ES cells exhibiting a SD pattern, the majority of the singlet and doublet FISH signals reflect equal amounts of DNA at the two alleles.

(9B) Coordination of FISH signals for X-chromosomal loci in MeOH-fixed ES cells. Frequencies of concordance and discordance for specified locus pairs were scored as in Figure 3B. p-values reflect the probability that the observed distributions are random. A high p-value for *Mecp2* and the *Xic* reflects the small sample size. As previously reported, the *Xic* exhibited a high proportion of concordant FISH signals with the linked *Mecp2* gene in samples fixed with MeOH (Gribnau et al., 2005), whereas in PFA-fixed samples this locus pair exhibited predominantly discordant FISH signals (Figure 3C).

(9C) Scoring of allele-specific FISH in MeOH-fixed *Tsix*-pA/+ ES cells. Cells displaying an SD pattern for the *Xic* were scored for identity of the allele displaying the singlet signal as in Figure 4B. p-values reflect the probability that the observed distributions are random. This analysis supports the conclusion that in MeOH-fixed ES cells, the *Xic* on the future Xi does not display an increased likelihood of exhibiting a singlet signal (Gribnau et al., 2005), in contrast to what was seen in PFA-fixed ES cells (Figure 4C).

signals, they demonstrate that the X chromosomes differ prior to X-inactivation in a manner that is predictive of Xi and Xa fates.

The *Xic* behaved oppositely to other loci on both the future Xi and the future Xa, mirroring the opposite expression patterns of *Xist* and most X-linked genes after X-inactivation and suggesting a correlation between the appearance of a locus by FISH and its future expression status. Azuara et al. (2003) reported a correlation between FISH signal appearance and current expression status. In that study, a replicated transgene tended to exhibit a doublet FISH signal when it was expressed and a singlet FISH signal when it was silenced. In our study, the replicated allele that more often appeared as a singlet signal in ES cells was the one that would be expressed after X-inactivation is triggered by differentiation. This result suggests that organization of sequences within the nucleus of a pluripotent embryonic cell may impact their expression after differentiation.

In wild-type ES cells, X chromosomes appear to switch between states in a concerted manner: when one X chromosome assumes the future Xi state, the other adopts the future Xa state. In heterozygous *Xist* and *Tsix* mutant ES cells, it appears that the X chromosomes no longer switch between states, suggesting that these non-coding RNAs are either required for switching to occur, or that they affect the likelihood that a chromosome will adopt the future Xi or future Xa state each time a switch occurs. The probability that a chromosome will become the Xi (or the Xa) is determined by the opposing activities of *Xist* and *Tsix* on that chromosome (Plath et al., 2002). These observations suggest that *Xist* and *Tsix* influence the fates of both X chromosomes by determining how effectively each chromosome competes to adopt the future Xi (or future Xa) state prior to X-inactivation. Both *Xist* and *Tsix* RNAs have been implicated in the regulation of chromatin structure (Heard, 2004; Navarro et al., 2005; Sado et al., 2005).

Prior to X-inactivation, these non-coding RNAs can be detected exclusively at the *Xic* on both transcriptionally active X chromosomes (Lee et al., 1999). Perhaps *Xist* and *Tsix* RNA mediate changes in chromatin structure at the *Xic* prior to X-inactivation. Such changes may in turn modulate the state of the entire X chromosome and direct its fate.

Our data show that loci on the X chromosomes in female ES cells differ in a manner that is predictive of future expression status. In addition to X-linked genes, several thousand autosomal genes are also subject to random monoallelic expression. It has been suggested that random monoallelic loci on autosomes and the X chromosome may share regulatory features (Ensminger and Chess, 2004; Singh et al., 2003). It will be interesting to determine whether a common mechanism of concerted switching between two states underlies the randomness of monoallelic expression throughout the genome.

Acknowledgements

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Joost Gribnau shared data and provided the Δ*Xist*/+ and 129-tet/*cas* ES cell lines prior to their publication; and Barbara Panning directed the entire project. BP, MRT, and MKA worked with me extensively on the text of the manuscript and deserve much of the credit for its present form. Together, we thank Rudolf Jaenisch and Nobuo Takagi for providing published cell lines, Tony Shermoen for developing the fluorescence intensity quantification methodology, and Martin Bigos and the staff of the Flow Cytometry Core Laboratory of the Gladstone Institutes for assistance with FACS sorting. Numerous faculty at UCSF provided valuable feedback on the data and the manuscript, including Hiten Madhani, David Morgan, Carol Gross, Gail Martin, Cori Bargmann, and Wallace Marshall. Pat O'Farrell was particularly generous with his time and advice, guiding us through the process of crafting our manuscript and getting it published. Thanks to all members of the Panning lab for their attentiveness, insight, and patience over the years that it took to bring this project to completion. Finally, I would like to thank Ting Wu at Harvard Medical School for her unwavering enthusiasm for and confidence in this work.

Chapter 2.

Evidence for homology sensing in X chromosome inactivation

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Summary

X chromosome inactivation, the mechanism by which X-linked gene dosage is equalized between the mammalian sexes, is regulated by chromosome number: in XX females, one of the two X chromosomes is subjected to chromosome-wide silencing, while in XY males, the single X escapes this process. Here we present evidence that, before initiation of silencing, an X chromosome senses the presence of its homolog. In female mouse embryonic stem (ES) cells, the future inactive and active X chromosomes (Xi and Xa) adopt distinct states. We find that the single X chromosome in male ES cells does not adopt either of these states, and is unaffected by mutations in the *X-inactivation center* (*Xic*) that control X-chromosome states in females, but is induced to adopt the future Xi state by an *Xic* transgene that causes ectopic X-inactivation. Two copies of *Xic*-deficient X-chromosomal sequences also adopt two states. Taken together, these results implicate chromosome-wide homology sensing in the mechanism of X-inactivation.

Introduction

It has long been hypothesized that X chromosomes communicate or interact *in trans* to regulate X-inactivation. The restriction of X-inactivation to cells with two or more X chromosomes indicated that silencing is controlled by a process that determines X chromosome number. Random and mutually exclusive assignment of Xa and Xi fates in

females implied that information about one X chromosome's fate is shared with the other. The *Xic*, a master regulatory locus on the X chromosome, is at the heart of the X-inactivation mechanism. The *Xic* encodes *Xist*, a nuclear RNA required for Xi silencing (Marahrens et al., 1997; Penny et al., 1996), whose expression is regulated by antisense transcription units, *Tsix* and *Xite*. Genetic analysis revealed that, in the presence of a wild-type X chromosome, an *Xist*-deficient chromosome always becomes the Xa (Gribnau et al., 2005; Marahrens et al., 1998), and a *Tsix/Xite*-deficient chromosome usually becomes the Xi (Clerc and Avner, 1998; Lee and Lu, 1999; Luikenhuis et al., 2001; Ogawa and Lee, 2003; Sado et al., 2001). These experiments demonstrated that heterozygous *Xic* mutations not only control the fates of the mutant X chromosomes *in cis*, they also control the fate of the wild-type X chromosome, which adopts the opposite fate, *in trans*. Thus, the two *Xic* loci in a female cell act in concert to control the fate of each X chromosome.

It has been proposed that a physical interaction between two *Xic* loci could activate a single *Xist* allele in a female cell (Marahrens, 1999; Muscatelli et al., 1992). Recent studies revealed that the *Xic* loci on homologous X chromosomes pair transiently at the initiation of silencing on the Xi (Bacher et al., 2006; Xu et al., 2006), providing the first direct evidence of a *trans* interaction in X-inactivation. A recent study from our lab raised the possibility of more general, X chromosome-wide communication, well before silencing is initiated. In female ES cells, which have not yet initiated X-inactivation, analysis of X-chromosomal sequences by fluorescence *in situ* hybridization (FISH) revealed that the future Xi and future Xa display distinctive patterns of singlet (S) and doublet (D) FISH signals that are independent of replication timing (Mlynarczyk-Evans et al., 2006). The differences in behavior of the future Xa and future Xi led us to propose that the X chromosomes adopt two states that anticipate their fates upon X-inactivation.

In the present study, we analyze the sequence requirements for these future Xi and future Xa states. Our findings indicate that X chromosomal sequences sense the presence or absence of homologous sequences, and implicate homology sensing in the control of X-inactivation.

Materials and Methods

Cell lines and culture

ES cells were cultured according to standard practice. Wild-type female (Marahrens et al., 1997) and male (E14) (Hooper et al., 1987) ES cell lines have been described. The XO ES cell clone "1/c16:3E" was isolated from a culture of wild-type female ES cells ("EL16," described in (Lee et al., 1999a)); 39,XO karyotype was confirmed by cytogenetic analysis (Figure 1A). The $11^{\beta\text{-}geo}11^{wt}$ ES cell line is "RRR379" (BayGenomics), in which a *β-geo* gene-trap cassette is integrated into the *Epn2* locus on chromosome 11. $X^{\Delta Xist}X$ (Gribnau et al., 2005), $X^{\Delta Xist}Y$ (Csankovszki et al., 1999), $X^{Tsix\text{-}pA}X$ and $X^{Tsix\text{-}pA}Y$ (Luikenhuis et al., 2001) ES cell lines have been described. The $X^{\Delta Tsix/Xite}X$ ES cell line has been described (Clerc and Avner, 1998); cytogenetic analysis revealed that the chromosome bearing the wild-type *Xic* lacks the *Mecp2* locus (Figure 1B). $X^{\Delta Tsix/Xite}Y$ (Morey et al., 2004) and $X^{\Delta Tsix/Xite}X^{trunc}$ (Clerc and Avner, 1998) ES cell lines have been described; cytogenetic analysis confirmed that in the latter cell line, the *Pctk1* locus is retained on the truncation chromosome (Figure 1C). The XY,A^{XicTg} ES cell line "166.6" has been described (Lee et al., 1996). To identify S-phase nuclei, cells were cultured with BrdU (Amersham) for 15 to 30 minutes before fixation.

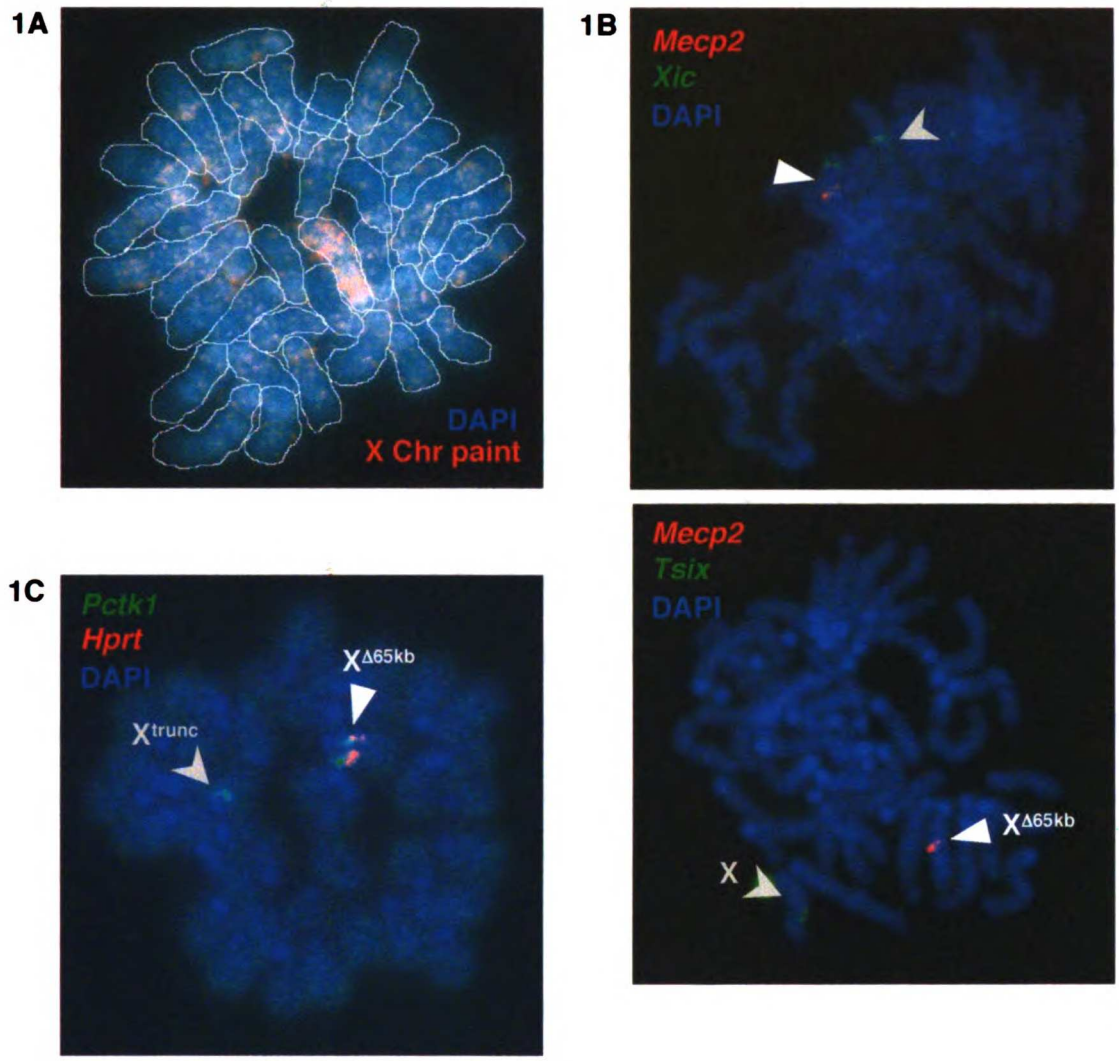


Figure 1. Cytogenetic characterization of the ES cell lines used in this study.

(1A) Mitotic chromosome spread of the XO ES cell clone "1/c16: 3E" demonstrates 39 chromosomes (stained blue with DAPI and outlined in white), one of which is detected with X chromosome paint (red).

(1B) Mitotic chromosome spreads of female ES cells heterozygous for the 65kb deletion ("$X^{\Delta Tsix/Xite}X$ ES") demonstrates that the *Mecp2* locus is absent from the wild-type X chromosome. Top: An *Xic* probe (green) marks the two X chromosomes, only one of which contains the *Mecp2* locus (red). Bottom: The *Mecp2* (red) -deficient chromosome contains the wild-type *Xic*, as determined using a *Tsix* probe (green) directed against the region deleted on the Δ65kb chromosome. White and grey arrowheads indicate the Δ65kb and wild-type chromosomes, respectively.

(1C) Mitotic chromosome spread of $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells demonstrates that the *Pctk1* locus (green) is retained on the truncated X chromosome, which does not hybridize with a probe to *Hprt* (red). White and grey arrowheads indicate the Δ65kb and truncated chromosomes, respectively.

Sample preparation and microscopy

ES cell metaphase chromosome spreads were prepared by standard methods, with the exception that all incubations were performed on ice. PFA-fixed ES cell samples were prepared as described (Marahrens et al., 1998). Biotinylated X chromosome paint was obtained from Cambio. BACs used for genomic probes have been described (Mlynarczyk-Evans et al., 2006), with the addition of *Pctk1* BAC RP23-362P12 (CHORI). The *Xist* exon 1 probe has been described (Mlynarczyk-Evans et al., 2006). For the *Tsix* probe, a collection of PCR products spanning the major *Tsix* CpG island and 4 kb of downstream sequence was used; for *β-geo*, the plasmid pGT2lxf (BayGenomics) was used. Probes were generated and FISH for genomic DNA was performed as described (Mlynarczyk-Evans et al., 2006). BrdU detection was performed as described through primary antibody incubation (Mlynarczyk-Evans et al., 2006), followed by secondary and tertiary incubations with rabbit α-mouse (Sigma) and AMCA-conjugated α-rabbit (Vector), respectively.

Samples were scored on an Olympus BX60 microscope. Images were collected with a Hamamatsu ORCA-ER digital camera using Openlab 4.0.1 software, assembled using Adobe Photoshop 7.0, and levels adjusted to enhance contrast.

Statistics

All p-values were determined by comparing the observed distribution of signal patterns at each allele to a random, 50/50 distribution (null hypothesis) using a chi-squared distribution test with one degree of freedom.

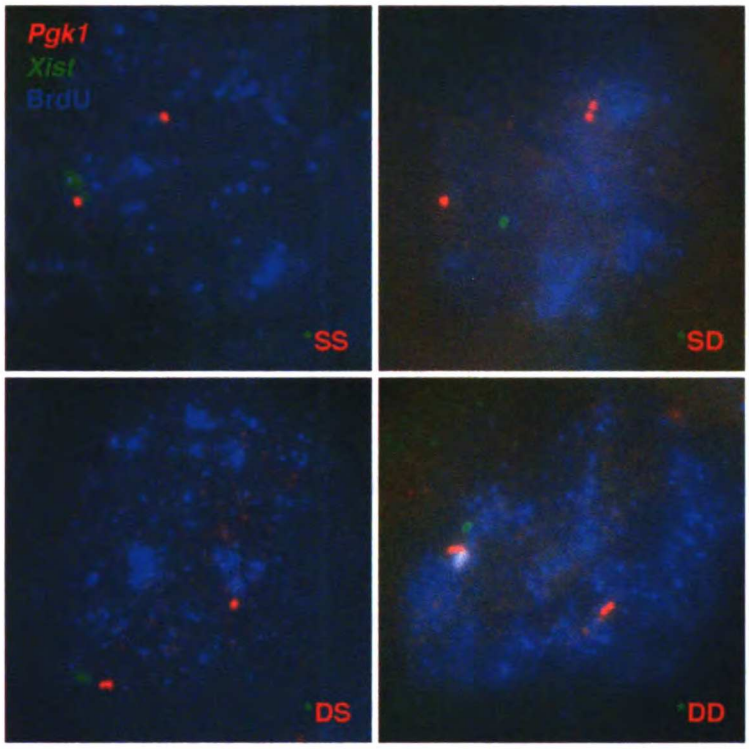
Results

The male X chromosome behaves differently from either X in females

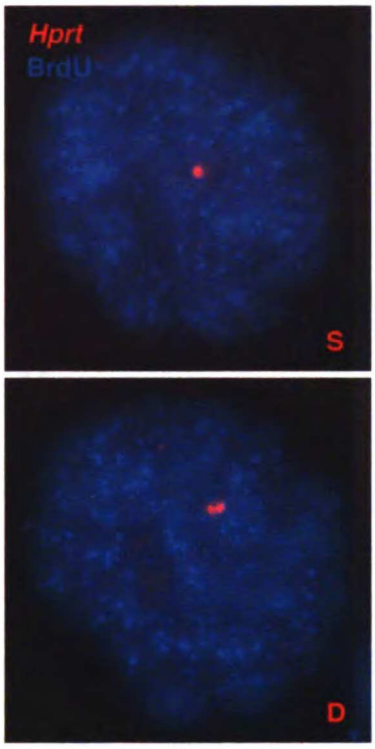
We developed a FISH assay for identifying the state of an individual X chromosome with the goal of analyzing the sequence determinants of the future Xa and future Xi states. First, we defined the typical future Xa and future Xi states in female ES cells. We performed allele-specific FISH for three X-chromosomal loci and scored each allele as displaying a S or D FISH signal (Figure 2A). We calculated the proportion of cells in which a locus appeared as a singlet, or "pS", on the future Xa or the future Xi (Figure 2B). Consistent with prior qualitative observations (Mlynarczyk-Evans et al., 2006), the future Xi displayed a high pS value at the *Xic* (0.43), and low pS values at *Mecp2* and *Pgk1* (0.23 and 0.27; Table 1). On the future Xa, these loci displayed opposite trends in pS values (Table 1). To summarize, the distinct pattern of pS values along a chromosome can be used to identify the future Xa and future Xi states.

Male cells have a single X chromosome, which always remains active. To determine if this chromosome adopts the future Xa state, we performed FISH for X-chromosomal loci in male ES cells. Nuclei were scored as showing a S or D FISH signal at each locus (Figure 2C), and pS values were determined (Figure 2D). For the *Xic*, the pS value was low in males (0.23) and similar to that of the *Xic* on the future Xa in females (Table 1). Five other loci along the length of the X chromosome also exhibited low pS values, ranging from 0.24 to 0.28, in contrast to the high pS values observed on the future Xa in females (Table 1). Thus, all loci exhibit a low frequency of singlets in male ES cells. Taken together, these data show that the X chromosome in male ES cells behaves differently from either X chromosome in females (Figure 2E).

2A



2C



2B

*XX, *marked allele: pS = [(*SS)+(*SD)]/(total)

*XX, unmarked allele: pS = [(*SS)+(*DS)]/(total)

2D

XY, XO: pS = (S)/(total)

Figure 2. Methodology for determining the frequency of singlet FISH signals on individual X chromosomes.

(2A) ES cells were briefly cultured with BrdU to label S-phase nuclei, fixed with PFA, and subjected to BrdU immuno-detection (blue) and FISH for genomic sequences. Analysis was restricted to cells in S phase because X-chromosomal loci replicate very early in ES cells(Mlynarczyk-Evans et al., 2006) ; thus, in most S-phase cells, loci are replicated and free, theoretically, to appear as either a S or D signal. In $X^{\Delta Xist}X$ female ES cells, the two X chromosomes can be distinguished cytologically and have known fates (Gribnau et al., 2005). Images show the four possible signal configurations for the X-chromosomal *Pgk1* loci (red): *SS, *SD, *DS and *DD. The future Xi (wild-type X) is detected by a probe to *Xist* exon 1 (green, *).

(2B) Formulas for calculating the pS value displayed by a locus on a given X chromosome in female cells, scored as in 2A.

(2C) FISH in male ES cell samples, prepared as in 2A. Images show the two signal classes (S, D) observed for the *Hprt* locus (red).

(2D) Formulas for calculating pS values in cells with one X chromosome, scored as in 2C.

(figure continues next page)

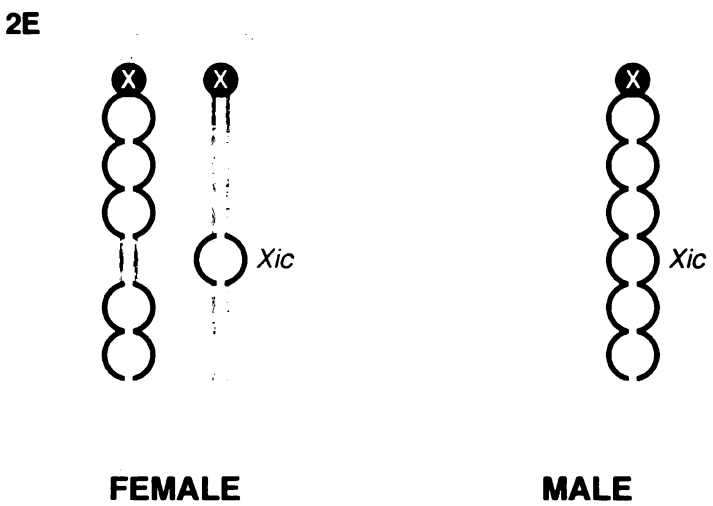


Figure 2. *(continued)*
(2E) Graphical interpretation of the data in Table 1. X chromosomes in female and male cells (grey ovals) are depicted as a replicated pair of sister chromatids joined at the centromere (black circle with "X"). Sequences exhibiting low pS values are depicted in dark blue (bubbles), and those showing high pS values are in light blue (straight lines). The future Xa, depicted on the right in both cells, differs between female and male cells.

	XX				XY		XO	
locus	**future Xi**	st. dev.	**future Xa**	st. dev.	**future Xa**	st. dev.	**future Xa**	st. dev.
Ccnb3	ND	-	ND	-	**0.257**	0.007	ND	-
Hprt	ND	-	ND	-	**0.270**	0.058	**0.269**	0.013
Mecp2	**0.233**	0.036		0.007	**0.235**	0.011	**0.229**	0.007
Xic		0.052	**0.231**	0.008	**0.227**	0.021	**0.206**	0.001
Pgk1	**0.268**	0.044		0.049	**0.278**	0.053	**0.280**	0.021
Grpr	ND	-	ND	-	**0.247**	0.028	ND	-

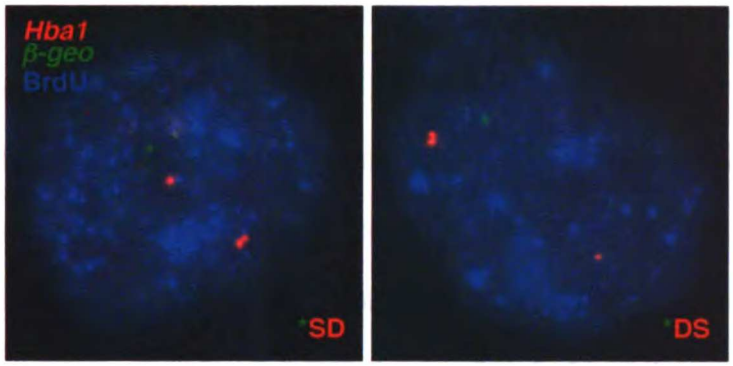
Table 1. A high frequency of singlet FISH signals on one X chromosome requires the presence of a second X chromosome. pS values for loci on X chromosomes in ES cell lines with the indicated sex chromosome constitutions. XX data are from $X^{\Delta Xist}X$ ES cells . Dark and light blue indicate low and high pS values, respectively. ND: not determined. Average values and standard deviations from 2-4 independent experiments ($n \geq 150$) are presented.

The sex-specific behavior of the X chromosome could be due to a Y chromosome effect, or to the X chromosome dosage difference between males and females. To distinguish between these possibilities, we examined the behavior of the single X chromosome in a 39,XO female ES cell line. FISH was scored and pS values determined as in male samples. The behavior of the single X chromosome in XO ES cells was indistinguishable from the single X in males, with low pS values (0.21 to 0.28) recorded at four X-chromosomal loci including the *Xic* (Table 1). These experiments suggest that when an X-chromosomal locus is present in a single copy, it will exhibit a low frequency of singlet signals, whereas when a second copy is present, one copy will exhibit a high frequency and the other a low frequency of singlets. The two distinct X chromosome states observed in normal female ES cells are both characterized by a high frequency of singlets at one or more loci, but a lone X chromosome in a male ES cell does not adopt either of these states. In conclusion, establishment of the future Xi and future Xa states appears to require two X chromosomes.

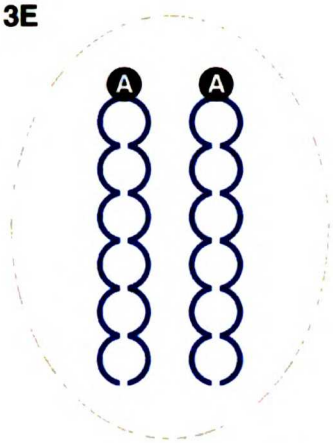
Biallelic autosomal loci behave similarly to X-chromosomal loci in males

The allele of an X-chromosomal locus that exhibits a high frequency of singlet FISH signals in females appears to be in a special state that can only exist when another X chromosome is present. We therefore wondered if the allele of an X-chromosomal locus that exhibits a low frequency of singlets represents "normal" behavior characteristic of loci that are not destined for X-inactivation. To define the behavior of "normal" genomic sequences, we performed FISH for biallelically expressed autosomal loci. In a prior study, the biallelic autosomal loci *Hba1* and *Fn1* did not exhibit a high proportion of SD FISH signals, suggesting that the two alleles behave in a comparable manner (Mlynarczyk-Evans et al., 2006). To confirm that this is the case, we performed

3A



3E

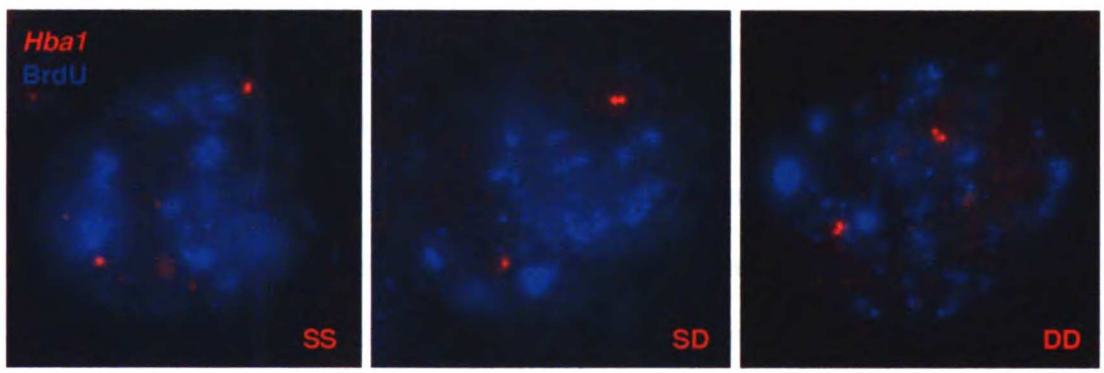


FEMALE or MALE

3B

	Hba1 singlet in SD cells			
	β–geo	wt	n =	p =
XY,11$^{\beta\text{-}geo}$11^{wt}	**50.0%**	**50.0%**	134	1.000

3C



3D

	female		**male**	
locus	**pS**	st. dev.	**pS**	st. dev.
Hba1	**0.240**	0.041	**0.230**	0.027
Fn1	**0.256**	0.024	**0.234**	0.021

Figure 3. Both alleles of biallelic autosomal loci show a low frequency of singlet FISH signals.
(3A) Alelle-specific FISH for a biallelically-expressed autosomal locus, *Hba1* (red), in male 11$^{\beta\text{-}geo}$11^{wt} ES cells. SD FISH signals in which the allele on the marked copy of chromosome 11, identified by a *β-geo* probe (green), appears as the singlet (left, *SD) or doublet (right, *DS) are shown.
(3B) Allele-specific scoring of *Hba1* SD FISH signals in male 11$^{\beta\text{-}geo}$11^{wt} ES as in 3A shows that the marked and unmarked alleles exhibit the singlet signal with equal frequency. p-value, determined using a chi-squared test, indicates that the data fit the null hypothesis of no bias in identity of the singlet allele.
(3C) FISH for *Hba1* (red) in wild-type male ES cells. SS, SD, and DD FISH signal patterns are shown in BrdU-stained nuclei (blue).
(3D) Average pS values for two biallelic autosomal loci, *Hba1* and *Fn1*, are low (dark blue) in both female and male wild-type ES cells. Cells were scored as in 3C and average pS values calculated as follows: [2(SS)+(SD)]/2[(SS)+(SD)+(DD)]. Average values and standard deviations from 2-4 independent experiments (n≥150) are shown.
(3E) Graphical interpretation of the data in 3B, 3D. Biallelically expressed sequences on a pair of autosomes ("A") show singlet FISH signals at low frequency in both male and female ES cells.

allele-specific FISH in an ES cell line in which one copy of the *Hba1* locus is closely linked to a *β-geo* marker (Figure 3A). Indeed, among the small fraction of cells displaying SD FISH signals at *Hba1*, the marked and unmarked alleles appeared as the singlet with equal frequency (Figure 3B). Thus, homologous alleles of a biallelic autosomal locus exhibit equivalent behavior by FISH.

We next performed FISH for *Hba1* or *Fn1* and determined the average frequency of singlet FISH signals at each locus (Figure 3C). Both biallelic autosomal loci exhibited low pS values (0.23 to 0.26, Figure 3D) that were comparable to the low pS values of loci on one copy of the X chromosome (Table 1). These observations support the idea that a low frequency of singlet FISH signals is the "normal" state of a genomic locus. The behavior of both copies of an autosome bearing biallelically expressed sequences is likely to be similar to the behavior of the single X chromosome in male ES cells (Figure 3E). In addition, we found that pS values for biallelic autosomal loci were comparable between male and female ES cells (Figure 3D). Thus, sex-specific differences in chromosome behavior may be restricted to sequences that differ in copy number between the sexes.

Effects of *Xic* mutations require the presence of a second X chromosome

The *Xist* and *Tsix* genes located within the *Xic* control the state adopted by an X chromosome in a female ES cell. In the presence of a wild-type homolog, an X chromosome bearing a loss-of-function mutation in *Xist* adopts the future Xa state, whereas an X chromosome bearing a loss-of-function mutation in *Tsix* adopts the future Xi state (Mlynarczyk-Evans et al., 2006). We wished to determine whether the mechanism by which the *Xic* controls the state of an X chromosome requires the presence of a second X chromosome. To address this question, we compared the

$X^{\Delta Xist}$ in:

	$X^{\Delta Xist}X$		$X^{\Delta Xist}Y$	
locus	pS =	st. dev.	pS =	st. dev.
Xic	0.230*	0.008	0.229	0.016
Pgk1	*	0.049	0.266	0.009

$X^{Tsix\text{-}pA}$ in:

	$X^{Tsix\text{-}pA}X$		$X^{Tsix\text{-}pA}Y$	
locus	pS =	st. dev.	pS =	st. dev.
Xic		0.006	0.218	0.006
Pgk1	0.345	0.016	0.230	0.001

$X^{\Delta Tsix/Xite}$ in:

	$X^{\Delta Tsix/Xite}X$		$X^{\Delta Tsix/Xite}Y$	
locus	pS =	st. dev.	pS =	st. dev.
Xic		0.076	0.236	0.015
Pgk1	0.269	0.041	0.229	0.021

Figure 4. An *Xic* mutation alters the X chromosome's behavior only when a second X chromosome is present.

pS values for two loci, the *Xic* and *Pgk1*, on X chromosomes bearing a large deletion including the *Xist* promoter and exon 1 (Δ*Xist*), a premature truncation of the *Tsix* transcript created by insertion of a polyadenylation cassette (*Tsix*-pA), and a 65 kb deletion in the *Tsix* and *Xite* region (Δ*Tsix/Xite*). Each chromosome was assayed in XX (presence of a second wild-type X chromosome indicated in grey) and XY (no other X chromosome present) ES cells. Allele-specific scoring in female cells used *Xist* ($X^{\Delta Xist}X$) or *Tsix* ($X^{Tsix\text{-}pA}X$, $X^{\Delta Tsix/Xite}X$) -specific probes, as in Figure 2A; male cells were scored as in Figure 2C. Average values and standard deviations from 2-3 independent experiments ($n \geq 150$) are presented. *Indicated data are also presented in Table 1.

effects of *Xic* mutations on the behavior of an X chromosome in female and male ES cells.

First, we established the effects of three different *Xic* mutations on the behavior of the mutant X chromosome in a female background. In the $X^{\Delta Xist}X$ ES cell line, the chromosome bearing a large deletion in the *Xist* gene will always become the Xa (Gribnau et al., 2005). In this cell line, the pS value at the *Xic* was low and that at *Pgk1* was high (Figure 4), indicating that the mutant chromosome adopted the future Xa state in a female cell. In the $X^{Tsix\text{-}pA}X$ and $X^{\Delta Tsix/Xite}X$ ES cell lines, which carry a premature polyadenylation signal in *Tsix* and a 65 kb deletion in the *Tsix/Xite* region, respectively, the mutant chromosomes always become the Xi (Clerc and Avner, 1998; Luikenhuis et al., 2001). pS values at the *Xic* and *Pgk1* (Figure 4) indicated that these X chromosomes adopted the future Xi state. (An apparent difference in the behavior of the *Pgk1* locus on the $X^{Tsix\text{-}pA}$ chromosome is believed to be attributable to technical difficulties in performing allele-specific FISH in this cell line, and will be re-examined before submission of this manuscript for publication.) Thus, all three *Xic* mutations cause the mutant X chromosome to adopt a specific state in the presence of a second, wild-type X chromosome.

Next, we analyzed the behavior of X chromosomes bearing these *Xic* mutations in a male ES cell background. We performed FISH for X-chromosomal loci in $X^{\Delta Xist}Y$ (Csankovszki et al., 1999), $X^{Tsix\text{-}pA}Y$ (Luikenhuis et al., 2001), and $X^{\Delta Tsix/Xite}Y$ (Morey et al., 2004) ES cell lines. In all three cell lines, we found that both the *Xic* and *Pgk1* exhibited low pS values (0.22 to 0.27, Figure 4), comparable to the pS values observed on a wild-type X chromosome in male ES cells (Table 1). These data show that *Xic* mutations that cause an X chromosome to adopt a specific state in female ES cells have no effect on

the behavior of an X chromosome in male ES cells. Therefore, the mechanism by which the *Xic* controls the state of an X chromosome seems to require the presence of a second X chromosome.

Homology at the *Xic* can trigger a change in X chromosome state

When ectopic copies of the *Xic* are introduced into male cells, the endogenous X chromosome sometimes undergoes X-inactivation in a regulated manner that parallels normal X-inactivation in females (Heard et al., 1996; Lee et al., 1996). We asked whether the single X chromosome adopts the future Xi state prior to this ectopic X-inactivation. We performed FISH for X-chromosomal loci in XY,A^{XicTg} ES cells, which contain 20-30 copies of a 450 kb *Xic* transgene integrated into an autosome (Lee et al., 1996). We scored the appearance of the *Xic* or *Pgk1* allele on the endogenous X chromosome (Figure 5A) and determined its pS value. *Pgk1* exhibited a low pS value in XY,A^{XicTg} ES cells, comparable to that seen in normal males, and also that seen on the future Xi in females (Figure 5B). At the *Xic*, however, the frequency of singlets differed between wild-type and transgenic male ES cells. The pS value for the *Xic* was significantly higher in XY,A^{XicTg} ES cells, approaching the high pS value at this locus on the future Xi in females (Figure 5B). This experiment indicates that the transgene can be sensed by the endogenous locus as a homologous copy of the *Xic*. This homology is sufficient to induce an increased frequency of singlets at the endogenous *Xic*, at least in a proportion of cells. It may be significant that by altering the configuration of the *Xic* alone, the single X chromosome in males now exhibits the distinct pattern of singlet frequencies characteristic of the future Xi in females (Figure 5C). Thus, the *Xic* transgene induces the single X chromosome in males to adopt the future Xi state prior to ectopic X-inactivation.

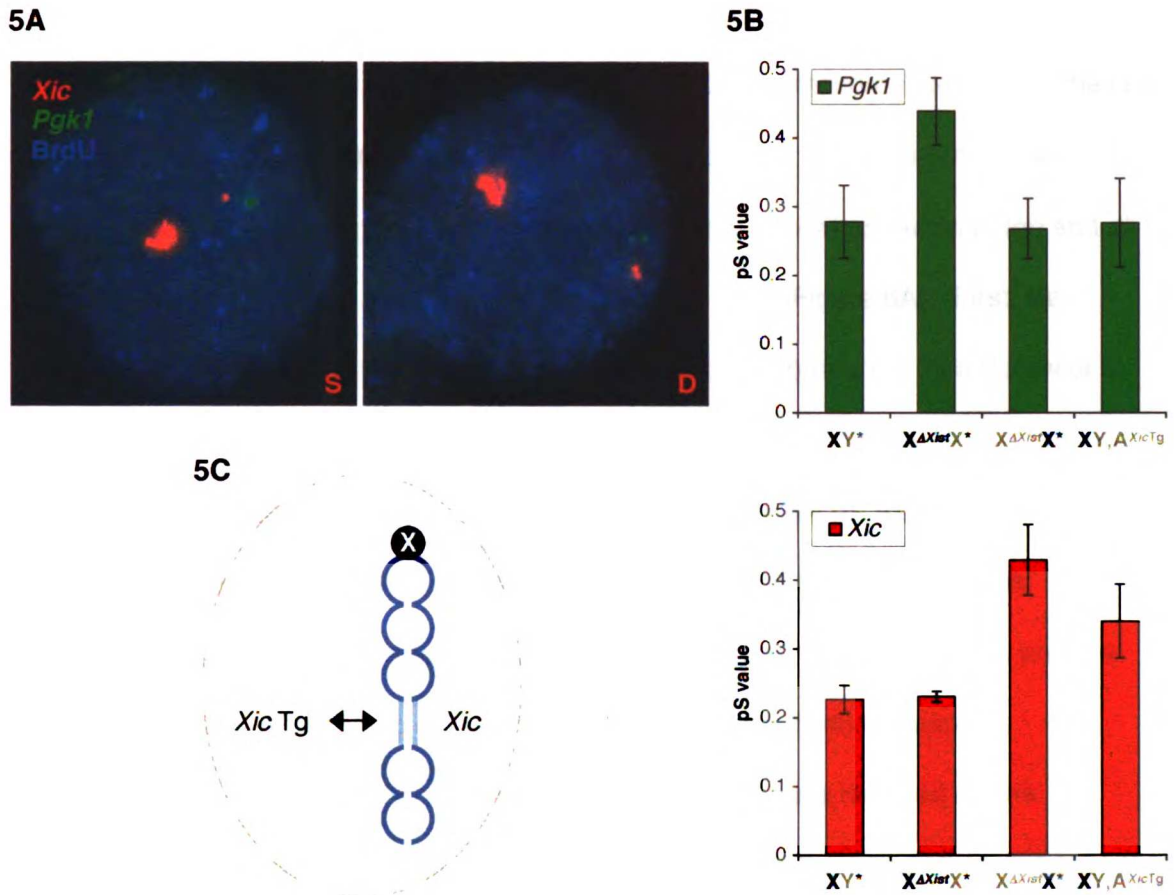


Figure 5. Ectopic *Xic* sequences are sufficient to induce a high frequency of singlets at the endogenous *Xic in trans*.

(5A) FISH for the *Xic* (red) and *Pgk1* (green) in male ES cells bearing an autosomally-integrated multi-copy *Xic* transgene (large domain of *Xic* signal). Examples of S-phase (BrdU-stained, blue) XY,A^{XicTg} cells in which the endogenous *Xic* appears as a singlet (left) and a doublet (right) are shown.

(5B) pS values at *Pgk1* (green) and the *Xic* (red) on the X chromosomes indicated in bold black (the single X in wild-type male, the future Xa and future Xi in female $X^{\Delta Xist}X$, and the endogenous X chromosome in XY,A^{XicTg} ES cells). Data represent average values from 2 to 5 experiments; standard deviations are shown. *Indicated data are also presented in Table 1.

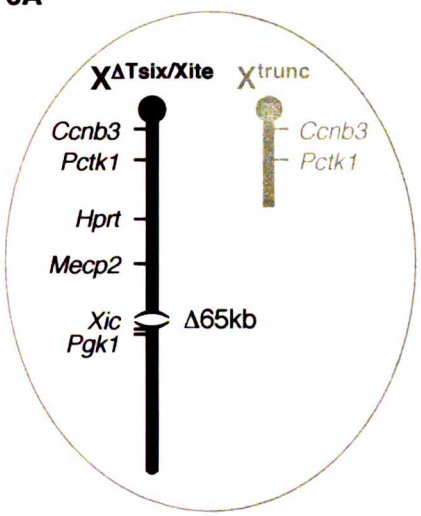
(5C) Graphical interpretation of the influence of an ectopic *Xic* transgene on the behavior of the endogenous X chromosome in a proportion of male ES cells. The transgene (Tg) causes an increased frequency of singlet FISH signals at the *Xic*. The configuration of the single X chromosome now resembles the future Xi in female ES cells.

Sequence requirements for establishing X chromosome states

We investigated the sequence requirements for the establishment of alternative X chromosome states using a cell line in which some X-chromosomal sequences have a homolog, and others do not. In the $X^{\Delta Tsix/Xite}X^{trunc}$ ES cell line, one X chromosome bears a deletion in the *Tsix/Xite* region, and the other is severely truncated, lacking *Hprt* and all distal sequences including the *Xic* (Clerc and Avner, 1998) (Figure 6A). First, we examined the behavior of sequences that lack a homolog, comparing their behavior to that of the same sequences on the $X^{\Delta Tsix/Xite}$ chromosome in a male ES cell background. In the truncation cell line, all three loci examined displayed low pS values (0.23 to 0.25,), comparable to those seen in the male cell line (0.22 to 0.24; Figure 6B). Thus, sequences that do not have a homolog behave like the male X chromosome, and the *Tsix/Xite* mutation is unable to confer the future Xi state on these sequences.

Next, we examined X-chromosomal sequences that are retained on the truncation chromosome. If these are sensed by the second X chromosome, we expect a high frequency of singlet FISH signals at one allele of each locus, resulting in an elevated proportion of SD FISH signals in the population (approximately 40 to 50%; Mlynarczyk-Evans et al., 2006). Alternatively, if sequences are not sensed by the second X chromosome, low frequencies of singlets should persist at both alleles, as seen for autosomal biallelic loci, leading to a low proportion of SD signals (less than 25%; (Mlynarczyk-Evans et al., 2006)). We examined the behavior of two X-chromosomal loci, *Ccnb3* and *Pctk1*, that are present in two copies in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells. Both loci displayed SD FISH signals at high frequency (approximately 45%) at levels indistinguishable between $X^{\Delta Tsix/Xite}X^{trunc}$ and the female control (Figure 6C). This observation suggests that the presence of two copies of these sequences triggers one

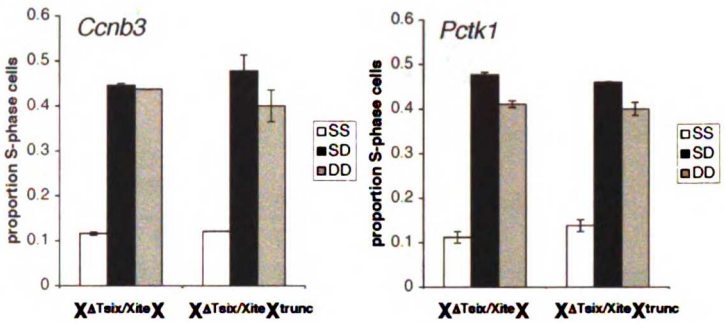
6A



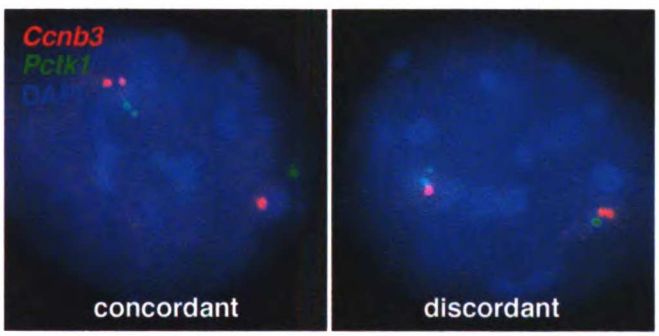
6B

	$X^{\Delta Tsix/Xite}$ in: XY		$X^{\Delta Tsix/Xite}$ in: XX^{trunc}	
locus	**pS =**	st. dev.	**pS =**	st. dev.
Hprt	**0.223**	0.004	**0.225**	0.036
Xic	**0.236***	0.015	**0.254**	0.026
Pgk1	**0.229***	0.021	**0.248**	0.046

6C



6D



6E

cell line	*Ccnb3* & *Pctk1* concordance	n =	p =
XX	65.3%	98	0.002
$X^{\Delta Tsix/Xite}X$	64.8%	128	0.001
$X^{\Delta Tsix/Xite}X^{trunc}$	66.3%	98	0.001

Figure 6. Sequence requirements for X chromosome behaviors.

(6A) Schematic depiction of the X chromosomes in the $X^{\Delta Tsix/Xite}X^{trunc}$ ES cell line.

(6B) pS values for loci on the $X^{\Delta Tsix/Xite}$ chromosome that lack homologous sequences in male and in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells. Average values and standard deviations from 2-3 independent experiments ($n \geq 150$) are presented. *Indicated data are also presented in Figure 4.

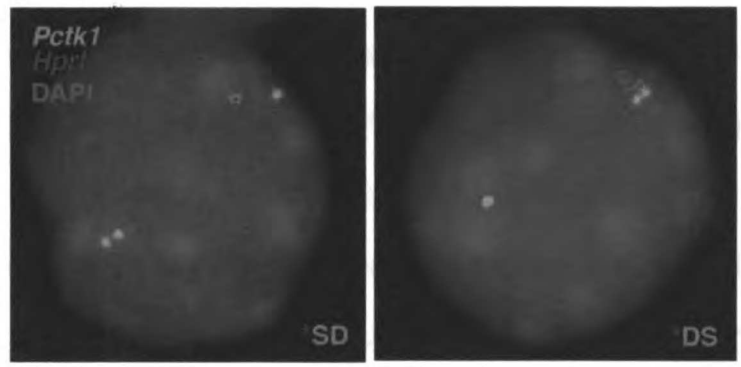
(6C) Frequency among S-phase ES cells of SS, SD, and DD FISH signals at *Ccnb3* (left) and *Pctk1* (right), two X-chromosomal loci that are present in two copies in female $X^{\Delta Tsix/Xite}X$ and $X^{\Delta Tsix/Xite}X^{trunc}$. Average data and standard deviations from 2-3 independent experiments ($n \geq 150$) are presented.

(6D) Left: Concordant and right: discordant SD FISH signals at *Ccnb3* (red) and *Pctk1* (green) in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells. DNA is stained with DAPI (blue).

(6E) Frequency of concordance for *Ccnb3* and *Pctk1* in wild-type female, $X^{\Delta Tsix/Xite}X$, and $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells. p-values, determined using a chi-squared test, indicate that the observed distributions are non-random.

(figure continues next page)

6F



6G

	Pctk1 singlet in SD cells			
	$X^{\Delta Tsix/Xite}$	$X^{(trunc)}$	n =	p =
$X^{\Delta Tsix/Xite}X$	32.4%	67.6%	170	<0.001
$X^{\Delta Tsix/Xite}X^{trunc}$	50.8%	49.2%	183	0.824

6H

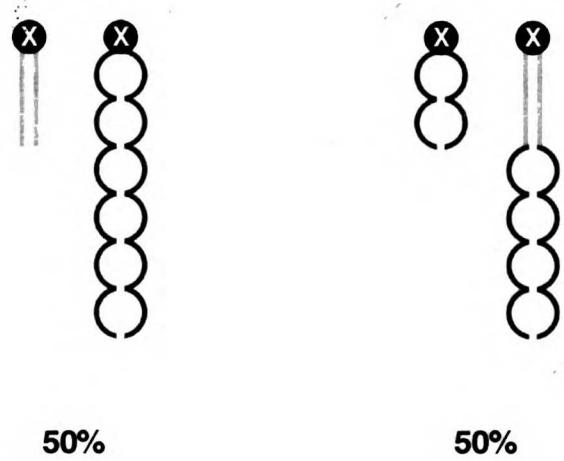


Figure 6. *(continued)*

(6F) Allele-specific FISH for *Pctk1* (red) in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells. SD FISH signals in which the allele on the full-length X chromosome, identified by a probe to *Hprt* (green), appears as the singlet (left, *SD) or doublet (right, *DS) are shown.

(6G) SD FISH signals for *Pctk1* in female $X^{\Delta Tsix/Xite}X$ and $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells were scored allele-specifically using probes to *Mecp2* and to *Hprt*, respectively, to determine the identity of the allele appearing as the singlet. (The *Mecp2* locus is missing from the wild-type X chromosome in the $X^{\Delta Tsix/Xite}X$ ES cell line; see Materials & Methods.) p-values reflect the probability that the observed distributions are random.

(6H) Graphical interpretation of the behavior of X-chromosomal sequences in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells. Sequences that are present in two copies coordinate and adopt two states at random relative to distal sequences that are present in one copy and show a low frequency of singlet FISH signals.

copy to exhibit a high frequency of singlets. Thus, the truncated chromosome appears to be sensed as a second X chromosome even in the absence of the *Xic*.

In female ES cells, linked loci exhibit coordinated behavior along each X chromosome: if one locus on a given X chromosome exhibits a high frequency of singlets, then an adjacent locus on that chromosome does so as well. To determine whether linked loci become coordinated in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells, we performed pair-wise FISH for *Ccnb3* and *Pctk1* and scored the frequency of concordant signals (Figure 6D). A concordance frequency of approximately 65% indicates that X chromosomal sequences are coordinated (Mlynarczyk-Evans et al., 2006). The locus pair displayed 66% concordance in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells, comparable to control female cell lines (Figure 6E). Thus, *Ccnb3* and *Pctk1* exhibit coordinated behavior such that both loci show a low frequency of singlets on one chromosome, and a high frequency of singlets on the other. These data indicate that the homologous portions of the X chromosomes in $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells adopt opposite states as in normal female ES cells.

Deletion of the *Tsix/Xite* region from one X chromosome in female ES cells causes the mutant chromosome to adopt the future Xi state (Figure 4). We asked whether the portion of the $X^{\Delta Tsix/Xite}$ chromosome that has a homolog in the truncation cell line also adopts the future Xi state. This state is characterized by a low frequency of singlets at X-chromosomal loci that are destined to be silenced (Table 1), such as *Pctk1* (Huynh and Lee, 2003). We performed allele-specific FISH for *Pctk1* in $X^{\Delta Tsix/Xite}X$ and $X^{\Delta Tsix/Xite}X^{trunc}$ ES cells and determined the frequency with which the $X^{\Delta Tsix/Xite}$ chromosome displayed the singlet signal (Figure 6F). In the presence of a full-length X chromosome, the $X^{\Delta Tsix/Xite}$ chromosome exhibited a significant bias against singlet FISH signals (Figure 6G), indicating that it adopts the future Xi state as expected. This result extends our

prior observations of a bias in singlet frequency between the future Xi and future Xa (Mlynarczyk-Evans et al., 2006) to a new cell line and a new locus. When the $X^{\Delta Tsix/Xite}$ chromosome is in the presence of the truncated X chromosome, however, both alleles of the *Pctk1* locus displayed singlet signals with equal frequency (Figure 6G). This result is consistent with the interpretation that the portion of the $X^{\Delta Tsix/Xite}$ chromosome with a homolog is in the future Xi state in half of the population, and in the future Xa state in the other half. Therefore, the *Xic* mutation does not act over a distance to control the state of linked sequences that have a homolog, and these sequences randomly adopt a state (Figure 6H). A further conclusion arises from the fact that the $X^{\Delta Tsix/Xite}X^{trunc}$ cell line is clonal, having been derived from a single $X^{\Delta Tsix/Xite}X$ cell in which the truncation event occurred (Clerc and Avner, 1998). In the founding cell, the $X^{\Delta Tsix/Xite}$ chromosome must have existed in the future Xi state, but in the expanded population this chromosome has adopted each state with equal frequency. Therefore, X chromosomal sequences that have a homolog must be able to switch between future Xi and future Xa states.

Discussion

The results of the present study indicate that the X chromosome has the ability to sense homology at multiple locations to regulate chromosomal state. In XX ES cells, the two X chromosomes adopt distinct states that are manifested by distinct frequencies of singlet FISH signals along the future Xi and the future Xa. Neither of these characteristic states is displayed by the single X chromosome in XY or XO cells, and *Xic* mutations that cause an X chromosome to adopt a particular state in an XX cell have no effect in an XY cell. In an XX cell, loss of homologous sequences from one X chromosome causes the

corresponding portion of the other X chromosome to lose its state-specific configuration; while in an XY cell, adding homology causes the corresponding sequence on the single X chromosome to adopt a configuration characteristic of a female-specific state. Together, these observations indicate that the X chromosome senses the presence of its homolog, perhaps on a domain-by-domain basis. In theory, the communication between X chromosomes could be mediated by direct interaction, or by asymmetric partitioning of diffusible factors. Limited access to a unique site in the nucleus, such as appears to be the case for olfactory receptor alleles exhibiting alternative expression states (Lomvardas et al., 2006), seems unlikely given the participation of the entire X chromosome in the communication process and the opposite behavior of the *Xic*. Recent studies demonstrating transient pairing of *Xic* loci upon initiation of X-inactivation did not detect significant interactions in undifferentiated ES cells (Bacher et al., 2006; Xu et al., 2006), or at other loci along the chromosome (Xu et al., 2006). However, interactions restricted to a brief window in the cell cycle, as for homologous copies of imprinted autosomal loci (LaSalle and Lalande, 1996), might not have been detected and remain possible.

In all female ES cell lines examined to date, the cytologically defined future Xi and future Xa states correlate with the fates of the chromosomes as Xi and Xa. In the *Xic* transgene-containing male ES cell line, the apparent shift of the single X chromosome to the future Xi state in at least a proportion of the population is consistent with the 37 to 45% frequency of ectopic X-inactivation these cells are reported to experience (Lee et al., 1999b; Lee et al., 1996). However, in several of the XY or XO cell lines which have mutations affecting *Tsix/Xite* function, none of which display evidence of a shift of the single X chromosome toward the future Xi state, varying frequencies of ectopic *Xist* expression have been reported shortly after induction of

differentiation: 10% to 35% in $X^{Tsix\text{-}pA}Y$ (Luikenhuis et al., 2001; Vigneau et al., 2006), 30% in $X^{\Delta Tsix/Xite}Y$ (Morey et al., 2004), and 60% in $X^{\Delta Tsix/Xite}X^{trunc}$ (Morey et al., 2004). While these observations may indicate that adoption of the future Xi state is not required prior to X-inactivation, an alternative possibility is that apparent ectopic X-inactivation in these XY or XO cell lines reflects deregulation of the *Xist* gene by disruption of *Tsix/Xite* function. In this view, *Xist* expression in these cell lines may not reflect the regulatory mechanisms that control normal X-inactivation in females (Ohhata et al., 2006). By extension, it may be significant that the single X chromosome in normal male cells, which will remain active, does not adopt the future Xa state. Lack of the future Xa state may indicate that the X-inactivation mechanism is not operational in male cells because no homolog has been sensed or "counted". Several recent reports have identified differences between male and female ES cells in levels of X-chromosomal histone methylation (O'Neill et al., 2003) and global DNA methylation (Zvetkova et al., 2005), but the present study is unique in demonstrating a difference between homologous alleles that is specific to females.

In theory, the capacity of a homologous pair of X chromosomes to adopt two states could be conferred by a master control locus for the chromosome, such as the *Xic*. However, a segment of homology between two X chromosomes results in two states even in the absence of the *Xic*. Therefore, the *Xic* is not required for X-chromosomal sequences to exhibit a high frequency of singlets on one homolog, to become coordinated into two distinct states, or to switch between these states such that they become randomized in the population. Instead, multiple *cis*-acting elements dispersed along the X chromosome could confer the ability to adopt two states on adjacent sequences. Indeed, repetitive elements have been proposed to play roles in monoallelic silencing on the X chromosome and autosomes (Allen et al., 2003; Lyon,

2006) both of which are chromosome-wide processes. Domains of monoallelically expressed genes are found distributed along each autosome pair, and in somatic cells these sequences exhibit coordinate replication timing that is early on one homolog and late on the other (Singh et al., 2003). Intervening biallelically expressed sequences exhibit equivalent replication behavior (Singh et al., 2003), and we have found that biallelic sequences do not adopt two states detectable by FISH in ES cells. However, the behavior of random monoallelic sequences remains to be investigated in pluripotent cells. Chromosome-wide homology sensing resulting in two states may turn out to be a general feature of the mammalian genome affecting monoallelic sequences on every chromosome.

Recent evidence suggests that *Xist* is a relative newcomer on the X chromosome, evolving in the mammalian lineage after the split from marsupials, which also achieve dosage compensation by X-inactivation (Duret et al., 2006). The data presented here raise the possibility that the proto-X chromosomes adopted mutually exclusive states before the arrival of *Xist*. Whether or not these two states were already used to achieve dosage compensation remains unclear; however, once *Xist* arrived on the X chromosome, it seems likely that it took control of a pre-existing homology sensing mechanism to achieve random, chromosome-wide gene silencing on a single X chromosome in females.

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Epilogue.

Causes and consequences of homologous alleles adopting alternative states

Epilogue.

Causes and consequences of homologous alleles adopting alternative states

Summary

In this chapter, I revisit several questions that were highlighted in the Prologue in light of the data presented in this thesis:

- How does the mechanism for assigning Xa and Xi fates operate?
- How could this mechanism have evolved?

I also place the data in the context of the literature on sex-specific X chromosome biology. The research presented in this thesis has raised a number of open questions, including:

- What is the molecular basis of the two states?
- How do linked sequences become coordinated in their behavior?
- What is the mechanism of interhomolog communication?
- Do all genes that are destined for random, monoallelic expression exhibit two states?

Approaching these broad questions will require starting with small experiments of limited scope. To this end, I present a number of experimental ideas in the disciplines of genetics, cell biology, and molecular biology that I consider to be feasible and likely to be informative. In some cases I have included unpublished experiments that provide an entrez into the project.

What do two states mean?

Rules that ensure mutually exclusive Xa and Xi fates

The following rules that govern the adoption of X chromosome states show how the system is tuned to ensure inactivation of one X in female cells:

(1) an X-chromosomal locus may adopt the state in which it exhibits a high frequency of singlets only when a homologous sequence is present;

(2) the two states, characterized at the cytological level by low and high singlet frequencies, are mutually exclusive; and

(3) a chromosome is competent for X-inactivation only when the linked *Xic* is in the state which exhibits a high frequency of singlets.

These rules governing X chromosome behavior highlight how the *Xic* employs the system for establishing X chromosome states to restrict the X-inactivation program to a single X chromosome in XX cells.

Beginning to build a new model

Using the data presented in Chapters 1 and 2 as a starting point, I created a model for the assignment of Xa and Xi fates using the standard format employed in the Prologue. In this model (Figure 1), there is asymmetry between two homologous X chromosomes along their lengths before the X-inactivation program is initiated. This asymmetry could be caused, for example, by asymmetric partitioning of a trans factor between them, or by establishment of an alternate conformation of the DNA or chromatin fiber of one of them. The particular configuration of an X chromosome in an undifferentiated cell is proposed to be responsible both for the frequency of singlet FISH signals we observed, as well as for directing silencing to the appropriate sequences

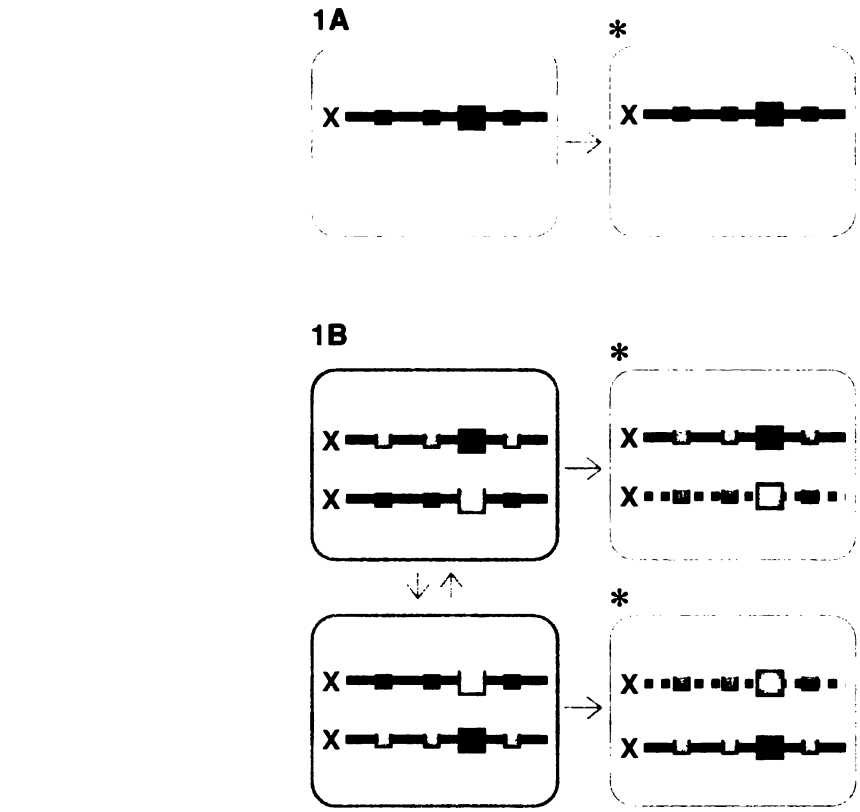


Figure 1. Starting point of a new model for designation of the Xa and Xi.
(1A) The behavior of the single X chromosome in a pluripotent male cell is attributed to coordinate assembly of a default factor/structure (dark blue) along its length. When packaged in this way, the *Xic* (large box) is unresponsive to *Xist* up-regulation upon receipt of a developmental cue (asterisk), and the single X chromosome remains active.
(1B) The differential behavior of the two X chromosomes in a pluripotent female cell is represented by the coordinate assembly of a homology-sensitive factor/structure (light blue) on one chromosome; the *Xic* is assembled oppositely compared to the rest of the X chromosome. The packaging of the two chromosomes is not stable in pluripotent cells, and X chromosomes appear to switch coordinately between states. The bold windows indicate that the identities of the future Xa and future Xi are assigned provisionally in each pluripotent cell. A developmental cue leads to up-regulation (yellow) of the *Xist* allele packaged in the homology-sensitive state, directing silencing factors (pink) to that chromosome, which becomes the Xi.

once a developmental signal triggers initiation of X-inactivation. When there is a single X chromosome present, a default conformation is assumed. When a second X chromosome is present, a homology-sensitive conformation is assumed by one of the two X's, while the other assumes the default conformation. The *Xic* is treated oppositely compared to the rest of the chromosome. In the context of this provisional model, I will reconsider the major themes that were identified in the Prologue as being important for the mechanism for designating one Xa and one Xi.

Regulation by X and autosome number. Under this model, competence for X-inactivation is associated with the presence of a second X chromosome (Chapter 2). It appears that regulation by X chromosome number involves sensing of homology between the two X's, and this occurs on a local rather than a centralized level. The means of sensing X chromosome homology is unclear at present. It is not clear either how this model would operate to ensure inactivation of all but one X in the case of supernumerary X chromosomes, or how ploidy of the cell should be able to direct the mechanism such that inactivation of the appropriate number of X chromosomes occurs. For this reason, future experiments in male and female tetraploid cells will be crucial, as proposed below. If X chromosome states follow the n-p/2 rule, then this will provide further support for the idea that the mechanism that establishes X chromosome states is responsible for the pattern of X-inactivation observed upon differentiation.

Breaking symmetry. Under the model in Figure 1, X chromosomes appear inherently asymmetrical when there are two of them. The ability of asymmetry to affect two entire chromosomes in a coordinate fashion suggests that cooperativity is involved in the establishment of each configuration. It is not clear whether symmetry needs to be broken afresh with each cell cycle, or whether a mechanism operates that keeps the chromosomes asymmetrical even when sister X chromatids segregate in (randomly

assorted) pairs to new daughter cells. Live cell analysis of X chromosome behavior, proposed below, may hold the key to understanding how asymmetry is established and/or maintained in this system.

Switchlike behavior. Under this model, adoption of the future Xa and future Xi states is mutually exclusive, thus ensuring all or nothing responses in assignment of Xa and Xi fates. Because we do not understand the molecular basis of X chromosome states, it is not clear how the system could be tuned to achieve this proposed exclusivity. Determination of whether or not states are always mutually exclusive is important to understanding the true mechanism and could be investigated by live cell analysis as described in detail further below. Once the states are assigned, they must be popagated properly; this could be accomplished by cooperative spreading of unknown factors or structures along each X chromosome, contributing to the switchlike nature of assignment of Xa and Xi fates.

Apparent randomness. In this model, apparent randomness of the assignment of Xa and Xi fates is achieved by the concerted alternation of the homologous X chromosomes between the future Xa and future Xi states in the pluripotent population. It is not clear whether this alternation is stochastic, or whether a reproducible pattern of state switching is followed. In theory, either could result in randomization of states in the pluripotent population (Williams and Wu, 2004). I suspect that the alternation will occur in a stochastic pattern because of the ability of the system to support partially non-random distributions such as the 80/20 129/*cas Xce* effect observed in Chapter 1—this could be achieved if the X chromosomes are competing to adopt a particular state, and their fitness is slightly different such that one of them "wins" more frequently than the other. If true, this idea would suggest that the state of a given X chromosome in a

daughter cell would not be predictable based on its state in a parent cell. An experimental approach to address this issue will be presented below.

Developmental regulation. The data on which the model in Figure 1 is based suggest that in each ES cell, X chromosome fates have been provisionally assigned even though the initiation of X-inactivation has not yet been triggered. Thus, the cellular mechanism for designating the Xa and Xi operates before the signal is received to initiate X-inactivation, and is mechanistically separable from the silencing phase. This finding suggests that pluripotent female cells may await a critical developmental cue that directly initiates the silencing program. This cue might, for example, lead to availability of trans factors that are necessary for *Xist* to spread and silence the designated future Xi. Recent studies indicate that the developmental cue may trigger *Xic* pairing (Bacher et al., 2006; Xu et al., 2006), raising the possibility that the critical trans factor may actually be the homologous *Xic* allele (Marahrens, 1999). The dependence of silencing on *Xic* pairing would provide a safeguard to ensure that X-inactivation is only initiated on one X chromosome in XX cells.

An evolutionary path from alternative states to exclusive inactivation of one X?

How could a system in which homologous X chromosomes have the capacity to adopt two distinct states have evolved? As suggested in the Discussion in Chapter 2, the possibility that alternative states are also a feature of autosomal genes that are destined to be expressed in a random monoallelic fashion could have provided an evolutionary starting point. Because the X and Y chromosomes were originally a pair of regular autosomes, one could posit that a system of two states existed, at least at a subset of loci, on the proto-X chromosome before the need for dosage compensation arose. As the proto-Y chromosome began to decay, male cells needed to upregulate X-

chromosomal genes that had lost their homologs. The ability to develop a long-range two-fold activation system on the X chromosome could have been facilitated by the posited pre-existing capacity to coordinate behavior of distant sequences along the chromosome. Perhaps distinct sequence features that marked the subset of loci that were originally subject to two states began to be acquired by additional loci that needed to achieve coordinate upregulation.

After the evolutionary step of X-upregulation was accomplished, the capacity for chromosome-wide alternative states would have come in handy again in female cells, which now found themselves suffering from two-fold hyperactivity of X-chromosomal genes. Female-specific two-fold downregulation of X-chromosomal gene expression could most easily be achieved by taking advantage of existing *cis*- and *trans*-regulatory features of the X chromosome. First consider the evolutionary path followed by placental mammals under this view. X chromosome behavior (adopting two states) was already regulated *in trans* by copy number, one of the most seemingly complex features of *Xic* locus regulation, before the silencing function of this locus evolved. In addition, the groundwork for *cis*-limited silencing by *Xist* was already laid by the prior existence of two regulatory features on the X chromosome: the mechanism for establishing alternative states, and the mechanism for spreading chromosome-wide hyper-expression to loci that did not have homologs on the Y. If these *trans*- and *cis*-regulatory features were already in existence, then less imagination is needed to envision how the *Xist*-mediated random and exclusive silencing of one X chromosome in females came into being.

It appears that different mammalian lineages independently solved the problem of how to counteract hyper-expression of X-chromosomal genes in females: whereas in placental mammals, a *cis*-acting silencing locus that acted randomly and exclusively on

a single X in females was invented, in marsupials, an imprinting mechanism that silenced the single paternally-inherited X in females evolved. The set of assumptions about ancestral X-chromosomal gene regulation (pre-existence of two states and hyper-expression) presented here leads to a parsimonious view of evolution of dosage compensation in mammals. The ancestral regulatory environment on the X chromosome could have led different mammalian lineages to separately invent chromosome-wide silencing of a single X in females—but using different approaches to assignment of Xa and Xi fates, and independent deployments of silencing machinery.

On the differences between one X and two

The research presented in this thesis appears to define a new category of sex-specific features of ES cells. As shown in Chapter 2, in pluripotent cells the X chromosome behaves differently depending on whether or not there is a homologous partner present, representing a difference between the cell biology of male and female ES cells. As shown in Chapters 1 and 2, the presence of two X chromosomes in female ES cells results in differentiation between them, such that wild-type X chromosomes differ from each another in females. While several features have been reported to differ between XX and XY ES cells, the non-equivalent behavior between the two genetically equivalent X chromosomes in XX cells appears to be unique in the literature. (Heterozygous mutations at the *Xic* can cause differences in chromatin composition between the two alleles prior to X-inactivation (Sun et al., 2006), but none of these differences has been reported to mark the X chromosomes differentially in wild-type cells.)

Several studies have noted differences between the X chromosomes of female and male ES cells. The presence of two (active) X chromosomes in ES cells has

recently been found to lead to hypomethylation of the entire genome (Zvetkova et al., 2005). As a result, DNA methylation is more complete in male ES cells than in females at X-chromosomal and autosomal sites, including at imprinting centers. This lost methylation is restored upon elimination of an X chromosome from the female ES cell lines. The authors proposed that double expression of a hypothetical X-linked modifier of DNA methyl-transferase activity could be responsible for the female-specific phenotype (Zvetkova et al., 2005). This study suggested a possible explanation for old observations of the methylation patterns at the promoter and 5' end of the *Xist* gene in ES cells. In several studies, these sites were found to be fully methylated in male ES cells (Norris et al., 1994), while they were approximately 50% methylated in female ES cells (Norris et al., 1994; Penny et al., 1996; Sado et al., 1996). An attempt to address the issue of whether hemimethylation represented one fully methylated allele and one unmethylated allele, or two mosaically methylated alleles, indicated that the latter interpretation was more likely (Sado et al., 1996). The new study (Zvetkova et al., 2005) provides a potential explanation of these results and suggests that the DNA methylation patterns at the *Xist* promoter are not a cause of future differential regulation of *Xist* alleles when X-inactivation is initiated.

Other studies examined histone modifications in female vs. male ES cells. It has been reported that the X chromosomes in female ES cells exhibit two-fold or greater hyperacetylation of H3 and H4, hyper(di)methylation of H3-Lys4, and hypo(di)methylation of H3-Lys9—all trends which correlate with gene activity—relative to the single X chromosome in males or to autosomal loci (O'Neill et al., 2003). The restriction of this trend to the X chromosome suggests that it is not accounted for by the global effects of the presence of two active X chromosomes in female ES cells (Zvetkova et al., 2005). Perhaps this specific pattern of chromatin marks on the X

chromosomes could be related to the proposed two-fold hyperactivity of X-linked genes relative to the autosomes, regardless of sex (Nguyen and Disteche, 2006). However, normalization of the data should have accounted for X chromosome copy number differences between females and males, leaving the two-fold difference between the sexes unexplained. A study from another group noted no significant difference between males (data not shown) and females in levels of hyper(di)methylation of H3-Lys4 at X-chromosomal genes, and argued that this mark was restricted to promoter regions of X-chromosomal genes as well as to autosomal imprinted genes, therefore representing a feature of monoallelically expressed genes (Rougeulle et al., 2003). In the study where differences were detected between males and females, analysis of allele-specific ratios of histone marks in female ES cells that will undergo partially or fully non-random X-inactivation due to the *Xce* effect (80/20) or to disruption of *Tsix* on one allele (100/0) showed no enrichment on either X, regardless of fate (O'Neill et al., 2003). Furthermore, the active chromatin features were common to genes that will exhibit three distinct expression profiles after X-inactivation: monoallelic from the Xi (*Xist*) or the Xa (X-linked genes subject to silencing), or biallelic (escape genes) (O'Neill et al., 2003). These active chromatin marks drop upon inactivation of one X chromosome until comparable levels are seen in males and females (O'Neill et al., 1999; O'Neill et al., 2003). These latter results show that the phenotype is a feature of both X chromosomes relative to autosomes and is unrelated to chromosome fate or future expression status.

A female-specific hotspot of H4–Lys8 and –Lys16 acetylation upstream of *Xist* has been reported in ES cells (O'Neill et al., 1999). This hotspot is present only in XX and not in XY ES cells, thus appearing to depend on X chromosome dosage. Heterozygous deletion of *Xist* exon 1 reduces acetylation levels by half, though they are still elevated above males, indicating that sequences in *Xist* exon 1 are required *in cis* to

achieve the hyperacetylation (O'Neill et al., 1999). Because the mutant X chromosome is likely to be designated the future Xa in a primary manner (Gribnau et al., 2005; Marahrens et al., 1998), contrary to the original interpretation (Penny et al., 1996), the result also suggests that levels of this histone mark may be comparable on the future Xa and future Xi in female ES cells.

One final thought on the differences between males and females bears mentioning. It was recently reported that the minimal *Xist* promoter becomes active in an X chromosome dosage-dependent manner. A randomly integrated transgenic construct in which a 540 bp *Xist* promoter fragment was fused to luciferase resulted in specific upregulation of luciferase activity upon differentiation of female, but not male ES cells (Sun et al., 2006). This result has only been reported by one group, with analysis of pools of transgenic colonies used to circumvent position effect differences due to differing integration sites in the male and female ES cell lines. The result is intriguing and bears repeating in a more controlled fashion using targeted integration into the same site in male and female ES cell lines, as well as comparison to a control promoter that should not exhibit sex-specific upregulation. If confirmed, this result would suggest that information about a cell's X chromosome dosage is used to control the availability of specific trans factors that upregulate *Xist*.

Where do we go from here?

Genetics of X chromosome behavior

Analysis of male and female tetraploid ES cells

Xa and Xi number are regulated by a cell's ploidy, according to the n-p/2 rule. If future Xa and future Xi states always correlate with X chromosome fates, then clear predictions

are made for X chromosome behavior in tetraploid ES cells (Figure 2). In female tetraploids, we should see two X chromosomes exhibiting the future Xa state, and two exhibiting the future Xi state. Analysis in male tetraploids is a very interesting case, because now two homologous X chromosomes will be present in the same nucleus, and yet no future Xi state should be established. The two X chromosomes in male tetraploids should behave similarly to biallelic autosomal loci as was characterized in Chapter 2. If this result is indeed observed, it would imply that the ability to sense homology requires a 1:1 ratio of autosomes to X chromosomes. Please also bear in mind that occasional Xi's were reported in male tetraploid embryos (Prologue, Table 1), suggesting some leakiness in the mechanism that assigns Xa and Xi fates—this result should be confirmed in differentiations of male tetraploid ES cells. The expected frequencies of FISH signal patterns for various possible outcomes of these experiments are modeled in Figure 2. For male cells, coordination analysis should also be performed for pairs of linked loci: if no states are adopted, then 50/50 distribution of concordant and discordant signals should be seen.

It should be straightforward to obtain or generate appropriate tetraploid ES cell lines for these experiments. Tetraploid (and presumably male) ES cells are commonly used in the production of chimeric mice to produce an extraembryonic component (e.g., Ohhata et al., 2006) and can be obtained from another lab. Because female tetraploids have more alleles to assay, the possible outcomes of the experiments under various models are less dramatically different; therefore, the ability to distinguish the future Xa and future Xi by allele-specific FISH is important (Figure 2B). This will require generating a new reagent, as such female tetraploid ES cells are unlikely to exist already. I don't think it will be difficult, though, as tetraploid ES cells arise spontaneously in my female ES cell cultures, and tetraploidy could also be induced by treatment with

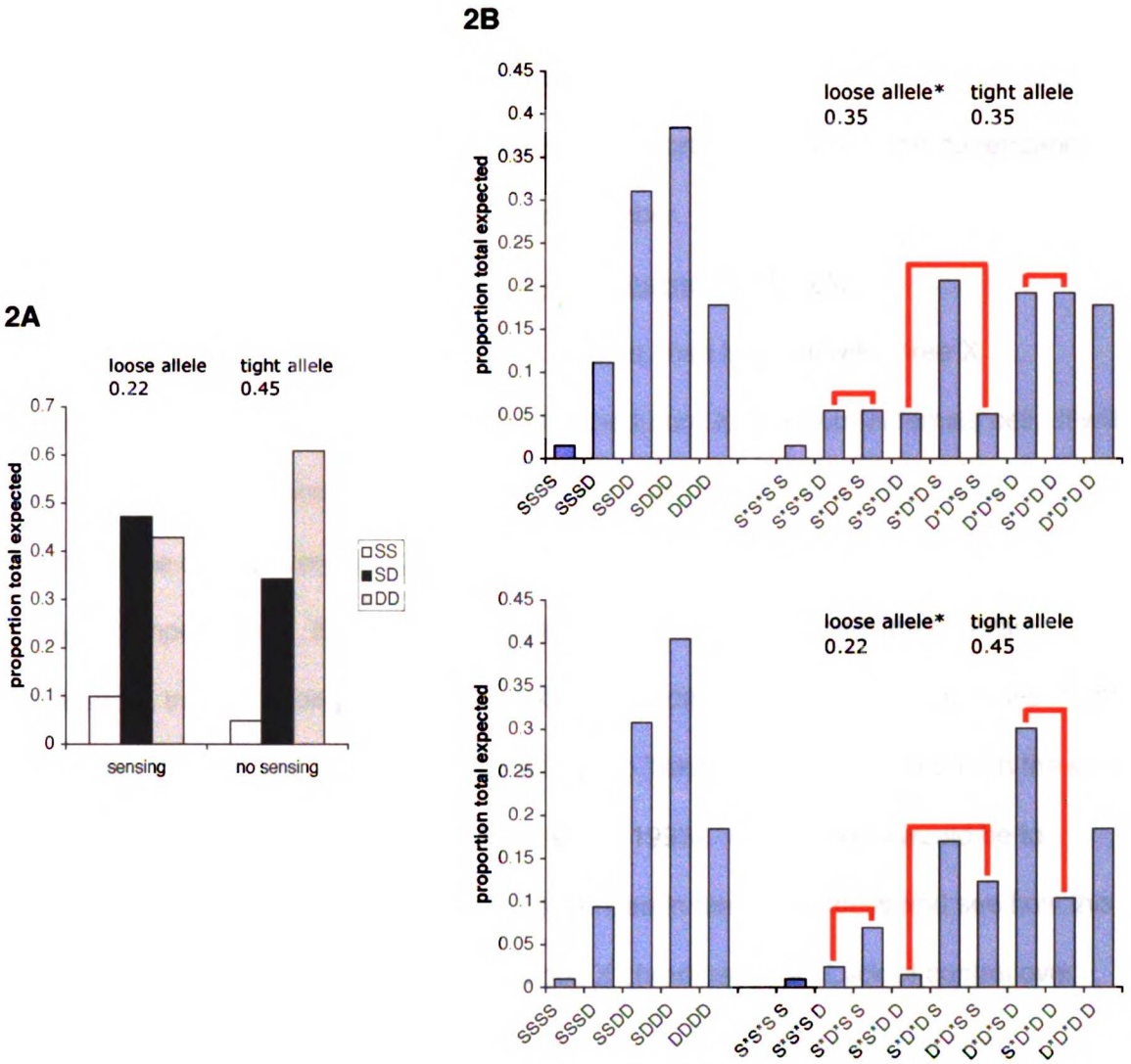


Figure 2. Predictions for behavior of X chromosomes in tetraploid ES cells.
(2A) For male tetraploid ES cells, the expected proportions of SS, SD, and DD FISH signals (based on the indicated pS values) at X-chromosomal loci are plotted under two models: sensing, in which the two X chromosomes in a male tetraploid cell behave like the two X chromosomes in a female diploid cell; and no sensing, where the two X chromosomes in a male tetraploid behave like biallelic loci on two autosomes in a diploid cell (both "loose alleles").
(2B) For female tetraploid ES cells, the expected frequencies of FISH signal patterns are depicted under models in which all alleles behave in an equivalent fashion (top, all alleles same average pS value), or in which two alleles adopt the future Xa and two the future Xi state (bottom, low and high pS values at two alleles each). The plots on the left show the expected distribution of the 5 signal patterns when alleles are not marked; these are essentially indistinguishable between top and bottom. The plots on the right show the equivalent analysis in an allele-specific fashion when alleles have fixed fates, with either the future Xi or the future Xa specially marked (*). Red brackets highlight important differences between signal classes under the two models.

cytochalasin B/D to block cytokinesis; it should therefore be possible to clone out tetraploid lines from any existing female ES cell line with marked alleles of fixed fate. The outcomes of these experiments will provide important insight into the dependency of X chromosome behavior on X and autosome number.

Can we assay a "trisomy" for the X chromosome in ES cells?

If X chromosome states determine Xa and Xi fates, then in a cell with three X chromosomes, the third X should behave like the future Xi in a normal female cell. It will be challenging to perform this experiment as ES cells do not tolerate increases in X chromosome dosage very well—even a normal XX cell is compromised by the lack of dosage compensation. It might be possible to obtain female ES cells that contain unbalanced translocation products of the X chromosome, where some sequences might be present in three copies. I would look at Nobuo Takagi's group's papers from the early 1990s (e.g., Shao and Takagi, 1990; Tada et al., 1993). Another idea would be to integrate transgenes of X-chromosomal sequences in female ES cells and see how this affects corresponding loci on the endogenous X chromosomes. Lack of control over copy number makes the latter experiment messy. Another alternative involving autosomal trisomies is discussed further below.

Identification of critical sequences for *trans*-sensing

The presence of an *Xic* transgene is sensed by the single X chromosome in male ES cells, affecting the behavior of the endogenous *Xic* (Chapter 2). To investigate the sequence requirements for the X chromosome homology sensing that we have detected in ES cells, a panel of transgenes could be tested in male ES cells. One would look for an increased frequency of singlet FISH signals at the endogenous locus that is homologous to the transgene used. This approach could be used to determine the

minimal size of homology and perhaps particular elements at the *Xic* and other loci that elicit a response in the endogenous X chromosome.

Is the *Tsix/Xite* region on one X critical *in trans* for the second X to upregulate *Xist*?

It has been suggested that the *Tsix/Xite* region is important for X chromosomes to communicate via physical pairing during the initiation of X-inactivation. Multicopy *Tsix/Xite* transgenes were reported to inhibit either X chromosome from inactivation in transgenic female ES cells (Lee, 2005), as well as to cause ectopic interactions between the transgene and the endogenous X chromosome that were proposed to interfere with X-inactivation (Xu et al., 2006). Heterozygous deletions of *Tsix* and/or *Xite* disrupted the transient colocalization between *Xic* alleles on homologous X chromosomes (Bacher et al., 2006; Xu et al., 2006), although X-inactivation still occurred in these cells. The inactivation that occurs in these cells is non-random, with the mutant X being inactivated, and in one view this X-inactivation is disregulated and abnormal, exhibiting advanced kinetics (see Appendix 1). Perhaps, therefore, these X chromosomes do not need to pair in order to be inactivated, and so that step does not occur. The wild-type and mutant X chromosomes in these cell lines do adopt the future Xa and future Xi states, respectively (Chapter 2), suggesting that the cells are subject to the usual early regulatory mechanisms. An important experiment to address whether the colocalization of *Xic* loci is required for establishment of Xa and Xi fates in a mutually exclusive manner would be to delete *Xist* from a *Tsix/Xite* mutant chromosome, such as in the $X^{\Delta Tsix/Xite}X$ (Δ65 kb) cell line (Clerc and Avner, 1998). (For comparison, it might be interesting to do this in the $X^{Tsix\text{-}pA}X$ ES cell line where *Tsix* function is disrupted without removal of the genomic *Tsix/Xite* sequence (Luikenhuis et al., 2001).) *Xist* mutations are epistatic to

Tsix (Sado et al., 2005; Sado et al., 2001), thus the $X^{\Delta Xist/Tsix/Xite}$ chromosome will not be able to become the Xi. Now, what happens to the wild-type X chromosome *in trans*? Does it adopt the future Xi state in ES cells (and the mutant X chromosome the future Xa state)? Does the wild-type X inactivate upon induction of differentiation? The answers to these questions will establish the connections between the X chromosome states observed in ES cells the transient pairing of *Xic*'s observed upon differentiaton. This information will provide important insights into the mechanism of mutually exclusive assignment of Xa and Xi fates.

Are two states really independent of the *Xic*?

The experiments in Chapter 2 indicated that when X-chromosomal sequences are present in two copies, they adopt two states even if the *Xic* is not linked. It would be nice to confirm that this is also the case in an otherwise normal female ES cell, by deleting the *Xic* from both alleles. This could be accomplished by deleting the major *Xic* region from the wild-type X chromosome in the $X^{\Delta Xist/Tsix/Xite}X$ cell line generated to address the previous question. The prediction would be that the X chromosomes would adopt and alternate between states, but that X-inactivation would be impossible.

Cell biology of SIAR

Background and rationale

The observations presented in this thesis show that in ES cells, distinct frequencies of singlet and doublet FISH signals characterize genomic sequences that are subject to random silencing on the X chromosome or to equivalent, biallelic gene regulation. What is the underlying cell biological cause of the appearance of a locus as a singlet or a doublet, and why does it vary between sequences with different expression

characteristics? The answers to these questions will be of interest to communities studying nuclear localization and chromosome dynamics.

The appearance of a locus as a singlet or doublet by FISH in samples in which nuclear structure is preserved may reflect the degree of cohesion between the two sister chromatids; alternatively, it may reflect stretching of the chromatin fiber, for example division of a genomic region into physically separated domains. The former possibility is supported in our cell cycle analysis of FISH signals by the general trend toward doublets appearing after replication of a locus, when the second sister is produced, and later increasing in abundance when sister chromatids condense and become physically partitioned in preparation for cell division (Chapter 1, Figure 2D). If FISH signals reflect the cohesion of sisters, then after replication, that locus may theoretically appear either as a doublet (sisters separated sufficiently to be distinguished by fluorescence microscopy) or as a singlet (sisters not separated sufficiently). Based on empirical evidence, I estimate that a FISH signal from hybridization of a BAC probe shows an approximate diameter of 50-100 nm, and that the centers of two foci must be at least 50 nm distant to be distinguished as a doublet.

Assuming the singlet and doublet FISH signals reflect sister proximity, then the frequency of singlets (pS value) at a locus could reflect that:

- after replication, there is a timed loss of cohesion such that sisters are tightly apposed before (singlet FISH signals) and separated after (doublet signals) this event.
- there is a constant degree of cohesion for the entire post-replication, pre-condensation cell cycle with loci exhibiting high pS values being more tightly apposed as diagrammed in Chapter 1, Figure 6A. Imagining this scenario in live cells, sisters may be fluctuating in their proximity throughout this period;

alternatively, they may not be fluctuating but the factors that hold sisters exhibiting high pS values together may be more resistant to disruption during the fixation and FISH procedure than loci that show low pS values.

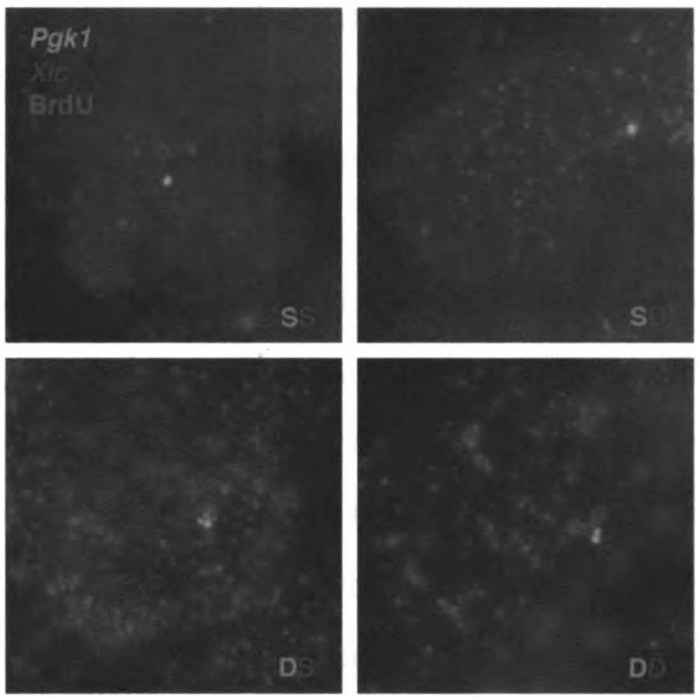
The data and modeling presented in Chapter 1 support this second interpretation. In the following section I will present further experiments geared at understanding the chromosome dynamics that underlie FISH signal patterns for X-chromosomal loci.

Linked loci in a given state appear as singlets or doublets independently *(experiment completed)*

The frequency of singlets observed by FISH at a particular locus may arise from a cohesive property of the sister chromatids. The pS value may reflect tight cohesion of sisters (appearance as a singlet by FISH) for a discrete portion of the cell cycle, and loss of cohesion (appearance as a doublet) for the remaining period. In the simplest version of this scenario, multiple loci on the same chromosome would be expected all to appear as singlets or as doublets in a dependent fashion, to the extent that their individual pS values allow. Alternatively, the pS value may reflect a constant degree of cohesion between sisters, with singlet and doublet FISH signals arising throughout the cell cycle due to chromatid breathing or sensitivity to disruption during sample preparation. In this second scenario, each locus should exhibit a singlet or a doublet FISH signal, at the frequency dictated by its individual pS value, independently of any other locus on the chromosome.

To determine whether sequences behave dependently or independently, we examined X-chromosomal loci pair-wise in male ES cells. S-phase cells were scored as showing a singlet FISH signal for both probes (SS), a singlet for one and a doublet for the other (SD or DS), or a doublet for both (DD) (Figure 3A). Using the pS values presented in Chapter 2, Table 1, we calculated the expected distribution of each signal

3A



3B

Dependent model

given: $(pS_{Pgk1}) > (pS_{Xic})$

SS = (pS_{Xic})

SD = $(pS_{Pgk1}) - (pS_{Xic})$

DS = 0

DD = $(1\text{-}pS_{Pgk1})$

Independent model

SS = $(pS_{Pgk1}) \cdot (pS_{Xic})$

SD = $(pS_{Pgk1}) \cdot (1\text{-}pS_{Xic})$

DS = $(1\text{-}pS_{Pgk1}) \cdot (pS_{Xic})$

DD = $(1\text{-}pS_{Pgk1}) \cdot (1\text{-}pS_{Xic})$

3C

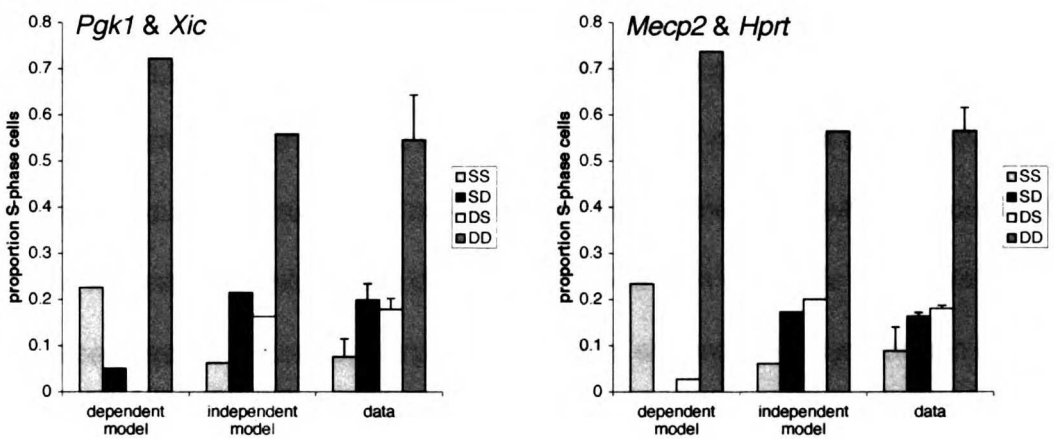


Figure 3. Loci exhibit singlet or doublet FISH signals independently along the chromosome. **(3A)** FISH for two loci, *Pgk1* (red) and the *Xic* (green), in male ES cells. BrdU immuno-staining (blue) identifies S-phase nuclei. The four possible FISH signal patterns are shown (*Pgk1* is listed first, the *Xic* second): SS, SD, DS and DD. **(3B)** Dependent and independent models for the behavior of two loci on a single X chromosome. In the dependent model, linked loci simultaneously appear as singlets or as doublets to the extent allowed by their individual pS values. In the independent model, each locus exhibits a singlet or a doublet FISH signal in accordance with its pS value independently of the other locus. Formulas show calculations of the proportions of cells that should display each signal class under the models. **(3C)** Scoring of FISH signal patterns in male ES cells for two locus pairs on the X chromosome (*Pgk1* and *Xic*; *Mecp2* and *Hprt*) closely fits the independent model for behavior of linked loci. The FISH signal proportions expected under each model were calculated as in 3B using the pS values presented in Chapter 2, Table 1. Data represent average values from 2 experiments; standard deviations are shown.

class under the dependent and independent models (Figure 3B) for two locus pairs, *Pgk1* and the *Xic*, and *Mecp2* and *Hprt*. The observed distribution of FISH signal patterns closely matched the independent model for behavior of linked loci (Figure 3C). These data are consistent with the idea that X-chromosomal loci exhibit a constant degree of cohesion throughout S-phase, and that each locus independently displays singlet and doublet FISH signals in accordance with its individual pS value. An important implication of this finding is that pS values can be strictly coordinated along a chromosome (for example, all low on the male X chromosome), but in any individual cell, the actual appearance of two loci will be discordant at a predictable frequency. This conclusion is consistent with prior observations of concordance and discordance frequencies in female ES cells (Mlynarczyk-Evans et al., 2006).

What do sister chromatid dynamics look like in live cells?

The independent behavior of linked loci in the FISH assay raises the possibility that sister chromatids are fluctuating or breathing *in vivo*. Furthermore, when a locus is in the future Xa state it should exhibit different dynamics than when it is the future Xi state. It should be possible to develop a system to monitor chromosome dynamics in live cells. Joost Gribnau has generated and provided to us female ES cell lines ("58Δ40" and "72Δ24"; Gribnau et al., 2005) with a multicopy array of tet operator sequences integrated at the *Xic* on the 129 allele in the female ES cell line 2-1, which is the future Xi in 80% of cells (Chapter 1). These cells could be transfected with a GFP-tet repressor fusion protein and subjected to live cell imaging to observe the dynamics of the tet-GFP spots, which ought to reflect sister chromatid dynamics as the FISH spots are thought to do. In 80% of cells, the dynamics of the *Xic* should reflect the future Xi state, where singlet FISH signals are common; in the remaining 20%, dynamics should reflect the

future Xa state, where singlets are rare. This analysis would provide important information about the cell biological basis for the frequencies of FISH signals that characterize the future Xi and future Xa in fixed samples. [Note: Edith Heard's group has developed a similar system for live cell imaging of the *Xic*, presented at the 2005 Gordon Conference on Epigenetics, which she is using to follow up on her lab's FISH-based observations that the two *Xic* alleles in female ES cells come into close proximity at the onset of X-inactivation triggered by ES cell differentiation (Bacher et al., 2006). Therefore, it might be possible to collaborate with her to address the issue of sister chromatid fluctuations.]

The above system could be adapted to simultaneously monitor both *Xic* alleles in live female ES cells, with differential labeling of the future Xa and the future Xi. A lac-operator array could be targeted to the *castaneus* X chromosome in Joost Gribnau's cell lines, at the same time introducing an *Xist* mutation that would fix the identities of the *castaneus* and 129 X chromosomes as future Xa and future Xi respectively (for example, by adapting Morgan's *castaneus* ΔA-repeat construct to contain a lac-operator array). Thus, use of two different fluorescent fusion proteins would allow simultaneous observation of *Xic* locus dynamics on the future Xa and the future Xi. This system would be ideal for examining relationships between the homologous chromosomes, though one should keep in mind that this area is already being explored by Edith's group.

Does X chromosome homology sensing involve direct interactions between homologs?

To begin to approach the question of how homology is sensed by the X chromosomes, it would make sense to perform careful analysis of interhomolog distances across the cell cycle. Perhaps homology is sensed by transient physical interactions that were not detected in ES cells in recent studies that did not control for the stage of the cell cycle

(Bacher et al., 2006; Xu et al., 2006). At imprinted loci, interchromosomal interactions were shown to occur in a cell cycle-regulated manner (LaSalle and Lalande, 1996). This question could be addressed in live cells using the two-color imaging system described above, or it could be done with fixed cells using FISH. One would measure inter-*Xic* distances across the cell cycle in female ES cells. I anecdotally noted a higher frequency of cells in G1-enriched fractions in which I detected only one spot (singlet or doublet) by FISH for an X-chromosomal locus in female ES cell samples. It is worth checking whether these cells represent XX ES cells in which the two alleles of X chromosomal loci are physically paired. Cell cycle synchronization is critical to this experiment, and I have tried several different approaches. I have found that fractionation of live ES cells by DNA content as in Chapter 1 produces cell cycle fractions that are reasonably well separated and amenable to microscopy. Synchronization by mimosine arrest and release (Gribnau et al., 2003; Chapter 1) gives finer cell cycle resolution, but results in lots of dead cells and leaves nuclei prone to autofluorescence, thus making it less than ideal prior to microscopy.

What are the rules for switching between states?

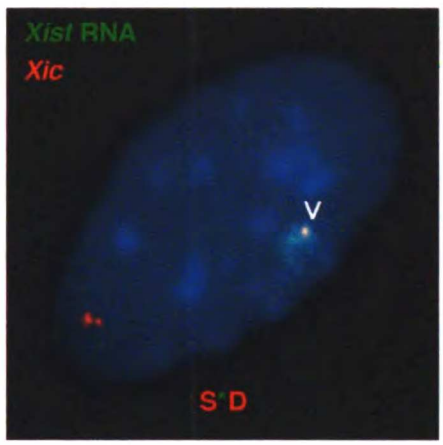
Analysis of clonal ES cell lines poised for random X-inactivation indicated that X chromosomes can switch states (Chapter 1). However, the timescale and pattern of this switching were not addressed. It was also not formally shown that X chromosomes always occupy states in a mutually exclusive manner. All of these questions could be addressed with a live cell imaging system as described above. Use of wild-type cells exhibiting 50/50 X-inactivation would be informative, though the experiments could also be done in the cell lines from Joost Gribnau that show 80/20 inactivation, because these cells still exhibit state switching (Chapter 1, Figure 5D). Only one allele need be monitored to begin this line of investigation.

First, by observing single live cells for long periods of time, one could determine whether an X chromosome exhibits a constant state in a given cell cycle, or whether it switches states within this timeframe. (The approach to defining characteristic behavior of each state was discussed two sections ago.) Then, by observing single cells through division, one could determine whether there is any pattern to the states the chromosome adopts in a pair of daughter cells. The possibilities are, relative to its state in the parent cell: same and same, opposite and opposite, or same and opposite. The relative frequencies of these patterns among daughter cell pairs would reveal the rules that govern switching. For example, switching might occur truly at random among daughters, one daughter or both daughters might always switch, or switching may be rare. This type of analysis was used to define the rules that govern mating type switching in yeast (reviewed in Broach, 2004). Finally, by using cells in which both alleles of an X-chromosomal locus are marked, one could determine whether X chromosome states are always mutually exclusive. Another fun idea would be to monitor both the *Xic* and another X-chromosomal locus on the same chromosome, and observe the dynamics of oppositely-behaving sequences on the same chromosome. I have tried to think of ways to address these questions by FISH in fixed cells, but the data that could be collected are unlikely to be conclusive. The problem is that because loci show singlets in both states, even though they do so at different frequencies, FISH in a single cell cannot definitively show a chromosome's state.

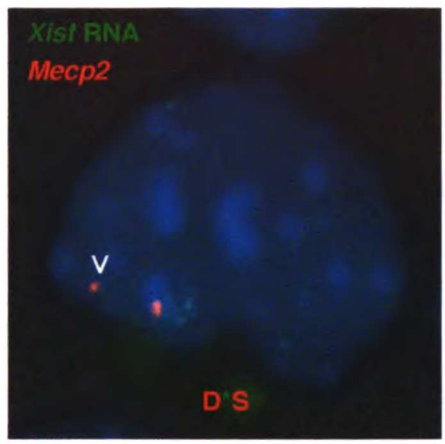
Switching stops when X-inactivation occurs *(experiment completed)*

We proposed that switching of X chromosomes between states in pluripotent cells generates a population in which each X chromosome is marked as the future Xi in 50% of cells (Chapter 1). Thus, switching could provide the basis for the apparently random pattern of X-inactivation in the population. One prediction of this hypothesis is that the

4A



4B



4C

	% singlet in SD cells			
locus	Xa	Xi	p =	(n)
Xic	30	70	0.001	(70)
Mecp2	67	33	0.004	(70)

Figure 4. X chromosome states become fixed upon initiation of X-inactivation.
(4A) Characteristic SD FISH signal pattern for the *Xic* (red) in differentiating day 3 EB cells. The accumulation of *Xist* RNA (green) on the Xi distinguishes it from the Xa. Carat marks the expressed allele, which tends to appear as a singlet.
(4B) Characteristic SD FISH signal pattern for *Mecp2* (red) in day 3 EB cells. *Xist* RNA is in green, and the carat marks the expressed, predominantly singlet allele.
(4C) Allele-specific scoring of SD FISH signals for *Xic* and *Mecp2* in day 3 EB cells.

state of an X chromosome in a wild-type population should correlate with the fate that is enacted upon initiation of X-inactivation. To test this prediction, we induced X-inactivation in wild-type female ES cells, and examined cells that had recently upregulated *Xist* to silence the Xi by FISH. Embryoid body (EB) differentiation was performed in suspension culture (Martin et al., 1977) for 3 days, at which time approximately 50% of cells have carried out X-inactivation. Day 3 EB cells exhibited an elevated proportion of SD signals (45%, n=125), suggesting that SIAR persists at this time. In wild-type female day 3 EB cells, the *Xic* allele on the Xi, now strongly expressing *Xist* RNA, preferentially appeared as the singlet (Figure 4A and 4C), as we had predicted from analysis of undifferentiated ES cells in which X chromosome fates were fixed by *Xic* mutations. The *Mecp2* allele tended to appear as the singlet on the Xa from which it is now exclusively expressed (Figure 4B and 4C), also consistent with our predictions from ES cells. Thus, in wild-type female ES cells, switching between states stops and the future Xi state is associated with *Xist* RNA upregulation upon initiation of X-inactivation.

SIAR persists at the time of initiation of X-inactivation, but it is lost in fully differentiated cells such as MEFs and TE cells (Chapter 1, Figure 7). I have been asked a few times about the timing of loss of SIAR, which could be addressed by performing FISH across an ES cell differentiation time-course and scoring the %SD signals.

Molecular biology of X chromosome states

Background and rationale

Something is clearly different between the future Xa and future Xi in pluripotent female cells, but what is the underlying molecular cause? The asymmetry between the two X chromosomes could be caused by asymmetric partitioning of a trans factor between

them, or by establishment of an alternate conformation of the DNA or chromatin fiber of one of them. Identification of the molecular players that establish the difference will provide great insight into the mechanism by which two X chromosomes are designated as the Xa and Xi in a mutually exclusive manner. From a practical experimental standpoint, identification of a mark that is always associated with one state or the other would greatly facilitate study of the process, since the current FISH assay for two states cannot be used to definitively determine the state of a chromosome in any single cell.

Identification of candidate trans factors

A number of candidate factors may be involved in establishing X chromosome states that later determine an X chromosome's fate. It makes sense to test the following categories:

Histone modifications, variants and the complexes that insert them into chromatin. While none of the chromatin marks tested so far shows differential distribution between the two X chromosomes at any of the chromosomal positions tested so far prior to X-inactivation, I expect that some combination of marks and positions *will* show a difference for the following reasons:

1. Chromatin marks are critical to later stages of the inactivation process, so it would be mechanistically parsimonious to use them in the early designation process.
2. *Xist* and *Tsix*, both of which modulate choice between the two X chromosomes manifested in ES cells, have both also been associated with modification of chromatin in undifferentiated ES cells (Morey et al., 2004; Navarro et al., 2005; Sun et al., 2006).

3. The composition of the chromatin fiber could be envisioned to affect its biophysical properties in live cells, and could thus affect the frequency of singlets and doublets that appear by FISH in fixed cells.

The list of known chromatin modifications and modifying complexes is growing all the time, so I will leave the task of assembling a list of candidates to whoever pursues this project.

Condensin and cohesin family. These structural components of chromatin are required to keep sister chromatids in close proximity and to organize them in preparation for mitosis (reviewed in Hagstrom and Meyer, 2003). They can be envisioned to affect the frequency with which sisters appear as a single or a double FISH signal. In fact, the significantly denser distribution of cohesin along the small budding yeast chromosomes may account for the fact that sisters cannot be distinguished as two spots by FISH. Cohesins and the molecularly similar condensins are therefore candidates for being the proximate cause of the distinctive frequencies of FISH signals we observe as the readout of two states. Perhaps the density of one of these molecules differs along the future Xi vs. the future Xa, or perhaps a variant is present on one chromosome—for example, along the length of the future Xa and at the *Xic* on the future Xi, where the unusually high frequency of singlets is observed. Most likely, condensin or cohesin distribution would be another consequence, and not the underlying cause, of the two X chromosome states. However, condensins play critical roles in dosage compensation in *C. elegans* and condensins and cohesins have been implicated in a number of other gene regulation systems (Hagstrom and Meyer, 2003).

One member of this family of proteins deserves special attention, because it has been implicated in X-inactivation—albeit later in development than one would expect for a factor that is critical for initial X-inactivation. As mentioned above, it could make sense

for factors to be used at several points in the X-inactivation program, so it is best to keep an open mind. The cohesin homolog *Smchd1* was identified in a screen for regulators of position effect variegation in the mouse (Blewitt et al., 2005). The dominant mutation referred to as *Momme D1* caused a reduction of the spread of silencing from a retrotransposon integration site into the agouti gene (the *agouti viable yellow* metastable epiallele), suggesting the mutation disrupted a dosage-dependent gene involved in silencing. *Momme D1* was female-specific homozygous lethal, with death by E14.5. At E10.5, females appeared macroscopically normal, but heterozygotes exhibited a reduction and homozyotes a complete absence of DNA methylation on the X chromosome, suggesting defects in establishment or, more likely given the timing of embryonic death, maintenance of X-inactivation. The mutation has been mapped to the *Smchd1* gene, and the mouse line has been sent to Neil Brockdorff for analysis of the X-inactivation phenotype (E. Whitelaw, personal communication). There is no published research on *Smchd1*, however, I have obtained a male ES cell line with a gene trap vector containing a neomycin resistance cassette integrated into the locus (XG144 ES, from BayGenomics; Stryke et al., 2003). I found that the wild-type and gene-trapped *Smchd1* alleles are expressed in ES cells by RT-PCR, apparently at high levels (data not shown). I attempted to generate a homozygous cell line by selecting for loss of heterozygosity by prolonged culture in high concentrations of G418 (Mortensen et al., 1992) without success, perhaps because the neomycin resistance cassette is already very highly expressed in the cell line. Other approaches, described below, could be taken to investigate whether this cohesin homolog plays a role in establishing the two X chromosome states in pluripotent female cells.

Meiotic factors. Because homology-sensing appears to be used to establish X chromosome states, it makes sense to test factors that could be involved in this process.

Barbara has proposed testing meiosis-specific genes, as sensing of homology is used to pair homologs in meiosis. She has heard from unpublished sources that meiotic genes are expressed in ES cells, which is encouraging as to the possibility that they may play a role in establishing X chromosome states.

Testing candidate factors for involvement in X chromosome states

There are a number of approaches to take in testing candidate trans factors for potential roles in establishing the two states. First, if antibodies are available, a simple test is to perform immunofluorescence and look for differential staining patterns on the two X chromosomes in female ES cells. Alternatively, GFP fusions could be constructed and transfected into female ES cells to assay localization. Even if these approaches do not reveal differential distribution between the chromosomes at a gross level, the candidate should be further tested by allele-specific ChIP. Using the methods worked out by Morgan Royce-Tolland in the lab, it is possible to assay chromatin association of particular factors on the future Xa and the future Xi using female ES cell lines in which the fates of the chromosomes are fixed and in which polymorphisms exist between alleles (e.g., $X^{Tsix\text{-}pA}X$ ES (Luikenhuis et al., 2001)). These approaches can show whether a candidate is in the right place at the right time to be related to X chromosome states.

To test for genetic requirement of a factor in establishing the two states, an siRNA approach can be taken. Using the methods worked out by Tom Fazzio in the lab, it is possible to select regions of a candidate transcript that are likely to produce a robust siRNA response, and to transfect the siRNA into ES cells. So far, the response in female ES cells has not been as robust as in the male E14 ES cell line (S.M.-E. and T.F., unpublished), perhaps in part because our female lines must be grown on a feeder layer, but optimization will undoubtedly improve the response (see Appendix 2). First,

one would look for a phenotype in which SIAR is lost. Second, one would induce differentiation and look for aberrance in X-inactivation, such as inactivation of none or both of the X chromosomes.

For some candidates, it may be possible to obtain mutant ES cell lines (from BayGenomics or elsewhere). One approach to generating a homozygous mutation in an originally heterozygous ES cell line was mentioned above. If female, these cell lines would then be examined for SIAR and X-inactivation phenotypes as described above for siRNA. It would be more straightforward to perform these assays and to interpret the results in stable cell lines than under siRNA conditions. The disadvantage of this approach is that in addition to being more time-consuming and expensive (to obtain/generate lines), most ES cell lines are likely to be male. Male cell lines are still of some use because, as will be described below, random monoallelic loci on the autosomes also appear to adopt two states like X chromosomes, so SIAR could be assayed at those loci. Ultimately, an X-inactivation phenotype will be the most convincing evidence for a candidate's involvement in chromosome states.

Developing a chromatid interaction assay for X chromosome states

If singlet and doublet FISH signals reflect the degree of proximity between two sister chromatids, then sisters on the future Xa and the future Xi should exhibit distinct frequencies of interactions. Chromosome conformation capture (3C) methodology has been developed to assay the frequency of intra- and inter-chromosomal interactions (Dekker et al., 2002). In 3C, nuclei are cross-linked to preserve native interactions, digested with a restriction enzyme to fragment genomic DNA, and then re-ligated to generate intra- and inter-chromosomal ligation products that reflect the proximity between these sequences in three-dimensional nuclear space; PCR is then used to assay for ligation products of interest. This methodology could be adapted to assay

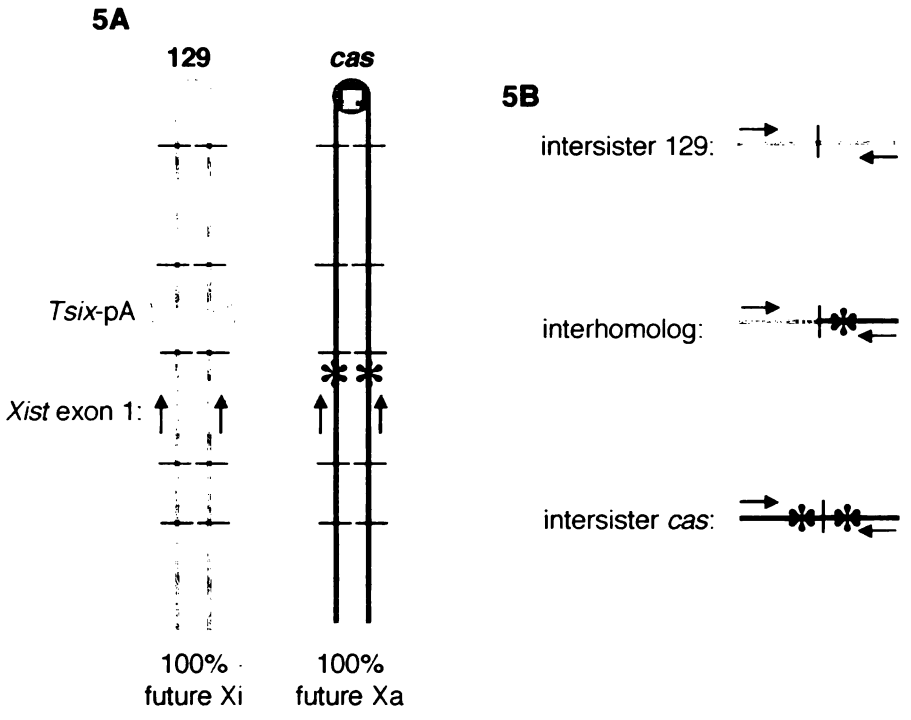


Figure 5. Allele-specific 3C strategy to probe intersister interactions.
(5A) In the female $X^{Tsix\text{-}pA}X$ ES cell line, the mutant X chromosome (pink, future Xi) is of 129 strain origin and the wild-type X (blue, future Xa) is of *castaneus* origin (Luikenhuis et al., 2001). Restriction site polymorphisms exist between the two strains. At the 3' end of *Xist* exon 1, there is a ScrFI site present in the *castaneus* allele (asterisk, Stavropoulos et al., 2001). BamHI sites, indicated by black tick marks, are distributed along the chromosomes and one is located 66 bp away from the ScrFI polymorphism. BamHI, which leaves a 4-base overhang, can be used as the restriction enzyme to digest the crosslinked nuclei in the first step of the 3C protocol. After dilution and religation, palindromic intersister (and interhomolog) ligation products will be produced **(5B)**. Palindromic intersister (and interhomolog) products can be specifically detected by PCR using a single primer that will anneal on both sides of the religated BamHI site. All three possible products (intersister 129 ("IS1"), intersister *cas* ("ISC"), and interhomolog ("IH") will amplify with this primer, providing an internal control for PCR efficiency. The ratio of each ligation product's contribution can be determined by digesting the PCR product with ScrFI, which releases fragments of unique sizes corresponding to each original ligation product. Possible primers I have considered (with amplicon/ScrFI digestion product sizes) include:
MRT3: TGGCAGCAAGTGCCTTTAC (340 bp; IS1: 340; IH: 236+104; ISC: 132+2x104)
3C-X2: TGCCATCTTGCCATCCCGCTGC (288 bp; IS1: 288; IH: 210+78; ISC: 132+2x78)
NS60: CCCGCTGCTGAGTGTTTGATATG (260 bp; IS1: 260; IH: ISC: 196+64; 132+2x64)
I was unable to get the PCR working in an initial attempt. This may be due to difficulty performing PCR over a completely palindromic sequence that can form a hairpin with itself. BamHI is also a finicky enzyme for nuclear digestion. If this strategy works at *Xist*, it can be adapted to take advantage of polymorphisms elsewhere on the X chromosome and autosomes.

intersister interactions in an allele-specific manner as outlined in Figure 5. By using female ES cells in which the identities of the future Xa and future Xi are fixed and in which polymorphisms exist between alleles (e.g., $X^{Tsix\text{-}pA}X$ ES (Luikenhuis et al., 2001)), it should be possible to distinguish interactions between sisters on the future Xa and sisters on the future Xi, as well as interactions between homologs. Based on the pS values presented in Chapter 2, I predict that the *Xist* locus should exhibit more frequent intersister interactions on the future Xi than on the future Xa. If a 3C assay can be developed, it may provide an independent approach to probing X chromosome states that would complement the microscopy assay. Ultimately, it may be possible to use such an assay in genetic screens to search in an unbiased manner for factors involved in establishing the two X chromosome states.

Random Monoallelic Regulation of Autosomal Loci

Background and rationale

It is generally believed that diploidy is an advantageous trait. Because two copies of each gene are present, one functional copy can compensate for loss-of-function of the homologous copy; thus, diploidy confers a survival advantage to individuals. Because diploids are relatively tolerant of reduced-function alleles, genetic diversity is maintained in the population, thereby providing the raw material for evolution of new alleles.

Although mammals are diploid organisms, a significant fraction of the mammalian genome is expressed from only one allele. X chromosome-inactivation was the first example of random monoallelic gene expression to be recognized in mammals (Lyon, 1961; Russell, 1961). Not long thereafter, random monoallelic expression of

immunoglobulin loci was inferred and proposed to contribute to the specificity of the immune response by ensuring production of a single species of antigen receptor by each B-cell (Pernis et al., 1965). This "allelic exclusion" principle was later extended to a number of other genes in the immune system, including T-cell receptors (Rajewsky, 1996), natural killer-cell receptors (Held et al., 1995), and several interleukins (Bix and Locksley, 1998; Hollander et al., 1998). Among approximately 1500 odorant receptor (OR) genes in the mouse (Zhang and Firestein, 2002), it has been discovered that a single allele of a single gene is expressed in each olfactory sensory neuron, underlying the extreme specificity of odorant recognition in the olfaction system (Chess et al., 1994). This type of regulation appears to be shared by the 300 or so pheromone receptors expressed in the vomeronasal system of the mouse (Rodriguez et al., 1999). The random monoallelic expression of many more mammalian genes may await discovery according to a recent study in which it was estimated that approximately 10% of autosomal loci exhibit allele-specific regulation (Gimelbrant and Chess, 2006). Monoallelic expression can also be imprinted, with expression exclusively from either the maternal or the paternal allele (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991). About 70 mouse genes, mostly expressed in the placenta and involved in fetal growth control, are now classified as being imprinted (da Rocha and Ferguson-Smith, 2004). Thus, monoallelic regulation is widespread in the mammalian genome and is critical to the function of many biological pathways.

The establishment of imprinted expression is conceptually straightforward: when the parental alleles are physically separate during oogenesis and spermatogenesis, different epigenetic marks are placed such that the two parental alleles arrive in non-equivalent states in the zygote. DNA methylation has been identified as the primary imprint used to control most imprinted genes (reviewed in da Rocha and Ferguson-

Smith, 2004). In contrast, mechanisms for randomly establishing expressed and silent alleles must operate after the two alleles meet in the embryo, and active and inactive fates must be assigned in a stochastic and mutually exclusive manner. The mechanisms that control this process are poorly understood, although it seems that the active and inactive alleles may be designated during embryogenesis, and then stably inherited (Mostoslavsky et al., 2001; Singh et al., 2003). While epigenetic differences, including differences in chromatin (Heard, 2004), replication timing (Mostoslavsky et al., 2001; Simon et al., 1999; Singh et al., 2003), and nuclear localization (Gribnau et al., 2003; Skok et al., 2001) distinguish expressed and silenced copies of genes in somatic cells, the mechanisms that initially designate active and inactive alleles at random are poorly understood.

Many of the features of the expressed and silent copies of monoallelically expressed genes are shared by the active and inactive X chromosomes, and it has been suggested that the mechanisms of monoallelic expression on the X chromosome and autosomes might be related (Ensminger and Chess, 2004; Ohlsson et al., 1998; Singh et al., 2003). Analysis of the genomic context of X-chromosomal and autosomal monoallelic genes has also revealed significant similarities in repeat content, with enrichment of full-length LINE-1 elements (Allen et al., 2003), suggesting that repeat content may be associated with regime of gene expression.

Results and future experiments

Random but not imprinted monoallelic loci adopt alternative states *(experiments completed and manuscript in preparation by M. K. A.)*

To determine whether similar mechanisms may underlie designation of expressed and silent alleles for X-linked genes and the rest of the genome, we assayed whether

autosomal genes that will be expressed monoallelically exhibit SIAR. We performed FISH in ES cells for several loci that exhibit random monoallelic expression in the olfactory system (several olfactory receptor arrays on chromosomes 2 and 11) and the immune system (*Igκ*, *Tcrβ*, and *Il4*), and scored the proportion of cells displaying SS, SD, and DD signals. Like X-chromosomal loci (Mlynarczyk-Evans et al., 2006), all future random monoallelic autosomal loci assayed displayed a high proportion of SD FISH signals in ES cells (approximately 40 to 50%, Figure 6A). In contrast, three imprinted monoallelic genes, *Igf2*, *Gnas*, and *Cdkn1c*, showed a lower frequency of SD cells that was comparable to biallelic genes, such as *GalNt3* (approximately 20%, Figure 6A). Thus, a high frequency of SD signals correlates with random, but not imprinted, monoallelic expression. Disruption of nuclear structure resulted in a decrease in the proportion of cells showing SD signals and increase in the proportion of cells showing DD signals for future random monoallelic autosomal genes (Figure 6B). This suggests that, as for X-linked genes, the singlets in SD FISH signals represent replicated loci. Thus, autosomal random monoallelic genes and X-linked genes share the unusual property of displaying SIAR in ES cells.

We next performed pair-wise FISH in ES cells for closely linked future random monoallelic loci on two autosomes (Figure 6C). Locus pairs exhibited approximately 65% concordance between SD signals (Figure 6D), even across regions containing biallelically expressed loci that do not exhibit SIAR (the random monoallelic loci *OR2-1* and *OR2-2* are coordinated across the biallelic locus *GalNt3*). Furthermore, different classes of random monoallelic genes wer coordinated on the same chromosome (see Il4 and OR11-1, Figure 6D). These results suggest that, like the X chromosomes, autosomes that contain future random monoallelic genes exist in two different states in ES cells. X chromosomes switch between states prior to random X-inactivation

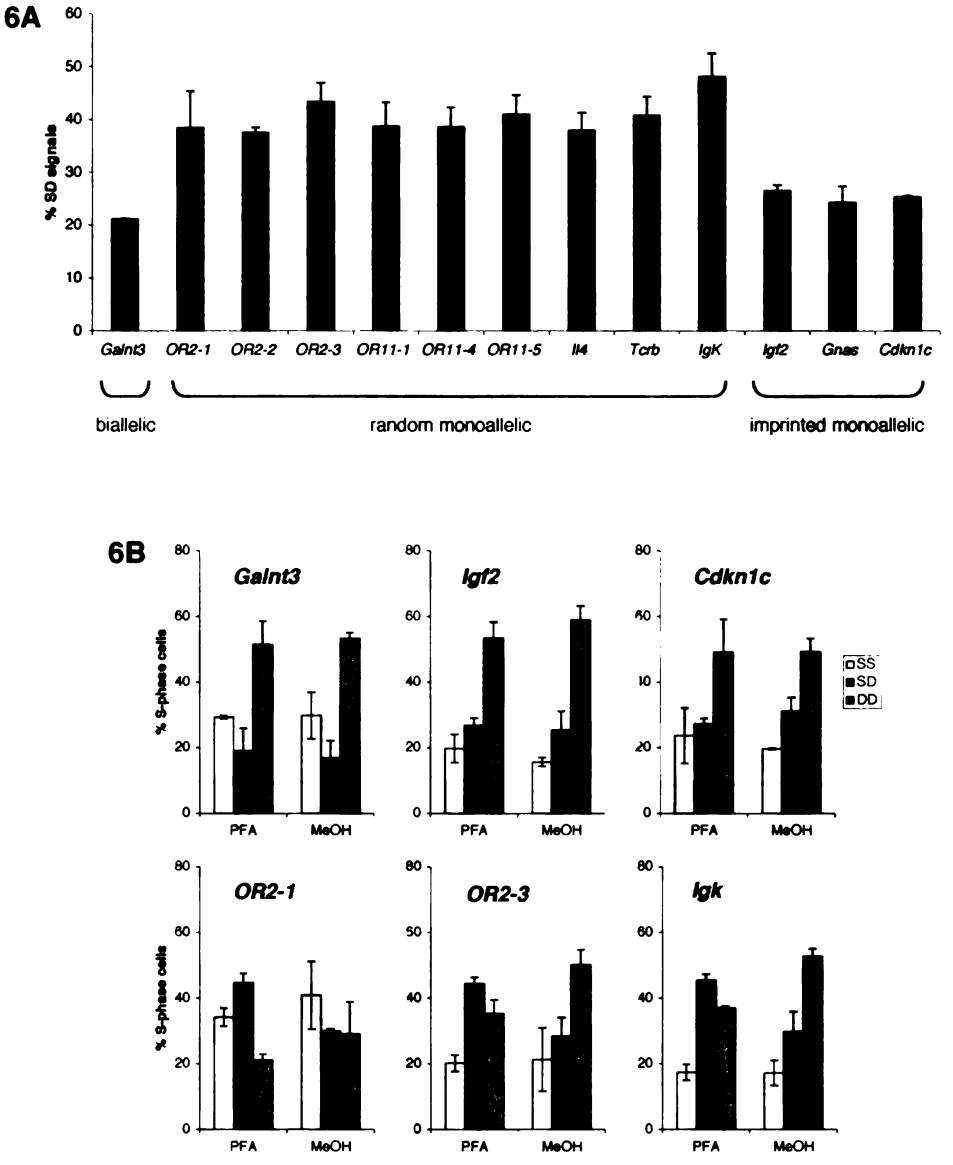
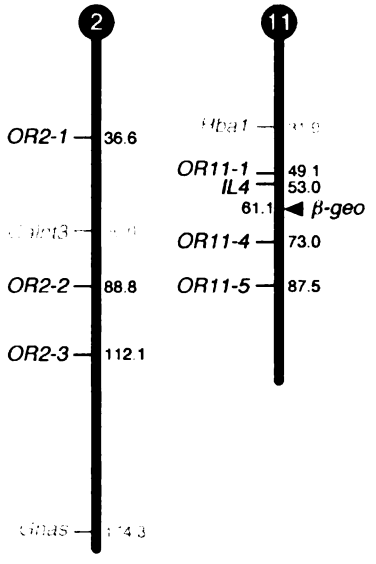


Figure 6. Autosomal sequences containing random monoallelic loci adopt two states.
(6A) FISH in ES cells reveals an elevated proportion of SD FISH signals at future random monoallelic but not imprinted loci. Nomenclature of OR arrays follows Zhang and Firestein, 2002. Average data from 2-4 experiments ($n \geq 150$) are presented; error bars indicate one standard deviation. BACs used for probes were: *Galnt3*: RP24-342H16; *OR2-1*: RP23-52P17; *OR2-2*: RP24-318N4; *OR2-3*: RP23-63C10; *OR11-1*: RP24-212H23; *OR11-4*: RP24-260A8; *OR11-5*(1): RP24-317D24; *IL-4*: RP23-226J16; *Tcr-β*(1): RP23-75E15; *Igκ*(1): RP24-174E21; *Igf2*(A): RP23-17N3; *Gnas*: RP23-147E9; *Cdkn1c*: RP23-124B2.
(6B) Comparison of the distributions of SS (white), SD (black) and DD (grey) FISH signal classes for a biallelic locus (*Galnt3*), two imprinted loci (*Igf2* and *Cdkn1c*) and three future random monoallelic loci (*OR2-1*, *OR2-3*, and *Igκ*) in S-phase ES cells upon fixation with PFA vs. MeOH. Data represent average values from 2-3 experiments; error bars indicate one standard deviation.
(figure continues next page)

6C



6D

locus pair	SD SD cells: concordant	discordant	p =	(n)
OR 2-1 & OR 2-2	**65%**	35%	0.002	(98)
OR 2-2 & OR 2-3	**68%**	32%	<0.001	(128)
OR 11-1 & OR 11-4	**65%**	35%	0.001	(133)
OR 11-1 & IL4	**62%**	38%	0.009	(125)

6E

		% singlet in SD cells: marked	unmarked	p =	(n)
RRR379	*OR11-1*	53	47	0.386	(225)
RRR379	*OR11-4*	48	52	0.612	(140)

6F

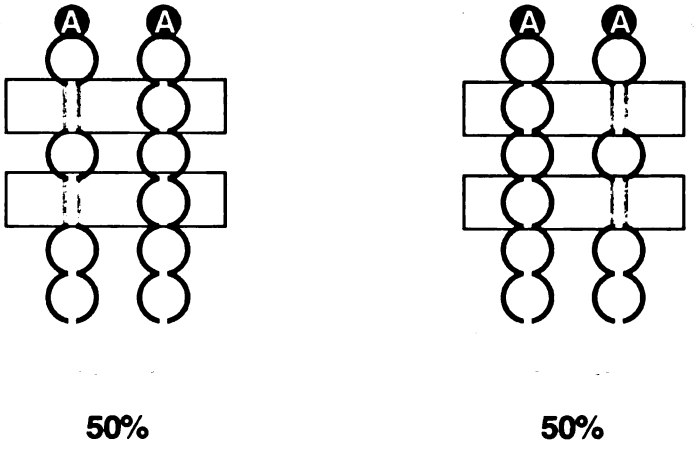


Figure 6. *(continued)*

(6C) Maps of chromosomes 2 and 11 showing positions in Mb of loci assayed in this study. Loci indicated in grey do not display SIAR. The location of a β-geo transgene insertion used in Figure 6E is indicated.

(6D) Coordination analysis for pairs of future random monoallelic loci in ES cells supports a model for autosomes adopting distinct states. Concordant and discordant signals were scored as in Chapters 1 and 2. p-values reflect the degree of certainty that the observed distributions are non-random.

(6E) Autosomes switch between states in pluripotent cells. Allele-specific scoring as in Chapter 2, Figure 3, of two future random monoallelic loci in the clonal $11^{\beta\text{-}geo}/11^{wt}$ ES cell line. Non-significant p-values indicate a random distribution.

(6F) Schematic interpretation of an autosome pair switching between two states. Random monoallelic loci are boxed.

(Mlynarczyk-Evans et al., 2006). To determine if autosomes can also switch between states, we performed allele-specific FISH for random monoallelic loci in $11^{\beta\text{-}geo}11^{wt}$, a clonal ES cell line in which one copy of chromosome 11 is marked by a *β-geo* cassette integration between the olfactory receptor arrays *OR11-1* and *OR11-4* (Figure 6C). The marked chromosome exhibited the singlet signal for each array (*OR11-1* and *OR11-4*) in approximately half of SD cells, and the unmarked chromosome displayed the singlet in the other half (Figure 6E). These results are consistent with the two chromosomes switching between states. Thus, in ES cells, future random monoallelic loci on autosomes behave like X-chromosomal loci prior to X-inactivation in the following ways: these loci exhibit SIAR; there is chromosome-wide coordination such that all affected loci on one chromosome show a high frequency of singlet FISH signals, indicating that they adopt distinct states; and homologous chromosomes switch between states in cycling cells (Figure 6F). These results raise the possibility that randomness of monoallelic expression on the X chromosomes and autosomes may be controlled by a common mechanism.

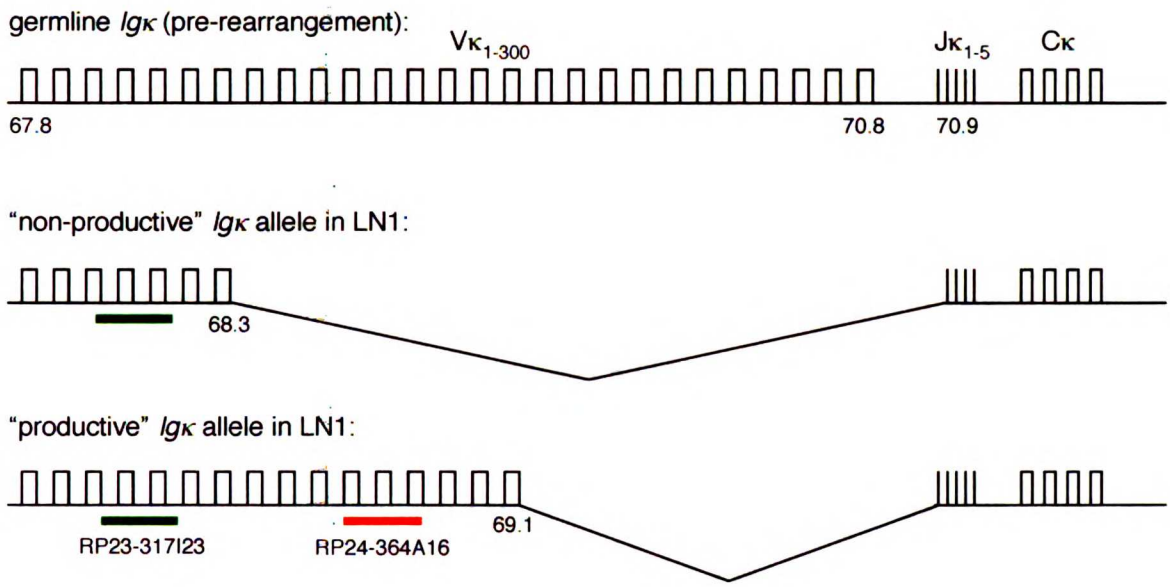
Does autosome state correlate with future expression status?

The frequency with which an X-chromosomal locus exhibits a singlet FISH signal is correlated with its fate upon initiation of X-inactivation: loci showing a low pS value are destined to be silenced, and those showing a high pS value will retain the potential to be expressed in the appropriate cellular context (Chapter 2). The similarities between the behavior of X-chromosomal and autosomal loci that are destined for random monoallelic expression raised the possibility that autosomal loci exhibiting a high pS value in ES cells are also destined to retain the potential for activity. I attempted to address this question using a special ES cell line that had been used to make mice in which the

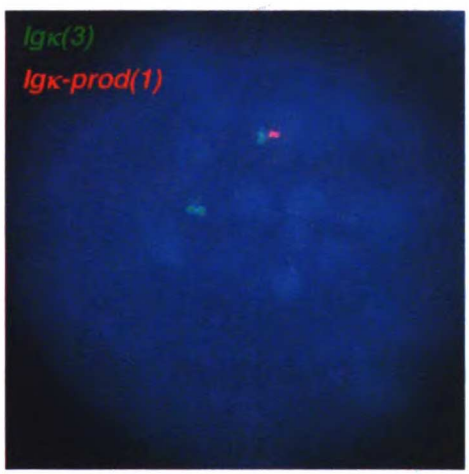
monoallelic expression status of the *Igκ* locus, which is normally random, had been reported to be invariant. This ES cell line, LN1, was generated by nuclear transfer cloning from a mature B-cell donor (Hochedlinger and Jaenisch, 2002). In mature B-cells, the immunoglobulin loci are rearranged and a single functional Igκ light chain allele is expressed. The process that leads to monoallelic expression of the immunoglobulin loci was the defining "allelic exclusion" mechanism, and it is thought to involve monoallelic activation of an allele, rearrangement, and low-level production of receptor that is expressed on the cell surface and screened for functionality and autoimmunity. If the chosen allele fails this test, further rearrangements are possible at the locus, or the other allele will be tried. If the chosen allele passes the test, positive feedback leads to reinforcement of its monoallelic expression (reviewed in Bergman and Cedar, 2004). Obviously, this process involves additional mechanisms that operate on top of the initial allele non-equivalence that the two states phenomenon in pluripotent cells seems likely to reflect. However, it seemed possible that the LN1 ES cell line represented a case in which the normally lengthy allelic exclusion mechanism was bypassed, and perhaps the ES cell line "knew" that the mouse would ultimately express this allele. Heck, stranger things have happened, including several times in my thesis work.

The prediction for the LN1 ES cell line was that the productively arranged Igκ allele should exhibit a high pS value, like future expressed loci on the X chromosome. As shown in Figure 7 and explained in greater detail in the figure legend, I performed allele-specific FISH for Igκ in the LN1 ES cell line, and found an equal frequency of singlets at both alleles. Thus, either there is no correlation between the appearance of this random monoallelic locus in ES cells and its future expression status, or the chromosomes are still switching between states because the cell does not yet know

7A



7B



7C

	singlet in SD cells: "prod"	"non-prod"	n
Igκ(3)	50.6%	49.4%	154

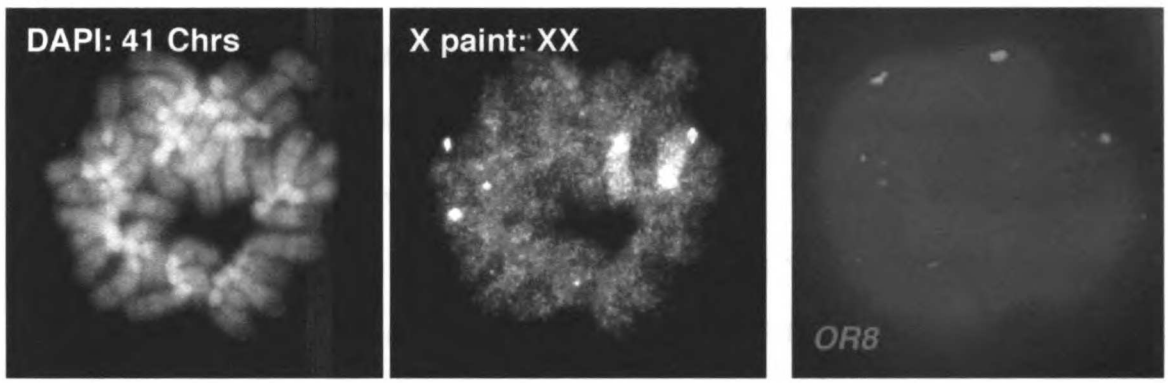
Figure 7. There is no bias in singlet appearance at the *Igκ* locus in the LN1 ES cell line.
(7A) Maps of the germline *Igκ* locus and the "productive" and "non-productive" alleles in the LN1 ES cell line (determined from data in Hochedlinger and Jaenisch, 2002; Gerdes and Wabl, 2004). Numbers below lines indicate approximate positions of sequence boundaries in Mb. I ordered the indicated BACs to use as probes to detect a region of the *Igκ* locus that is common to both alleles, and one that is present only in the "productively" arranged allele. It was later shown that the "non-productive" allele is expressed in a distinct sub-set of B-cells in mice derived from this cell line, and that these cells are eliminated because the receptor is auto-reactive (Gerdes and Wabl, 2004).
(7B) Allele-specific FISH in LN1 ES showing the *Igκ* loci (green) with the "productive" allele also detected by the second probe (red). Nuclei were stained with DAPI (blue).
(7C) Table showing allele-specific scoring of singlets among cells displaying the SD signal pattern at *Igκ* in LN1 ES cells.

which allele will be expressed. A subsequent paper describing mice cloned from this cell line argues for the latter interpretation, as both Igκ alleles were found to be expressed in different B-cells; cells expressing the allele previously reported as not expressed were eliminated because that receptor is autoreactive (Gerdes and Wabl, 2004).

Do chromosome states follow the n-1 rule?

While analysis of the behavior of supernumerary X chromosomes in ES cells is problematic due to toxicity of hyperexpressed X-chromosomal genes, certain autosomes are tolerated in additional copies. Trisomy of chromosome 8 is common in ES cells and results in vigorous growth and retention of differentiation potential (Park et al., 1998). The 41,XX ES cell line I isolated in the Panning lab (1/c16: 5H) appears to carry trisomy 8 (Figure 8A). Chromosome 8 is relatively poor in random monoallelic genes, however there is one cluster of three OR genes. This small OR cluster does appear to exhibit SIAR (Figure 8B) in normal diploid cells. Based on the scoring in diploid cells, I extrapolated hypothetical pS values for the presumed predominantly singlet and predominantly doublet alleles of the *OR8* locus. Using these values, I calculated the proportion of each of the four FISH signal classes (SSS, SSD, SDD, and DDD) that would be expected in the trisomic cell line under several different models: all alleles are equivalent (pS values the same for all), one is future active, and two are future active (Figure 8C). It may be possible to distinguish between these models by scoring the frequency of these four signal classes in the trisomic line. This experiment may allow us to answer the question of whether the two states of autosomes in ES cells follow the n-1 rule defined for the number of inactive X chromosomes in female somatic cells. It has already been suggested that autosomal random monoallelic loci do follow this rule in somatic cells (Ensminger and Chess, 2004).

8A



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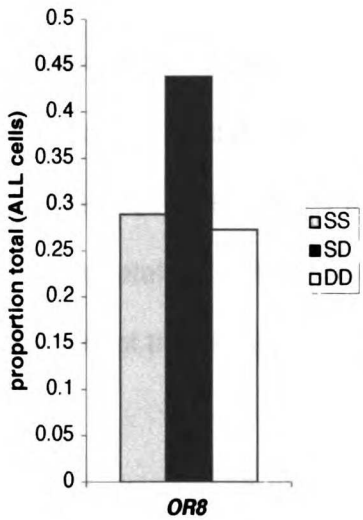

8C

	hypothetical pS values		
	allele 1	allele 2	allele 3
three equivalent	0.51	0.51	0.51
one fut active	0.41	0.41	0.61
two fut active	0.41	0.61	0.61

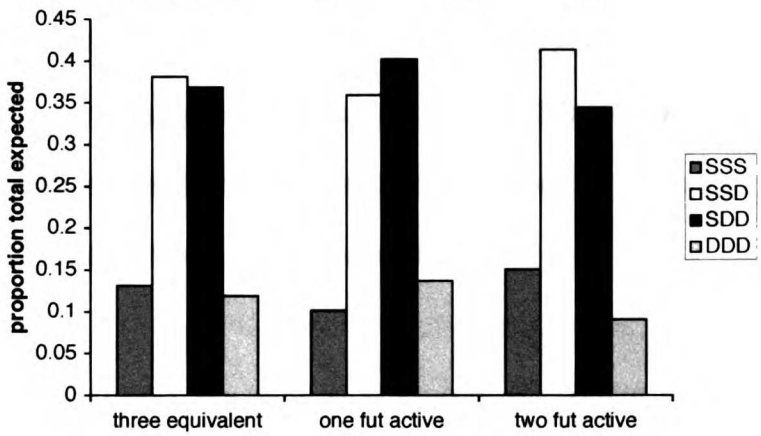

Figure 8. Testing the n-1 rule with an OR array on a trisomic autosome.
(8A) Cytogenetic characterization of 1/c16:5H indicates it is 41,888. A mitotic spread shows 41 DAPI-stained chromosomes (left) with two X chromosomes detected by X chromosome paint (middle). A PFA-fixed interphase cell hybridized with the *OR8(B)* probe (red; RP23-183L2) detects three foci, suggesting three copies of chromosome 8 are present.
(8B) Scoring of %SS, SD, and DD FISH signals for *OR8* in normal diploid cells suggests the locus displays SIAR like other OR arrays. The experiment was performed once (n=121); it should be repeated in BrdU+ cells.
(8C) Hypothetical pS values (table) were used to calculate models (graph) for %SSS, SSD, SDD, and DDD FISH signals expected in the 41,888 cell line if: all three alleles are equivalent; one is "future active;" or two are "future active." The models must be re-calculated when data in diploid BrdU+ cells is obtained (8B). Note that because we do not have a way of fixing the fate of the autosome, there is no way to empirically determine the pS values for the presumed high and low pS value-displaying alleles; thus, they must be estimated from the average pS of the two alleles in the normal diploid cell line. I tried the experiment in the 41,888 cell line once (not BrdU+), and the data most closely matched the expectation for three equivalent alleles; however, I do not trust this experiment. Scoring should be repeated in BrdU+ cells.

Do imprinted loci behave differently in male and female ES cells?

A recent study raises the issue that imprinting may be differentially maintained in male vs. female ES cells, due to progressive DNA demethylation in the latter associated with the presence of two active X chromosomes (Zvetkova et al., 2005). It has always been somewhat surprising that we saw such a low %SD FISH signals at imprinted loci in ES cells in our experiments (it is comparable to biallelic loci); Gribnau observes a higher %SD in his 2003 paper (20% to 41% for imprinted vs. 12% to 19% biallelic). The initial experiments that I did were in female ES cells, but further experiments that I performed for Mary Kate's paper were not carefully controlled as to the sex of the cells. Therefore, the fact that we did not see a high %SD may be related to the fact that some samples were scored in female ES cells, where imprinting may be lost, vs. male ES cells, where it may be retained. This issue merits re-examination in a methodical manner. I performed one trial of this comparison, and the results are presented in Table 1.

Table 1. Scoring of %SD signals for imprinted loci in male and female ES cells. PFA-fixed, BrdU+ E14 and 2-1.10B cells were scored for *Igf2* and a control random monoallelic locus, *Tcrβ*.

	%SS	%SD	%DD	n
random monoallelic (Tcrβ)				
male	19.4%	45.2%	35.5%	155
female	17.9%	44.2%	37.8%	156
imprinted (Igf2)				
male	11.9%	37.7%	50.3%	159
female	14.7%	28.8%	56.4%	156

Concluding thoughts

In somatic cells, autosomal and X-linked genes that are subject to random monoallelic expression exhibit several similarities: the homologous alleles replicate asynchronously, early or late replication timing of each allele is mitotically stable, and all random monoallelic loci on the same chromosome are coordinated for either early or late replication (Ensminger and Chess, 2004; Singh et al., 2003). In pluripotent cells, we have shown that the two X chromosomes exhibit a system of alternation between distinct states (Mlynarczyk-Evans et al., 2006), and here we have presented evidence that the two autosomes in each homologous pair also exhibit such a system affecting the behavior of random monoallelic loci. For the X chromosomes, we have shown evidence that the two states may be used to direct the fates of the chromosomes as Xa and Xi. Although it has not yet been possible to establish such a connection for autosomal random monoallelic loci, it is tempting to speculate that similar mechanisms may operate at all loci exhibiting allele-specific regulation, at any chromosomal location. Perhaps as for X chromosomes, the state of each allele of a random monoallelic locus in a pluripotent cell provides a template for locking in different gene regulatory programs on each allele upon differentiation.

Most autosomal genes that are subject to random monoallelic regulation are ultimately expressed only in highly specialized differentiated cells. For these genes, it has been proposed that the stable, early-replicating allele observed in all somatic cells is the one that retains the potential for future expression (Ensminger and Chess, 2004; Singh et al., 2003). In this view, early differences between alleles would affect the expression potential in the distant future. Seemingly inconsistent with this idea, a recent study showed that for one OR gene, both alleles were available for expression by the same cell, albeit at different times (Shykind et al., 2004).

What is the significance of an initial differentiation between two homologous alleles for regulation of autosomal random monoallelic genes? It has long been clear that regulation of random monoallelic loci involves additional regulatory layers that are not shared with X-chromosomal genes and that differ between classes of random monoallelic genes. The feedback mechanisms taking place during allelic exclusion in the immune receptor system have been described above. In the olfactory system, stochastic monoallelic expression of one out of 1500 OR genes is apparently accomplished by interaction of a unique, monoallelically active enhancer sequence with one allele of one OR gene (Lomvardas et al., 2006), then reinforced by feedback mechanisms that depend on the function of the chosen receptor (Shykind et al., 2004). A random, stable non-equivalence between alleles in the olfactory system could perhaps be responsible for regulating this enhancer in a monoallelic fashion. And perhaps at the immunoglobulin loci, a random non-equivalence between alleles may help to promote initial rearrangement and expression of a single antibody chain in a monoallelic manner.

Acknowledgements

I would like to thank Barbara for her fountain of creative ideas for this projects, and members of the Panning lab for helping me to work out many of the experimental outlines and protocols suggested here (especially Jason Huff for 3C, and Tom Fazzio for siRNA). I am excited that Morgan Royce-Tolland is pursuing the testing of candidate trans factors for roles in establishing two states using immunofluorescence, ChIP, and siRNA. Mary Kate Alexander produced almost all of the random monoallelic locus data in Figure 6. I am very pleased and fortunate that she is pursuing that line of research, and I want to thank her for her generosity and helpfulness throughout our collaboration.

Angela Andersen provided assistance in characterizing the 41,trisomy 8 cell line and performed the EB differentiation from which the data in Figure 4 were obtained. I thank Joost Gribnau for suggesting the LN1 experiment, and Konrad Hochedlinger in Rudolph Jaenisch's lab for providing the ES cell line.

Appendix 1.

Cis-elements involved in random X-inactivation

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Cis-elements involved in random X-inactivation

Summary

In this Appendix, I undertake a detailed review of genetic studies of *Xic* function. I focus on the roles of sequence elements within the *Xic*—including *Xist*, *Tsix*, *Xite*, *DXPas34*, and the *Xce*—in the so-called random X-inactivation mechanism, although some discussion of imprinting is incorporated for clarity. I also include a comparative discussion of mouse *Tsix* vs. human *TSIX* function. Finally, I present the results of an 8.5 kb targeted deletion in the *Xic* that I undertook as part of my original thesis project. Throughout this Appendix I suggest areas where future research is likely to inform our understanding of the mechanism for achieving random and exclusive X-inactivation.

Introduction

The *X-inactivation center* is a master regulatory element responsible for initiating silencing on the appropriate number of X chromosomes to achieve a ratio of one Xa to two autosomal complements in each cell. The *Xic* contains the *Xist* gene, which produces a non-coding RNA that is upregulated specifically from the Xi-elect upon initiation of X-inactivation, and associates with the chromosome *in cis*, spreading silencing along its length. After initial X-inactivation, which is locked in after a few cell cycles and transition to a differentiated status (Kohlmaier et al., 2004; Wutz and Jaenisch, 2000), *Xist* continues to be expressed and to be associated with Xi chromatin, playing a role in maintenance of silencing (Csankovszki et al., 2001). It is important to

point out the critical aspects of the regulatory circuit that operates at the initiation of X-inactivation:

(1) It results in *Xist* upregulation;

(2) Upregulation affects one of the two copies of the *Xic* in each XX female cell (often referred to as "counting" in the X-inactivation field);

(3) The affected copy of the *Xic* appears to be selected at random (usually referred to as the "choice" process in the field); and

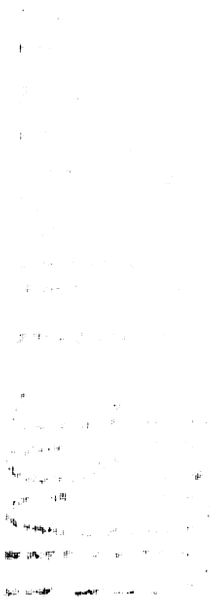
(4) The process is set in motion by an unknown developmental cue that coincides with differentiation.

Multiple *Xic* sequence elements appear to contribute to these prominent regulatory features, thus ensuring proper dosage compensation.

Overview of* Xic *elements

The *Xic* can be thought of as the *Xist* gene and the cis-elements that ensure its proper regulation (Plath et al., 2002). An overview of transcribed elements at the mouse *Xic* is shown in Figure 1. A major regulatory element is *Tsix*, a large antisense transcription unit that completely overlaps *Xist* (Lee et al., 1999a; Luikenhuis et al., 2001; Mise et al., 1999; Sado et al., 2001). Most *Tsix* transcripts originate from a major promoter located near a prominent CpG island, *DXPas34* (Debrand et al., 1999; Sado et al., 2001). *Tsix* also has a minor upstream promoter which splices into the downstream exons and contributes minimally to *Tsix* RNA levels (Sado et al., 2001; Shibata and Lee, 2003). A region between these promoters in which very low levels of transcripts originate has been dubbed *Xite* (Ogawa and Lee, 2003).

The genes surrounding *Xist* and *Tsix/Xite* include several pseudogenes, non-coding transcription units, and protein-coding genes, most of which are subject to X-



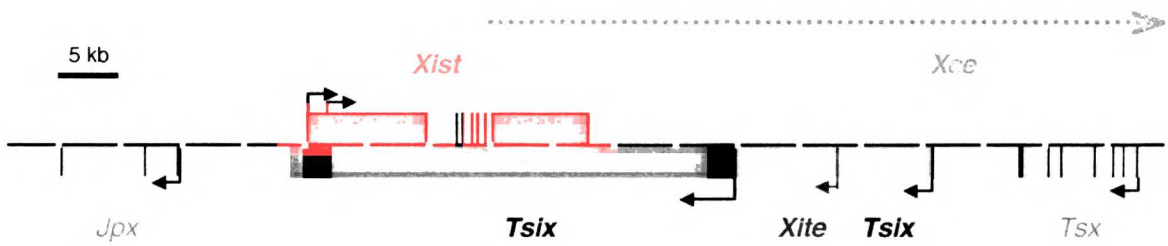


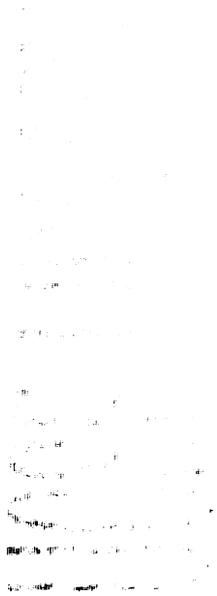
Figure 1. Transcribed elements of the mouse *X-inactivation center*.
The region of the mouse *Xic* surrounding the *Xist* gene is depicted. Transcription units on the top strand are depicted above and those on the bottom strand below the black line. Dark vertical lines and boxes indicate exons, and light regions between them indicate introns. For *Xist*, the first promoter indicates major transcription start site in embryonic cells, and the second indicates a somatic promoter (Johnston et al., 1998; Sado et al., 2005). For *Tsix*, the major promoter is associated with a region of heterogeneous splicing (Sado et al., 2001; Ogawa and Lee, 2003) in the vicinity of the *DXPas34* CpG island (asterisk; Debrand et al., 1999), and the upstream promoter is a minor one (Sado et al., 2001); both can splice to the downstream exon overlapping the 5' end of *Xist*, though half of all transcripts may not be spliced (Shibata and Lee, 2003). *Xite* is associated with low-level intergenic transcription, and mutations in this region can skew X-inactivation away from 50/50 (Morey, 2001; Ogawa and Lee, 2003). The *Xce* element which mediates skewed X-inactivation in inter-strain mouse crosses (Cattanach, 1975) has not been cloned but maps roughly to a 1.85 Mb region that includes the 3' end of *Xist* and downstream sequences (Chadwick and Willard, 2006). *Jpx* is a non-coding gene (Chureau et al., 2002), and *Tsx* is a poorly conserved testis-specific protein-coding gene (Simmler at al., 1996); neither has been shown yet to play a role in *Xic* function.

inactivation in female somatic tissues (Chureau et al., 2002; Johnston et al., 2002; Simmler et al., 1996; Simmler et al., 1997); however, none of these genes has been implicated in the regulation of X-inactivation. Distinctive chromatin marks (acetylation of H4-Lys8 and -Lys16, and dimethylation of H3-Lys9) characterize a ~100 kb domain upstream of *Xist* before X-inactivation is initiated (Heard et al., 2001; Morey et al., 2004; O'Neill et al., 1999), but the dependence of this domain on any of the specific DNA elements in the region has not been investigated.

The *X-controlling element*, which modifies the randomness of X-inactivation in certain mouse strain intercrosses (Cattanach, 1975), has been roughly mapped to a region downstream of *Xist*, which includes *Tsix*/*Xite* (Chadwick et al., 2006; Simmler et al., 1993). The existence of polymorphisms in the *Xite* region between divergent mouse strains, in addition to the phenotypes of targeted genetic mutations (discussed below), led to the suggestion that *Xite* might represent the *Xce* locus (Ogawa and Lee, 2003). Autosomal loci that abrogate the *Xce* effect have been identified: mutations in these loci allow divergent X chromosomes to be inactivated at random, rather than in the skewed fashion characteristic of the wild-type autosomal background (Percec et al., 2002; Percec et al., 2003). These loci may encode trans factors that bind in the *Xce* region, modulating the frequency with which the linked *Xist* allele is designated for upregulation.

Expression patterns of Xist *and* Tsix *before and during random X-inactivation*

Reciprocal expression patterns of *Xist* and *Tsix* during initiation of X-inactivation suggested that *Tsix* might be a negative regulator of *Xist*. In female pluripotent cells in which random X-inactivation is destined to take place, *Tsix* is expressed in a biallelic fashion with RNA localized to a strong pinpoint signal at the *Xic* on both X chromosomes; the single X chromosome in pluripotent male cells likewise shows a *Tsix*



pinpoint (Debrand et al., 1999; Lee et al., 1999a). *Tsix* is expressed at quite high levels in undifferentiated cells (Luikenhuis et al., 2001), and antisense transcripts may outnumber *Xist* by 100-fold (Shibata and Lee, 2003) even though *Tsix* is turned over at least 10-fold more rapidly (Panning et al., 1997; Sheardown et al., 1997a; Sun et al., 2006). When *Tsix* is present, very low levels of the *Xist* transcript (Sun et al., 2006) can sometimes be detected as a faint pinpoint colocalizing with *Tsix* (Lee et al., 1999a).

Upon initiation of X-inactivation in female cells, levels of the *Xist* transcript from the Xi quickly rise (Kay et al., 1993; Sun et al., 2006) as the RNA spreads to coat that chromosome *in cis*, followed rapidly by gene silencing (Panning et al., 1997; Sheardown et al., 1997a). Upregulation of *Xist* is accompanied by simultaneous shutoff of *Tsix* on the Xi (Debrand et al., 1999; Lee et al., 1999a). Meanwhile, *Tsix* continues to be expressed from the Xa in both males and females for a period of several days *in vitro* and until approximately E11.5 *in vivo* (Sado et al., 2001), and is eventually shut off after low-level *Xist* transcription ceases from that chromosome (Lee, 2000; Lee et al., 1999a; Sado et al., 2001). In somatic cells, where high-level monoallelic *Xist* expression stably persists on the Xi, *Tsix* is no longer expressed from either X chromosome (Debrand et al., 1999; Lee et al., 1999a; Sado et al., 2001). These expression dynamics suggested that asymmetric shutoff of *Tsix* on one of the two X chromosomes is related to upregulation of *Xist* and spreading of chromosome-wide silencing on the Xi.

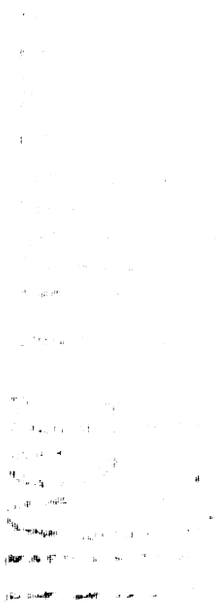
Molecular Genetics of *Xic* Function

Upon the discovery of the *Xist* gene, multiple groups began working to dissect the functions of sequences within the *Xic*, such that today it is one of the most extensively analyzed loci in the mouse genome. Studies have taken two general approaches:

supplying additional copies of all or part of the *Xic* as a transgene, or making a targeted genetic change at the endogenous *Xic*. Because X-inactivation is regulated by X chromosome number, differences in phenotypes between male and female cells can provide insight into to the mechanisms that are operating. In this section, I will focus on the basic classes of phenotypes that result from manipulation of the *Xic* constitution of male and female cells.

Xic *transgenes in male cells*

Transgenes containing genomic *Xic* sequences have been tested for *Xic* function in males (Figure 2, top). Two prominent phenotypes can be observed in transgenic male cells. First, the autosome in which the transgene integrates can exhibit *Xist* upregulation in a differentiation-dependent manner similar to normal X-inactivation in females. Transgenes ranging in size from 460 kb down to 35 kb of genomic sequence have been reported to function in this assay (Heard et al., 1999a; Herzing et al., 1997; Lee and Jaenisch, 1997; Lee et al., 1999b; Lee et al., 1996; Matsuura et al., 1996). The phenotype indicates that the transgene contains all of the *cis*-regulatory information needed for developmentally-controlled upregulation of *Xist*. The function of the 35 kb transgene that contains only the *Xist* gene flanked by minimal upstream and downstream sequences suggests that the critical regulatory elements are closely linked to *Xist* (Herzing et al., 1997), although some members of the X-inactivation community consider the 80 kb transgene tested by Lee et al. to be the definitive minimal transgene showing *Xic* function (Lee et al., 1999b). Expression of an inducible *Xist* cDNA transgene in male ES cells indicated that controlling the gene at a transcriptional level, coupled with the developmental regulation of trans-silencing factors, was a viable means for controlling *Xist* RNA function that may be relevant in female cells (Wutz and



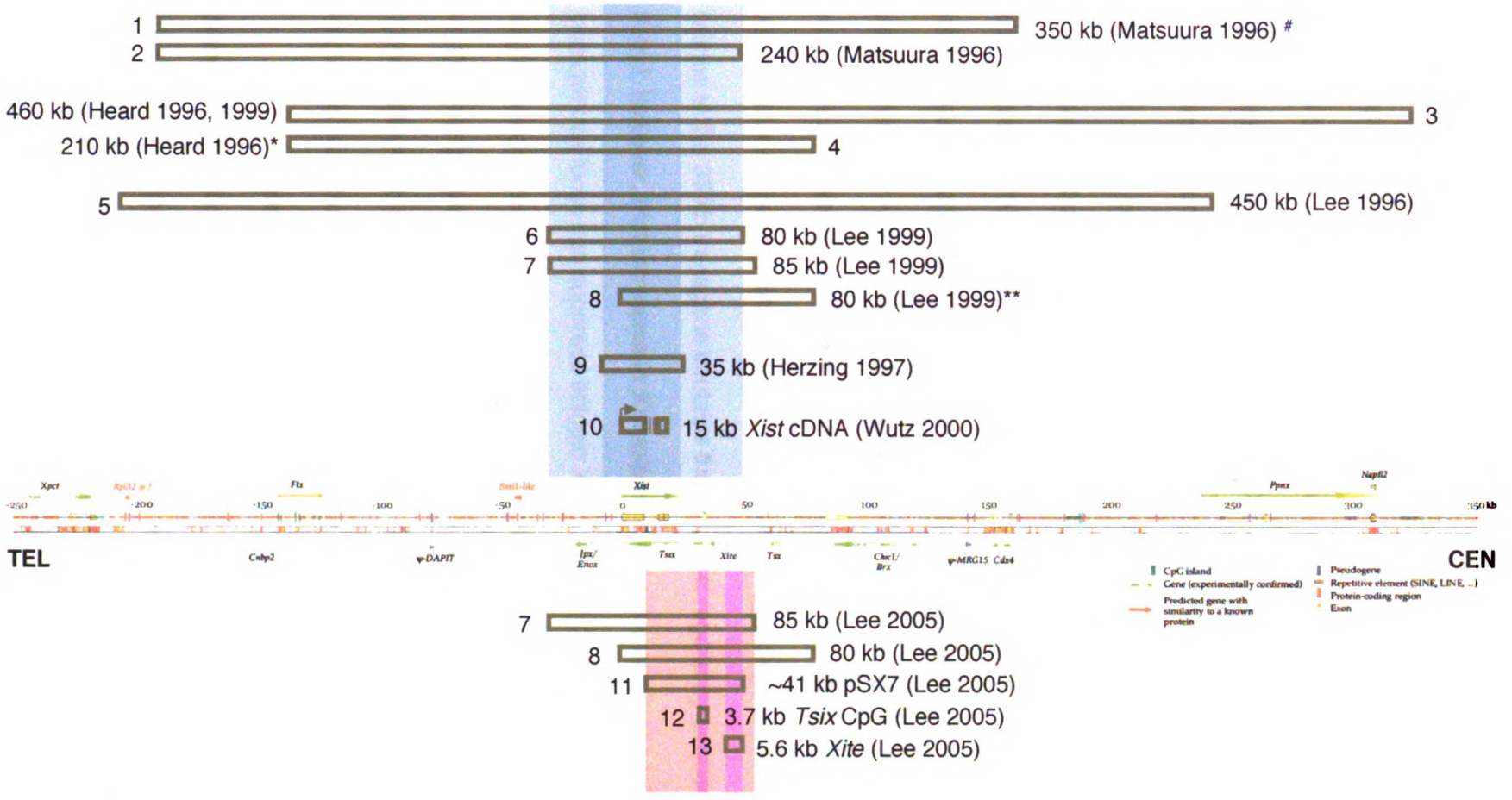


Figure 2. Selected *Xic* transgenes.
Graphical depiction of the mouse genomic region containing the *Xic* (adapted from Chureau et al., 2002). Regions assayed as transgenes in male (top) or female (bottom) ES cells/mice are shown. All supported *Xist* upregulation and *cis*-silencing in males, except as indicated. Minimal regions reported to trigger ectopic inactivation of the single X in males (blue) or to inhibit X-inactivation in females (pink) are highlighted. #one multicopy autosomal Tg did not exhibit *Xic* function. *single copy, functioned only in imprinted X-inactivation in pre-implantation embryos (Okamoto et al., 2005). **initiated but did not maintain X-inactivation. Identification numbers next to each construct are for reference in the text and tables.

Jaenisch, 2000). Further transgenic analysis of sub-domains of the *Xist* gene in male ES cells has been useful in identifying functional modules within the RNA (or DNA) sequence (Wutz et al., 2002).

A second phenotype of *Xic* transgenes in male cells that is sometimes observed is a *trans* effect: *Xist* from the endogenous *Xic* on the single X chromosome becomes upregulated in some cells (Heard et al., 1999a; Herzing et al., 1997; Lee et al., 1999b; Lee et al., 1996; summarized in Table 1). This phenotype is interpreted to indicate that the cell senses the presence of more than one *Xic*, and designates the endogenous X chromosome as Xi at a certain frequency. The smallest transgene that exhibited this phenotype was again the 35 kb region, suggesting that the critical sequences needed for autonomous *Xic* function are tightly linked to *Xist* (Herzing et al., 1997). It appears that no ectopic X-inactivation occurred upon differentiation of male cells containing the 15 kb *Xist* cDNA transgene (Wutz and Jaenisch, 2000), suggesting that the 20 kb of sequence unique to the 35 kb transgene contains these critical regulatory sequences. It should be noted, however, that increases in *Xist* expression from a transgene relative to the endogenous locus can suppress ectopic X-inactivation (Debrand et al., 1999). Thus, it might be worth revisiting the possible *trans* effects of an uninduced *Xist* cDNA transgene on the endogenous *Xic* in differentiating male ES cells. Truncation of the 3' end of the *Xist* cDNA (Wutz and Jaenisch, 2000) would facilitate distinguishing *Xist* RNA that originates from the transgene compared to the endogenous locus.

It has been observed that *Xic* transgenes only function in these assays when present as multicopy arrays (Heard et al., 1999a), although multicopy arrays do not ensure function (Matsuura et al., 1996). It should also be noted that, when transgenes do cause endogenous X-inactivation, cells are found in which *Xist* expression is exhibited by both the transgene and the X, and by one or the other (Table 1). Thus, the

Tg line	construct	copy #	% inactivation			reference
			Tg	X	X+Tg	
L4 12	2-3	2	93%	**2%**	**5%**	Heard, 1999
L1 17*	2-3	5-8	88%	**2%**	**10%**	Heard, 1999
116.6	2-5	20-30	62%	**2%**	**35%**	Lee, 1996
116.6	2-5	20-30	55%	0%	**45%**	Lee, 1999
116.7	2-5	**6-8**	76%	0%	**24%**	Lee, 1996
116.13	2-5	**3-4**	53%	**2%**	**44%**	Lee, 1996
zHβ2	2-9	6	72%	**20%**	**8%**	Herzing, 1997
zHβ10	2-9	8	77%	**9%**	**14%**	Herzing, 1997
πJL1.4	2-6	>10	65%	0%	**35%**	Lee, 1999
πJL2.4	2-7	>10	50%	0%	**50%**	Lee, 1999
πJL3.10	2-8	>10	80%	0%	**20%**	Lee, 1999

Table 1. Ectopic X-inactivation in male cells bearing autosomally-integrated *Xic* transgenes. *Xist*-coated chromosomes were scored on day 7-12 of EB differentiation, depending on the study. *tetraploid cells censored from data. Construct identification numbers correspond to the figures in this Appendix (e.g., 2-3 is Figure 2, construct 3).

normal, tight regulation of one Xa and one Xi observed in female cells is not recapitulated. Copy number dependence could be attributed to position effects resulting in heterogeneous function of *Xic* transgenes or to important X-chromosomal elements that are missing from the transgenes (Heard et al., 1999a), and disregulation could result from the foregoing effects coupled with high copy number of the transgenes. Effects relating to copy number have been noted in several studies: *Xist* expression level correlated with copy number in one (Lee et al., 1996), did not correlate with copy number in another (Herzing et al., 1997), and there was evidence for expression from only a single copy of *Xist* in yet another (Matsuura et al., 1996). This latter result is consistent with an earlier suggestion that multiple cis-linked copies of the *Xic* are not recognized as separate *Xic*'s (Muscatelli et al., 1992).

Xic *transgenes in female cells*

The effects of *Xic* transgenes in female cells (Figure 2, bottom) have not been studied extensively. In the only study to date, large *Xic* transgenes in female ES cells behaved similarly to males in that *Xist* expression was exhibited upon differentiation in some cases. Notably, simultaneous *Xist* upregulation from both endogenous X chromosomes was rarely observed, and it was argued that overall X-inactivation and differentiation were less robust than in control female cells (Lee, 2005). Smaller transgenes that encompassed the *Tsix* and *Xite* promoter regions, which cannot themselves express *Xist*, were reported to cause a complete lack of endogenous X-inactivation and poor differentiation of female ES cells (Lee, 2005). It was this phenotype, in combination with the argument that spread of silencing from *Xic* transgenes in male cells is not as robust as normal X-inactivation in females (Lee et al., 1999b), that led to the suggestion that X chromosome dosage increases the

competence of one X chromosome to be inactivated by *Xist* spreading (Prologue, Figure 4B). An X-encoded "competence factor" is hypothesized to bind in the *Tsix/Xite* promoter regions, and titration of this factor by the transgenes in female cells was postulated to prevent X-inactivation on the endogenous X chromosomes because neither was "competent" to inactivate (Lee, 2005). This study raised the issue of potential differences in the responses of male and female cells to ectopic *Xic* sequences, and further investigations will surely be illuminating. Taken together, analysis of *Xic* function through transgenic studies demonstrates that additional copies of the *Xic* can affect the outcome of the regulatory program controlling X-inactivation.

Loss-of-function analysis of the* Xic *sense strand in females

Targeted genetic analysis of the *Xic* began in the mid 1990's with the first deletion in the *Xist* gene published in 1996 (Penny et al., 1996). Since that time, several large deletions in *Xist*, carried on one of the two X chromosomes in female ES cells and mice, have demonstrated that *Xist* is required *in cis* for X-inactivation to occur because the mutant X chromosome is never inactivated (Penny et al., 1996; Marahrens et al., 1997; Csankovszki et al., 1999; Sado et al., 2005; Figure 3, bottom).

Two small deletions within the *Xist* gene have been published, providing insight into functional modules in this large transcript. A repeated element at the 5' end of *Xist*, the "A repeat," has been shown to be required for silencing through inducible expression of a deletion-bearing *Xist* allele from the single X in males (Wutz et al., 2002). In contrast, the small, highly conserved exon 4 showed no phenotype in heterozygous female cells (Caparros et al., 2002).

In mice bearing heterozygous *Xist* deletions, the kinetics and completeness of X-inactivation are the same as in wild-type mice, suggesting that inactivation of the wild-

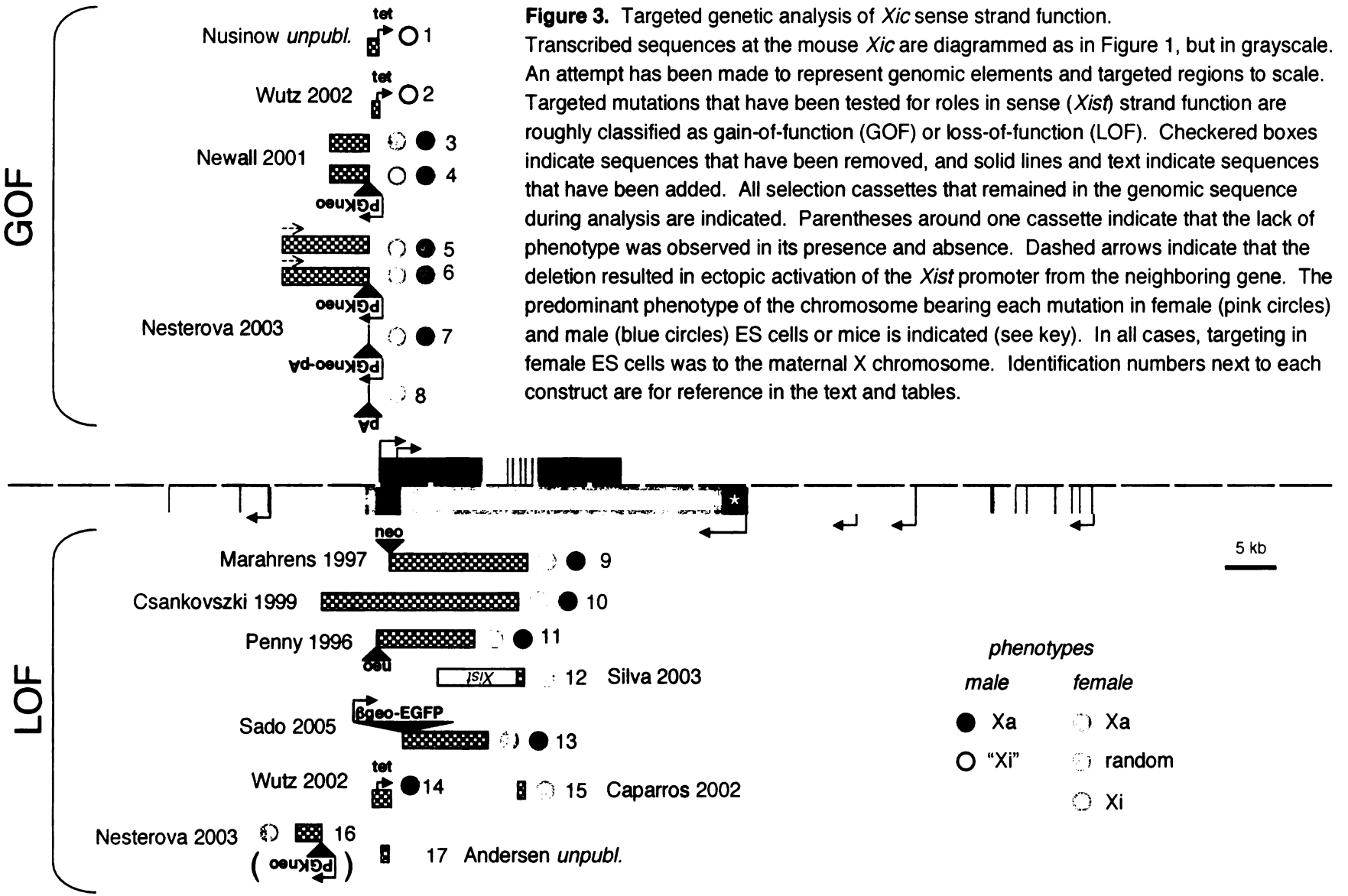


Figure 3. Targeted genetic analysis of *Xic* sense strand function.
Transcribed sequences at the mouse *Xic* are diagrammed as in Figure 1, but in grayscale. An attempt has been made to represent genomic elements and targeted regions to scale. Targeted mutations that have been tested for roles in sense (*Xist*) strand function are roughly classified as gain-of-function (GOF) or loss-of-function (LOF). Checkered boxes indicate sequences that have been removed, and solid lines and text indicate sequences that have been added. All selection cassettes that remained in the genomic sequence during analysis are indicated. Parentheses around one cassette indicate that the lack of phenotype was observed in its presence and absence. Dashed arrows indicate that the deletion resulted in ectopic activation of the *Xist* promoter from the neighboring gene. The predominant phenotype of the chromosome bearing each mutation in female (pink circles) and male (blue circles) ES cells or mice is indicated (see key). In all cases, targeting in female ES cells was to the maternal X chromosome. Identification numbers next to each construct are for reference in the text and tables.

type X chromosome occurs in 100% of cells in a primary fashion (Gribnau et al., 2005; Marahrens et al., 1998), contrary to the original interpretation that skewing was likely a secondary effect caused by loss of cells failing to inactivate the mutant X (Penny et al., 1996). Thus, *Xist* mutations reveal that the wild-type *Xic* is responsive to the inactivation potential of its mutant homolog, suggesting communication between *Xic* alleles is involved in the designation of the Xa and the Xi. Deletion of the A repeat element in female ES cells also appears to skew choice in a primary manner, suggesting that the silencing and choice functions of *Xist* may be mechanistically related (A. A. Andersen, unpublished).

Attempts to analyze the function of *Xist* regulatory elements have been made. Two small sequences within 8 kb upstream of the major *Xist* promoter (a promoter-proximal region containing a cluster of DNase hypersensitive sites (Sheardown et al., 1997b), and the site of a pseudogene once incorrectly mapped as an alternative *Xist* promoter (Johnston et al., 1998; Romer and Ashworth, 2000; Warshawsky et al., 1999)) have also been deleted with no effect on *Xist* expression, X-inactivation potential, or randomness of choice of the mutant allele in female ES cells (Nesterova et al., 2003; Newall et al., 2001). Together, these mutations eliminate the majority of the sequence upstream of *Xist* that was present in the smallest transgene reported to exhibit autonomous *Xic* function (Herzing et al., 1997) as being important to the regulation of *Xic* function.

It has been difficult to generate female mice homozygous for *Xist* mutations because paternal inheritance of an *Xist* loss-of-function allele is lethal due to disruption of imprinted X-inactivation in the extraembryonic lineages (see Prologue, Figure 3; Marahrens et al., 1997; Sado et al., 2001) and no homozygous deletion has yet been reported in female ES cells.

Loss-of-function analysis of the* Xic *sense strand in males

Loss-of-function of *Xist* is reported to have no effect in male ES cells or mice (Marahrens et al., 1997; Figure 3, bottom).

Gain-of-function analysis of the* Xic *sense strand in females

Gain-of-function mutations in *Xist* have been produced intentionally and unintentionally by targeted mutations at the *Xist* promoter (Figure 3, top). Inadvertent gain-of-function *Xist* alleles produced during deletion of promoter sequences have been tested in heterozygous female mice. Bidirectional activity of nearby promoters reading away from *Xist* (a PGK promoter in a selection cassette, or the *Jpx* gene promoter brought into proximity by a deletion) has led to upregulation of *Xist in cis* (Nesterova et al., 2003; Newall et al., 2001). This *Xist* upregulation leads to non-random X-inactivation with the mutant allele becoming the Xi in 80% to 90% of cells in heterozygous female mice, again apparently in a primary fashion as it is observed very early in development (Nesterova et al., 2003; Newall et al., 2001).

Gain-of-function analysis of the* Xic *sense strand in males

Hyper-expression of *Xist* also has a phenotype in male cells (Figure 3, top). In undifferentiated male ES cells carrying the inadvertent over-expressing alleles, *Xist* transcripts accumulate at low levels around the single *Xic*, and in a proportion of cells this localization resembles the mature *Xist* domain of a somatic female cell (Newall et al., 2001; Nesterova et al., 2003; Table 2). Intentional upregulation of *Xist* has also been accomplished in male cells by placing the *Xist* gene under the control of an inducible promoter. Induction of *Xist* in male ES and differentiating cells causes ectopic X-

cell line	construct	% Xi	differentiated?	reference
wt female				
a	-	75%	D4 EB	Lee and Lu, 1999
b	-	17%	D3 EB	Lee, 2005
c	-	30-50%	diff in vitro	Vigneau, 2006
wt male				
d	-	1-2%	undiff	Nesterova, 2003
e	-	2%	D2 LD	Morey, 2004
f	-	0%	diff in vitro	Vigneau, 2006
***Xist* gof male**				
Δ5'+neo	3-6	15%	undiff	Nesterova, 2003
Δ5'	3-5	10%	undiff	Nesterova, 2003
Δhs+neo	3-4	12%	undiff	Newall, 2001
***Tsix* lof male**				
ΔCpG+neo	4-20	0%	D4 EB	Lee and Lu, 1999
ΔCpG+βgeo-pA	4-18	0%	mice	Ohhata, 2006
pA	4-21	10%	D3 RA	Luikenhuis, 2001
pA	4-21	38%	D3 RA	Vigneau, 2006
ΔAV	4-7	39%	D3 RA	Vigneau, 2006
ΔDXPas34	4-10	15%	D3 RA	Vigneau, 2006
Δ65kb	4-3	30%	D2 LD	Morey, 2004
Δ65kb (XO)	4-3	60%	D3 LD	Morey, 2004
Δ65kb+37 (XO)	4-6	9%	D3 LD	Morey, 2004

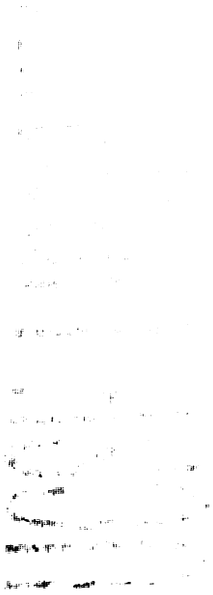
Table 2. Ectopic *Xist* expression in male cells bearing targeted alterations of the *Xic*. High-level *Xist* expression was scored in undifferentiated or differentiated mutant male (or XO) cells, as indicated. Differentiation methods included EB (embryoid body), RA (retinoic acid), LD (low-density plating -LIF), and analysis in mouse somatic tissues. Scoring in wild-type male and female ES cells (in some cases from the same studies) is provided for comparison. Construct identification numbers correspond to the figures in this Appendix, as in Table 1.

inactivation and cell death (Wutz et al., 2002; K. Worringer, S. M.-E., D. A. Nusinow, unpublished).

Loss-of-function analysis of the* Xic *antisense strand in females

In 1998, a deletion encompassing 65 kb of sequence downstream of *Xist* was reported to exhibit an opposite phenotype to deletion of *Xist* in female cells: the mutant X chromosome always became the Xi (Clerc and Avner, 1998). With the identification shortly thereafter of *Tsix* transcription originating downstream of *Xist*, a number of groups began to analyze antisense function with smaller deletions in this region (Figure 4, bottom). In female cells, deletion of the major *Tsix* promoter leads to primary non-random X-inactivation (Lee and Lu, 1999; Sado et al., 2001) as observed in the original large deletion. By contrast, deletion of the minor upstream *Tsix* promoter does not affect randomness (Ogawa and Lee, 2003; Sado et al., 2001). Insertion of a polyadenylation cassette to trap and cause premature termination of *Tsix* transcripts from both promoters before reaching the *Xist* gene has the same effect as deletion of the major promoter, indicating that *Tsix* RNA or transcription that is antisense to *Xist* is responsible for antisense function (Luikenhuis et al., 2001; Sado et al., 2001; Shibata and Lee, 2004). A *Tsix* cDNA minigene expressed downstream of *Xist* without traversing the sense gene did not complement loss of *Tsix* function (Shibata and Lee, 2004), indicating that *Tsix* RNA may not be important to the repressive action exerted by the antisense gene against *Xist*. A large deletion of *Xist in cis* acts dominantly over a *Tsix* loss-of-function allele, with the mutant X chromosome always remaining active *in trans* to a wild-type X chromosome (Sado et al., 2005).

Heterozygous deletion of *Xite* also affects the randomness of X-inactivation in females, with the mutant chromosome preferentially becoming the Xi, similar to *Tsix*



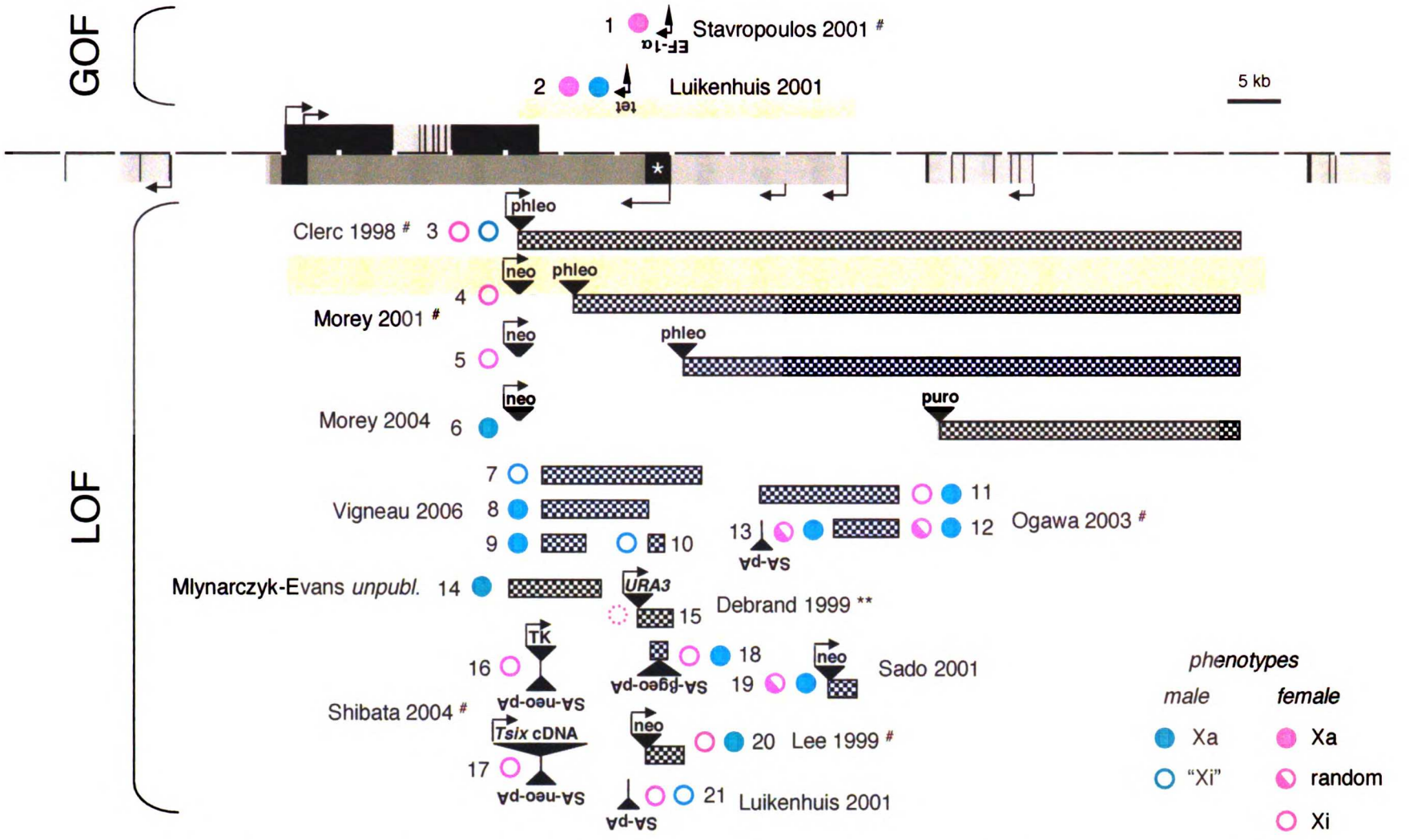


Figure 4. Targeted genetic analysis of *Xic* antisense strand function. Targeted mutations that have been tested for roles in *Tsix/Xite* function are depicted as in Figure 3. Regions that were added back in ES cells are shown in light gray. The 3' end of the *Brx* gene appears to the far right in the genomic diagram. **Targeting was to a transgene tested in male ES cells; # Targeting was to the paternal X chromosome in female ES cells.

mutations (Morey et al., 2001; Ogawa and Lee, 2003), although the skewing is not complete and partially non-random ratios in the range of the *Xce* effect are seen (Ogawa and Lee, 2003). Unlike *Tsix*, premature truncation of *Xite* RNA in the antisense direction does not recapitulate the phenotype of *Xite* deletion, and X-inactivation remains random (Ogawa and Lee, 2003). The latter result leaves open the possibility that *Xite* functions as a DNA element.

Unlike for Xist, it has been possible to generate female mice that are homozygous for a *Tsix* mutation, although they are born at low frequency (Lee, 2002). Inheritance of a maternal *Tsix* deletion disrupts imprinted X-inactivation in the extraembryonic tissues of both male and female embryos such that, depending on the mutation analyzed, heterozygous $X_m^{\Delta Tsix} X_p$ and $X_m^{\Delta Tsix} Y_p$ embryos are born at 10 to 20% (Lee, 2000) or 2% (Sado et al., 2001) of the expected frequency. The lethality of maternal inheritance of a *Tsix* mutation can be partially complemented by concomitant inheritance of a paternal *Xist* deletion, which reverses the pattern of X-inactivation in the extraembryonic tissues (Sado et al., 2001), and can be almost completely rescued by complementing the defective extraembryonic component of the embryos with tetraploid ES cells (Ohhata et al., 2006). In the single study in which $X_m^{\Delta Tsix} X_p^{\Delta Tsix}$ mice have been generated, which did not employ any rescue technique, female offspring inheriting the homozygous deletion were born at 20 to 60% the expected frequency compared to their hemizygous brothers (Lee, 2002). It is unclear whether this sex ratio distortion stems from an imprinting defect, a failure of random X-inactivation, or both, although the author favored a defect in random X-inactivation (Lee, 2002). Use of tetraploid ES cell complementation (Ohhata et al., 2006) could clarify this issue. X-inactivation was random in the somatic tissues of homozygous mutant females (Lee, 2002) as expected

since cells must achieve dosage compensation to survive and the two *Tsix*-mutant *Xic* alleles are genetically identical. Upon differentiation *in vitro* for 3 days, homozygous *Tsix* loss-of-function female ES cells exhibited upregulation of *Xist* from either one or two X chromosomes, and the frequency of cells lacking an Xi was comparable to that in a parallel differentiation of wild-type cells (Lee, 2005). While this result was interpreted to imply disruption of random X-inactivation, several alternative possibilities have been raised by other work (see discussion of the phenotype in males below, and of imprinted X-inactivation *in vitro* in Appendix 2), such that, in my opinion, the impact of homozygous loss-of-function of *Tsix* on random X-inactivation in females remains an open question.

Loss-of-function analysis of the* Xic *antisense strand in males

In XY or XO cells bearing the 65 kb deletion of the entire *Tsix/Xite* region and beyond, the single mutant X chromosome was reported to exhibit *Xist* upregulation and X-inactivation (Clerc and Avner, 1998; Morey et al., 2004). Therefore, it was expected that some smaller region would be identified in males that was responsible for this ectopic *Xist* expression/X-inactivation phenotype. Subsequent analyses have shown that deletion of most sequences within the original 65 kb region has no phenotype in male ES cells (Figure 4, bottom). The sequences that have been tested and shown not to be essential include the 5' 13 kb of the original 65 kb deletion up to the *DXPas34* GpG island (Vigneau et al., 2006; S. M.-E. unpublished), the 10 kb including *Xite* and the minor *Tsix* promoter (Ogawa and Lee, 2003; Sado et al., 2001), and the 3' 28 kb of the original large deletion (Morey et al., 2004).

Reports differ concerning the phenotypes of deleting the major known remaining elements, the *Tsix* promoter and associated *DXPas34* GpG island, in male cells. For one loss-of-function mutation in the *Tsix* promoter/*DXPas34* region in male ES cells, it

has been reported that *Xist* is properly shut off and the single X chromosome remains active upon differentiation *in vitro* (Lee and Lu, 1999). For another mutation, which likely abrogates *Tsix* function more completely because it consists of a splice acceptor and polyadenylation cassette that traps antisense transcripts originating from all promoters (and a similar allele results in a stronger imprinting phenotype (Sado et al., 2001)), ectopic *Xist* expression has been noted upon differentiation *in vitro* (Luikenhuis et al., 2001). The fact that different groups detect effects of different degrees with the same cell lines subjected to the same differentiation procedure (Table 2; Luikenhuis et al., 2001; Vigneau et al., 2006) suggests that the reported differences in phenotype among the several *Tsix* loss-of-function constructs tested in male ES cells may relate in part to variations between the methods of each lab. The extensive *Xist* hyper-expression phenotype of the male *Tsix*-pA ES cell line (Luikenhuis et al., 2001) as measured by one lab (Vigneau et al., 2006) resembles the strong phenotype of the original 65 kb deletion in XO or XY cells (Table 2; Clerc and Avner, 1998; Morey et al., 2004). It has been argued that this degree of ectopic X-inactivation is comparable to the degree of normal X-inactivation in differentiating female ES cells (Clerc and Avner, 1998; Vigneau et al., 2006). The ectopic *Xist* expression has been shown to result in silencing of X-linked genes in a proportion of affected cells at early timepoints (Morey et al., 2004). A less severe but still significant effect of *Xist* hyper-expression has also been noted for a precise deletion of *DXPas34* that does not alter the minimal *Tsix* promoter (Vigneau et al., 2006).

Two interpretations of this *Xist* hyper-expression phenotype in *Tsix* mutant male cells have been offered: either sequences in the *Tsix* region contain a critical cis-element needed for a cell to "count" its X chromosomes and "choose" one to remain active (Clerc and Avner, 1998; Morey et al., 2001; Morey et al., 2004; Vigneau et al., 2006), or

disruption of *Tsix* function leads to disregulation of *Xist* that does not reflect the regulatory mechanisms controlling X-inactivation in normal females (Ohhata et al., 2006; Sado et al., 2002). Several pieces of data support the latter interpretation. First, a similar phenotype of hyper-expression and ectopic spreading of *Xist* is seen upon disregulation of the *Xist* promoter (Table 2; Newall et al., 2001; Nesterova et al., 2003). As in those cases, *Xist* spreading caused by *Tsix* disruption is observed even in undifferentiated ES cells and it proceeds with advanced kinetics when differentiation is triggered (Morey et al., 2001), suggesting that normal regulatory mechanisms are bypassed. Second, it has been noted that the frequency of male cells exhibiting ectopic *Xist* expression decreases at later timepoints of *in vitro* differentiation, and many cells survive beyond the X-inactivation window and do not exhibit *Xist* coating (Morey et al., 2001; Vigneau et al., 2006; S. M.-E. and C. de la Cruz, unpublished observations). Furthermore, in the somatic tissues of hemizygous *Tsix* mutant male mice, *Xist* expression is absent and the *Xist* promoter is stably methylated as in normal males (Ohhata et al., 2006). From these results, it has been suggested either that mutant male cells exhibiting *Xist* upregulation die such that their numbers are reduced at later timepoints (Morey et al., 2004; Vigneau et al., 2006), or that a counting/surveillance mechanism that operates during random X-inactivation rescues the viability of mutant cells by *Tsix*-independent repression of *Xist* (Ohhata et al., 2006). Based on my own experience with constructs 4-3 (Clerc and Avner, 1998) and 4-21 (Luikenhuis et al., 2001), I favor the latter explanation. Cell death analysis during differentiation of male cells bearing these profound *Tsix* loss-of-function mutations has not yet been undertaken in a quantitative manner, and should be performed to help distinguish between the possibilities.

Gain-of-function analysis of the* Xic *antisense strand in females

Two gain-of-function constructs have been targeted to the *Tsix* region to further investigate antisense function (Figure 4, top). In separate studies, an inducible and a constitutive promoter were used to drive persistent *Tsix* expression from one allele in female ES cells (Luikenhuis et al., 2001; Stavropoulos et al., 2001). When differentiation was induced by monolayer culture in the presence of retinoic acid or by aggregation into embryoid bodies, the *Tsix* hyper-expressing allele was never inactivated, and all cells which underwent inactivation showed a wild-type Xi (Luikenhuis et al., 2001; Stavropoulos et al., 2001). No changes in the kinetics or completeness of X-inactivation were noted in one study (Luikenhuis et al., 2001), suggesting that hyper-expression of *Tsix* had a primary non-random effect on the choice process similar to heterozygous loss-of-function and gain-of-function *Xist* mutations, and to heterozygous loss-of-function *Tsix* mutations. However, in the other study, the majority of EBs failed to undergo X-inactivation or to differentiate in the presence of one *Tsix* hyper-expressing allele (Stavropoulos et al., 2001). Due to the *Xce* effect in the female ES cell background in which the mutation was introduced, the wild-type allele is normally designated as the Xi in a minority of cells. The authors suggested that hyper-expression of *Tsix* did not affect the choice process, and that the minority of cells designating the wild-type allele as Xi were able to complete X-inactivation, while the majority of cells designating the targeted allele as Xi-elect were unable to carry out silencing because persistent *Tsix* expression blocked *cis*-upregulation of *Xist* (Stavropoulos et al., 2001).

The apparent discrepancy between the two reports of heterozygous *Tsix* gain-of-function alleles may be a result of incomplete analysis in the former study (definitive data supporting the claim of a primary non-random effect were not shown in Luikenhuis et al., 2001). In this case, the results of the latter study (Stavropoulos et al., 2001) may

indicate that enhancing *Tsix* transcription beyond its already high expression level may not be possible (which was suggested by Luikenhuis et al., 2001) or may not further repress the cis-linked *Xist* allele. If it is the level of functional *Xist* transcript produced by each *Xic* that is compared during the process which designates the future Xa and the future Xi (Mlynarczyk and Panning, 2000), then hyper-expression of *Tsix* from one allele might not affect normal random choice between the two X chromosomes. It would be informative to assay SIAR in an allele-specific fashion in *Tsix* hyper-expressing ES cells (Luikenhuis et al., 2001) to determine whether the chromosomes adopt future Xa and future Xi states in a skewed manner. If the states are not skewed (if skewing occurs it should be dramatic as it would be in the opposite direction of the *Xce* effect in the wild-type ES cell background), the conclusion of secondary non-randomness would be supported. Careful kinetic analysis of *Xist* upregulation under RA and EB differentiation conditions should also be performed to determine whether skewing of X-inactivation is primary or secondary.

An alternative interpretation of the discrepancies between these two reports is that the different results are real, and related to the fact that the *Tsix* hyper-expression construct was targeted to the maternal allele in one study (Luikenhuis et al., 2001), and to the paternal allele in the other (Stavropoulos et al., 2001). High-level expression of *Tsix* from the paternal X chromosome would be predicted to block imprinted X-inactivation in the extraembryonic lineages, like a paternally-inherited *Xist* deletion (Marahrens et al., 1997), whereas high-level *Tsix* on the maternal X is the normal case and facilitates imprinted X-inactivation. If the extraembryonic lineages produced *in vitro* during EB differentiation also undergo paternal X-inactivation, then a *Tsix* hyper-expressing paternal X, as in the study by Stavropoulos et al., might block X-inactivation in these early-differentiating cell types *in vitro*. Therefore, an imprinting defect in the

critical outer cell layer of the embryoid bodies might account for the poor overall EB differentiation these authors observed. If so, aggregation of the paternal *Tsix*-mutant ES cells (Stavropoulos et al., 2001) with a wild-type extra-embryonic XEN cell lineage (Kunath et al., 2005) might rescue EB differentiation and allow primary non-random X-inactivation to proceed in the embryonic lineages formed by mutant cells. See Appendix 2 for an in-depth discussion of the apparent maintenance of the X-inactivation imprint in female ES cells.

Gain-of-function analysis of the* Xic *antisense strand in males

One *Tsix* hyper-expression construct has been introduced on the single X chromosome in male ES cells (Figure 4, top). No X-inactivation phenotype was noted (Luikenhuis et al., 2001).

Of Mice and (Wo)men

Inter-species transgenic analysis of *Xic/XIC* function indicates that *Xist* expression is regulated differently in humans and mice, with human *XIST* being prematurely upregulated in the mouse (Heard et al., 1999b; Migeon et al., 2001b). This observation may be related to the fact that key aspects of the antisense *Tsix* gene structure and developmental regulation that result in cis-repression of mouse *Xist* are not shared by the analogous human *TSIX* gene (Chow et al., 2003; Lee et al., 1999a; Migeon et al., 2001a; Migeon et al., 2002).

Antisense transcripts initiating downstream of the last exon of *XIST* have been detected in humans (Chow et al., 2003; Migeon et al., 2001a; Migeon et al., 2002), but the structure of the human *TSIX* gene differs from mouse. The site of initiation of the

transcript has varied in different studies/cell types, but in all cases the antisense transcription appears to terminate before reaching the large first exon of *XIST* (summarized in Chow et al., 2003, Figure 5). This gene structure is different from mouse *Tsix*, which overlaps with the entire *Xist* transcript, including the A-repeats. Shibata and Lee speculate that, by analogy to mouse, human *TSIX* corresponding to the 5' end of *XIST* might be less abundant than that corresponding to the 3' end, and therefore might not have been detected in these studies; however, the authors did not perform any experiments to detect human *TSIX* (Shibata and Lee, 2003).

Like mouse *Tsix*, human *TSIX* has not been detected in adult female or male cells (Chow et al., 2003; Migeon et al., 2002). *TSIX* expression has been detected by RT-PCR in female placental, fetal, and neonatal cells (Migeon et al., 2002), in mouse ES cells containing a human YAC transgene (Migeon et al., 2002), in a male embryonic carcinoma line (Chow et al., 2003) and a male cancer cell line (HT-1080) transformed with an *XIC* PAC (Chow et al., 2003). Although neonatal expression of human *TSIX* seems a bit late compared to mouse, these studies generally indicated that human *TSIX* may be expressed in a similar developmental context to mouse *Tsix*.

The localization pattern of human *TSIX* is markedly different from mouse. By FISH, *TSIX* appears to colocalize with *XIST*, including possibly coating the chromosome when *XIST* is doing so (Chow et al., 2003; Migeon et al., 2002). The authors use double-stranded probes that could potentially detect both sense and antisense transcripts, which is troubling, although they provide controls indicating their probes are specific to *TSIX* and *XIST*, respectively. In mouse, the only pattern ever observed for *Tsix* is a pinpoint signal, and mouse *Tsix* is never co-expressed with high-level *Xist* coating of the same chromosome (Lee et al., 1999a). It should be noted that in human ES cells, *XIST* expression may also differ compared to mouse, with patterns detected by

FISH in two different cell lines of no expression vs. coating signals that do not appear to change upon differentiation (Hoffman et al., 2005).

Human *TSIX* is less abundant than *XIST*, both in cell types in which it appears as a pinpoint signal as well as when it appears as a coating signal (Chow et al., 2003), whereas in mouse ES cells *Tsix* is vastly more abundant than *Xist* (Shibata and Lee, 2003). Human *TSIX* has a comparable half-life to *XIST* of approximately 5 hours (Chow et al., 2003), whereas in mouse, the half-life of *Tsix* is much shorter than *Xist* (Sun et al., 2006).

Based on the available data, it appears that human *TSIX* may not play an analogous role to mouse *Tsix* in the control of *XIST* expression during initiation of X-inactivation. (See the exchange between Barbara Migeon and Jeannie Lee on this subject (Migeon, 2003).) In mouse, the primary role of *Tsix* appears to be in the control of imprinting. Perhaps in humans, where the issue of imprinting in the placenta is still unresolved (for a review, see Plath et al., 2002), the *TSIX* gene is "degraded;" alternatively, a non-functional human *TSIX* may be a more "ancestral" form of the gene that has not yet been co-opted into a functional role in imprinting or cis-repression of *XIST* as in mouse. In a recent study, cow *XIST* and *TSIX* were reportedly co-expressed in female somatic cells, and cow *TSIX* but not *XIST* was variably expressed in male cells (Farazmand et al., 2004), suggesting that cow *TSIX* also does not function the way mouse *Tsix* does. Because the cow and human lineages diverged before mouse and human, a common lack of function for *TSIX* in humans and cows would support the suggestion that a functional role for *Tsix* evolved specifically in the rodent lineage. In addition, a report that cows exhibit imprinted X-inactivation in the placenta (Xue et al., 2002), suggests that imprinting can be achieved without *TSIX*.

The upshot is, *caveat emptor*: not everything we learn in the mouse may turn out to be relevant to human X-inactivation. In particular, *Tsix* may be an evolutionary add-on to the fundamental *Xist*-dependent random X-inactivation mechanism shared among all placental mammals. In this regard, I will remind the reader of several core findings that suggest it will be possible to move beyond *Tsix* in studying the random X-inactivation mechanism. First, the fundamental aspects of the random X-inactivation mechanism seem to be conserved between humans and mice, with dependence of the X-inactivation mechanism on *Xist/XIST* and *Xic/XIC* copy number in both systems. It has even been suggested that the presence of a human *XIC* can trigger inactivation from a mouse *Xic in trans* (Migeon et al., 2001b). Second, in mouse, the minimal transgene that functions as an autonomous *Xic* is just 35 kb in size and includes *Xist* but not *Tsix* (Herzing et al., 1997). Third, recent evidence suggests the existence of a *Tsix*-independent "surveillance" mechanism, which requires *Xist* function, that responds to X chromosome number and shuts off *Xist* on one chromosome to ensure an appropriate 1Xa:2A expression ratio (Ohhata et al., 2006; Sado et al., 2005). Thus, we may yet unravel a fundamental mechanism in mouse for designation one Xa and one Xi in each female cell that is shared by all mammals.

An 8.5 kb Region Downstream of *Xist* Does Not Contain the "Counting Element"

Background and rationale

In 2000, when I was beginning to consider thesis proposal ideas, Barbara gave me the opportunity to co-author with her a short commentary piece on a new paper by Jeannie Lee showing that *Tsix* was critical for imprinted X-inactivation in the mouse

(Lee, 2000). In the process of working on that piece (Mlynarczyk and Panning, 2000), Barbara and I developed a "yin-yang" model for competitive *Xist* and *Tsix* function to control random choice in X-inactivation (Figure 5). In this model, binding of a blocking factor to a cis-acting counting element would repress *Xist in cis*, and a proposed chromatin remodeling activity of *Xist* acted locally at the counting element sequence, decreasing its affinity for blocking factor. Meanwhile, *Tsix* repressed *Xist* function *in cis*, keeping the counting element sequence available for binding blocking factor.

We hypothesized a location for the counting element invoked in our model based on the combination of two critical pieces of data:

(1) A 35 kb transgene containing genomic *Xist* plus 9 kb of upstream sequence and 5 kb of downstream sequence integrated in male ES cells was reported to trigger inactivation of the endogenous X chromosome *in trans* (Herzing et al., 1997). We reasoned that the transgene must contain the counting element sequence, such that it titrates away blocking factor from the endogenous X, leading to its inactivation.

(2) A 65 kb region including the 5'-most 3 kb of the *Xist* gene and downstream sequences was reported to cause inactivation of the single X chromosome in an XO cell line in virtually every cell (Clerc and Avner, 1998). We (and they) interpreted this result as indicating that the counting element had been removed by the deletion, leading to a situation in which it was impossible for blocking factor to bind and choose this X to remain active. The major known element in the 65 kb region was *Tsix*, but as the first *Tsix* promoter deletion was reported *not* to cause ectopic inactivation of the single X chromosome in male cells (Lee and Lu, 1999), the *Tsix* promoter could not be the counting element.

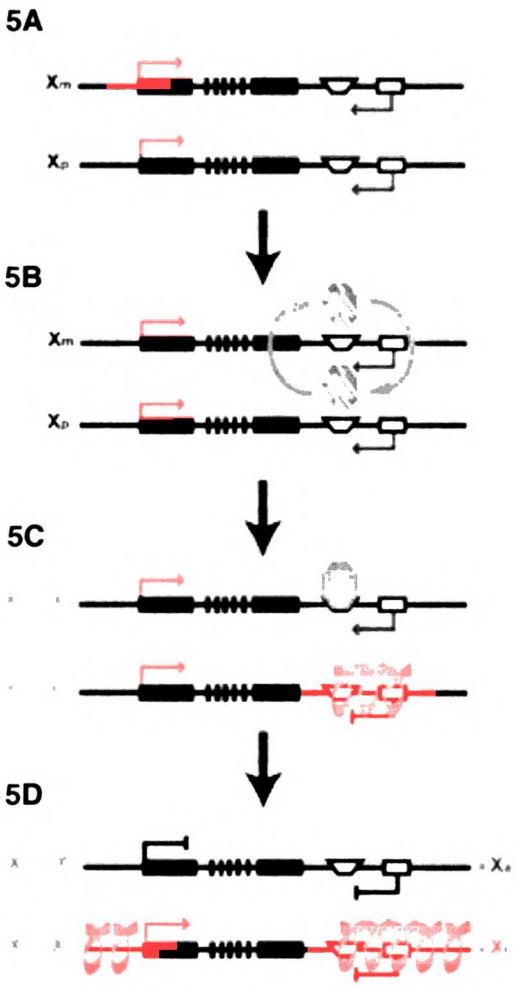


Figure 5. The yin-yang model for *Xist* and *Tsix* function in random X-inactivation.
The black line diagrams represent elements of the X-inactivation center: solid rectangles represent the exons of the *Xist* gene; the open trapezoid represents the counting element (CE); the open rectangle represents the *Tsix* CpG which includes the major transcriptional start site of *Tsix*. The purple octagon represents a single, autosomally encoded blocking factor. Figure not drawn to scale. Position of the CE has not been mapped and is tentative. (BF, purple octagon)
(A) Before differentiation, future embryonic cells express *Tsix* (green arrow) and *Xist* (red arrow) from both X chromosomes. *Tsix* counters *Xist* function *in cis*.
(C) A single, autosomally encoded blocking factor (BF, purple octagon) assembles and tests binding at the available CE's before stably binding to one of them.
(D) Stable binding of the BF to the CE of either the Xm or the Xp blocks *Xist*'s ability to silence that chromosome (red spirals), 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*. Please note that BF 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.
(E) When a spreading or stabilizing factor becomes available, the X expressing functional *Xist* is rapidly coated and silenced and the X which has bound the BF 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.

Putting these pieces of information together, we reasoned that the overlapping region between the sequences that were required (65 kb) and sufficient (35 kb) for an X chromosome to be chosen to remain active was the most likely location for the counting element.

We set out to delete an 8.5 kb region including the 5' end of *Xist* and downstream sequences, which did not include *Tsix* (Figure 4, construct 14). There were several interesting repeated elements in this region which we imagined could nucleate blocking factor in a cooperative manner. Furthermore, one of these repeated elements was a 17-mer present in an estimated 150 copies, with a core element that matched the consensus binding site for the *Drosophila* Zeste protein (Simmler et al., 1996). Zeste is responsible for mediating pairing between distant chromosomal regions, including homologs, resulting in the phenomenon of transvection, whereby *cis*-elements on one homolog can regulate the allelic gene on the other (Pirrotta, 1999). In the mouse genome, the 17-mer repeat was nearly unique, appearing at just one other locus, the *pre-T cell receptor alpha* gene, at low copy number (Reizis et al., 2000).

Generation of* Xist-ms2 *and* Xist-ms2-*Δ8.5kb* *ES cell lines

The genetic targeting scheme to generate the 8.5 kb deletion is depicted in Figure 6 with further experimental details in the figure legend. Two separate targeting constructs would be used to introduce 5' and 3' loxP sites, and then the intervening region, including selection cassettes, would be removed by Cre-mediated recombination. During the first targeting event, a "tag" consisting of 4 copies of the ms2 RNA hairpin would be introduced into the end of the *Xist* gene, allowing the targeted allele to be distinguished by FISH or by an ms2 coat protein-GFP fusion that would bind to the tagged RNA. The deletion would remove the 3' end of *Xist* exon 7 which had been

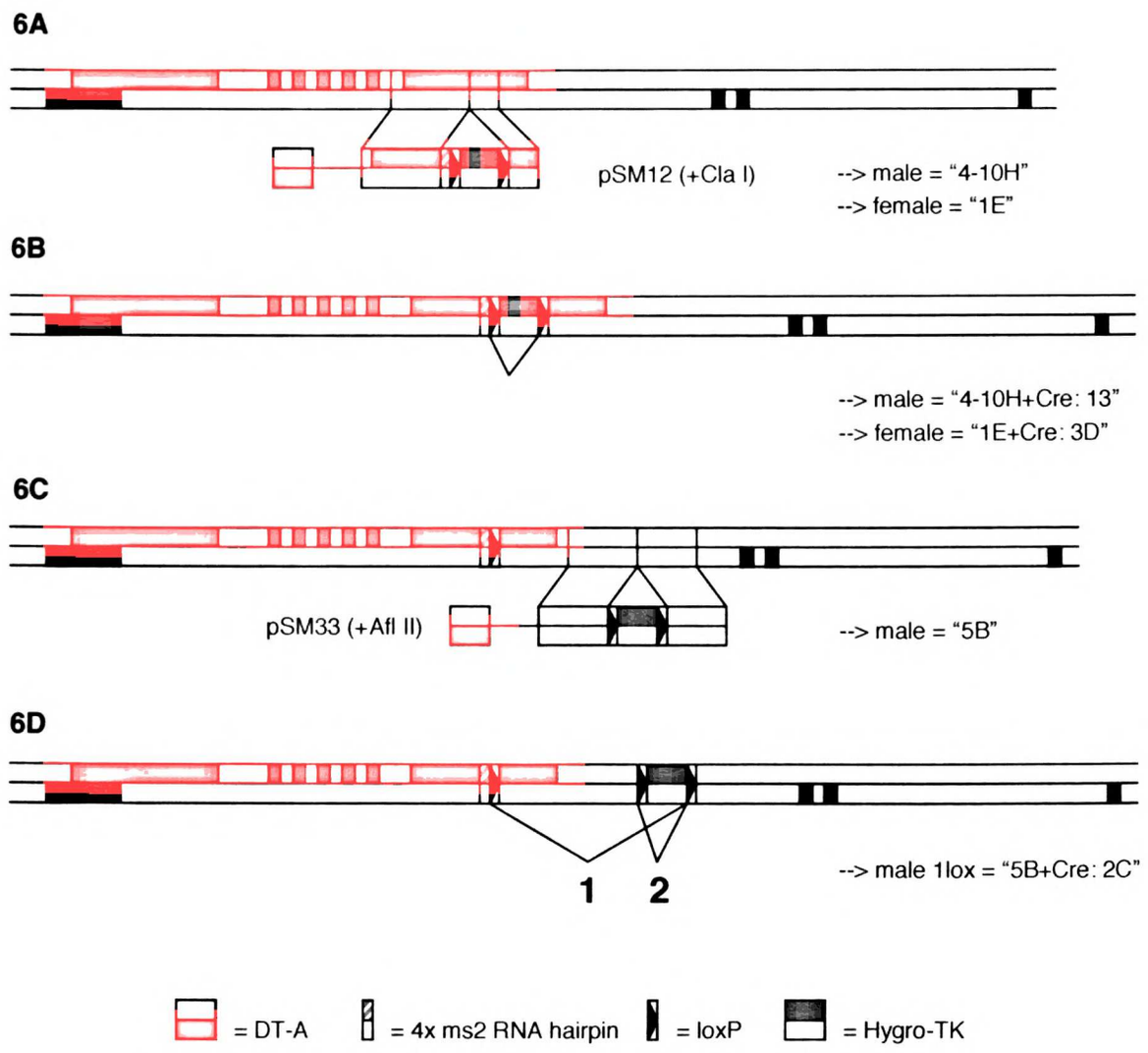


Figure 6. 8.5 kb deletion genetic targeting strategy.
Approximate positions of *Xist* (red) and *Tsix* (green) exons are depicted (not to scale). Targeting constructs are indicated including linearization sites. Recombinant cell line names are indicated.
(6A) Targeted insertion of 4xms2 RNA hairpin tag, 5' loxP site and hygro selection cassette. Clones were screened for targeting by PCR (ex6-4.1a x ex6-6.1b: 1.8kb = not tgted; hyTK-8F x ex6-6.1b: 1.8kb = tgted).
(6B) Cre-mediated excision of hygro selection cassette, screened by PCR (hyTK-1.1F x hyTK-6R: 750bp = non-recombinant; hyTK-1.1F x ex6-4b: 500bp = recombinant).
(6C) Targeted insertion of 5' loxP site and hygro cassette. Clones were screened by Southern blot as shown in Figure 7.
(6D) Cre-mediated excision of hygro selection cassette (2) and hygro + 8.5 kb region (1) confirmed by Southern blot as shown in Figure 7.

shown not to be essential for *Xist* function in a transgene assay (Wutz and Jaenisch, 2000). The ms2 tag and a triple polyadenylation sequence would remain at the end of the truncated exon 7 after the deletion.

The chosen starting ES cell lines were E14 (male, feeder free, 129 strain X chromosome) and 2-1 (female, feeder-dependent, 129 strain maternal and *castaneus* paternal X chromosomes). The first targeting step (Figure 6A) was to insert the ms2 tag and 5' loxP site into the middle of *Xist* exon 7. Linearized targeting construct was electorporated into ES cells according to the standard lab protocol. ES cell colonies resistant to 200 μg/mL hygromycin were picked after 11 to 14 days of selection and screened by PCR for integration of the targeting construct. A targeting efficiency of approximately 1 in 80 was observed in the E14 ES background. Targeted clones were obtained in male and female backgrounds. In the next step (Figure 6B), closed circular Cre plasmid was electroporated into the targeted clones and limiting dilutions were plated and counterselected with 4.5 μg/mL acycloguanosine. Colonies were screened by PCR and recombinants that had excised the hygro cassette were found at a frequency of approximately 1 in 8. Loss of hygro resistance was confirmed in a titration. The resulting cell lines are referred to as "male *Xist-ms2* ES" and "female *Xist-ms2* ES."

The second targeting step (Figure 6C) was to insert the 3' loxP site. Electroporation and selection were performed as with the first targeting step and colonies were screened by Southern blot (Figure 7). 1 in 100 colonies was correctly targeted in the male background, but no targeted female clone was identified in over 200 colonies screened. Next (Figure 6D), the male clone was transfected with Salk crippled Cre and selected as previously; recombinant clones that had excised hygro (2lox) or hygro and the 8.5 kb region (1lox) were confirmed by Southern (Figure 7). (The 2lox

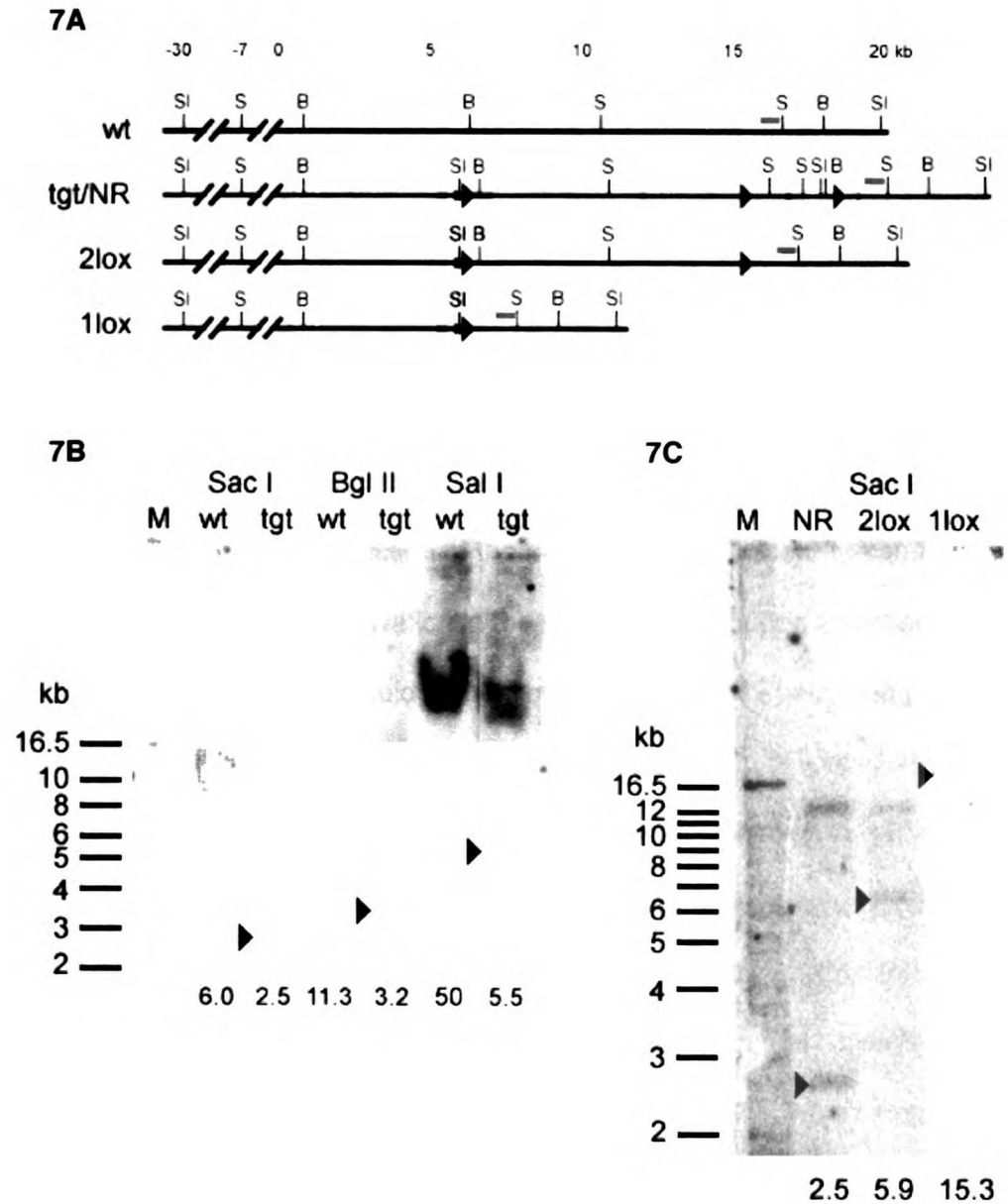


Figure 7. Confirmation of 8.5 kb deletion by Southern blot.

(7A) Genomic restriction maps for wild-type, doubly targeted (tgt/NR, as in Figure 5C), 2lox and 1lox recombinant (as in Figure 6D) clones. Arrowheads: loxP sites; S: Sac I; B:Bgl II; SI: Sal I. Location of the probe (760 bp amplicon from pSM26 using S26.2-f: taccctggaaaactctatct and S26.2-r: tttaaatatgtgtgtctgag) is indicated by the red line.

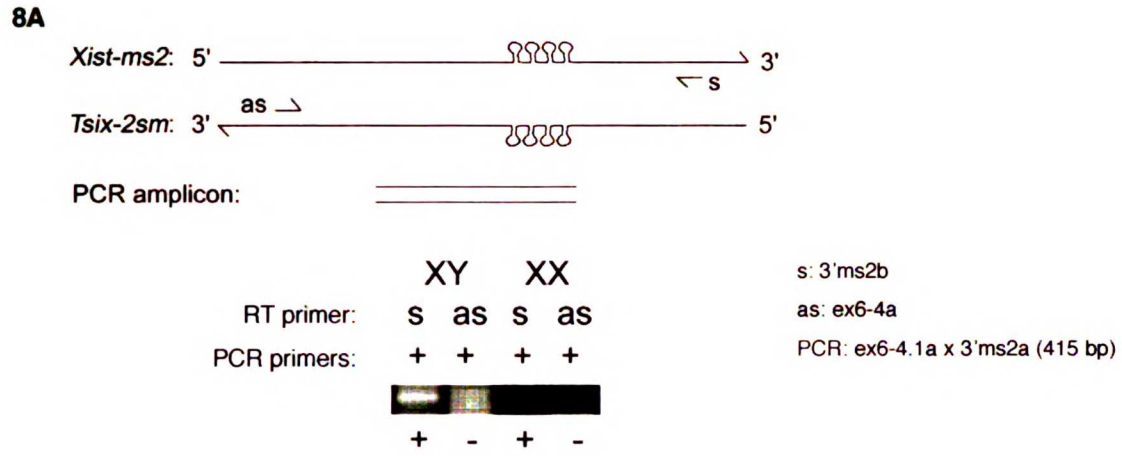
(7B) Three restriction enzymes were used to confirm the second targeting event; diagnostic band sizes (arrows) are indicated below the blot. M: markers.

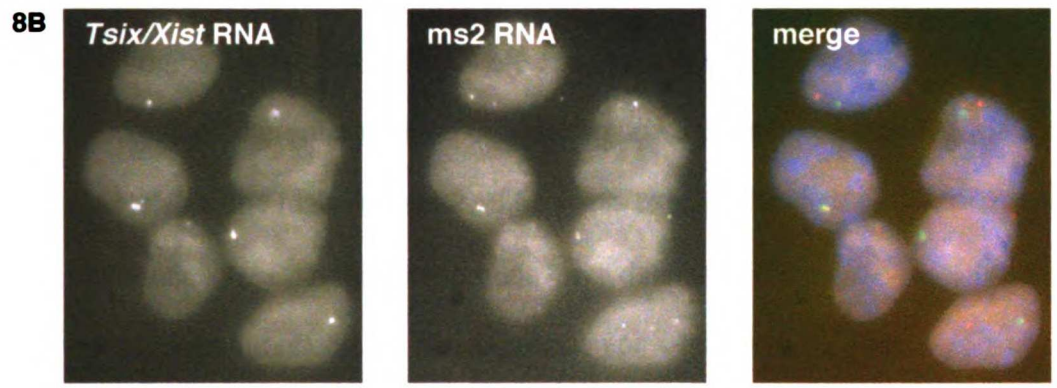
(7C) Confirmation of 1lox and 2lox recombination events.

clone was lost in the liquid nitrogen failure in 2003.) The resulting cell line is referred to as "male *Xist-ms2* Δ8.5kb ES."

ms2-tagged Xist *RNA is expressed and does not perturb male and female ES cells*

The male and female *Xist-ms2* ES cell lines did not exhibit any significant changes in overall *Xist* or *Tsix* RNA levels compared to the parental cell lines by RT-PCR (data not shown). Expression of the *Xist-ms2* fusion RNA was detectable in both cell lines by RT-PCR (Figure 8A), and in one FISH experiment in the male ES cell line I was able to detect pinpoint expression with a double-stranded ms2 probe (probably the *Tsix* strand; Figure 8B). Introduction of the ms2 tag did not appear to cause reproducible skewing of X-inactivation in the female cell line upon induction of differentiation, although this should be confirmed. The 4x ms2 hairpin tag therefore represents a useful reagent potentially allowing discrimination of *Xist* allele expression in fixed female cells by FISH, or visualization of *Xist* RNA localization in live female cells through use of an ms2 coat protein-GFP fusion. I created several versions of this fusion protein but found bright nuclear signal to obscure any possible specific localization of the protein bound to the RNA. My best lead was with a construct that contained a nuclear export sequence that reduced the nuclear background (pSM41, which may have been mixed up with another plasmid and should be remade according to my notes if thought to be useful). Use of the ms2 tag to analyze *Xist* expression during female ES cell differentiation could prove very interesting, allowing one to address the timing of each allele's upregulation in this female cell line that exhibits the *Xce* effect, as well as allowing visualization of the dynamics of *Xist* spreading during initial coating of the Xi.





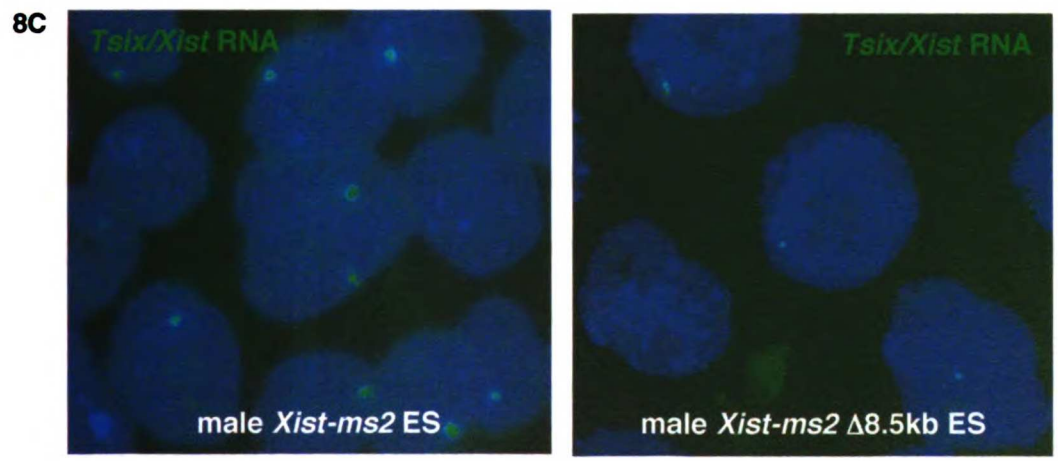


Figure 8. Phenotypes of *Xist-ms2* and Δ8.5kb ES cell lines.

(8A) Strand-specific RT-PCR scheme to detect sense and antisense transcription of the *Xist-ms2* fusion RNA in male and female *Xist-ms2* ES cell lines. +/- symbols below the agarose gel image indicate amplification of a specific product. Primer names are indicated to the right.

(8B) FISH for *Tsix/Xist* (left) and ms2 tag (center) in male *Xist-ms2* ES cell line.

(8C) FISH for *Tsix/Xist* (green) in male *Xist-ms2* (left) and *Xist-ms2* Δ8.5kb (right) ES cell lines.

The 8.5 kb deletion does not cause a "counting element" phenotype in male cells

Prior to differentiation, the male *Xist-ms2* Δ8.5kb ES cell line exhibited slightly weaker *Tsix/Xist* pinpoint RNA expression than the *Xist-ms2* control (Figure 8C). Induction of differentiation with retinoic acid, which triggers X-inactivation in female ES cells (Paterno and McBurney, 1985), leads to *Xist* upregulation on the Xi and shutoff of *Tsix* on the Xa (Lee et al., 1999a). In the male *Xist-ms2* Δ8.5kb (1lox) ES cell line, retinoic acid differentiation led to progressive loss of pinpoint *Tsix/Xist* expression on the single X chromosome with faster kinetics than the 2lox control male cell line (data not shown), perhaps because of initially weaker expression. There was no increase in cell death across the differentiation time-course compared to control male cells (data not shown). Notably, the male *Xist-ms2* Δ8.5kb cells exhibited no ectopic *Xist* upregulation upon differentiation. Several other groups have deleted this region in males and reported no profound effects on *Xist* expression or X-inactivation. Two separate deletions were made by Vigneau et al., 2006 (see constructs 4-8 and 4-9), and without showing their construct or data, Reizis et al., 2000, mentioned that the region did not show a phenotype in males. No group has yet analyzed the function of this region in female cells, and it is certainly worth completing the deletion in the female ES cell background not only for the sake of completeness, but also because, as I have tried to point out throughout this chapter, phenotypes of *Xic* mutations are frequently sex-specific. Because *Tsix* levels may be reduced by the mutation (Figure 8C), and another group has observed a slight increase in *Xist* expression in males when the region is deleted (Vigneau et al., 2006), I predict that in heterozygous females, the Δ8.5kb allele will cause the mutant X chromosome to more frequently become the Xi.

Closing remarks

At the time that I performed my experiments in the male Δ8.5kb ES cells in 2002, we interpreted the results to mean that the deleted region did not contain the counting element which we believed had been removed by the larger 65 kb deletion (Clerc and Avner, 1998). I no longer "believe" in the counting element as a discrete DNA sequence that is located within the 65 kb region, and I do not recommend basing future targeted genetic analysis on this hypothesis. However, many very interesting genetic experiments are still viable, as I have tried to note throughout this Appendix. I remain confident that genetic analysis of *Xic* function through transgenes and deletions, tested in male and female ES cells and mice, holds the key to unraveling the complex regulation of *Xist* that is at the heart of the mechanism by which one Xa and one Xi are designated in each female cell.

Acknowledgements

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cell line that he made using the targeting construct designed and partially constructed by Katie Worringer while I was supervising her rotation in the lab; and Cecile de la Cruz's findings of *Xist* downregulation in the Δ65 kb XO ES cell line after prolonged differentiation in culture. Thanks also to Jason Huff for prompting me to write down my thoughts about differences between mouse and human *Tsix/TSIX*. I thank Philippe Clerc, and Sandra Luikenhuis from Rudolph Jaenisch's lab, for sharing ES cell lines that informed my thinking on the subjects considered in this chapter.

Appendix 2.

The imprint controlling paternal X-inactivation is retained in female embryonic stem cells

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Summary

In this Appendix, I have collected information pertaining to my observation that ES cells retain the imprint that controls paternal X-inactivation in the extraembryonic lineages of the mouse. I present background information pertaining to the location of the X-inactivation imprint—that is, which chromosome (Xm or Xp) and what sequences within the *Xic* are likely to bear the parental mark. Then I provide information on the production of extraembryonic cell types from ES cells *in vitro*, and review my experiments that suggest the imprint is retained. I suggest follow-up experiments that could give mechanistic insight into imprinted X-inactivation.

Introductory Material

The phenomenon of genomic imprinting was originally defined by differential phenotypes of embryos inheriting DNA sequences exclusively from the mother or the father (Crouse, 1960). It has been shown that imprinted loci exhibit transcriptional differences between maternally- and paternally-inherited chromosomes (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991). Approximately 70 autosomally imprinted genes have been identified, and most are expressed in the placenta and regulate fetal growth (da Rocha and Ferguson-Smith, 2004). The prevailing theory for the evolution of imprinting is the "parental conflict theory," positing that in mammals, which exhibit considerable maternal

investment in carrying the developing young, the mother's and father's interests are not perfectly aligned (Moore and Haig, 1991). In this theory, there is selective pressure for the father's genome to promote the growth of the offspring at all costs, as larger offspring exhibit better survival and reproduction in the next generation, whereas pressure on the mother's genome is to limit invasive growth of the placenta and fetal size to ensure her own survival and fecundity for future pregnancies.

The observation of exclusive inactivation of the paternal X chromosome in the somatic tissues of female kangaroos was among the first examples of genomic imprinting (Sharman, 1971). X-inactivation in the mouse presents a special case of imprinting, as it is the only example in which the same locus is subject to both imprinted and random monoallelic expression (Takagi and Sasaki, 1975). During embryogenesis, X-inactivation occurs in three sequential waves, each one corresponding to differentiation of a distinct cell lineage within the early embryo (Monk and Harper, 1979; Sugawara et al., 1985). Imprinted X-inactivation occurs in the first two waves, which generate trophectoderm and primitive endoderm cells, extraembryonic lineages that contribute to the placenta. Random X-inactivation occurs in the third wave within the epiblast lineage that gives rise to the embryo proper (reviewed in the Prologue).

Like random X-inactivation, the imprinted process requires the *X-inactivation center*, including the two key non-coding RNA genes, *Xist* and *Tsix*. The expression patterns of *Xist* and *Tsix* in the pre-implantation embryo suggested that these RNAs function in imprinted X-inactivation. On the Xm, which will remain active in the extraembryonic lineages, a strong pinpoint of *Tsix* is expressed (Lee, 2000; Sado et al., 2001), while only a weak pinpoint of *Xist* transcription, if any, is detected *in cis* (Debrand et al., 1999; Okamoto et al., 2005). On the Xp, *Tsix* is not transcribed and strong expression of *Xist* RNA appears to progressively spread and coat the chromosome

(Okamoto et al., 2005; Okamoto et al., 2004; Sheardown et al., 1997). Thus, during the first wave of imprinted X-inactivation, a chromosome expressing *Tsix* exhibits cis-repression of *Xist*, whereas on a chromosome which does not express *Tsix*, *Xist* spreads and silences X-linked genes *in cis*. Genetic studies have confirmed critical roles for *Xist* and *Tsix* in imprinted X-inactivation. While maternal inheritance of an *Xist* deletion is tolerated, paternal inheritance is lethal due to deficient development of extraembryonic tissues caused by a lack of X-inactivation (Marahrens et al., 1997). Thus, *Xist* is required for imprinted inactivation of the Xp, and the functional *Xist* allele on the Xm apparently does not become upregulated to compensate for the lack of paternal X-inactivation. *Tsix* promoter deletions have the opposite parent-of-origin-specific effects. *Tsix* deletions are lethal only when inherited from the mother (Lee, 2000; Sado et al., 2001) due to ectopic de-repression of *Xist* and cis-inactivation of the Xm in addition to the Xp in most extraembryonic cells. As expected for genes with opposite effects on the same process, a paternally-inherited *Xist* deletion can be rescued by maternal inheritance of a *Tsix* loss-of-function allele (Sado et al., 2001).

The mechanisms for parent-specific gene regulation in genomic imprinting rely upon differential placement of chromatin marks on a given sequence during oogenesis or spermatogenesis; these marks later direct differential expression of the homologous alleles in the embryo. The primary imprinting mark that directs paternal X-inactivation in the trophectoderm and primitive endoderm lineages of the mouse has not been discovered. A 180 kb *Xic* transgene integrated into an autosome, when inherited from the father but not from the mother, perfectly recapitulates imprinted X-inactivation in the pre-implantation embryo, indicating that this region contains the site of the primary imprint and all the necessary cis-elements to read it out (Okamoto et al., 2005).

In theory, the imprint could be located at the *Xic* of either the Xm or the Xp. If on the Xm, the imprint would be predicted to result in repression of *Xist*, either directly or indirectly though activation of *Tsix*. If on the Xp, the imprint would be expected to exert the opposite effects on *Xist* or *Tsix*. Several experiments bear upon the subject of whether the Xm or the Xp is the site of the imprint. Aberrant inheritance of X chromosomes from the parents can lead to defects in dosage compensation and embryonic lethality. Embryos with exclusively paternal X chromosomes exhibit *Xist* RNA coating of all X chromosomes during the cleavage stages of development. However, XpXp androgenetic embryos (Latham et al., 2000; Okamoto et al., 2000) and XpO embryos (Matsui et al., 2001) are progressively able to repress *Xist* expression from the Xp over time, and ultimately achieve nearly correct dosage compensation. Embryos with extra maternal X chromosomes resist *Xist* expression and X-inactivation during early development. Correction of dosage compensation in XmXm parthenogenetic embryos by upregulation of *Xist* on at least one X chromosome was highly variable between embryos (Latham et al., 2000; Matsui et al., 2001; Nesterova et al., 2001). Whereas XpO embryos had shut off *Xist* expression from the single Xp in nearly 50% of cells by the blastocyst stage, XmXmY embryos had activated *Xist* expression from one Xm in at most 10% of cells at this stage (Matsui et al., 2001). The greater lability of the epigenotype of the Xp compared to the Xm may explain why some embryos survive maternal inheritance of *Tsix* deletions (Lee, 2000; Sado et al., 2001), while paternal inheritance of an *Xist* deletion is always lethal (Marahrens et al., 1997): *Xist* may be downregulated on the Xp in the former more easily than it may be upregulated from the Xm in the latter. This differential ability to reprogram *Xist* expression patterns on the Xp and the Xm suggests that the imprint controlling X-inactivation is located on the Xm, protecting it from inactivation. Nuclear transplantation experiments with cytologically

marked X chromosomes suggest that the Xm's resistance to inactivation is acquired during oocyte growth between prophase of meiosis I and meiosis II, a time when other imprints are placed on the maternal genome (Tada et al., 2000).

Many imprints in mammals require DNA methylation, differentially acquired in the male or female germline, for their establishment. Differential DNA methylation allows differential regulation of maternal and paternal gene copies in the embryo. Methylation of gene promoters is also associated with gene silencing outside of the context of genomic imprinting. Several groups have searched for differential methylation of the *Xist* gene that could serve as the X-inactivation imprint. In the male germline, CpG dinucleotides at the *Xist* promoter are methylated prior to meiosis, but this methylation is erased during spermatogenesis and is absent in mature spermatids (Ariel et al., 1995; Zuccotti and Monk, 1995). In the female germline, CpG dinucleotides at the *Xist* promoter and in the first exon become methylated during oogenesis and this methylation was thought to be retained upon fertilization as determined by methyl-sensitive restriction analysis (Ariel et al., 1995; Zuccotti and Monk, 1995). Another group failed to detect methylation in oocytes and early embryos using bisulfite sequencing (McDonald et al., 1998). It is therefore unclear whether the *Xist* gene on the Xm is methylated in the early embryo, which would correlate with lack of *Xist* transcription from the Xm. All reports concur that *Xist* on the Xp is demethylated, as expected for a highly expressed gene.

Another attractive target for the X-inactivation imprint is the *Tsix* gene. A maternal imprint should direct high maternal *Tsix* expression, while a paternal imprint would direct low or absent expression. It has been suggested that the CpG island at the *Tsix* promoter, *DXPas34*, is an imprinting center involved in keeping the *Xist* allele on the Xm silent during early embryogenesis (Lee, 2000). Although *DXPas34* is

differentially methylated on the Xa and Xi (Courtier et al., 1995), a bisulfite sequencing study designed to detect differential CpG methylation in oocytes and spermatocytes failed to find any differences between the two (Prissette et al., 2001). Subsequently, putative binding sites for CTCF have been identified in *DXPas34* (Chao et al., 2002). While CpG methylation has relatively little effect on CTCF binding to *DXPas34 in vitro*, non-CpG methylation abolishes CTCF binding in this assay.

Recently, clear differential methylation in gametes was identified at *Xite*, a transcriptional element upstream of *Tsix*, and the CTCF sites in the *Tsix* promoter region. These sites were unmethylated in oocyte DNA, and methylated in sperm DNA (Boumil et al., 2005). The methylation at *Xite* and the *Tsix* CTCF sites has the potential to serve as a primary mark for imprinted X-inactivation, with paternal methylation perhaps leading to repression of *Tsix* expression on the paternal X chromosome, in turn leading to paternal X-inactivation. Arguing against this possibility is the fact that the differential methylation was lost during pre-implantation development (Boumil et al., 2005), when imprinted *Tsix* and *Xist* expression leading to paternal X-inactivation is observed. Both maternal and paternal alleles are unmethylated in ES cells (Boumil et al., 2005), potentially correlating with the reprogramming of this cell type in preparation for random X-inactivation. Analysis of methylation patterns at these sites on autosomal *Xic* transgenes that become imprinted during gametogenesis (Okamoto et al., 2005) will help to discern whether this differential methylation is closely associated with the imprinting status of an allele. Finally, it should be borne in mind that the X-inactivation imprint may not necessarily consist of a primary DNA methylation mark, even though all other characterized imprinting systems employ differential methylation.

Observations on X-Inactivation in Extraembryonic Cell Types *in vitro*

Prior investigations of imprinting in ES cells

In the X-inactivation field, it is generally believed that the imprint controlling paternal-specific X-inactivation in the extraembryonic lineages is erased prior to random inactivation in the embryonic ectoderm. Furthermore, it is assumed that the imprint is not maintained in embryonic stem cells cultured *in vitro*. One study addressed the issue of possible retention of the X-inactivation imprint in ES cells. In a study which employed cytologically marked X chromosomes, a late-replicating Xp was detected in ES cell-derived extraembryonic cell types, providing evidence that the X-inactivation imprint may actually be maintained (Tada et al., 1993). A later attempt by the same group to reproduce these experiments was unable to confirm preferential paternal X-inactivation or *Xist* expression in these cell types (Sado et al., 1996), at variance with the prior study (Tada et al., 1993). Wishing to clarify the issue of whether the X-inactivation imprint is retained or erased in ES cells, we set out to examine the mode of X-inactivation exhibited by extraembryonic cell types produced by *in vitro* differentiation of ES cells.

ES *cell differentiation* in vitro *generates primitive endodermal cell types*

Embryonic stem cells are derived from the inner cell mass (ICM) of blastocyst stage mouse embryos, and are the closest *in vitro* correlate of these pluripotent cells. In a teratoma assay in mice and during EB differentiation *in vitro*, ES cells are able to give rise to the same lineages that the ICM gives rise to *in vivo*: the extraembryonic primitive endoderm lineage, and the definitive germ layers of endoderm, mesoderm and ectoderm. *In vivo*, imprinted inactivation of the paternally-inherited X chromosome is carried out in the primitive endoderm of female embryos, while the germ layer precursors

inactivate the maternal and paternal X chromosomes at random. *In vitro*, embryoid body (EB) differentiation of ES cells is commonly used to assay random X-inactivation, and it is widely believed that the imprint driving paternal X-inactivation has been erased in ES cells. However, the mode of X-inactivation—imprinted or random—that is followed in primitive endodermal cell types produced by *in vitro* differentiation of ES cells has not been adequately studied.

Two common *in vitro* differentiation methods are used that produce primitive endoderm derivatives (Figure 1). In embryoid body (EB) differentiation, ES cells are cultured in the absence of pluripotency-sustaining leukemia inhibitory factor (LIF) under non-adherent conditions in which they aggregate into small clumps; these clumps then commence differentiation, with the outer cells taking on a primitive endoderm-like fate within a few days (Martin and Evans, 1975). The primitive endoderm cells promote cavitation of the EB, wherein programmed cell death is triggered in the innermost cells and the remaining inner cells become organized into a columnar epithelium (Coucouvanis and Martin, 1995). If, after 3 to 4 days, these EBs are allowed to attach to a substrate, embryonic-like cell types arising from the inner cells grow out and can form a number of distinctive differentiated cell types and morphological structures (Martin and Evans, 1975). If, on the other hand, the EBs are maintained in suspension culture, cystic balloon-like structures often begin to expand out from the EB mass by approximately the 8th day (Martin et al., 1977). The mural region of cystic EBs is analogous to the yolk sac of the mouse embryo, consisting of an outer layer of visceral endoderm (a derivative of primitive endoderm) lined sparsely with a mesodermal component. Thus, cystic EB culture is a system in which primitive endoderm derivatives formed *in vitro* can be easily identified and studied. Although it is more difficult to isolate primitive endoderm cells from attached EB cultures, it is important to note that these

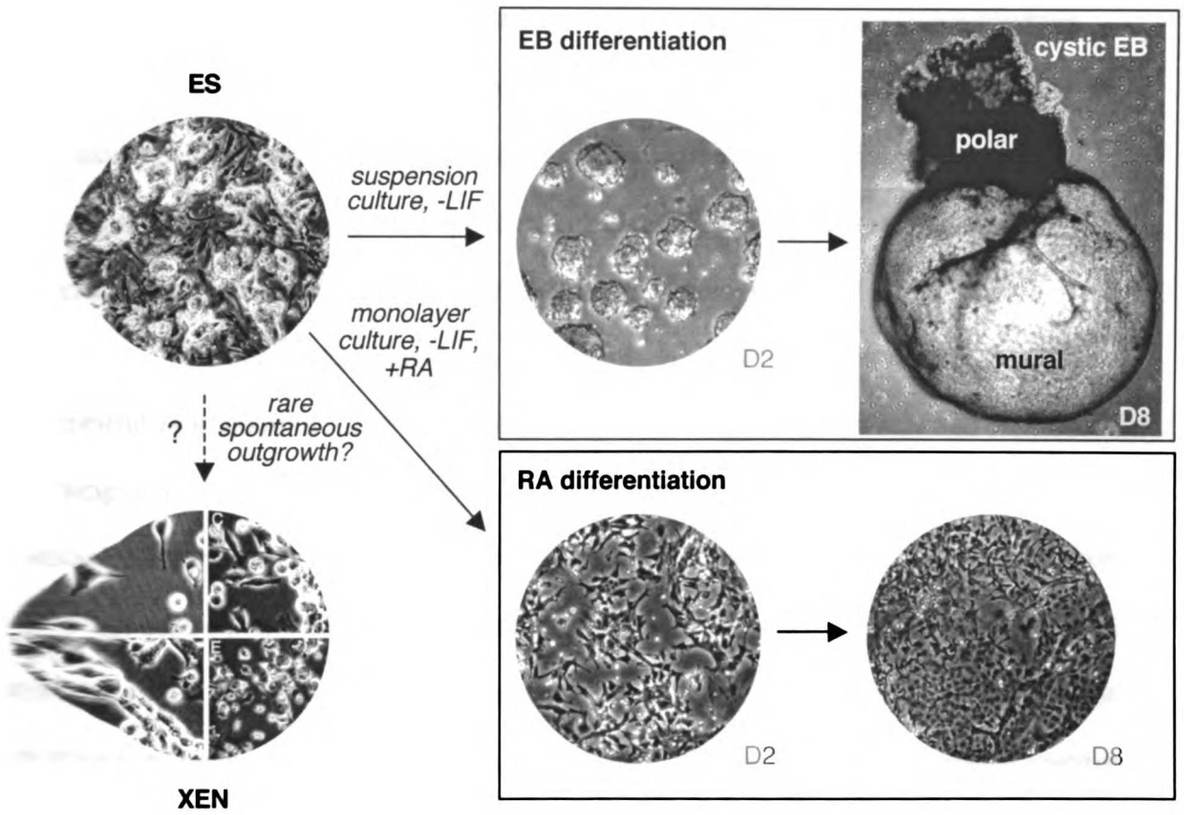

Figure 1. ES cell differentiation *in vitro* generates extraembryonic cell types. Depiction of three differentiation pathways for ES cells that generate extraembryonic cell types. In embryoid body (EB) differentiation, the simple EBs (left) form in which the outer cell layer appears to correspond to primitive endoderm. If simple EBs are cultured in suspension for approximately 8 days, cystic balloon-like structures (mural region) sometimes form that are the correlate of the mouse yolk sac with an outer primitive endodermal layer and a sparse inner mesodermal layer; the polar region of the EB primarily consists of embryonic-like derivatives. Retinoic acid (RA) differentiation produces visceral and parietal endoderm-like cell types. Finally, our observations in the Panning lab suggest that extra-embryonic endoderm stem (XEN) cells may sometimes arise spontaneously from ES cell cultures. XEN cell image was taken from Kunath et al., 2005.

cells play critical roles in this context: they are responsible both for substrate attachment and for signaling to induce differentiation of the inner cells into embryonic derivatives.

Another common differentiation method that produces primitive endoderm derivatives consists of plating ES cells onto an adherent surface at low density in the absence of LIF and the presence of retinoic acid (RA). Retinoid signaling triggers differentiation into the primitive endoderm lineage, and if cells remain sparse, further differentiation into parietal endoderm-like cells occurs (Hogan et al., 1981).

The late mouse blastocyst consists of three cell types: trophectoderm (TE), primitive endoderm (PrE), and epiblast, the latter two having been formed from the ICM population of the early blastocyst. Stem cell populations can be derived *in vitro* from the extraembryonic cell populations of the blastocyst (Rossant, 2001). Trophoblast stem (TS) cells can be derived from trophectodermal cells of the early blastocyst (Tanaka et al., 1998), and extraembryonic endoderm (XEN) stem cells have recently been isolated and characterized as well (Kunath et al., 2005). In both TS and XEN cells, X-inactivation has already taken place in an imprinted fashion, and the paternal X chromosome is silenced in all cells (Kunath et al., 2005; Mak et al., 2002).

Both ES and XEN cells are derivatives of the ICM lineage. The ability of ES cell lines to give rise to primitive endodermal cell types upon differentiation suggests that ES cells may retain the full developmental potential of both the primitive endoderm lineage and the epiblast lineage, whereas XEN cells are restricted to the primitive endoderm lineage. Thus, it is formally possible that ES cells could give rise to XEN cells *in vitro* (Figure 1), providing another route to primitive endoderm generation from versatile pluripotent ES cells.

Experimental Methods and Preliminary Results

A XEN-like cell line spontaneously arising from female ES cells exhibits exclusively paternal X-inactivation

In the Panning lab, we had noticed the appearance of a distinct subpopulation of cells in one of our female ES cell lines. These cells were small and refractile, appearing much like XEN cells in morphology (Figure 1; Kunath et al., 2005). When they were present in EB suspension cultures, cavitation was dramatically enhanced (data not shown), suggesting that the contaminating cells had properties of the primitive endoderm that normally stimulates this process (Coucouvanis and Martin, 1995). The contaminating cells proliferated more rapidly than the undifferentiated ES cells, and were overtaking the culture. As the ES cell cycle is already remarkably short, we considered the possibility that these cells had achieved a fitness advantage over the undifferentiated female cells by carrying out dosage compensation through X-inactivation, which is true of XEN cells (Kunath et al., 2005). The contaminating cells that had spontaneously arisen from our ES cell line therefore appeared to resemble XEN cells in a number of respects.

To investigate the nature of the ES 2-1 derived cell type, we isolated a clonal cell line, F5. The F5 cell line should be tested for a panel of XEN cell markers (Table 1) to confirm its identity. We determined that X-inactivation has taken place in F5. FISH for *Xist* RNA revealed a single, very intensely hybridizing *Xist*-coated domain in every cell (data not shown). We confirmed that this was an inactive X chromosome by showing that one of the two X's replicated asynchronously relative to the autosomes and its homolog (Figure 2A). Note that the Xi in XEN cells has not yet been tested for

	expression										
marker	ES	EB diff	RA diff	PrE	VE	PE	XEN	XEN diff	primer F	primer R	amplicon	reference
Oct4	+	+/-		-	-	-	-	-	Oct3a	Oct3b	313	
Afp	-	+		+/-	+	+/-	+/-	+	Afp-f	Afp-r	294	Kunath et al., 2005
Sox7	-	-					+	+/-	Sox7-f	Sox7-r	201	Kunath et al., 2005
Hnf4	-	+/-					+	+	Hnf4-f	Hnf4-r	357	Kunath et al., 2005
Hhex	-			+	+/-	+/-			Hex-f	Hex-r	396	SME notes 12-10-02
Fn1	+		-	+	+	-			Fnn1-f	Fn1-r	467	SME notes 12-10-02
B3gt5	-			+	+	-			B3gt5-f	B3gt5-r	227	SME notes 12-10-02
Thbd	-		+	-	-	+			Thbd-f	Thbd-r	273*	SME notes 12-10-02

Table 1. RT-PCR markers of primitive endodermal cell types.
These primer sets are intron-spanning unless marked with an asterisk. All are located in my primer stocks collection. PrE: primitive endoderm; VE: visceral endoderm; PE: parietal endoderm.

	expression markers						
marker	**f primer**	**r primer**	**amplicon**	**enzyme**	**buffer,T**	**129**	**cas**
Xist	Scr-f	Scr-r	307	ScrFI	4,37	307	186+131
Pgk1	Pgk-1f	Pgk-2r	425	MseI	2B,37	425	275+150
Hdac6	Hdac6-f	Hdac6-1r	272	BbvCI	4,37	166+106	272

Table 2. Primers and restriction enzymes for allele-specific analysis of X-linked gene expression. cDNA restriction enzyme site polymorphisms between 129 and *castaneus* mouse strains useful for assaying expression of X-chromosomal markers. References/sources for these polymorphisms are listed in the legend to Table 4.

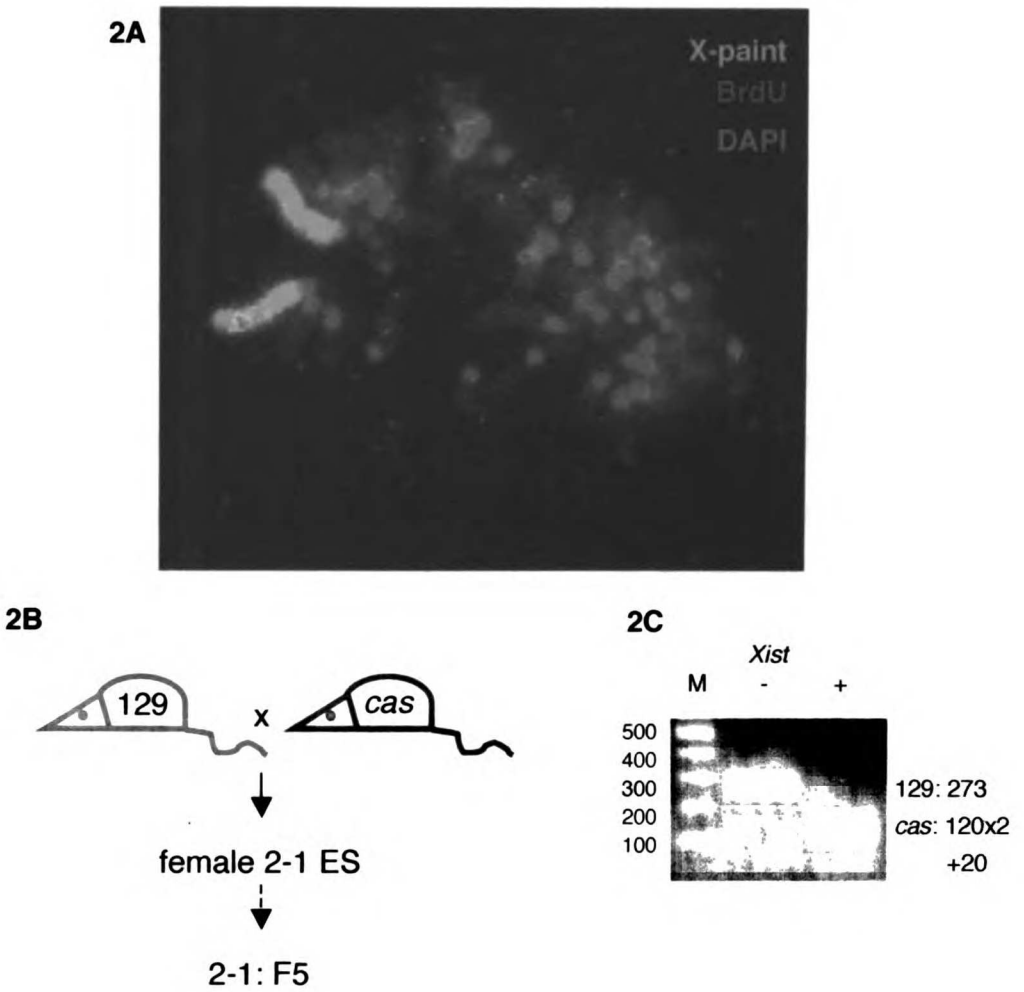

Figure 2. A XEN-like female ES cell derivative exhibits paternal X-inactivation.
(2A) The 2-1:F5 cell line has one asynchronously replicating X chromosome. Cultures were pulsed briefly with BrdU, washed and medium replaced, and incubated for 2-3 hours before harvesting and preparing mitotic chromosome spreads. Cells were subjected to FISH with X chromosome paint (red) followed by BrdU detection (green), which shows that one X incorporated BrdU at a different time than the rest of the genome. DNA was stained with DAPI (blue).
(2b) The mouse cross that generated the 2-1 ES cell line, from which the F5 XEN-like clone spontaneously arose. Mother: pink; father: blue.
(2C) Allele-specific RT-PCR shows that *Xist* is expressed exclusively from the paternal X chromosome in 2-1:F5. Primers were NS66 and NS67, and the enzyme (- or +) was ScrFI. Note: this amplicon is not intron-spanning.

asynchronous replication, so if F5 is confirmed to be a XEN line, then ours will be the first demonstration of this particular Xi feature in this cell type.

In XEN cells, the paternally-inherited X chromosome is exclusively inactivated. The ES cell line from which F5 is derived was generated from a cross between a female mouse carrying a 129 strain X chromosome, and a male mouse carrying a *castaneus* X chromosome (Figure 2B; Marahrens et al., 1997). The existence of polymorphisms between these two strains allows discrimination of gene expression from the maternal vs. the paternal X chromosome (Table 2). By allele-specific RT-PCR, the *Xist* allele on the *castaneus* X chromosome was exclusively expressed in the F5 cell line (Figure 2C), indicating that the paternal X chromosome has been inactivated in every cell. Thus, it appears that in an ES cell culture, a XEN cell line was spontaneously produced and underwent imprinted X-inactivation. Because the XEN cells originally contaminating the ES 2-1 cell line may all have been derived from a single original cell that could have inactivated the paternal X by chance, although because of the *Xce* effect in the 129/*cas* cross, the *cas* X should be inactivated in only 20% of cells (Cattanach and Rasberry, 1994). It will be important to extend this observation by isolating XEN cells from other female ES cell lines and determining if they also exhibit imprinted X-inactivation.

The X-inactivation imprint is maintained in reciprocal crosses of female ES cells

The observation that an ES-derived XEN cell line exhibited paternal X-inactivation raised the possibility that the imprint specifying paternal X-inactivation is preserved in ES cells. Therefore, we assayed whether X-inactivation also occurred in an imprinted manner upon purposeful production of extraembryonic cell types by *in vitro* differentiation of ES cells. We induced differentiation of ES cells into cystic EBs (Figure 1) and confirmed that the primitive endoderm derivatives in the mural region had

completed X-inactivation (Figure 3A). Next, taking advantage of the known parentage of the 2-1 ES cell line (Figure 3B), we assayed X-linked gene expression by allele-specific RT-PCR (Table 2). In samples from the polar region of cystic EBs, which consists of cell types that correspond to embryonic lineages and exhibit random X-inactivation, *Xist* was expressed from both the 129 and the *castaneus* X chromosomes (Figure 3C). The X-linked *Pgk1* gene, which is silenced on the Xi, was also biallelically expressed (Figure 3C), consistent with random X-inactivation. In contrast, samples from the mural region of primarily primitive endodermal cells exhibited almost exclusive expression of *castaneus Xist* and 129 *Pgk1* (Figure 3C), indicating that a maternal Xa and a paternal Xi were present in the majority of cells. These data are consistent with X-inactivation occurring in an imprinted fashion in primitive endodermal cell types produced by EB differentiation of ES cells *in vitro*.

To confirm and extend these results, we tested the pattern of X-inactivation in an ES cell line generated from the reciprocal cross of mouse strains. In the 1/c16 ES cell line, the maternal X chromosome is of *castaneus* origin, and the paternal X chromosome is from the 129 strain background (Figure 3B; Lee et al., 1999b). In samples from the mural region of cystic EBs, the paternal 129 strain *Xist* allele was exclusively expressed (Figure 3D), demonstrating imprinted X-inactivation in a second female ES cell line. At the time that these experiments were performed, this cell line was contaminated with XpXp (di-paternal X chromosome) ES cells, which explains how exclusive paternal *Xist* expression can be reconciled with the biallelic *Pgk1* expression that can be seen in Figure 3D (in the XmXp cells, the Xm is the Xa; in the XpXp cells, one of the Xp's is the Xa). This experiment should be repeated using the 16.7 cell line obtained from Jeannie Lee's lab, which is an XmXp clone of the 1/c16 ES cell line (confirmed in Figure 6A).

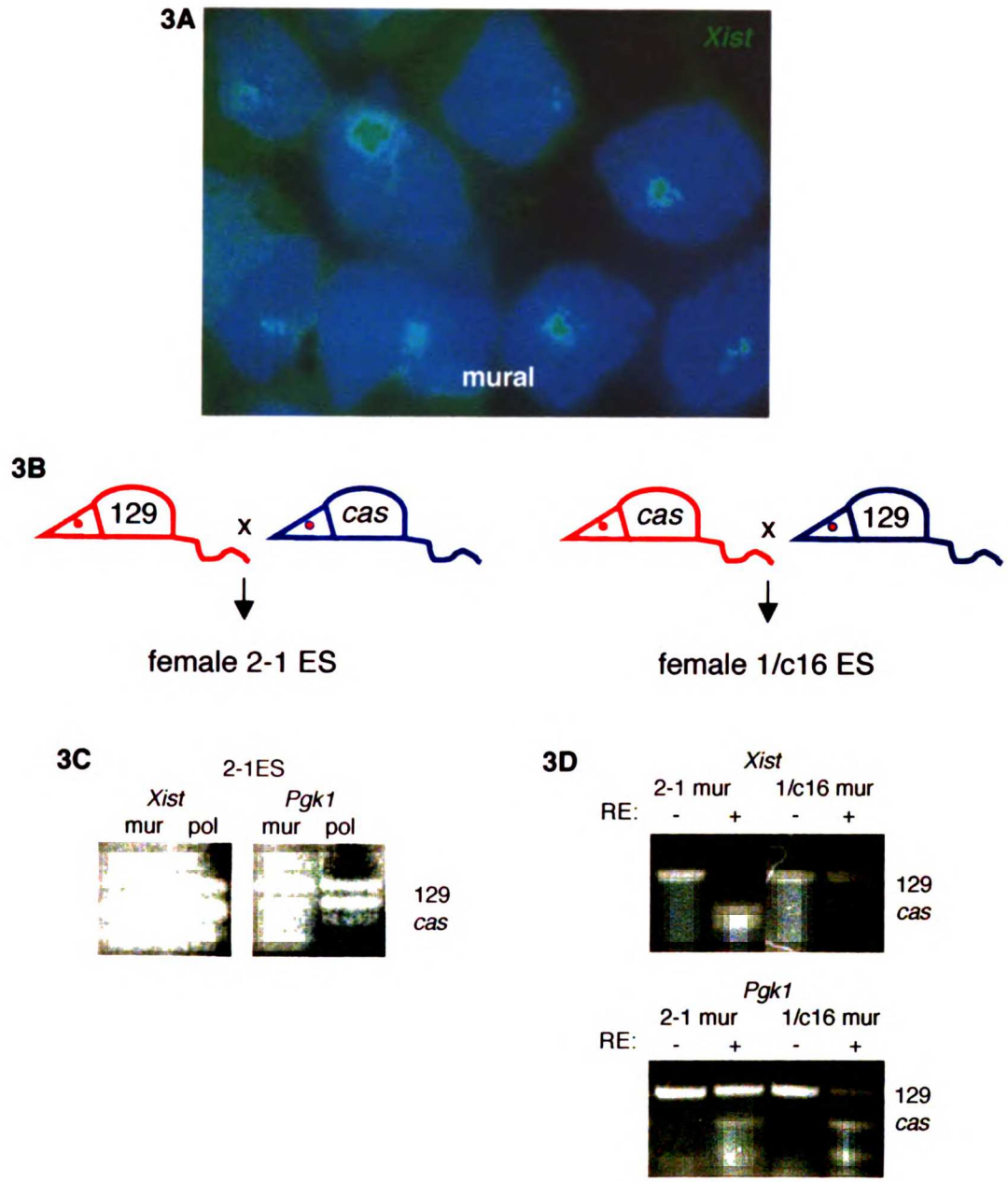

Figure 3. The mural region of cystic EBs exhibits imprinted X-inactivation.

(3A) Mural cells of cystic EBs have undergone X-inactivation as shown by FISH for *Xist* RNA (green).

(3B) Reciprocal mouse crosses were used to derive the ES cell lines 2-1 and 1/c16.

(3C) Allele-specific RT-PCR in 2-1 ES cell-derived cystic EB tissues. *Xist* and *Pgk1expression* in mural and polar region RNA samples were assayed by PCR using the primers indicated in Table 1 and were digested with the appropriate restriction enzyme.

(3D) Allele-specific RT-PCR in mural region RNA samples from 2-1 and 1/c16 ES cell-derived cystic EBs. PCR was performed as in C and the undigested (-) and digested (+) PCR products are shown. The expression of some 129 allele *Pgk1* is due to the contamination of the 1/c16 cell line with XpXp cells which express *Pgk1* from the 129 allele.

Taken together, these data show that imprinted X-inactivation occurs *in vitro*. Therefore, the imprint controlling paternal X-inactivation is preserved in female ES cells.

Imprinted X-inactivation does not occur upon retinoic acid differentiation

Differentiation of ES cells in monolayer in the presence of retinoic acid (RA) induces the formation of primitive endoderm derivatives (Hogan et al., 1981) and is accompanied by X-inactivation (Paterno and McBurney, 1985). Therefore, I wondered whether the imprint could also drive paternal X-inactivation during RA differentiation. In preliminary experiments, differentiation of the 2-1 ES cell line in monolayer in the presence of 100 nM RA essentially as in Paterno and McBurney (1985) resulted in morphological changes consistent with primitive and parietal endoderm specification (see Figure 1), but both maternal and paternal *Xist* and *Pgk1* appeared to be expressed (data not shown). This experiment indicated that X-inactivation may not occur in an imprinted manner in RA-differentiated cultures, even though primitive endoderm cell types appeared to be produced. Random X-inactivation upon RA-induced differentiation would be consistent with early findings in an embryonal carcinoma cell line (Paterno and McBurney, 1985). This experiment should be performed again carefully, paying attention to the following things: First, the completeness of X-inactivation in RA-differentiated cultures should be confirmed by FISH for *Xist* RNA coating the chromosome and for silencing of an X-linked gene (*Pgk1*; my BAC probe works great for detecting nascent RNA pinpoints). I have anecdotally found that in some RA-induced samples where the cells look completely differentiated, I have been unable to detect *Xist* coating the chromosome. If X-inactivation hasn't happened, then of course *Xist* and *Pgk1* expression will be biallelic. Second, RA-differentiated cells should be subjected to

testing by a panel of RT-PCR markers (Table 1) to confirm that they are indeed primitive/parietal endoderm.

If the preliminary finding of random X-inactivation in RA-differentiated PrE derivatives is upheld, then this may suggest that readout of the imprint is depends upon the cellular context in which differentiation occurs. What is common to differentiation events in the early embryo *in vivo* and in EB differentiation *in vitro*, both of which trigger imprinted X-inactivation in primitive endodermal lineages, that is different in RA differentiation? In the former developmental systems, cells differentiate within a three-dimensional context in which signaling with their neighbors plays an important role in specifying cell fates and morphological changes (Coucouvanis and Martin, 1995; Coucouvanis and Martin, 1999). By contrast, in RA differentiation, ectopic retinoid signaling drives cells into the primitive endoderm lineage in the absence of significant cell-cell contacts. In Figure 4, I show a very speculative model in which cell-cell signaling in EBs (and the early embryo) leads to primitive endoderm specification and activation of the X-inactivation imprint. In the absence of reading out the imprint, the ability of RA-differentiated ES cells to undergo X-inactivation in a random fashion is consistent with my observations that undifferentiated ES cells already have a mechanism in place for randomly assigning future Xa and future Xi fates (Mlynarczyk-Evans et al., 2006). RA can also promote primitive endoderm to visceral endoderm differentiation in the context of ES cell aggregation into EBs in suspension culture (Hogan et al., 1981). Using this RA differentiation protocol in which cell-cell signaling can occur may inform the above hypothesis, and will distinguish between a dominant role for retinoid signaling in overriding the imprint, versus the involvement of cell-cell contacts in promoting its readout.

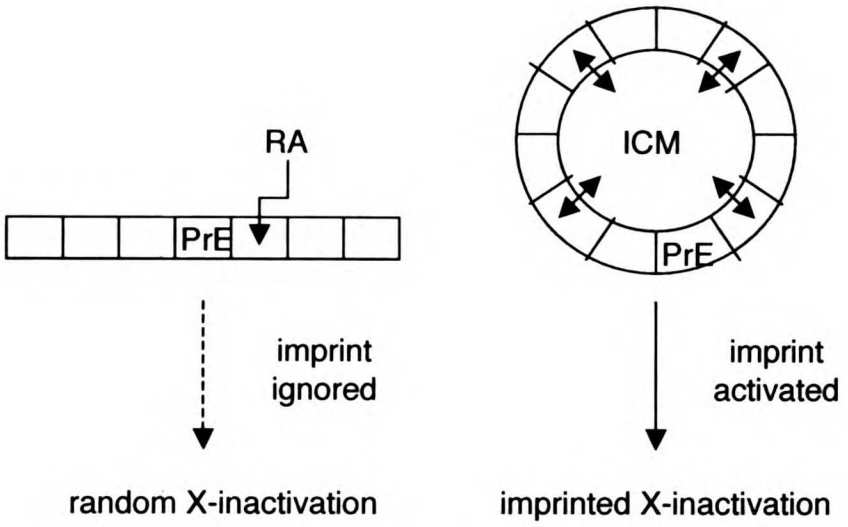

Figure 4. A speculative model for activation of the X-inactivation imprint.
A model in which cell-cell signaling is required for activation of the imprint driving paternal-specific X-inactivation. In monolayer culture, ectopic retinoid signaling is sufficient to induce primitive endodermal specification without activating the imprint, whereas in the three-dimensional context of the embryoid body, signaling between the internal and external cells triggers primitive endoderm specification and activation of the imprint.

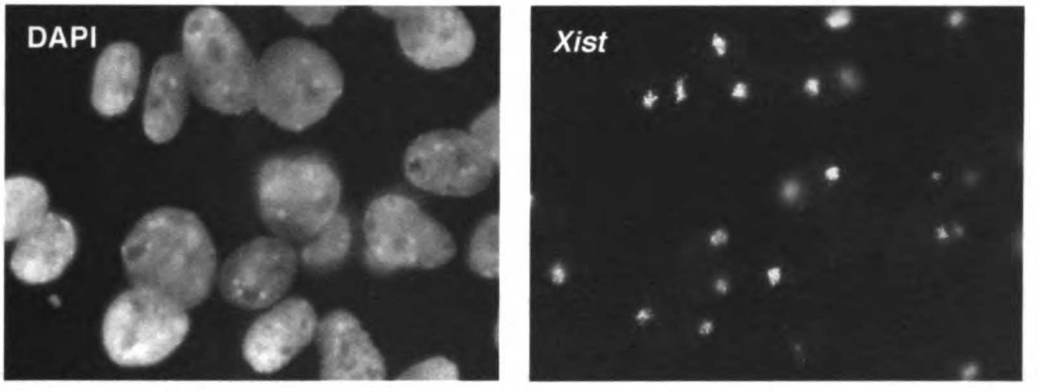

Figure 5. De-differentiation of ES into TE cells is accompanied by X-inactivation.
2-1 ES cells plated onto coverslips were treated with siRNA against Oct4 and cultured for 5-6 days before fixation. Patches of cells with TE-like morphology were visible. FISH for *Xist* RNA (right) confirmed the presence of an *Xist*-coated chromosome in these cells, which exhibited a distinct flattened and elongated nuclear morphology (DAPI, left) compared to ES cell nuclei, which are smaller in diameter, spherical, and more intensely staining.

Does imprinted X-inactivation occur upon de-differentiation of ES cells into TS/TE cells?

In the early mouse embryo, ICM cells arise after specification of the trophectoderm (TE) lineage, and the ICM and ES cells do not normally differentiate into TE cells. However, it has recently been found that interruption of certain pluripotency signaling pathways can cause ES cells to de-differentiate into TE-like cell types (Niwa et al., 2000; Rossant, 2001). Treatment of low-density ES cell cultures with siRNA directed against *Oct4* can cause ES cells to de-differentiate into cells that appear morphologically like trophectoderm (TE) cells and express TE markers (Velkey and O'Shea, 2003). TE cells, which are the first cells to differentiate and undergo X-inactivation in the early embryo, and the trophoblast stem (TS) cell lines that can be cultured *in vitro*, both exhibit imprinted X-inactivation (Mak et al., 2002). As the X-inactivation imprint appears to be read out upon "forward" differentiation into extraembryonic cells, it makes sense to determine whether ES cells undergo imprinted X-inactivation upon de-differentiation into TE cells. Because siRNA treatment is performed essentially in monolayer, the answer to this question will also have bearing on the issue of whether cell-cell signaling is involved in readout of the imprint, as discussed above.

Tom Fazzio has optimized a system for production of siRNA (Yang et al., 2002) and transfection into ES cells (using an Invitrogen protocol, similar to Hay et al., 2004). With his help, I made one quick attempt at Oct4 siRNA transfection into female 2-1.10B ES cells to cause de-differentiation into TE cells. The outcome of this experiment was uninformative, and some troubleshooting will be necessary to increase the efficiency of siRNA in female ES cells. Five days after Oct4 siRNA transfection, approximately 5 to 10% of female ES cells appeared differentiated and TE-like, compared to the near complete effect Tom observes in male E14 ES cells. This may be related to cell line

differences, or specifically to the feeder-dependency of the female cell line which contrasts with the feeder-independence of the E14 cell line. The attachment of ES cells in monolayer appears to be important for siRNA efficiency, and it would be worth trying to transfect female ES cells in the absence of feeders, as this may promote monolayer attachment. Feeders may interfere with siRNA effects and also may complicate later analysis of the specific phenotype of the ES cells. If feeders must be used, one could try pre-treating them with Oct4 siRNA to "block" them the day before plating and Oct4 siRNA transfection of the female ES cells.

I did obtain one clear result pertaining to the X-inactivation phenotype of female ES cells de-differentiated into TE cells: X-inactivation does occur upon de-differentiation into TE (Figure 5). When this experiment is repeated, the identity of the TE-like cells should be confirmed by assaying them for TE markers (Tanaka et al., 1998). Angela Andersen, who has expertise in the derivation and culture of TS cell lines, has useful primer sets for this characterization. She plans to derive TS cells from ES cells *in vitro*, and will have useful input on experimental design. It is really worth pursuing this experiment as an answer to the question of whether ES-derived TE cells exhibit imprinted X-inactivation should be forthcoming.

Phenotypes of ES cells with aberrant X chromosome parentage

To begin to analyze the functional consequences *in vtiro* of retention of the X-inactivation imprint, it will be informative to test the imprinted X-inactivation phenotypes of ES cells with abnormal parental contributions of the X chromosome(s). A basic characterization of X-inactivation phenotypes in imprinted tissues produced by XmO, XpO, XmXm, and XpXp ES cell lines will provide an important foundation of information. We have or could obtain all four of these cell lines, as indicated in Table 3. They should

cell line	description	reference	sex chrs	imprinting prediction
2-1(.10B) ES	129/cas cross	Marahrens et al., 1997	Xm Xp	**Xa Xi**
1/c16 (16.7) ES	cas/129 cross	Lee et al., 1999 *Nat. Genet.*	Xm Xp	**Xa Xi**
parthenogenetic ES	parthenogenetic	Gribnau et al., 2003	Xm Xm	**Xa Xa****
1/c16: 3E	39,XO derived from 1/c16 ES	S. M.-E.	Xm O	**Xa O**
1/c16: 5H	41,trisomy 8 derived from 1/c16 ES (also LOH for Chr 16???)	S. M.-E.	Xp Xp	**Xi Xi***
			Xp O	**Xi Y***
Fa2L (female TpA)	*Tsix* -pA insertion in 129 allele of 2-1 ES	Luikenhuis et al., 2001	$Xm^{Tsix\text{-}pA}Xp$	**Xi Xi***
Ma2L (male TpA)	*Tsix* -pA insertion in male J1 ES	Luikenhuis et al., 2001	$Xm^{Tsix\text{-}pA}Y$	**Xi O***
F.ind1a (female tet-Tsix)	tet-*Tsix* insertion in 129 allele of 2-1 ES	Luikenhuis et al., 2001	$Xm^{tet\text{-}Tsix}$ Xp	**Xa Xi**
M.ind1a (male tet-Tsix)	tet-*Tsix* insertion in male J1 ES	Luikenhuis et al., 2001	$Xm^{tet\text{-}Tsix}$ Y	**Xa Y**
H12	EF1α-*Tsix* insertion in 129 allele of 16.7 ES	Stavropoulos et al., 2001	$Xm\ Xp^{EF1\alpha\text{-}Tsix}$	**Xa Xa****
D102	65 kb deletion in Pgk1a allele of HP3.10 ES (129/Pgk1a cross)	Clerc and Avner, 1998	$Xm\ Xp^{\Delta 65kb}$	**Xa Xi**
XLD	truncation of most of Xm in D102 ES	Clerc and Avner, 1998	$(Xm^{trunc})\ Xp^{\Delta 65kb}$	**(Xa) Xi*****
CK35Δ65	65 kb deletion in male CK35 ES	Morey et al., 2004	$Xm^{\Delta 65kb}$ Y	**Xa Y**

Table 3. Parental origins of X chromosomes in ES cell lines and imprinting predictions. The predicted imprinted X-inactivation outcomes *in vitro* are based on the Xm always remaining active and the Xp always being inactivated, the known effect of loss of *Tsix* function on the Xm leading to its inactivation in imprinted X-inactivation, and the inference that *Tsix* hyper-expression on the Xp would block its inactivation in imprinted X-inactivation. *imprinting phenotype would lead to inappropriate X-inactivation if *Xist* cannot be down-regulated on the wt Xp. **imprinting phenotype would lead to lack of X-inactivation if *Xist* cannot be up-regulated from the wt Xm. ***imprinting predicted to enhance ectopic X-inactivation phenotype of this mutation. Cell line in grey has been lost, but could possibly be re-derived from the original Jaenisch lab stock.

be subjected to cystic EB differentiation, or to another mode of differentiation in which the imprint is read out *in vitro* (de-differentiation into TE?).

XmO ES cells should not undergo X-inactivation, just like normal male XmY ES cells do not. I have cloned out a 39,XO ES cell line from 1/c!6 (clone 3E; Chapter 2, Figure 1A), and using a panel of X-chromosomal markers to genotype it (Table 4), have shown that the single X chromosome is of maternal (*cas*) origin (Figure 6A). I have not yet confirmed lack of X-inactivation in imprinted lineages, but this experiment should be straightforward.

XpO ES cells may be expected to display an imprinted X-inactivation phenotype, because the Xp is normally inactivated in imprinted lineages *in vivo*. I had isolated an XpO ES cell line from a stock of 2-1-derived ES cells (Table 3). Unfortunately, both this cell line and the parental stock were lost in the liquid nitrogen disaster of 2003. Before it was lost, I had tested the XpO cell line for imprinted X-inactivation by EB differentiation. I expected that if imprinted inactivation of the Xp occurred, the cell line might be unable to form cystic structures. Instead, this cell line exhibited an enhanced ability to generate cystic balloon structures compared to the parental line, and ectopic X-inactivation was never observed, arguing against the functional significance of any imprint on the Xp. At early stages of EB differentiation, I did note a low frequency of cells that showed limited "sparkles" of *Xist/Tsix* emanating from the *Xic* (probably *Xist*; Figure 6B), which is not observed in XmY ES cell lines, suggesting that the Xp might exhibit greater expression of *Xist* than the Xm during specification of the X-inactivation pattern in the primitive endoderm lineage *in vitro*. Given evidence that the Xp epigenotype is less rigid than the Xm for imprinted X-inactivation, perhaps the lack of ectopic X-inactivation in XpO ES cells should be expected, and this result may support the idea that the Xm is the site of an imprint that protects it from inactivation in extraembryonic lineages.

	genomic DNA markers						
marker	**f primer**	**r primer**	**amplicon**	**enzyme**	**buffer,T**	**129**	**cas**
X Chr:							
Hdac6 (6.19 Mb)	Hdac6-1f	Hdac6-1r	634	XmaI	4B,37	634	412+222
13.58 Mb X	13.58-f	13.58-r	163	HpyCH4III	4,37	163	110+53
20.93 Mb X	20.93-f	20.93-r	180	FokI	4,37	69+111	180
44.85 Mb X	44.85-f	44.85-r	200	Bsp1286I	4B/37	200	118+82
64.33 Mb X	64.33-f	64.33-r	199	BfaI	4/37	98+101	199
Xist	NS66	NS67	264	ScrFI	4,37	25+239	25+132+107
Pgk1	Pgk-gf2	Pgk-gr2	285	MseI	2B,37	285	153+132
Autosomes:							
Mor240-2	Mor240-2f	Tsp-r	499	Tsp45I	1B,65	404+95	239+165+95
Igf2	Igf2-f	Igf2-r2	313	HincII	3B,37	313	171+142?

Table 4. Primers and restriction enzymes for (X) chromosome genotyping.
Genomic restriction enzyme site polymorphisms between 129 and *castaneus* mouse strains useful for assaying chromosomal origin. I discovered the numerical markers (which correspond to position, in Mb, on the X chromosome) by analysis of a database of *cas* sequences (Lindblad-Toh et al., 2000); *Xist* marker is from Stavoropoulos et al., 2001; *Pgk1*, *Hdac6*, and *Mor 240-2* markers are based on my own sequencing and confirmation of polymorphisms in these genes; *Igf2* is from Gribnau et al., 2003.

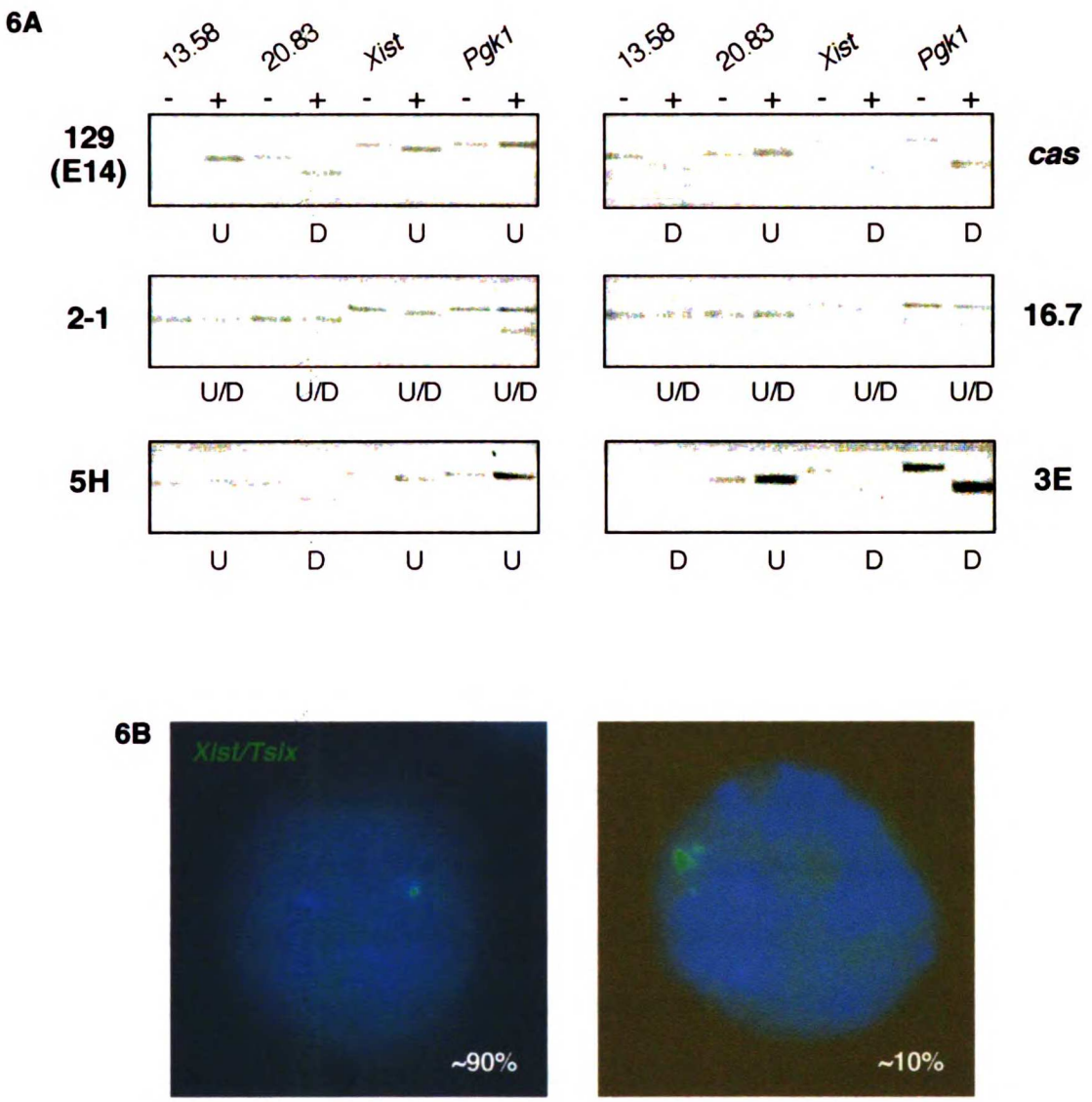

Figure 6. Analysis of ES cell lines of aberrant Xm/Xp constitution.

(6A) PCR genotyping of ES cell lines using the primers shown in Table 4. +/- addition of restriction enzyme; U/D undigested/digested. The two wild-type female ES lines (2-1, 16.7) are heterozygous at all markers, while 5H (XX, Epilogue, Figure 8A) shows exclusively 129-derived X chromosomes (paternal in the parental 1/c16 ES), and 3E (XO, Chapter 2, Figure 1A) shows a *cas* X chromosome (maternal in the parental 1/c16).

(6B) EB differentiation of XpO cells was performed for 2-4 days in suspension culture. By FISH for *Xist/Tsix* RNA (green), most cells showed a normal pinpoint signal (left), while a minority of showed "sparkles" (right) that are rare in differentiating XmY ES cells.

Testing the imprinted X-inactivation phenotype of XmXm ES cells *in vitro* will be highly informative. The bulk of the evidence indicates that the Xm is where the imprint resides, so a phenotype is most likely to be observed with this X chromosome parentage. The prediction is that neither X chromosome will be able to be inactivated in the primitive endodermal lineage *in vitro*. This could be manifested in cystic EB culture as an inability to form cysts, or possibly not to undergo differentiation at all. Parthenogenetic ES cells are formed from embryos in which both sets of chromosomes derive from the mother, so they have an XmXm constitution. A parthenogenetic ES cell line has been obtained from the Jaenisch lab (Gribnau et al., 2003), and should be tested in the cystic EB assay. The presence of di-maternal copies of every chromosome may be problematic, such that any poor differentiation phenotype could be due to mis-expression of other imprinted genes. It is also possible that parthenogeneic ES cells may not exhibit rigid maintenance of imprinting (Nesterova et al., 2001). Thus, a lack of phenotype may not be conclusive either. In that case, another idea for how to generate a di-maternal ES cell line in a controlled manner will be presented further below.

To complete analysis in a logically complete set of cell lines, it will be informative to assay ES cells with XpXp constitution, such as 1/c16: 5H (Figure 6A; Epilogue, Figure 8A). This cell line has additional chromosome abnormalities, such as trisomy for chromosome 8; fortunately, this trisomy has been reported not to affect differentiation potential (Park et al., 1998). Based on the lack of phenotype in the XpO ES cell line, I would predict that an XpXp ES cell line should exhibit appropriate X-inactivation, with inactivation of a single X. This result would indicate that in the absence of an imprint protecting a chromosome from inactivation, the random X-inactivation mechanism can operate. In fact, the apparent contribution of XpXp cells to the cystic tissue of embryoid bodies in my early experiments with the 1/c16 ES cell line (Figure 3D) suggests that

appropriate X-inactivation is achieved in the 5H cell line, and that the primitive endoderm lineage can form normally. To support these conclusions, the experiment should be repeated using the clonal population of 5H cells.

To sum up, assaying this informative panel of ES cell lines represents a logically complete set of experiments. I have not yet analyzed any of the cell lines of exclusive Xm chromosomes, and the XmXm parthenogenetic ES cell line will be very interesting as the imprint is hypothesized to reside on this chromosome. I have very preliminarily assayed the cell lines of exclusive Xp origin, and my sense is that ectopic inactivation of the Xp does not occur during generation of the lineage that produces the cystic structures of EBs. After establishing this basic foundation of understanding, analysis of X-inactivation patterns in the imprinted lineages of ES cells carrying specific *Xic* mutations (Table 3) will prove informative. Please see the table and legend for detailed information.

Use of XEN cells to rescue primitive endodermal defects

It can be difficult to analyze a phenotype that results in cell death. If the imprint is found to have a functional effect *in vitro* that is manifested as lack of X-inactivation where it would be appropriate and/or presence of X-inactivation where it is inappropriate, then the affected cells may die. Alternatively, cells that are unable to undergo X-inactivation may not be able to differentiate (Stavropoulos et al., 2001). Either cell death or lack of differentiation may prevent proper differentiation of EBs—even of the randomly inactivating population that is internal to the outer primitive endodermal layer, since the inner cells receive differentiation cues from the outer cells. It is very unlikely that cystic EBs would be able to form from dying or undifferentiated cells, and yet the mural cells provide the only context in which I have been able to assay imprinted X-inactivation so

far. Furthermore, the late stage at which cystic EBs begin to appear (D8) suggests that secondary selection effects could strongly contribute to the X-inactivation patterns observed in those cells, and rare cells that manage to achieve appropriate X-inactivation would likely found any cystic balloons that are observed, even if an imprinting defect was present. Thus, it may be very difficult to directly show an inappropriate X-inactivation phenotype using (cystic) EB differentiation. If imprinted X-inactivation can be assayed by RA differentiation into primitive endoderm derivatives (which is unlikely, as discussed above) or by Oct4 siRNA-mediated de-differentiation into TE derivatives, then phenotypes may be more easily observable. Even if these other assays are an option, it would be very useful to be able to show that any non-cystic or poor differentiation phenotypes in EBs are specific to extraembryonic endoderm defects. To this end, developing a XEN cell rescue assay will be very useful.

Although nothing has been published on the ability of XEN cells to rescue the extraembryonic defects of ES cell differentiation, I have found anecdotally that when my female ES cell cultures were contaminated with the XEN-like cells from which F5 was cloned out, the efficiency of cavitation in my simple EBs at D3 of differentiation was remarkably enhanced. Thus, I believe addition of XEN cells to an ES cell population that is unable to differentiate well is likely to rescue extraembryonic defects and to allow proper substrate attachment and outgrowth of randomly inactivating cell types. XEN cells should be mixed with the compromised ES cell line and composite EBs allowed to form in suspension. To assay rescue of differentiative potential, the the composite EBs should be allowed to attach to a substrate and assayed for outgrowth of embryonic derivatives exhibiting random X-inactivation. Either the XEN cell line or the ES cell line should be marked, for example by GFP expression, to allow determination of the contribution of each cell type to the differentiated population. Use of male XEN cell lines

would ensure that any *Xist* expression detected originates from the ES cells. If it could be obtained, the cell line from the Lee lab which contains a constitutive *Tsix*-expressing allele on the Xp would be very interesting to test in this assay (Stavropoulos et al., 2001). This cell line was unable to differentiate well as EBs, perhaps due to failure of imprinted X-inactivation (discussed in Appendix 1). If aggregation with XEN cells allows X-inactivation to occur in the internal cells, the interpretation that an extraembryonic imprinting defect was the cause of the poor differentiation phenotype would be supported.

Use of Cre-lox recombination to generate "di-maternal" and "di-paternal" ES cell lines

The importance of analyzing ES cell lines containing two copies of the Xm ("di-maternal") or two copies of the Xp ("di-paternal") was discussed above. The di-maternal and di-paternal cell lines that currently exist have additional chromosomal abnormalities, and so it will be desirable to produce such cell lines in a controlled manner. An approach to generating di-maternal and di-paternal ES cell lines is diagrammed in Figure 7. This approach takes advantage of Cre-lox recombination between homologous chromosomes to stimulate loss of heterozygosity for part of the X chromosome. In ES cells, interhomolog recombination occurring after a locus has replicated frequently results in loss of heterozygosity for markers distal to the recombination site. Loss of heterozygosity occurs when one recombinant sister segregates with one non-recombinant sister in the so-called "X" segregation pattern (Figure 7; Liu et al., 2002). To plan the targeting strategy roughly outlined in Figure 7, it will be helpful to refer to the paper by Liu et al. (2002), which describes useful constructs that could form the basis for a selection scheme. If this approach works well to generate di-maternal and di-paternal

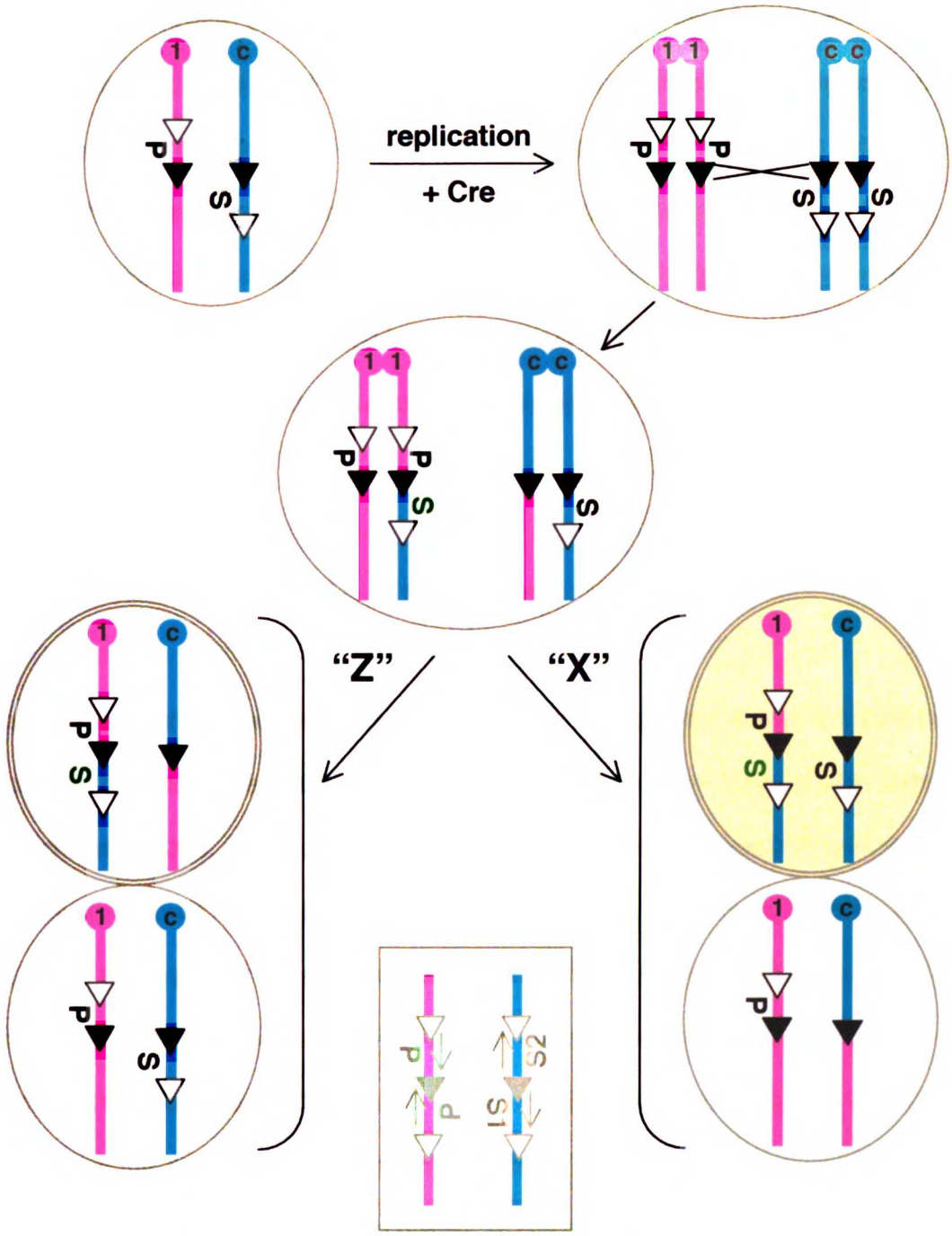

Figure 7. An approach to generating di-maternal and di-paternal XX ES cell lines. Maternal 129 (pink, "1") and paternal cas (blue, "c") X chromosomes are targeted with constructs that introduce loxP sites (black triangles) at allelic positions, and a promoter (P) and promoterless selectable marker (S) on opposite chromosomes. If Cre acts between homologs after replication, then only one of each sister pair is recombinant. "Z" segregation of chromatids preserves heterozygosity, whereas "X" segregation leads to loss of heterozygosity of distal sequences. The double-circled cells will be selected based on marker expression (green), and partially di-paternal cells (yellow) can be identified by genotyping. Frt sites (white triangles) allow subsequent selection cassette removal. The scheme can be designed to facilitate selection of di-maternal cells from the same starting cell line using two different selectable markers (inset).

ES cell lines of congenic background, then it could be used in the future as a tool for locating the imprint. One could "walk" down the *Xic* with a series of targeting constructs to generate loss of heterzygosity distal to various positions. In the resulting cell lines, assessment of whether imprinted inactivation in the extraembryonic lineages occurs normally or is disrupted would allow mapping of the imprint location. This experimental idea highlights how valuable it would be to have an *in vitro* system for studying imprinted X-inactivation, such that constructs could be tested in ES cells without the need to generate mice.

Discussion

Our experiments show that the X-inactivation imprint is retained in female ES cells, and can be read out upon differentiation into primitive endoderm derivatives *in vitro* under certain circumstances. So far, we have not shown an aberrant X-inactivation phenotype caused by the imprint *in vitro*, but this may simply be because we have not yet assayed a cell line in which proper dosage compensation requires reprogramming the maternally inherited X chromosome, which is hypothesized to bear the imprint. If a phenotype of two active X's is observed in differentiating XmXm ES cells, it would provide strong evidence that the X-inactivation imprint is located on the Xm. The ability to assay imprinted X-inactivation *in vitro* would greatly facilitate the study of the molecular mechanism of imprinted *Xist* expression in the extraembryonic lineages. In addition, one interesting question that could be addressed *in vitro* would be, in the absence of X-inactivation, can female cells take on restricted cell fates?

The finding of a functional readout of imprinted X-inactivation *in vitro* would have important implications for the interpretation of many experiments in the field of X-

inactivation. The possible contribution of imprinted X-inactivation to the poor differentiation phenotype caused by over-expression of paternal *Tsix in vitro* has already been discussed (Stavropoulos et al., 2001). A similar phenotype was seen upon introduction of multicopy transgenes of *Xite* and the *Tsix* CpG region into female ES cells: no X-inactivation and poor attachment to the substrate were reported (Lee, 2005). Could this phenotype also reflect a failure in imprinted X-inactivation?

During cloning of mice by nuclear transfer of ES cells, most imprints become unstable (Humpherys et al., 2001). However, one can see some residual imprint in extraembryonic tissues of mice cloned from 2-1 ES cells (see Eggan et al., 2000: compare Figure 3B, ratio of mus/*cas Xist* in placentae from ES cell clones 1 & 2 (~0.66/1) to Figure 3C, ratio of mus/*cas Xist* in embryonic tissue and MEFs from ES cell clone 1 (~2.5/1); a ratio of ~0.16/1 mus/*cas Xist* was observed in the placentae of natural offspring of the equivalent cross). This observation implies that the imprint governing paternal X-inactivation is unusually stable. Previously, it had been suggested that the imprint governing paternal X-inactivation is unusual in that it appears to be erased (Sado et al., 2005), as most germline imprints survive the wave of demethylation in the ICM during pre-implanation development (Morgan et al., 2005). Demonstration of the persistence of the imprint for paternal X-inactivation is therefore significant in that X-inactivation re-joins other autosomal imprints in being maintained in somatic lineages.

Acknowledgements

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TS cell biology, and for her willingness to collaborate on TS/TE cell generation from ES cells *in vitro*; and Alexander Gimelbrant in Andy Chess's lab for pointing me to resources on 129/*castaneus* genomic polymorphisms. Thanks as always to Barbara for her experimental ideas and help in obtaining reagents for this project. Based on Barbara's guidance, Cecile de la Cruz investigated X-inactivation patterns in sectioned cavitating female EBs, and Katie Worringer pursued the roles of Dnmts and CTCF as candidate molecular players in maintaining and reading out the imprint as her initial thesis proposal; both of their work has informed my thinking. Some of the introductory material was adapted from a section of a review that I wrote (Plath et al., 2002). Special thanks to Tom for providing references and information on siRNA techniques that were included in this Appendix.

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