X Chromosome Inactivation
Counting and Choice

Chance or Design

Erasmus MC
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Chance or Design

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List of abbreviations

Dnmt	DNA methyl transferase
ES cells	Embryonic stem cells
MSCI	Meiotic sex chromosome inactivation
PRC	Polycomb repressive complex
Tsix	Gene antisense to *Xist*
Xa	Active X chromosome
Xce	X chromosome controlling element
XCI	X chromosome inactivation
Xi	Inactive X chromosome
Xic	X chromosome inactivation center
Xist	X-inactive specific transcripts
Xite	X chromosome intergenic transcripts element
Xm	Maternal X chromosome
Xp	Paternal X chromosome

Chapter 1

Introduction

Introduction

Sex determination

Many species use environmental cues such as egg incubation temperature to determine whether the progeny develops to a male or female. Other species utilize genetic sex determination, but do not have distinguishable sex chromosomes. Relatively few species have two distinguishable sex chromosomes. In XY systems the male is heterogametic and in ZW, the female.

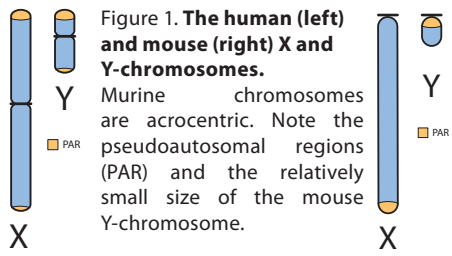

Figure 1. **The human (left) and mouse (right) X and Y-chromosomes.** Murine chromosomes are acrocentric. Note the pseudoautosomal regions (PAR) and the relatively small size of the mouse Y-chromosome.

It is commonly accepted that the mammalian Y and X chromosomes once started as two autosomes. After one of the autosomes acquired a sex-determining gene, an inversion including the sex-determining gene probably prevented recombination. As a consequence more male advantage alleles accumulated in the non-recombining area during further evolution. This unpaired part of the sex-determining chromosome (the future Y chromosome) rapidly degraded because of deletions and mutations in this non-recombining male specific region. Genes that gained a male specific function or new genes with a male specific function acquired from autosomes survived the degradation of the Y chromosome. The Y chromosome therefore harbours an unusual functionally coherent set of genes that have functions in sex determination and fertility. This cascade of events explains the small size of the Y chromosome and the small pseudo-autosomal region that can still pair with the X chromosome and is necessary for proper meiosis (Figure 1) (Graves, 2006).

A consequence of the loss of gene content from the Y chromosome is an unequal dosage of unpaired genes between males and females. Since this inhibits proper evolution of dosage sensitive genes, it became advantageous to apply a sort of dosage compensation. So, dosage compensation for the X chromosome is associated with Y chromosome gene loss. (Graves, 2006). In the animal kingdom a variety of X dosage compensation mechanisms emerged. In placental mammals, one of the two X chromosomes in females is transcriptionally inactivated. In worms, expression of both X chromosomes in female cells is reduced by half, and in flies the single X chromosome in males is up regulated two-fold compared to the X chromosome in females (Figure 2). In mammals, there are genes that escape

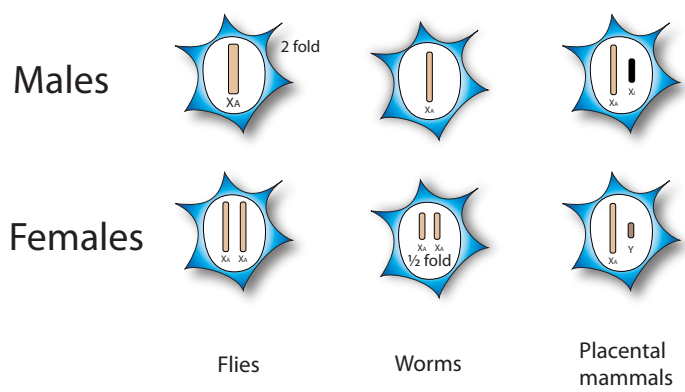

Figure 2. **Different ways of dosage compensation in flies, worms and placental mammals** The male fly up regulates genes located on the single X chromosome twice. In worms, females down regulate genes on both X chromosomes. Placental mammals achieve dosage compensation by random X chromosome inactivation of either one of the two X chromosomes in the embryo. * Some flies have a neo-Y chromosome, others have no Y-chromosome

X chromosome inactivation and, interestingly, most of these are clustered on the small tip of the X chromosome, called the pseudo-autosomal region (Figure 1). The majority of this region consists of autosomal sequences that were added at later stages of the evolution of placental mammalian sex chromosomes.

Since placental mammals (eutherians) diverged from monotremes and marsupials about 210 and 180 million years ago (MYA), respectively, studies of the dosage compensation mechanism in these mammals may reveal important information with regard to X inactivation in eutherians. Both monotremes and marsupials show X chromosome inactivation (XCI) but the mechanisms driving the process are unknown. In marsupials, XCI is imprinted and results in inactivation of the paternal X chromosome. Interestingly, XCI appears to be tissue specific in marsupials and only parts of the X chromosome are inactivated (Figure 3) (Graves, 1996). Interestingly, the region homologous to the inactivation center (the essential locus for XCI in placental mammals) is disrupted by independent rearrangements in marsupials and monotremes. This indicates that the eutherian XCI process evolved after the divergence of marsupials and monotremes (Hore et al., 2007).

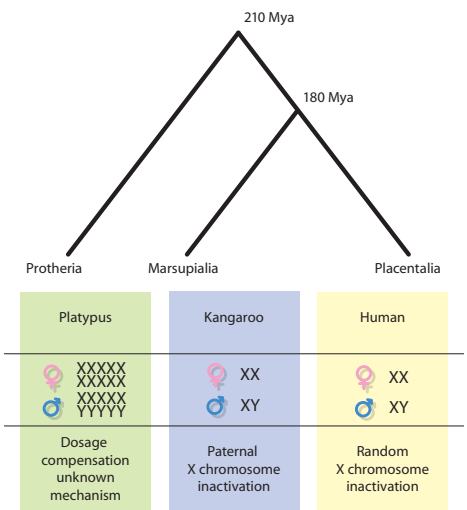

Figure 3. **Mammalian phylogenetic tree**

Figure 4. **Duck-billed platypus.**

The duck-billed platypus, an egg-laying mammal that diverged 210 MYA from the rest of the mammals (Figure 4), shows a strange sex-chromosome configuration. First, there are 5 Y chromosomes and 5 X chromosomes in each male nucleus that form a chain of linked chromosomes during male meiosis. The linking of the sex chromosomes prevents random combinations of Xs and Ys and the formation of unbalanced sperm. Second, the oldest pair, the one with the almost entirely degenerated Y chromosome, showed at least some homology to the bird Z chromosome. The youngest pair, located on the other end of the chain, shows homology to the human X chromosome. These data raise the possibility that bird and mammalian sex-chromosome systems are somehow linked (Carrel, 2004; Grutzner et al., 2004). Platypus compensates the dosage of (at least) the X chromosome that resembles the human X chromosome, but the mechanism is unknown (Grutzner and Graves, 2004).

The molecular mechanism behind the chromosome-wide silencing of the X chromosome in placentalia is complex and involves an untranslated RNA molecule, designated *Xist* (X chromosome inactive specific transcript), that coats the future inactive X chromosome in cis, and marks the onset of XCI. Accumulation of modified histone variants and DNA methylation play a key role in the establishment of this extraordinary epigenetic silencing. Epigenetic

changes that lock in the silent state will be discussed in more detail later in this chapter (Heard, 2004).

Functional X chromosome monosomy

In contrast to autosomal monosomies, which have hardly been described, all male mammals are monosomic for almost all X linked genes. The tolerance for monosomic expression of the X chromosome is intriguing because all autosomal monosomies are lethal. No autosomal monosomic embryo has been observed so far, even among first-trimester miscarriages. To compensate the loss of X-linked genes in males Ohno proposed in 1967 that genes on the X chromosome should be upregulated to be at equal level with the autosomes. Indeed, recent studies using genome wide expression analysis confirms this hypothesis and shows that the single (active) X chromosome in male and female cells is expressed at twice the level compared to genes on autosomes (Nguyen and Disteche, 2006). In haploid cells the X chromosome is not upregulated, maintaining a balanced expression compared to the rest of the genome. Upregulation therefore must occur rapidly in early development. Taken together, the loss of regions of the Y chromosome is compensated by transcriptional up-regulation of the X chromosome. The dosage differences between male and female X encoded genes is compensated by the XCI process (Lahn and Page, 1999).

The discovery of X chromosome inactivation (XCI)

The first indications for a difference between the two X chromosomes in female cells came from observations made by Barr en Bertram in the late forties. Studies with male and female neuronal cat cells showed a structure in female nuclei not present in male nuclei. This structure could be detected through a normal microscope as a deeply stained body close to the nucleolus. Because male cells did not show this nucleolar satellite, Barr suggested that this structure was the heterochromatin of one of the sex chromosomes (Barr, 1949).

Several years later a heteropyknotic chromosome was described in murine mammary cancer cells, that according to some was the result of an infection by the 'Bittner milk agent' and was the cause of the malignancy. However, Ohno and Hauschka discovered that this heteropyknotic chromosome could not be attributed to viral parasitism of the Bittner MTA virus. In fact 'the milk agent tumors' had no relation with the observed chromatin body. They reasoned that they were looking at the now well known sexual dimorphism of somatic interphase nuclei issued by Barr, and concluded it had to be the X chromosome (Ohno and Hauschka, 1960).

In the year 1961 Mary Lyon put forward the hypothesis that the heteropyknotic X chromosome described by Ohno and Hauschka in mouse diploid cells is the inactive X chromosome (Xi) and that it can be either paternal or maternal. Mouse genetic studies, in particular the existence of normal fertile XO mice and the mosaic phenotype of X chromosome linked coat color mutants, indicated that one of the two X chromosomes in female cells had to be randomly inactivated at an early stage in development and that females were functionally X chromosome mosaics (Lyon, 1961; Ohno and Hauschka, 1960). (This hypothesis had already been mentioned by Ohno in a paper, but unfortunately was depicted as insignificant by a reviewer and therefore was removed from the manuscript (Migeon, 2007; Ohno and Hauschka, 1960)). The X chromosome inactivation hypothesis implied that early in development one of the X chromosomes in a cell was inactivated and that the Xi was clonally propagated. Therefore XCI had to be stable and heritable throughout multiple mitotic cell divisions. Evidence for this hypothesis came from studies showing that expression of two X linked G6PD variants were clonally propagated when cultured from a single cell (Davidson et al., 1963).

Studies with early implantation mouse embryos showed that XCI in extra embryonic tissues is complete before 6.0d of gestation and the XCI in epiblast cells is complete between 6.0 and 6.5d of gestation at the onset of gastrulation, indicating that X chromosome inactivation was coupled to cellular differentiation (Monk and Harper, 1979).

Supporting evidence for the X chromosome inactivation hypothesis came from a different line of research. A few years earlier Jacobs had added a fourth group of human beings with abnormal chromosome numbers in addition to 'Down syndrome', Klinefelter syndrome (XXY) and Turner syndrome (XO), the ' XXX diploid super female'. This female was characterized by a chromosomal number of 47 and three X chromosomes and had two chromatin bodies in her nuclei (Jacobs et al., 1959). A survey on 4515 mental defectives revealed more males and females with supernumery X chromosomes. Sex-chromosome complement in this group consisted in about 0.5-1% of XXY, XXXY, XXXXY, XXX and XO. Studies with buccal smears of these patients indicated that in all subjects all but one X chromosome was heteropyknotic. This result suggested a counting mechanism, in which the cell counts the number of X chromosomes and let Harnden pose the equation: number of chromatin bodies (B) = X – Ploidy / 2 or, the number of chromatin bodies is one less then the number of X chromosomes in diploid cells (Harnden, 1961; Jacobs et al., 1959; Maclean and Mitchell, 1962). Grumbach confirmed this observation (Melvin M. Grumbach, 1963).

Similar to studies with aneuploidy embryos and individuals, many studies have been conducted with cells containing an extra set of chromosomes. Triploid and tetraploid human embryos spontaneously abort and die at day 100 or 75 after conception respectively. Studies with cell lines derived from XXX triploid embryos showed that a variable number (1 or 2) of X chromosomes is inactivated. This result is in line with the Harnden equation because XXX triploid cells have a X:ploidy ratio of 1.5, which can never result in one active X per diploid genome (see above). Interestingly, the number of inactive X chromosomes also varies within an embryo, so these XXX females are mosaics with cells harboring different inactivation patterns. The number of spontaneously aborted tetraploids is about one fifth as common as triploid embryos. Studies with cell lines derived from these embryos indicated that two X chromosomes are appropriately inactivated as predicted by Harndens equation (Carr, 1970). Nevertheless tetraploidy appears to have a more serious effect on development. The same is reported for mouse development (Carr, 1970).

PRIMARY NON-RANDOM XCI AND SECONDARY NON-RANDOM XCI (POST-CHOICE SELECTION)

Mary Lyon hypothesized that every X chromosome has a 50% chance to be inactivated. However, several studies indicate that the distribution between the paternal and maternal X chromosome is not always equal but skewed e.g. in some mice in 70% of the cells the paternal X chromosome is inactivated and in the remaining 30% the maternal X chromosome. This observation can be explained by two different mechanisms. First, primary non-random inactivation explains skewing of XCI by a mechanism in which cells primarily choose a particular distribution and clonally propagate this to their progeny. Second, secondary non-random XCI, or post-choice selection XCI occurs random but the skewing is the result of cell selection. Both forms of non-random inactivation patterns seem to exist and will be discussed hereafter (Figure 5).

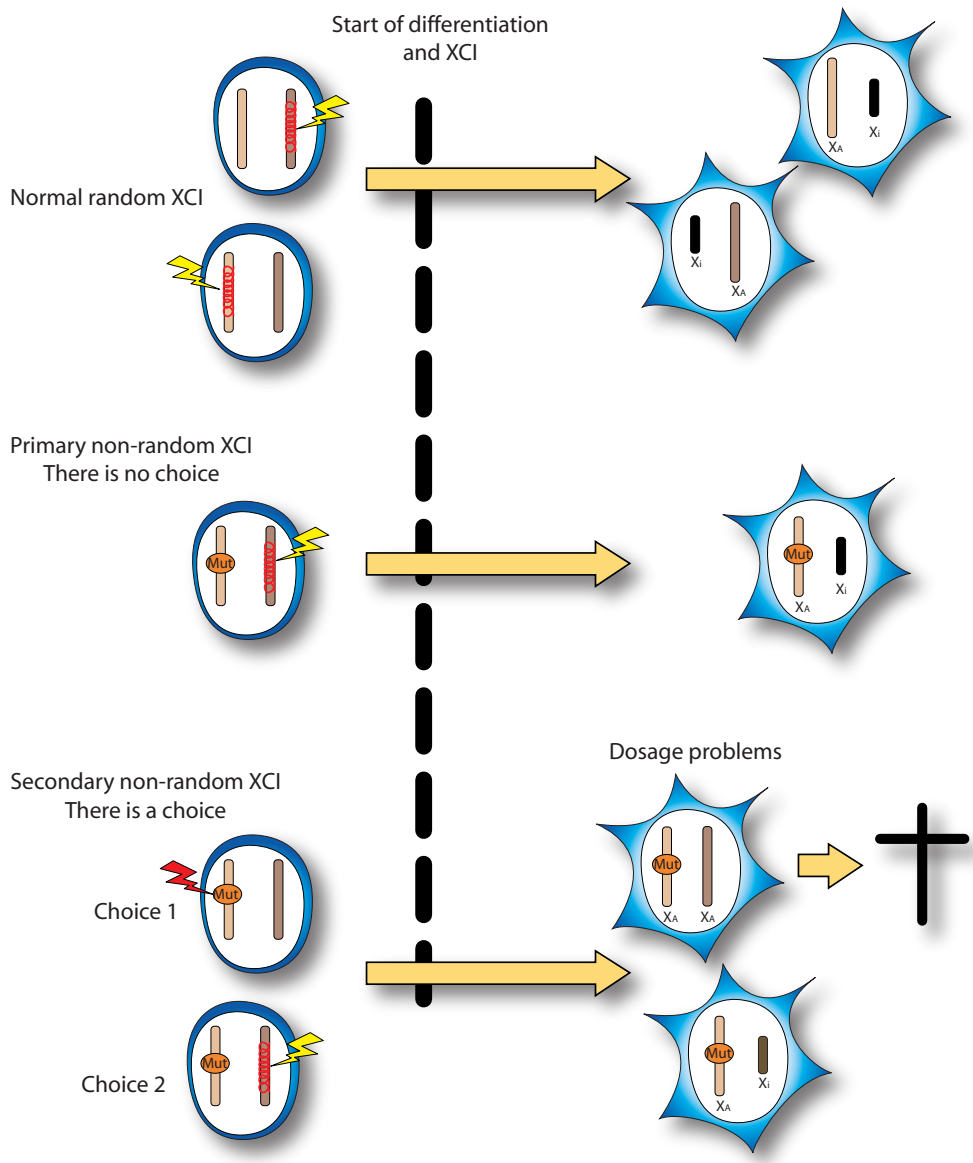

Figure 5. **Primary and secondary non-random XCI**
(A) Normal random XCI in female cells. Both alleles are chosen randomly to be inactivated. (B) Primary non-random XCI in female cells. In all cells the same allele is inactivated. (C) Secondary non-random XCI in female cells. Both alleles can be chosen to be inactivated randomly. Cells that have chosen the mutated X chromosome to inactivate will have two active X chromosomes after the XCI process because the mutated allele cannot initiate XCI. These cells will die because of dosage problems. Note that the end-result is the same for primary and secondary non-random XCI except for cell death in the latter.

Primary non-random XCI

Coat color variation in mice heterozygous for X-encoded coat color markers indicated skewing of XCI. Cattanach attributed this skewed distribution to a genetically defined region called the X chromosome controlling element (Xce). The Xce was located near the breakpoint of the X:autosomal Cattanach translocation based on identification of alleles with different ratios of coat color skewing. He postulated that the Xce locus influenced the probability of the whole chromosome to be inactivated or remain active, a form of primary non random XCI (Cattanach and Isaacson, 1965, 1967; Cattanach and Williams, 1972).

Hitherto, there was no evidence discriminating whether the skewed pattern is a result of selection after a primarily random inactivation or is the result of primary non-random choice of the alleles. Based on the distribution of the two different Xi's during development among a large number of mice, the resulting binomial distribution argued in favor for the latter. Selection would show a departure from a binomial distribution at least at some point during differentiation. Cattanach confirmed his postulate using a PGK1 (X-linked) allozyme assay that allowed discrimination between proteins from two different alleles. His experiments showed that skewing in the XCI pattern was already detectable in E7.5 embryos and did not change during later development or adulthood (Johnston and Cattanach, 1981). A different study confirmed this finding showing that it was primary non-random skewing opposed to selection of cells that was responsible for the skewed phenotype. Since skewing of XCI was already detectable in E6.5 embryos it was concluded that the Xce influenced the initial choice moment which of the two X chromosomes to inactivate (Rastan, 1982). So far, 4 different Xce alleles have been described in mice. Similar to findings in the mouse, skewing is also reported for humans. In humans the amount of skewing follows a bell shaped curve throughout the population suggesting that the humans have more Xce's than imbred mice (Amos-Landgraf et al., 2006). Alternatively, this distribution can be the result of secondary events or both (Minks et al., 2008). Interestingly, a point mutation in the minimal promoter of the *Xist* gene (that results in almost complete skewing) in two independent families suggests that the cause of the skewed pattern is linked to *Xist* expression (Plenge 1997).

Imprinted XCI

Another form of primary non-random XCI is observed in the extra embryonic lineages of the embryo. Takagi and colleagues showed that in the extra embryonic tissues of a E7.5 embryo, the paternal X chromosome was inactive and heteropyknotic whereas the maternal X chromosome was active (Figure 6). To identify the parental origin of the X chromosomes, Takagi used a Cattanach's translocation chromosome (an X chromosome with an insertion of a segment of chromosome 7 (Xt)). This longer Xt chromosome can be recognized in metaphase spreads, and therefore allows parental discrimination of the X chromosomes in females. In contrast to imprinted non-random inactivation in extra-embryonic tissues, XCI is random with respect to the parental origin of the X chromosome in the developing embryo (Takagi and Sasaki, 1975).

The cause of imprinted XCI was unknown. It could be explained by four different phenomena. First, skewed XCI can be due to primary non-random XCI in which the X chromosomes are parentally marked or imprinted. Second, it can be due to secondary selection for cells with the Xm as Xa after primary random XCI. Third, the Xm could be reactivated after random XCI, and fourth, a selection pressure exerted by the phenotype of the maternal reproductive tract against cells which express Xp.

Cytogenetic studies excluded the possibility of reversal of XCI on the Xm, ruling out the third possibility that random XCI process is followed by reactivation of the maternal X chromosome and the subsequent inactivation of the Xp (Takagi, 1976). The influence of

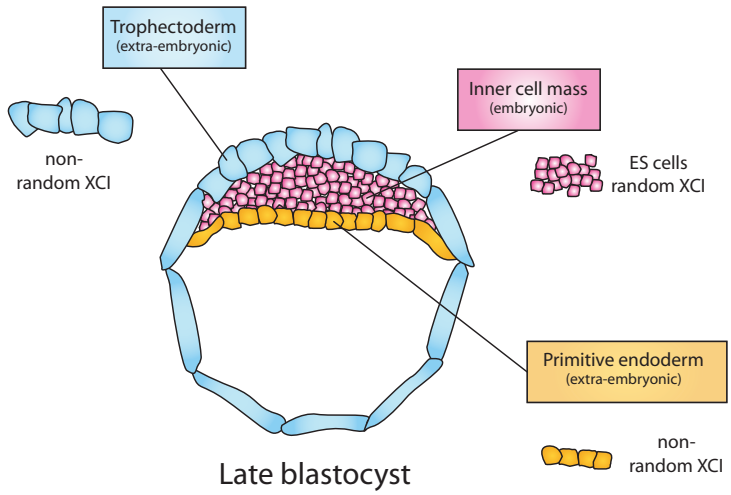

Figure 6. **The late blastocyst stage of the developing embryo**
The extra embryonic tissues (primitive endoderm and throphectoderm) are charcterized by imprinted XCI. The inner cell mass, the part destined to be the embryo proper, shows random XCI.

the maternal reproductive system on the randomness of XCI in the yolk sac has also been excluded by transplantation studies (West et al., 1977).

The second option of secondary selection has to be the result of a dramatic cell selection process leading to elimination of 50% of the cells, which have inactivated the Xm and are expressing the Xp. This however has never been observed. Moreover, successful propagation of XpO mice from XO mothers made this probability unlikely. Since in XpO progeny from these parents the X is always paternal it indicated that the expression of the paternal X chromosome is not lethal to the embryo. It therefore is difficult to see how expression of the paternal X chromosome could result in such a disadvantage for cells.

Hence the first option is most likely. The X chromosomes had parental marks that resulted in exclusive expression of the maternal X chromosome in the trophectoderm and the primitive endoderm in the developing embryo.

Secondary non-random XCI

In X-autosome translocations a part of an autosome is fused to a part of an X chromosome and vice versa. The resulting embryo is diploid (balanced) for all its genes. Further mating with these animals, results in a variety of genotypes. Depending on the crossing there will be balanced and unbalanced (monosomic for parts of chromosomes) genotypes. Studies with mice bearing X:autosome translocations showed that only one part of the broken X chromosome is able to initiate XCI and inactivate itself and parts of the attached autosomes. Even though mice are balanced carriers of an X-autosome translocation, in half of the cells of the developing embryo a part of the translocated X chromosome is not inactivated and is functionally diploid for that region of the X chromosome. Furthermore the part of the translocated autosome that is attached to the X chromosome that is inactivated results in a functional monosomy for this particular region of the autosome. This functionally aneuploidy is believed to give varying amounts of skewing in different X-autosome translocations as a consequence of selection for functionally euploid (with the normal X chromosome inactive) cells (Russell and Montgomery, 1969, 1970).

Of particular interest is the Searle's translocation. That is a reciprocal translocation between the X chromosome and chromosome 16 with the breakpoint in a more or less central position in the X chromosome. This translocation results in almost totally skewed inactivation of the wild type X chromosome. To find out the nature of non-random XCI in mice with the Searle's translocation, McMahon made use of a PGK1 isozyme expression assay. The X-linked Pgk1 gene has two isoforms that are distinguishable and can be used as a tool to asses the amount of skewing of XCI. Based on this assay it was found that cells with an inactivated wild type X chromosome were selected for during embryogenesis. Cells that initiated XCI on the translocated X chromosome disappeared from the population. This indicates a secondary non-random XCI pattern (McMahon and Monk, 1983; Rastan, 1983). Interestingly, before the secondary non-random XCI selection process is initiated the distribution of the Xi's is actually inverse because of primary non-random XCI caused by different Xce alleles. The Searle's translocation is therefore an example of both forms of non-random XCI.

THE X CHROMOSOME INACTIVATION CENTER (XIC)

Partial inactivation of the autosome in X-autosome translocations indicated that the presence of a region on the X chromosome that is responsible for XCI. Genetic studies suggested that the amount of XCI spreading into the adjacent autosome was dependent on the distance to the breakpoint. Moreover, only one part of the broken X chromosome had the ability to become inactivated. These observations provided clear evidence for a single region on the X chromosome responsible for XCI and also showed that the X chromosome is different from autosomes because these could not propagate spreading of XCI to the same extent as the X chromosome (Russell, 1963).

Translocation studies narrowed the region to a locus whose borders are defined by the Searle's translocation and the HD3 truncation. Rastan et al. showed that the X inactivation center (Xic), was required for XCI to occur on a particular X chromosome. She hypothesized that the Xic is necessary and sufficient to induce XCI on a chromosome but that one Xic per cell is randomly blocked by an autosomally encoded blocking factor preventing XCI (Brown et al., 1991b; Rastan, 1983; Rastan and Robertson, 1985; Takagi, 1980). What exactly was responsible for XCI to occur remained unknown.

Xi-SPECIFIC TRANSCRIPTS *(XIST)*

After XCI most of the genes on the Xi are silenced and the genes on the Xa continue to express. In contrast, Brown and colleagues isolated a gene located within the human XIC with an inverse expression pattern. This gene, designated Xi-specific transcripts (*Xist*), is exclusively expressed from the Xi and silenced on the Xa. The gene contains no long open reading frames and on a northern blot appeared as a smear because of extensive alternative splicing (Brown et al., 1991a; Brown et al., 1991b). The same year, the mouse *Xist* gene was identified and located to the mouse Xic, and expression analysis showed the same pattern as its human homologue (Figure 7). Moreover, the level of *Xist* expression is inversely correlated with the

Figure 7. **The *Xist* gene**
The *Xist* gene is a non-coding gene that consists of 7 exons and spans approximately 23kb in mice.

strength of the Xce allele (Borsani et al., 1991; Brockdorff et al., 1991). Analysis of human *XIST* revealed a consensus cDNA of 17 kb, 8 exons and comparison with the mouse *Xist* sequence indicated a limited overall sequence similarity (~60%). Two blocks of sequence show strong similarity, one 5′ and one more 3′ in *XIST*, showing nine and eight direct repeats respectively. Interestingly, using fluorescent in situ hybridization analysis (FISH), the localization of *XIST* RNA was confined to a site in the nucleus that precisely corresponded with the position of the heterochromatic Barr body. *XIST* RNA is only present in the nucleus and is not associated with the translational machinery. In aneuploid cells the number of sites of *XIST* accumulation matches the number of Xi's; always one less than the number of X chromosomes. In interphase nuclei mouse *Xist* associates with the Xi through an interaction with the non-chromatin nuclear matrix and occupies the same 3-D nuclear territory as the whole Xi and is therefore not limited to the surface of the X chromosome. The bulk of the *Xist* molecules seen in the clouds are spliced. Furthermore during mitosis the *Xist* molecules dissociate from the Xi and disperse into the cytoplasm. In early G1 new transcripts associate with the Xi (Brown et al., 1992; Clemson et al., 1996; Melvin M. Grumbach, 1963). These observations supported a direct role for the *XIST*/*Xist* RNA in XCI.

If *Xist* is responsible for XCI, expression of *Xist* should precede XCI. Expression analysis of *Xist* in early embryos revealed that it does. *Xist* RNA is detected at the 4 to 8-cell stage of the embryo one day prior to differentiation of the trophectoderm and subsequent initiation of XCI. Allele specific analysis revealed imprinted paternal *Xist* expression at this stage. In the epiblast (embryo proper) the imprint on the X chromosomes is erased to allow random XCI in the embryo proper. At E6.5 the first maternal *Xist* RNA was detected by Kay indicating the start of (random) XCI in embryonic tissues (Kay et al., 1993).

Panning confirmed that the inner cell mass (ICM) of mouse embryos did not contain an inactivated X chromosome in preimplantation blastocysts in contrast to the trophectoderm. RNA FISH analysis with early female embryos (d 6.5) showed that three patterns of *Xist* expression can be discerned, a minority of cells with biallelic low level *Xist* expression; about 15% had differential biallelic expression (one highly expressed *Xist* gene and one gene with low expression) and the majority had high-level monoallelic expression. Male embryos showed only monoallelic low-level expression of *Xist* (~15%) or no expression at all. Unfortunately, because double stranded probes were used for the analysis, these RNA FISH studies could not distinguish between sense or antisense transcription. Later studies indicated that the low-level *Xist* transcription actually turned out to be the antisense transcription of *Tsix* (see below) and only the high level *Xist* transcription represents sense *Xist* RNA (Panning et al., 1997). Quantitative RT PCR analysis now shows that undifferentiated male ES cells express *Xist* at a very low level (three to four copies per nucleus). Female undifferentiated ES cells have about ten copies of *Xist* per nucleus which could be due to contamination of differentiated ES cells in the population. The half-life of an *Xist* RNA molecule ranges from 3.5h to 6h and does not change during differentiation. During the XCI process the amount of *Xist* RNA increases 30 fold. Increased transcription is responsible for at least a factor 2 to 10 of this increase (Sun et al., 2006).

Sequence homology analysis has indicated that *Xist* shows partial homology to the protein coding Lnx3 gene in chicken. Also in marsupials the Lnx3 gene is protein coding, whereas in eutherians Lnx3 has lost its protein-coding function. This loss of an ORF appears concomitant with the pseudogenization of at least two other genes close to the *Xist* (Duret et al., 2006). No other sequence similarities could be detected for *Xist* in marsupials, indicating that *Xist* emerged after the divergence between marsupials and eutherians (Duret et al., 2006).

Xist RNA has different domains with separate functions. One domain designated, the A repeat located 5′ in *Xist*, is a stemloop repeat that is required for inactivation. The silencing function of *Xist* is completely dependent on this 5′ repeat element. Other domains in the RNA are functionally redundant and act cooperatively in the localization of *Xist* to the X chromosome (Wutz et al., 2002).

DNA methylation of *Xist*

In male and female somatic tissues, CpG sequences in the *Xist* promoter are methylated on the Xa. In contrast, the *Xist* promoter on the Xi in female somatic tissues is not methylated. Interestingly, the absence of methylation on the paternal *Xist* promoter during male gametogenesis corresponds with the imprinted *Xist* expression and XCI in the extra embryonic tissues. Therefore, an unmethylated promoter is associated with an active *Xist* gene. These observations indicate that the methylation status of the *Xist* promoter correlates inversely with the transcriptional status of the *Xist* gene in differentiated tissue that represents the maintenance phase of XCI. (McDonald et al., 1998; Norris et al., 1994; Zuccotti and Monk, 1995)

Although initial analysis of *Xist* promoter methylation indicated a role for methylation in imprinted XCI several other studies analyzing the methylation status of the *Xist* locus in oogenesis and spermatogenesis led to contradicting results. Different groups have published data using different techniques analyzing different regions in the *Xist* promoter. Taken together in sperm DNA methylation is absent just upstream of the transcriptional start site of *Xist*. In contrast, a region just 150 base pairs upstream of the start site, designated the MluI cluster, is methylated in sperm (Norris et al., 1994). The data concerning DNA methylation in the oocyte is more confusing. Some reports indicate that both regions upstream of the *Xist* promoter are methylated in the oocyte in contrast to other reports which claim the opposite (McDonald et al., 1998; Zuccotti and Monk, 1995). Analysis of the region just downstream of the promoter also showed contradicting results in oocytes (Ariel et al., 1995; McDonald et al., 1998). More locus wide CpG methylation studies are needed to establish whether oocyte specific DNA methylation is present in the *Xist* locus.

Preemptive differential methylation?

Whether differential methylation is already present prior to initiation of random XCI is an interesting question because it could reveal the mechanism of counting and choice. In male ES cells the *Xist* promoter is fully methylated as expected, because *Xist* is inactive in these cells. In contrast, in female ES cells approximately 50% of the methylation sensitive restriction sites are digested with methylation sensitive restriction enzymes. Although these results suggested that one allele was already preemptively methylated in female ES cells, additional studies indicated that these findings have to be explained by overall hypomethylation of both *Xist* promoters (~50% of normal value on both alleles) in female ES cells compared to male ES cells (Norris et al., 1994; Sado et al., 1996). This is consistent with the finding that total CpG methylation in female ES cells is reduced compared with male ES cells.

DNA METHYL TRANSFERASES, *XIST* EXPRESSION AND COUNTING AND CHOICE

Currently, three DNA methyl transferases have been identified. Two of these enzymes, Dnmt3a and Dnmt3b, are de novo DNA methyl transferases, the third enzyme, Dnmt1 is a maintenance DNA methyl transferase which copies a methyl group on the unmethylated CpG residue of a hemimethylated dinucleotide. Despite the important role of DNA methylation in regulating gene expression Dnmt1 deficient ES cells have been generated. Analysis of Dnmt1

deficient male ES cells after 9-12 days of differentiation indicated that the *Xist* gene was ectopically active in 90% of the cells. In E9.5 Dnmt1 deficient male and female embryos ~5% of the cells showed aberrant *Xist* clouds, confirming the finding that hypomethylation correlates with aberrant *Xist* expression in vivo. DNA methylation analysis of the *Xist* promoter in male embryos showed demethylation of the region 5' *Xist* (Beard et al., 1995; Li et al., 1993; Li et al., 1992; Panning and Jaenisch, 1996).

Ectopic *Xist* RNA expression is also reported in male and female Dnmt3a-/-; Dnmt3b-/- double knockout male embryos in 5% and 17.7% of the cells respectively at day 9.5 of development. However, analysis of XCI in these embryos during the early stage of the XCI process indicated that they induce proper random and imprinted XCI but revealed that *Xist* repression is not properly maintained leading to reactivation of the silent *Xist* gene (Sado et al., 2004). These results were confirmed in differentiating Dnmt3a-/- and Dnmt3b-/- male ES cells which showed delayed appearance of *Xist* clouds in up to 68% of the cells. Interestingly, X chromosomes accumulating *Xist* are not transcriptionally inactivated because *Xist* has to be expressed in a critical window of differentiation to initiate XCI (Sado et al., 2004; Wutz and Jaenisch, 2000). These results suggest that DNA methylation is not required for establishing epigenetic differences, driving the counting and choice process, but is involved in the repression of *Xist* during the maintenance phase of XCI.

XIST DELETIONS AND THE COUNTING AND CHOICE PROCESS

Definitive evidence that *Xist* is absolutely required for the XCI process to occur came from several studies analyzing *Xist* deletions in female ES cells. To study XCI in the context of the deletion embryonic stem cells (ES cells) are used to mimic in vivo differentiation of the embryo proper. Undifferentiated ES cells have two active X chromosomes in culture mimicking the preimplantation stage of the embryo. Induction of differentiation results in rapid initiation of XCI and ES cells are therefore an excellent model-system to investigate XCI. Analysis of XCI in a female cell line with a 7 kb deletion of exon 1 of the *Xist* gene, including the promoter, showed complete non-random XCI towards inactivation of the wild type X chromosome. This result indicated that XCI is absent on the mutated allele implying a direct role for *Xist* in *in cis* inactivation. Whether complete skewing was caused by primary or secondary non-random XCI remained unclear, although the authors favored the latter because expression of X-linked genes was not completely restricted to the mutated allele. Primary non-random XCI of all cells would result in expression of X encoded genes located on the mutated X chromosome. This is true if indeed all cells are differentiated and if the XCI process is synchronous. Since cells could be identified with two Xa's it was concluded that selection against cells with two active X chromosomes was still ongoing in their analyzed 10.5-12.5 day differentiated embryonic stem cells. The authors concluded that the choice process was unaffected and that selection against XaXa cells that attempted to inactivate the mutated X chromosome caused the skewing of XCI (Penny et al., 1996). In contrast, in vivo studies with a different deletion encompassing part of the *Xist* gene but leaving the promoter intact showed that in day 7.5 female embryos 99% of the cells had a wild type *Xist* cloud. This result indicates that a null mutation of the *Xist* allele results in primary non-random inactivation towards the wild type allele (Marahrens et al., 1998).

Further, analysis of these mice indicated that *Xist* is not required in spermatogenesis. Male mice were fully fertile although they did not give any female offspring, because paternal transmission of *Xist* is required for imprinted XCI in the trophectoderm. After maternal inheritance of the mutated allele, the paternal allele could be normally inactivated in female

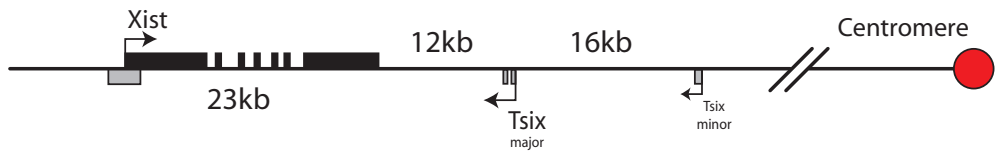

Figure 8. ***Tsix*, a gene antisense of *Xist***
Tsix is transcribed in the antisense direction of *Xist*. The major promoter is located approximately 12kb downstream of *Xist*.

embryos. The embryo proper of these females showed total non-random XCI towards the wild type allele; the maternal X is always the Xi. Interestingly, male mice with the *Xist* deletion could produce female XpO offspring when they were crossed to XO females. This supports the hypothesis that the lethal phenotype could be attributed to a failure of extra embryonic lineages to develop in XX embryos inheriting the mutated allele through the paternal germ line (Marahrens et al., 1997).

A conditional *Xist* deletion has also been generated. In mouse fibroblasts this conditional deletion revealed that the *Xist* RNA is not required for maintenance of the silent state of the Xi. While only sporadic reactivation of X linked genes was reported from the Xi, histone macro H2A colocalization to the Xi was disrupted after conditional deletion of *Xist*. Because studies indicate that *Xist* is bound to the nuclear matrix and not to chromatin, macroH2A, a histone H2A variant that is incorporated into the nucleosome, may represent the link between Xi chromatin, *Xist* and the nuclear matrix (Clemson et al., 1996; Csankovszki et al., 1999). However, the observation that *Xist* can be detected in metaphase spreads argues against this hypothesis.

TSIX, A GENE ANTISENSE TO *XIST*

Initial indications for the presence of an additional gene transcribed antisense to *Xist* came from fluorescent in situ hybridization (FISH) experiments with strand specific probes in the *Xist* gene. These studies showed antisense expression relative to *Xist* and in reference to its antisense orientation it was designated *Tsix*. *Tsix* completely overlaps *Xist*, does not contain an open reading frame and like *Xist* remains in the vicinity of the Xic as determined by RNA FISH. In contrast to *Xist, Tsix* does not spread along the rest of the X chromosome. *Tsix* contains four exons and two transcription start sites (Figure 8), and is expressed in undifferentiated female and male ES cells detectable as two pinpoints in RNA FISH experiments. Upon differentiation *Tsix* expression becomes mono allelic in female ES cells, and is only expressed on the future Xa. *Tsix* expression on the Xa is eventually repressed between days 4-11 in male and female cells (Lee, Davidow et al. 1999). Analysis of *Tsix* expression using a *Tsix* specific DXPas34 DNA probe revealed *Tsix* transcripts inside the immature *Xist* domains in differentiating ES cells and also in a small part of the cells in E6.5-7.5 embryos. These observations indicated that *Tsix* transcription is inversely correlated with the onset of *Xist* RNA coating of the X chromosome but its extinction does not precede *Xist* coating in all cells (Debrand et al., 1999; Lee et al., 1999; Sado et al., 2001).

Roughly half of the *Tsix* transcripts is spliced and *Tsix* RNA is 10-100 fold more abundant than *Xist* RNA in undifferentiated ES cells. Interestingly, *Tsix* appears to be present in a gradient with 100 fold more *Tsix* than *Xist* in the 5′ portion of *Tsix* and only 10 fold more in the 3′ portion of *Tsix*. *Tsix* expression thus represents a gradient that decreases towards the 5′ portion of *Xist,* which may indicate transcriptional interference as a mechanism to suppress *Xist* (Shibata and Lee, 2003; Sun et al., 2006). *Tsix* does not require splicing for its function, as a

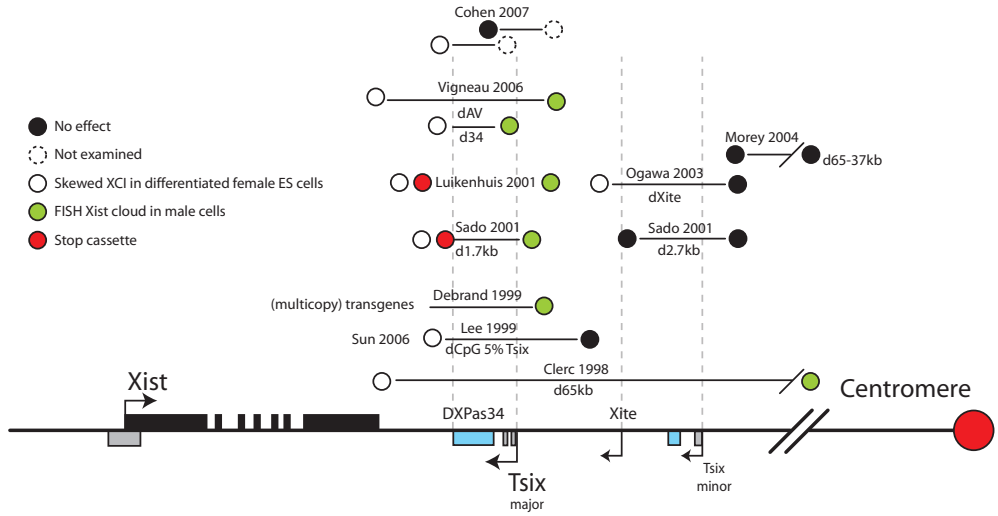

Figure 9. ***Tsix* deletions and resulting phenotypes**
The *Xist*/*Tsix* locus with *Tsix* deletions. Almost all deletions that encompass DXPas34 cause ectopic expression in male cells, indicated by the green dots. The white circles represent deletions that cause skewing in female cells. Red dots are stop cassetes in the *Tsix* gene that trap *Tsix* transcription. Red dots indicate stop cassetes in the *Tsix* gene. Blue squares are CpG islands with hypersensitive sites. *Xite* transcription starts downstream of the *Tsix* minor promoter but upstream from the *Tsix* major promoter. See text for details.

cell line with a locus containing a mutated *Tsix* gene, which is splicing defective, is still able to stably repress the *Xist* locus in cis (Sado et al., 2006).

Tsix deletions

A 65 kb targeted deletion of the region 3' of *Xist* that abolished *Tsix* expression leads to complete non-random XCI towards the mutated chromosome indicating that *Tsix* is a negative regulator of *Xist*. This mutation was generated prior to the discovery of *Tsix* and because the deletion resulted in male *Xist* expression it was concluded that the deleted region harbored the binding site for the blocking factor proposed by Rastan. (Rastan, 1985; Clerc and Avner, 1998). DXPas34 is a CpG island located at the 3' end of *Xist* but inside the *Tsix* gene and is located just downstream of the major *Tsix* promoter (Figure 9) (Courtier et al., 1995). Deletion of this element, including the major transcriptional start site of *Tsix*, results in 5% *Tsix* expression compared to wild type and complete non-random XCI (Lee and Lu, 1999) (Figure 9). Interestingly another deletion of DXPas34 and the major promoter (including a poly A stop insertion) caused aberrant *Xist* expression in a small proportion of male ES cells indicating that *Tsix* plays a role in repression of *Xist* in male and female cells (Figure 9) (Sado et al., 2001). The deletion did not cause increased cell death or *Xist* expression from both alleles in differentiating female cultures. This indicates that complete skewing is the consequence of the (primary) non-random XCI. Deletion of the minor promoter and exon 1 did not show any skewing, indicating it does not affect the choice process (Figure 9) (Sado et al., 2001). When the *Tsix* major promoter is deleted separately it only causes reduced expression of *Tsix*, but no increased skewing of XCI, suggesting promoter activity for DXpas34 itself. Indeed DXPas34 carries bi-directional promoter activities in luciferase reporter assays and deletion of DXPas34 alone causes skewing towards inactivation of the mutated allele (similar to a *Tsix* mutant). Interestingly, this deletion shows

persistent *Tsix* expression in day 12 differentiated cells suggesting that Dxpas34 also has a role in silencing *Tsix* expression (Cohen et al., 2007). Different mutant *Tsix* alleles (deletions or a stop cassette in the *Tsix* gene) result in *Xist* expression in male cells (Figure 9) (Debrand et al., 1999; Luikenhuis et al., 2001; Sado et al., 2001; Vigneau et al., 2006). This is in contrast with the dCpG deletion for which *Xist* expression was not reported in differentiated male ES cells (Lee and Lu, 1999). Whether *Tsix* transcription per se, the RNA molecule or the deletion of a crucial DNA element is responsible for *Tsix* function remained unresolved. *Tsix* seems to act through shutting down the *Xist* promoter, although transcription interference can not be excluded (Sun et al., 2006). A transcriptional stop signal (triple poly-A) introduced downstream of the DXPas34 region into *Tsix* indicated that the lack of transcription through the locus or the RNA itself causes primary non-random XCI towards the mutated chromosome. Also in somatic tissues of female mice the XCI pattern is non-randomly skewed towards the mutated allele (Luikenhuis et al., 2001). So, different deletions or 'stop cassettes' in the *Tsix* gene that abolish or lower *Tsix* transcription cause skewing of XCI and (not always) ectopic *Xist* expression in male cells. *Tsix* transcription inhibits *Xist* expression and is a negative regulator of X chromosome choice by modulating *Xist* expression.

Xite

RT PCR analysis in the 5' region of *Tsix* revealed extensive but low-level 'antisense transcription' in the direction of the *Tsix* major and *Xist* promoter. The transcripts surprisingly initiate downstream of the minor *Tsix* promoter and a CpG island that is located between the minor and major promoter of *Tsix* (Figure 9). Two intergenic (actually intragenic) transcription start sites were identified and were designated 'X chromosome Intergenic Transcripts Element' (*Xite*) (Ogawa and Lee, 2003). In differentiated male and female ES cells a deletion of *Xite* did not affect counting i.e. no *Xist* in XY cells and proper XCI in female cells. The *Xite* deletion does result in skewing ranging from 85:15 to 99:1 (mutant X: wild type X) in different cell lines. Note the difference with the *Tsix* mutants that show exclusive *Xist* expression of the mutant alleles and cause ectopic *Xist* expression in male cells (Ogawa and Lee, 2003). The antisense expression from the *Xite* locus could represent intergenic transcription as reported for the β-globin locus (Gribnau et al., 2000). The hypothesis states that intergenic transcription opens a chromatin domain and is required for the domain to function properly.

Prolonged expression of *Tsix*

Tsix expression can be detected from the blastocyst stage up to embryonic day 15.5, where it is barely detectable. In ES cells *Tsix* expression is repressed between day 4-11 in male and female cells. When expression is prolonged using an inducible knock-in TET or CMV promoter in front of *Tsix*, primary non-random XCI is observed towards inactivation of the wild type X chromosome. Thus constitutive *Tsix* expression results in permanent inhibition of XCI and that allele will never be inactivated, consistent with the idea that *Tsix* is a negative regulator of *Xist* (Luikenhuis et al., 2001; Sado et al., 2001).

Tsix and imprinted expression in extra-embryonic tissues

In the extraembryonic tissues at the blastocyst stage *Tsix* transcripts can only be detected from the maternally inherited X chromosome but not from the paternally derived X chromosome. *Xist* expression is expressed only from the paternal allele. Moreover, inheritance of a *Tsix* deletion is embryonic lethal only when inherited through the mother because it causes aberrant *Xist* expression from the maternal allele in the extraembryonic tissues (*Xist* expression from the single X chromosome in males and from two X chromosomes in females is observed

in embryos in these tissues). Female as well as male embryos die due to abnormal formation of these tissues prior to or during gastrulation. Since female Δ*Tsix* mice die because of ectopic maternal *Xist* expression, an *Xist* deletion inherited though the father could therefore theoretically rescue this phenotype. Indeed, when XX female mice are crossed to $X^{\Delta Xist}$/Y males (*Xist* deletion) the females in the litter all have an $X^{\Delta Xist}/X^{\Delta Tsix}$ genotype (Sado et al., 2001).

Because *Tsix* is a negative regulator of *Xist* this suggests a key role for *Tsix* in imprinted XCI. This however, can be interpreted in two ways. First, *Xist* is expressed from the paternal allele only because there is no *Tsix* expression, implying that *Tsix* is imprinted and regulates imprinted XCI. This is however not likely because *Tsix* is not expressed at the onset of imprinted XCI. *Xist* is expressed from 4-8 cell stage embryos onward but *Tsix* expression is first detected around the blastocyst stage. Second, *Xist* is only expressed from the paternal allele (because of an *Xist* imprint) and prevents expression of *Tsix* on the Xp in extra embryonic cells when it is developmentally induced in the blastocyst stage. The observation that *Tsix* expression is absent as soon as *Xist* is upregulated in random XCI, suggests that *Xist* negatively regulates *Tsix* expression, supporting this hypothesis. Taken together, *Tsix* plays a role in X chromosome choice since deletion of *Tsix* causes XCI skewing. Nevertheless, *Tsix* is not likely to be involved in the maternal imprint because it is not expressed synchronously with the onset of imprinted XCI and no differential methylation has been demonstrated between the male and female germ line. However, *Tsix may* have an effect on imprinted XCI because deletion of it results in aberrant inactivation of the Xm.

XIST TRANSGENES AND XCI COUNTING

One important feature attributed to the Xic is that this region contains an element required for counting the number of X chromosomes to generate one active X chromosome per diploid genome. This hypothetical counting element can be defined as a region, which will induce XCI on the single X chromosome in male cells when additional copies are present in the male genome. Additional copies of the putative counting element in female cells would lead to induction of XCI on both X chromosomes.

The first study reporting a 450 kb transgene encompassing part of the genetically determined Xic indicated that multicopy arrays can induce aberrant *Xist* expression from both the transgenes and the endogenous Xic in male ES cells. About one-third of the cells showed *Xist* accumulation on the autosome and X chromosome, whereas two-thirds show accumulation of *Xist* on the autosome only (Lee et al., 1996). *Xist* expression in fibroblasts isolated from embryos generated with transgenic ES cells is exclusively transcribed from the transgenes, indicating selection against male cells with an inactive X chromosome. In differentiating transgenic ES cells an increased transgene copy number correlated with increased cell death in these cultures. This result may reflect cell death as a consequence of ectopic XCI induced on the X chromosome, but could also be the consequence of induced monosomy of part of an autosome or both. Close examination of an ES cell line with ~24 tandemly integrated transgenes shows long range silencing in cis, delayed replication and hypoacetylation of the transgenic autosome. These results show that a transgene containing part of the Xic including *Xist* is capable of driving long-range heterochromatin formation (Lee and Jaenisch, 1997; Lee et al., 1996). Taken together this data supports the presence of a counting element on the transgenes. However, one should realize that these results can also be explained by the existence of an X encoded XCI activator. The

transgene may harbor the XCI activator resulting in *Xist* expression from the endogenous locus and the transgenic locus.

Several other studies with *Xist* transgenes integrated in male and female ES cells have been reported so far. Interestingly, these studies indicate that the amount of cells that show *Xist* expression from the transgenes is copy number dependent. In these multicopy transgenes endogenous *Xist* cloud formation does not exceed 11%. Surprisingly *Xist* accumulation on the endogenous X chromosome was abolished in transgenic male ES cell lines with a deletion of the *Tsix* promoter region DXPas34. In these cell lines no X chromosome-derived *Xist* RNA domain was observed implying a role for the DXPas34 element in counting (Debrand et al., 1999; Heard et al., 1999).

In contrast to multicopy *Xist* transgenes, single copy transgenes do not induce aberrant *Xist* cloud formation in male cells. This is not due to position effect variegation (PEV) judged by the normal expression of the Brx gene located within the transgene only 75kb downstream of *Xist*. Moreover, the transgenes have to be integrated in the host genome in a tandem repeat since two single copy transgenes in the same cell do not lead to aberrant *Xist* induction.

Similar to the transgenes containing all known genes involved in XCI, *Xist*, *Tsix* and *Xite*, a small *Xist* transgene with 15kb flanking sequence is also proficient in induction of XCI in cis on autosomes, despite the fact that these transgenes lack the DXPas34 element previously shown to be required for ectopic XCI. Studies with doxycyclin inducible single copy *Xist* cDNA transgenes showed long range silencing in cis on different autosomes in undifferentiated and differentiated ES cells. Withdrawal of doxycyclin in undifferentiated ES cells leads to reactivation of the silenced genes. In contrast, when the ES cells are induced when they are differentiating, the time point of doxycyclin mediated induction of *Xist* expression determined whether first, *Xist* was able to cause long range silencing; and second, whether this silencing was reversible or not. *Xist* can mediate silencing only when induced within the first 24 hours of differentiation. After 72 hours of differentiation silencing becomes irreversible. So *Xist* has to be expressed in a window between 48 and 72 hours after the initiation of differentiation to lock the silent state. (Herzing et al., 1997; Kohlmaier et al., 2004; Wutz and Jaenisch, 2000)

IMPRINTED X CHROMOSOME INACTIVATION

In the mouse extraembryonic tissues the paternal X chromosome is always inactivated. A paternal imprint resulting in exclusive inactivation of the Xp, or a maternal imprint repressing XCI on the Xm or both paternal and maternal imprints acting synergistically could govern imprinted XCI. Imprinted XCI is dependent on *Xist* as paternally inherited *Xist* deletions are embryonic lethal as a consequence of the inability to inactivate the Xp or the Xm in the extraembryonic lineages (Marahrens et al., 1997; Penny et al., 1996). Furthermore, proper imprinted XCI is also dependent on *Tsix* as *Tsix* mutations, that abolish *Tsix* function, inherited through the maternal germ-line are also embryonic lethal. A *Tsix* promoter deletion results in ectopic *Xist* accumulation on the Xm in XmΔ/Xp and XmΔ/Y extraembryonic tissues. This is consistent with the hypothesis that *Tsix* is an inhibitor of *Xist*. Furthermore, the *Tsix* deletion phenotype can be partially rescued by a paternal *Xist* deletion that results in a mouse with an inversed pattern of XCI in extra-embryonic tissues (Lee, 2000; Sado et al., 2001).

In wild type mice, *Xist* is first expressed exclusively from the paternal allele around the 2-4-cell stage of the embryo (Kay et al., 1993; Zuccotti et al., 2002). *Tsix* expression can be first detected around the blastocyst stage but not during the cleavage stages were imprinted XCI is initiated (Debrand et al., 1999; Lee, 2000; Sado et al., 2001). Concomitant with XCI mediated down regulation of X-linked gene transcription, which starts at the 8-cell stage (Okamoto

et al., 2005), the first histone modifications are detectable on the Xp. A Chromatin modification complex (Eed/Enx1) and incorporation of histone variants can be detected from the 16-cell stage onwards (Okamoto et al., 2004).

Parthenogenetic (two genomes inherited from the mother), androgenetic (two genomes inherited from the father) and digynic (triploid with two genomes inherited from the mother and one from the father) embryos are interesting to investigate with respect to imprinted XCI. Nonetheless, analysis of XCI in these embryos should be taken with caution because the development of these embryos can be perturbed due to X chromosome dosage problems or the improper dosage of the imprinted genes. Parthenogenetic embryos die around day 10 of mouse development because of hampered development of extra-embryonic lineages (McGrath and Solter, 1984; Surani et al., 1984). In 10-12-cell stage embryos a variable amount of *Xist* clouds is detected. Some do not have any *Xist* cloud but in some embryos even cells that attempt to inactivate both X chromosomes can be detected. At the 30-cell stage cells most embryos display no *Xist* clouds. Nonetheless, some embryos show up to 80% of the cells with a *Xist* cloud (Matsui et al., 2001). This variability in XCI in the extraembryonic lineage is most likely to the cause of the reported early lethality of most embryos in utero, because most cells do end up with two Xa's. These cells will eventually die and disrupt placental function.

Studies with triploid digynic embryos, analyzed at day 10 of gestation, indicated that XmXmXp and XmXmY embryos have equal survival rates. Interestingly, in the XmXmY embryos, random XCI was found in the extra-embryonic tissues where normally imprinted XCI occurs indicating that both Xm alleles have an equal probability to initiate XCI (Speirs et al., 1990). Matsui found in blastocysts with more than 50 cells at most 10% of the XmXmY cells expressing *Xist*. This suggests that XCI can occur randomly in the extra-embryonic tissue in XmXmY embryos. However, the absolute probability for the imprinted Xm alleles to initiate XCI is low but equal. In contrast, almost all cells of an XmXmXp blastocyst showed *Xist* expression from the 8-64 cell stage from the Xp, suggesting a high probability on the imprinted Xp allele (Matsui et al., 2001). The size of the XmXmXp embryos seems to correlate with the amount of inactive X chromosomes. The higher the percentage of XiXiXa cells, the higher weight, indicating a growth advantage for XiXiXa cells over XiXaXa cells (Endo et al., 1982; Speirs et al., 1990).

Thus far, the origin of the imprint and the molecular mechanism involved in imprinted XCI remain elusive. Nonetheless, studies performed with parthenogenetic embryos generated early (non-growing) and full-grown oocytes support the presence of a maternal mark. Non-growing oocytes inactivate their Xm in the extraembryonic tissues, but full-grown oocytes do not. The Xm in the early oocyte nucleus thus behaves as an Xp in that it is preferentially inactivated, indicating that a maternal imprint required for suppressing XCI is acquired during oogenesis (Tada et al., 2000).

Androgenetic embryos display ectopic *Xist* RNA cloud formation preferentially showing double and single *Xist* clouds in XpXp and XpY embryos respectively. At the four-cell stage both Xp express *Xist* and this gradually decreases from 97% to 20% (8-16 cell stage to blastocyst) most likely because of cell selection. In 6.5 day embryos only in 7% of the cells two *Xist* clouds can be detected (Okamoto et al., 2000; Takagi, 2003). These studies indicate that the paternal allele also carries an imprint, but in contrast to the maternal imprint this mark is required to induce *Xist* expression. Nonetheless, the nature of the paternal imprint is still under debate. Imprinted *Xist* expression could result from passage of the Xic through spermatogenesis as a consequence of meiotic sex-chromosome inactivation (MSCI) or the protamine to histone replacement after fertilization (Huynh and Lee, 2003). Evidence against a role for MSCI in imprinting comes from studies with *Xist*

transgenes, which do not undergo MSCI in the male germ line but properly express *Xist* during early development when inherited through the male germline. Moreover, maternally inherited transgenes do not show *Xist* cloud formation but show pinpoint signals in blastocysts, probably representing *Tsix* expression (Okamoto et al., 2005; Okamoto et al., 2004).

It is tempting to speculate that the Dxpas34 repeat has a role in imprinted XCI because DXpas34 is methylated on alleles that do not express *Tsix* at day 12 of development (Cohen et al., 2007). The DXpas34 deletion also results in the improper imprinted XCI, when maternally inherited, resulting in ectopic *Xist* expression form the Xm.

Similar to other imprints that are established in oogenesis and are dependent on the de novo DNA methyl transferase Dnmt3A, the maternal mark suppressing XCI on the Xm could be provided by the same DNA methyl transferase. However, evidence against a role for Dnmt3A in maternal imprinting comes from studies with embryos derived from double homozygous Dnmt3A and Dnmt3B female knockout mice that show proper imprinted and random XCI. In addition, despite the reported differential DNA methylation of the DXpas34 element in somatic tissues no differential CpG methylation was detected during oogenesis, spermatogenesis and the early cleavage stages, also precluding a role for DNA methylation in imprinted XCI (Boumil et al., 2006; Prissette et al., 2001). Because Dnmt3A homozygous knockout males are infertile a role for Dnmt3A in establishing the male imprint remains an open question (Kaneda et al., 2004a; Kaneda et al., 2004b). Initial studies indicated that in male germ cells the promoter of the (silent) *Xist* gene is hypomethylated compared to male adult tissue, which led to the hypothesis that differential DNA methylation directs the imprinted expression of *Xist* on the Xp (Norris et al., 1994). However, extensive bisulfite sequencing studies indicated that there is no differential methylation of the *Xist* promoter region between oocytes en sperm (McDonald et al., 1998). Therefore, *Xist* promoter methylation is not likely to be involved in establishment of the putative paternal imprint.

MODELS FOR COUNTING AND CHOICE

The blocking factor model

Observations in humans carrying supernumery X chromosomes suggest that one X chromosome is kept active per diploid genome. One model to explain this counting phenomenon is the existence of a autosomally encoded blocking factor, which was put forward by Rastan et al. (Rastan and Robertson, 1985) The blocking factor prevents one X chromosome from inactivation because one blocking factor is present per diploid genome (Figure 10). The factor could be a limited protein complex or a nuclear episome. The symmetry-breaking model postulated that diffusible molecules build up the blocking factor, to be sequestered to one binding site in the nucleus assembled through intermolecular interactions between these proteins (Nicodemi and Prisco, 2007). A blocking factor model predicts that the blocking factor binds one of the X chromosomes randomly via a region determined the 'counting element' which is located within the Xic (Rastan and Robertson, 1985).

Different groups have attempted to identify the counting element by deletion studies. A 65kb deletion 3' of *Xist* that abolished counting, as detected by *Xist* cloud formation in male ES cells, suggested that the counting element was located within the deleted region. Different smaller deletions have been made over the past 10 years and the remaining candidate element is a 1.2kb (Debrand et al., 1999). All deletions affecting putative blocking factor binding cause ectopic *Xist* accumulation in male cells and result in primary non-random XCI in female cells, because in female cells the mutated X is not protected in contrast to the wild type X chromosome.

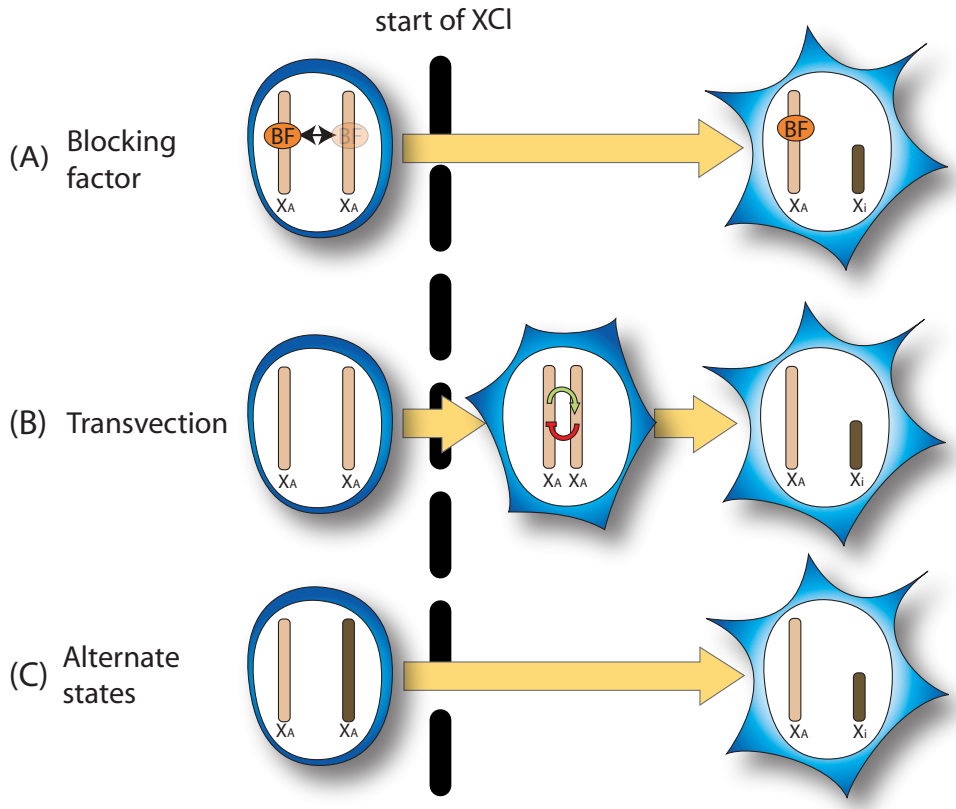

Figure 10. **Different models for XCI counting and choice**
Three popular models that explain XCI counting and choice. (A) Exclusive binding of a blocking factor results in one active X chromosome in female cells randomly. (B) Cross-talk between the two XIC's orchestrates XCI counting and choice. (C) Alternate states of the two X chromosomes in female cells determine which X chromosome is inactivated.

An important note concerning this and other reported counting defects is that these deletions all abolish *Tsix* transcription. Interestingly, integration of a stop cassette that abolished *Tsix* transcription without deleting DNA sequences resulted in the same phenotype as found for the cell lines with the specific deletions suggesting that a DNA element could not be responsible for the observed phenotype. Furthermore, neither the blocking factor itself nor its binding site has been identified over the past 20 years.

Polyploidy and the blocking factor model

Analysis of XCI in polyploid embryos could help dissect the mechanism driving the XCI counting and choice process. As mentioned earlier, polyploid embryos die during development around day 10 (Carr, 1970). The blocking factor model would predict that a tetraploid embryo should inactivate all but two X chromosomes. A triploid embryo however would run into trouble because cells in these embryos will have 1.5 blocking factor. As expected for a blocking factor model, XCI in triploid XXX embryos is variable and one or two X chromosomes

are kept active. In tetraploid XXXX embryos, X chromosome counting works properly as day 10 embryos preferentially maintain two active X chromosomes per tetraploid cell. Nonetheless, a significant number of cells with one or three inactive X chromosomes were reported. (Carr, 1970; Speirs et al., 1990; Webb et al., 1992). To study XCI in more detail tetraploid cells were generated by fusion of embryonic carcinoma cells with lymphocytes. The Xi provided by the lymphocytes reactivates in these hybrid cell lines. Analysis of XCI after differentiation showed inactivation of one X chromosome in ½ to ¾ in tetraploid XXXX cells. In about ¼ of the cells two X chromosomes were inactivated. Three Xi's were also seen in varying amounts. Important to note is that these cell hybrids tend to loose X chromosomes during the differentiation process. In addition, analysis of XCI was performed by a BrdU incorporation assay. If cells do not divide (cells with 3 and 4 Xi's will not or only scarcely) they will not be detected by this assay. This data must therefore be interpreted with caution (Takagi, 1993).

Transient colocalization of X inactivation centers

Different groups have proposed that cross talk between the Xic's in a nucleus is orchestrating the counting and choice mechanism. This theory is based on the observations that in early differentiating EB's the two Xic's show non-random spatial distributions (they come closer to each other) (Figure 10). In one study it was found that in 8-15 % of the cells the two Xic's in female cells are within 1 µm of each other. A different study found that around day 2-4 of differentiation the relative distance of the two Xic's in female cells change from 0.41 ND to ~0.32 ND (ND is the distance relative to the nuclear diameter) (Bacher et al., 2006; Xu et al., 2006). The first study concluded that cross talk precedes XCI counting and choice because transient pairing was observed prior to *Xist* RNA accumulation. In complete contrast however the second study found that cells, in which the Xic's transiently move closer, have accumulated *Xist* RNA. Interestingly, male XY cells carrying multicopy Xic transgenes are capable of inducing approximation events comparable to wild type XX cells. Nonetheless, in a female cell line with a heterozygous 65kb deletion 3' of *Xist* including *Tsix* and *Xite* (Clerc and Avner, 1998) this spatial association is lost, despite the fact that this cell line does not show a counting defect. This result indicates that additional parameters are required to explain counting and choice by transient movement of the Xic's during differentiation of female cells and may be the consequence of the XCI process itself.

Other models for XCI counting and choice

Several other models explaining the XCI counting and choice process have been postulated. For instance, X chromosomes in female cells might be distinguished prior to the onset of XCI by methylation marks or other alternative epigenetic states (Figure 10). Although preemptive methylation marks were reported to control random *Xist* expression in the promoter region (Norris et al., 1994), bisulfite genomic sequencing revealed that the parental *Xist* alleles are not differentially methylated (McDonald et al., 1998). One specific feature of genes with the potential to be mono-allelically expressed, including imprinted genes and genes located on the X chromosome is the presence of asynchronous replication timing. In addition, differences in cohesion of sister chromatids has been reported in female ES cells prior to the start of XCI, although it is unclear what this means on a molecular level (Mlynarczyk-Evans et al., 2006). These distinct epigenetic states may play a role in the XCI counting and choice process.

XCI ESTABLISHMENT AND MAINTENANCE

The establishment of transcriptional silencing on the inactivated X chromosome remains elusive. Recent studies focus on the chromatin modifications during the initiation of XCI to provide some insight into the action of *Xist*. The earliest chromatin changes are the loss of 'active chromatin histone modifications' such as Histone 3 Lysine-9 acetylation (H3K9ac) and Histone 3 Lysine 4 mono and di-methlation (H3K4me1/2). The mechanism that removes these epigenetic marks remains unclear. Soon after these changes, repressive marks appear such as H3K27me3, H4k20me1 and Histone 2A mono-ubiquitnation (H2Aub1) on the future Xi. Polycomb Repressive Complexes (PRC's) are histone modification complexes involved in gene silencing and their association to the Xi appears to be *Xist* dependent. The H2Aub1 mark and the H3K27me3 mark are catalized by the PRC1 and PRC2 complexes respectively and will be discussed in more detail below.

H3K27me3 and H2AK119u1 are *Xist* dependent, reversible and not sufficient for silencing

Xist has to be expressed in a critical window to induce irreversible silencing during early differentiation. Analysis of H3K27 tri-methylation by the Polycomb repressive complex 2 (PRC2) in early differentiating ES cells indicates that tri-methylation of H3K27 is independent of silencing i.e. the A repeat in *Xist*, which is required for silencing, is not necessary to establish H3K27 trimethylation. The same accounts for mono-ubiquitination of H2A on lysine 119 that parallels H3K27me3 and is mediated by Ring 1b, a protein that is part of the PRC1 complex. Studies with *Xist* A-repeat mutant cells show proper ubiquitination, precluding a role for this histone mark in establishing the silent state.

Interestingly *Xist* has to be expressed in the window that is necessary for irreversible silencing to occur (Wutz and Jaenisch, 2000) and to establish appropriate levels of H3K27 tri-methylation and H2AK119ub1. In addition both marks are reversible, in that if *Xist* is shut off, they disappear. However if *Xist* is turned on again, it restores the marks to wild type levels. This phenomenon is designated 'the chromosomal memory' for these marks (triggered by *Xist*) and shows that these modifications are reversible and *Xist* dependent (Kohlmaier et al., 2004; Schoeftner et al., 2006).

The open question remains, what causes the transcriptional silencing properties of the *Xist* molecule? The H3K27me3 mark was a candidate for a role in transcriptional silencing because it accumulates early on the Xi after initiation of XCI. However, an Eed (PRC2 complex) homozygous knock out cell line showed that *Xist* preserves its long range silencing capabilities and even properly maintains the silent state. PRC2 and H3K27me3 are thus not sufficient for the initial silencing mechanism during establishment. Also the early H2AK119ub1 mark did not show a role in the initiation of silencing since the homozygous knockout of the responsible protein, Ring1b or its homologue Ring1a did not show defective XCI initiation (Csankovszki et al., 1999; de Napoles et al., 2004; Kohlmaier et al., 2004; Schoeftner et al., 2006; Wang et al., 2004).

The Xic is not required for maintenance

Brown and Willard analyzed a series of mouse/human somatic cell hybrids in which they deleted the XIC after the XCI process. Their data revealed no reactivation of 8 inactivated X linked genes and indicates that the XIC is not required for the maintenance of XCI (Brown and Willard, 1994).

Deletion of *Xist* causes low-level reactivation

Studies with a doxycyclin inducible *Xist* transgene demonstrated that *Xist* cannot induce silencing after a initiation time window. A conditional knock out of *Xist* showed that *Xist* RNA disappeared from the Xi as expected after Cre mediated recombination after XCI was established. The inactive X chromosome however did not show robust reactivation of its genes indicating that *Xist* is not essential for maintenance of XCI and confirmed the aforementioned experiments with the cell hybrids (Brown et al., 1994; Csankovski et al., 1999). Furthermore the Xi remained late replicating and hypo acetylated on histone H4. (Penny et al., 1996; Wutz and Jaenisch, 2000). To investigate this in more detail cells with a conditionally deleted *Xist* gene from the Xi, were analyzed in combination with an X linked GFP transgene *in cis*, which served as a marker for reactivation. Very low frequencies of reactivation were detected. However, the reactivation rate was elevated after treatment with the DNA demethylating agent 5-azadC or the inhibitor of histone deacetylases, TSA (both known for their reactivation properties). Both treatments show synergistic effects when combined with the conditional deletion of *Xist* indicating that different epigenetic modifications act synergistically in maintaining the silent state (Csankovszki et al., 2001).

A role for PRC2 in early maintenance

Trophoblast cells form part of the extra-embryonic tissues and show imprinted XCI. In trophoblast cells imprinted XCI is not properly maintained when a critical component of the complex responsible for it (PRC2) was mutated. RNA-immunofluorescence experiments show that in wild type trophoblast, but also ES cells H3K27 trimethylation colocalized with *Xist* accumulation. In ES cells PRC2 itself is only transiently recruited to the inactivated X and its presence peaks between 2-8 days of differentiation. After that, it diminishes until all cells have lost PRC2 at embryonic day 13. A silencing deficient *Xist* also recruits the PRC2 complex precluding a role for the complex in the establishment of the Xi. This suggests a role for H3K27 tri methylation in the early maintenance phase of XCI (Plath et al., 2003; Wang et al., 2002).

A synergistic role in silencing for MacroH2A

MacroH2A is a histone H2A variant that localizes to the inactive X chromosome in the maintenance phase of XCI. *Xist* is dispensable for XCI maintenance at this time but the localization of macroH2A is still dependent on *Xist* since *Xist* conditional knockout cell show a loss of localization of macroH2A to the Xi. A silencing deficient *Xist* cDNA transgene can still localize macroH2A to the active, but *Xist* coated X chromosome. Recruitment of macroH2A is therefore not likely to be involved in the initiation of silencing (Csankovszki et al., 1999; Wutz and Jaenisch, 2000). MacroH2A can be ubiquitinated by the CULLIN3/SPOP ubiquitin ligase complex. Loss of this ubiquitination mark by RNAi experiments shows loss of macroH2A localization to the Xi and Xi reactivation when combined with 5-Aza-dC and TSA treatments. This is a strong indication that macroH2A plays a synergistic role in the maintenance of XCI together with DNA methylation and histone de-acetylation and provides an additional epigenetic layer of transcriptional silencing (Csankovszki et al., 2001; Csankovszki et al., 1999; Hernandez-Munoz et al., 2005).

Interestingly, macroH2A has been found to be bound to the nuclear matrix and could represent a *Xist* dependent link between chromatin of the Xi and the nuclear matrix (Clemson et al., 1996; Csankovszki et al., 1999).

DNA methylation and maintenance of the active and inactive state of the X chromosome

DNA methylation is one of the last modifications established on the inactive X chromosome and is regarded to be the 'lock in' of the silent state. In the extraembryonic tissues, where maintenance of the inactive X chromosome is less stringent, there is indeed a reduced level of DNA methylation. It is believed that the temporal nature of this tissue does not require stringent maintenance of the Xi as it is discarded after birth.

Dnmt1 deficiency results in ectopic *Xist* expression

In somatic cells the *Xist* promoter is fully methylated on the active X chromosome and completely demethylated on the inactive X chromosome correlating with *Xist* gene activity. In Dnmt1 knockout ES cells after differentiation and in embryos, ectopic *Xist* RNA expression can be detected indicating that maintenance of *Xist* promoter silencing on the active X chromosome is at least in part dependent on DNA methylation. This finding is consistent with the finding that genes that are in an off state are fully methylated in their promoter region and those that express are not (Beard et al., 1995; Li et al., 1992; Norris et al., 1994). Initial studies showed that this ectopic *Xist* expression, which was induced relative late during the differentiation process, was accompanied by inactivation of X linked genes and resulted in increased cell death in differentiating cultures due to aberrant gene dosage. These results are in contrast with the 'initiation window' published by Wutz and Jaenisch because the reported upregulation of *Xist* happens outside this window and therefore cannot cause silencing (Panning and Jaenisch, 1996; Wutz and Jaenisch, 2000).

Dnmt3A/B has a role in the regulation of *Xist* expression

Dnmt1 is 'the maintenance' DNA methyltransferase. In addition, two proteins, Dnmt3A and Dnmt3B have been identified which accomplish de novo methylation. Differentiated double knock out ES cells for these proteins show a hypomethylated *Xist* promoter region on the active X chromosome that is normally fully methylated. However, despite the absence of differential methylation of the *Xist* promoter in differentiated cells, the homozygous double knockout embryos present only a mild phenotype with respect to XCI. About 5% of the cells in male and slightly more in female embryos show ectopic *Xist* expression. This percentage is comparable with the amount of ectopic expression of homozygous Dnmt1 knockout embryos. Interestingly the double homozygous Dnmt3A/B KO embryos show proper silencing of one X chromosome in almost all female cells despite the presence of two *Xist* clouds indicating that the ectopic expression does not induce silencing, and is apparently induced beyond the critical window for XCI (Okano et al., 1999; Sado et al., 2004). In differentiating double homozygous Dnmt3A/B ES cells the effect was more pronounced. At day 12 almost 70% of female cells showed ectopic *Xist* although *Xist* expression did not induce inactivation in concordance with the initiation widow mentioned above. Taken together, DNA methylation plays a role in the regulation of the expression of *Xist*, but is not required for establishment of the silent state (Sado et al., 2004).

Xist, DNA methylation and histone acetylation have synergistic roles in XCI

The role of DNA methylation in maintaining the silent state became evident when a conditional knock out of *Xist*, which showed reactivation of some of the inactive X chromosomes. This effect was more pronounced when it was combined with DNA demethylating agents as 5-azadC or an inhibitor of histone deacetylases

TSA and indicates synergism between the *Xist* RNA, DNA methylation and histone de-acetylation in the maintenance of the silent state of the X chromosomes. A Dnmt1 homozygous knockout cell line interestingly has a much more pronounced effect on reactivation of the Xi than the conditional *Xist* deletion, indicating that methylation is a more important contributor to maintenance of the silent state than *Xist* (Beard et al., 1995; Csankovszki et al., 2001; Kaneda et al., 2004a; Li et al., 1992; Okano et al., 1999; Panning and Jaenisch, 1996; Sado et al., 2004).

HUMAN XCI AND DISEASE

XCI in humans is different from mouse XCI in a few ways. In human females the XCI pattern is random with respect to the parental origin, as it is in the mouse, but there is no definitive proof for imprinted XCI in the human extraembryonic tissues. The XIC in humans is also different. Interestingly, in human, TSIX does not overlap with the entire XIST gene as it does in mice but only overlaps the last few exons of XIST. Tsix, the repressor of Xist in mice can be cotranscribed in humans with XIST and therefore is unlikely a repressor of XIST. Indeed, the particular overlap has been shown to be crucial for its function of Tsix in mice (Luikenhuis et al., 2001).

As in mice, female humans also show skewed XCI. The amount of skewing varies, and the distribution of these amounts follow a bell shaped curve. Approximately 30% displays more than 75% skewing towards one locus and around 5% shows even more than 90% skewing. The amount of skewing is also tissue dependent, most likely due to secondary selection in these tissues during development. In mice, different Xce's cause different amounts of skewing (primary non-random XCI). Important to note is that mice are inbred and only F1s display skewing because two different Xce's are now combined. Humans are not inbred and thus have potentially many more Xce's in the population. The existence of an Xce in humans however is uncertain but this could explain the bell shaped curve mentioned above. Nonetheless, skewing does not seem to correlate between mothers and their neonates (Bolduc et al., 2008). This data should be interpreted with caution because the amount of skewing was determined in born neonates and adults. Secondary effects, such as cell selection, can mask primary non-random XCI patterns. Increases in somatic XCI skewing are reported for a variety of diagnosis, including premature ovarian failure and recurrent spontaneous abortion, several autoimmune disorders and some cancers. Understanding the mechanism of XCI counting and choice is important to judge the clinical relevance of these observations (Minks et al., 2008).

Females are mosaics with respect to the parental origin of their active X chromosomes. This has important implications for X-linked mutations and disease. Both males and females have only one active copy of the X chromosome. In females, any significant mutation in a gene would result in a defective protein or no protein at all, depending on the status of the X chromosome carrying the mutation (active or inactive). Half of the female cells have the healthy copy active but, the other half has the mutated copy of the gene active. One can imagine that the amount of skewing, primary or secondary, has important effects on the severity of the disease. The cells with the proper protein can usually carry out the required function and compensate although obviously, this is dependent on the function of the protein. This makes females often less susceptible to X linked diseases but also makes clear why females do have a phenotype when they inherit a mutated X linked gene (although mildly compared to their male counterpart) (Migeon, 2007).

Extra X chromosomes

Different syndromes are the result of extra X chromosomes in a diploid nucleus. In the case of an extra X chromosome in diploid males (XXY Klinefelter) or diploid females (XXX or even XXXX) the disease phenotype is caused by dosage problems despite inactivation of all but one of the X chromosomes. This is possible because a variable part of the X chromosome escapes inactivation. This pseudoautosomal region (PAR), which harbours around 50 genes, is necessary for pairing with the Y chromosome during meiosis and is not inactivated in somatic cells. In Klinefelter disease the male has 3 PARs instead of two. Furthermore, other isolated regions on the X chromosome escape inactivation and are thus expressed at levels that are either too high or too low in cells with an aberrant X chromosome number. Note that these XCI escaping genes are also responsible for a dosage difference between normal males and females (Migeon, 2007).

One X chromosome in females

The above also helps to understand why XO individuals (Turner syndrome) display a Turner phenotype. A single X is sufficient for development, but the XO individuals carry a number of Turner stigmata. For example, two X chromosomes (one inactivated and one active) are needed for proper development of the human female gonads. With only one active X chromosome, the loss of oocytes from the third month of gestation is accelerated until no oocytes are left at birth, resulting in fibrotic 'streak gonads'

Ring X chromosomes

Truncation of an X chromosome and the subsequent rejoining of the ends results in a ring like X chromosome. Because a cell cannot survive with an active ring chromosome due to dosage problems, only cells survive with inactivated ring chromosomes. Since these individuals miss a PAR (located at the telomeres) and other XCI escaping genes, these individuals have a Turner phenotype. In a more severe phenotype the ring chromosome does not inactivate because the XIC is also deleted and gives an even greater dosage problem. Very interestingly, some of these ring chromosomes do contain the Xist locus but do not inactivate, indicating that at least one additional DNA element is necessary for XCI (Migeon et al., 1994; Migeon et al., 1993; Wolff et al., 1994).

AIM AND SCOPE OF THIS THESIS

Placental mammals achieve dosage compensation of X chromosome encoded genes between females and males by inactivation of one of the X chromosomes in female cells. Initiation of this process is characterized by counting the number of X chromosomes relative to the ploidy of the cell and choosing the future active X chromosome(s). The counting and choice process ensures that only one of the two X chromosomes in female diploid cells is randomly active per diploid genome. The aim of this thesis was to unravel the molecular mechanism behind the X chromosome inactivation counting and choice process.

Different models have been posed to explain counting and choice. The prevailing 'blocking factor' model explains counting and choice by a limited factor in the cell nucleus that can only block one X chromosome from inactivation. Unfortunately, despite extensive research over the last two decades no such factor has been isolated nor has its binding site on the X chromosome been identified.

Experiments described in Chapter 2 demonstrate that when the other allele contains different *Xist* deletions the wild type allele is primarily chosen for inactivation in female embryos. The absence of a post choice selection mechanism argues against the existence of a blocking factor. Asynchronous replication timing is known to be associated with imprinted and non imprinted mono-allelically expressed genes. Although the X chromosomes in female ES cells display asynchronous replication timing before XCI is initiated, this does not correlate with X chromosome choice after initiation of XCI. Asynchronous replication timing therefore is not likely to play a major role in the counting and choice process.

In Chapter 3 we describe the analysis of diploid and tetraploid ES cells and find that after initiation of XCI these cells show numbers of *Xist* clouds that do not comply with the n-1 rule (all but one X is inactivated per diploid genome). The experimental data can be simulated by assuming a certain probability for each X chromosome in a nucleus to inactivate, indicating a stochastic nature for the XCI counting and choice process. Further, cells with a deletion of all elements known to be involved in the counting process demonstrate proper initiation of XCI on the wild type allele. This locates a gene encoding a novel XCI-activator outside the deleted region and supports a probabilistic mechanism for X chromosome counting and choice.

In Chapter 4 we describe the generation and analysis of triploid XXY cells. These cells have an X:ploidy ratio of ⅔ and show borderline XCI initiation. The results support a stochastic model and show that threshold levels of the XCI-activator must be just below the concentration present in XXY cells.

Chapter 5 describes the simulation of X chromosome inactivation counting and choice. A stochastic model deals with probabilities, probability time frames and cell division parameters. We used computer simulation to show that all our observations and observations made by others can be explained with a stochastic model. We conclude that the sensitivity of a Xic allele for the activating stimulus and the XCI-activator concentration together orchestrate X chromosome inactivation counting and choice.

REFERENCES

Amos-Landgraf, J.M., Cottle, A., Plenge, R.M., Friez, M., Schwartz, C.E., Longshore, J., and Willard, H.F. (2006). X chromosome-inactivation patterns of 1,005 phenotypically unaffected females. Am J Hum Genet 79, 493-499.

Ariel, M., Robinson, E., McCarrey, J.R., and Cedar, H. (1995). Gamete-specific methylation correlates with imprinting of the murine *Xist* gene. Nat Genet 9, 312-315.

Bacher, C.P., Guggiari, M., Brors, B., Augui, S., Clerc, P., Avner, P., Eils, R., and Heard, E. (2006). Transient colocalization of X-inactivation centres accompanies the initiation of X inactivation. Nat Cell Biol 8, 293-299.

Barr, M.L., and Bertram, E.G. (1949). A Morphological distribution between Neurons of the Male and Female and the Behavior of the Nucleolar satellite during Accelerated Nuclear Protein Synthesis. Nature 163, 676-677.

Beard, C., Li, E., and Jaenisch, R. (1995). Loss of methylation activates *Xist* in somatic but not in embryonic cells. Genes Dev 9, 2325-2334.

Bolduc V, Chagnon P, Provost S, Dubé MP, Belisle C, Gingras M, Mollica L, Busque (2008). No evidence that skewing of X chromosome inactivation patterns is transmitted to offspring in humans. J Clin Invest. 2008 Jan;118(1):333-41.

Borsani, G., Tonlorenzi, R., Simmler, M.C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., et al. (1991). Characterization of a murine gene expressed from the inactive X chromosome. Nature 351, 325-329.

Boumil, R.M., Ogawa, Y., Sun, B.K., Huynh, K.D., and Lee, J.T. (2006). Differential methylation of *Xite* and CTCF sites in *Tsix* mirrors the pattern of X-inactivation choice in mice. Mol Cell Biol 26, 2109-2117.

Brockdorff, N., Ashworth, A., Kay, G.F., Cooper, P., Smith, S., McCabe, V.M., Norris, D.P., Penny, G.D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. Nature 351, 329-331.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991a). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature 349, 38-44.

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

Brown, C.J., Lafreniere, R.G., Powers, V.E., Sebastio, G., Ballabio, A., Pettigrew, A.L., Ledbetter, D.H., Levy, E., Craig, I.W., and Willard, H.F. (1991b). Localization of the X inactivation centre on the human X chromosome in Xq13. Nature 349, 82-84.

Brown, C.J., and Willard, H.F. (1994). The human X-inactivation centre is not required for maintenance of X chromosome inactivation. Nature 368, 154-156.

Carr, D.H. (1970). Chromosome studies in selected spontaneous abortions. 1. Conception after oral contraceptives. Can Med Assoc J 103, 343-348.

Carrel, L. (2004). Evolutionary biology: chromosome chain makes a link. Nature 432, 817-818.

Cattanach, B.M., and Isaacson, J.H. (1965). Genetic control over the inactivation of autosomal genes attached to the X chromosome. Z Vererbungsl 96, 313-323.

Cattanach, B.M., and Isaacson, J.H. (1967). Controlling elements in the mouse X chromosome. Genetics 57, 331-346.

Cattanach, B.M., and Williams, C.E. (1972). Evidence of non-random X chromosome activity in the mouse. Genet Res 19, 229-240.

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

Clerc, P., and Avner, P. (1998). Role of the region 3' to *Xist* exon 6 in the counting process of X chromosome inactivation. Nat Genet 19, 249-253.

Cohen, D.E., Davidow, L.S., Erwin, J.A., Xu, N., Warshawsky, D., and Lee, J.T. (2007). The DXPas34 repeat regulates random and imprinted X inactivation. Dev Cell 12, 57-71.

Courtier, B., Heard, E., and Avner, P. (1995). Xce haplotypes show modified methylation in a region of the active X chromosome lying 3' to *Xist*. Proc Natl Acad Sci U S A 92, 3531-3535.

Csankovszki, G., Nagy, A., and Jaenisch, R. (2001). Synergism of *Xist* RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J Cell Biol 153, 773-784.

Csankovszki, G., Panning, B., Bates, B., Pehrson, J.R., and Jaenisch, R. (1999). Conditional deletion of *Xist* disrupts histone macroH2A localization but not maintenance of X inactivation. Nat Genet 22, 323-324.

Davidson, R.G., Nitowsky, H.M., and Childs, B. (1963). Demonstration of Two Populations of Cells in the Human Female Heterozygous for Glucose-6-Phosphate Dehydrogenase Variants. Proc Natl Acad Sci U S A 50, 481-485.

de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M., et al. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell 7, 663-676.

Debrand, E., Chureau, C., Arnaud, D., Avner, P., and Heard, E. (1999). Functional analysis of the DXPas34 locus, a 3' regulator of *Xist* expression. Mol Cell Biol 19, 8513-8525.

Duret, L., Chureau, C., Samain, S., Weissenbach, J., and Avner, P. (2006). The *Xist* RNA gene evolved in eutherians by pseudogenization of a protein-coding gene. Science 312, 1653-1655.

Endo, S., Takagi, N., and Sasaki, M. (1982). The Late-Replicating X chromosome in Digynous Mouse Triploid Embryos. Developmental Genetics 3, 165-176.

Graves, J.A. (1996). Mammals that break the rules: genetics of marsupials and monotremes. Annu Rev Genet 30, 233-260.

Graves, J.A. (2006). Sex-chromosome specialization and degeneration in mammals. Cell 124, 901-914.

Gribnau, J., Diderich, K., Pruzina, S., Calzolari, R., and Fraser, P. (2000). Intergenic transcription and developmental remodeling of chromatin subdomains in the human beta-globin locus. Mol Cell 5, 377-386.

Grutzner, F., and Graves, J.A. (2004). A platypus' eye view of the mammalian genome. Curr Opin Genet Dev 14, 642-649.

Grutzner, F., Rens, W., Tsend-Ayush, E., El-Mogharbel, N., O'Brien, P.C., Jones, R.C., Ferguson-Smith, M.A., and Marshall Graves, J.A. (2004). In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. Nature 432, 913-917.

Harnden, D.G. (1961). Nuclear sex in triploid XXY human cells. Lancet 2, 488.

Heard, E. (2004). Recent advances in X chromosome inactivation. Curr Opin Cell Biol 16, 247-255.

Heard, E., Mongelard, F., Arnaud, D., and Avner, P. (1999). *Xist* yeast artificial chromosome transgenes function as X-inactivation centers only in multicopy arrays and not as single copies. Mol Cell Biol 19, 3156-3166.

Hernandez-Munoz, I., Lund, A.H., van der Stoop, P., Boutsma, E., Muijrers, I., Verhoeven, E., Nusinow, D.A., Panning, B., Marahrens, Y., and van Lohuizen, M. (2005). Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase. Proc Natl Acad Sci U S A 102, 7635-7640.

Herzing, L.B., Romer, J.T., Horn, J.M., and Ashworth, A. (1997). *Xist* has properties of the X chromosome inactivation centre. Nature 386, 272-275.

Hore, T.A., Koina, E., Wakefield, M.J., and Marshall Graves, J.A. (2007). The region homologous to the X chromosome inactivation centre has been disrupted in marsupial and monotreme mammals. Chromosome Res 15, 147-161.

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

Jacobs, P.A., Baikie, A.G., Brown, W.M., Macgregor, T.N., Maclean, N., and Harnden, D.G. (1959). Evidence for the existence of the human "super female". Lancet 2, 423-425.

Johnston, P.G., and Cattanach, B.M. (1981). Controlling elements in the mouse. IV. Evidence of non-random X-inactivation. Genet Res 37, 151-160.

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., and Sasaki, H. (2004a). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 429, 900-903.

Kaneda, M., Sado, T., Hata, K., Okano, M., Tsujimoto, N., Li, E., and Sasaki, H. (2004b). Role of de novo DNA methyltransferases in initiation of genomic imprinting and X chromosome inactivation. Cold Spring Harb Symp Quant Biol 69, 125-129.

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

Kohlmaier, A., Savarese, F., Lachner, M., Martens, J., Jenuwein, T., and Wutz, A. (2004). A chromosomal memory triggered by *Xist* regulates histone methylation in X inactivation. PLoS Biol 2, E171.

Lahn, B.T., and Page, D.C. (1999). Four evolutionary strata on the human X chromosome. Science 286, 964-967.

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

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

Lee, J.T., and Jaenisch, R. (1997). Long-range cis effects of ectopic X-inactivation centres on a mouse autosome. Nature 386, 275-279.

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

Lee, J.T., Strauss, W.M., Dausman, J.A., and Jaenisch, R. (1996). A 450 kb transgene displays properties of the mammalian X-inactivation center. Cell 86, 83-94.

Li, E., Beard, C., and Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. Nature 366, 362-365.

Li, E., Bestor, T.H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69, 915-926.

Luikenhuis, S., Wutz, A., and Jaenisch, R. (2001). Antisense transcription through the *Xist* locus mediates *Tsix* function in embryonic stem cells. Mol Cell Biol 21, 8512-8520.

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

Maclean, N., and Mitchell, J.M. (1962). A survey of sex-chromosome abnormalities among 4514 mental defectives. Lancet 1, 293-296.

Marahrens, Y., Loring, J., and Jaenisch, R. (1998). Role of the *Xist* gene in X chromosome choosing. Cell 92, 657-664.

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

Matsui, J., Goto, Y., and Takagi, N. (2001). Control of *Xist* expression for imprinted and random X chromosome inactivation in mice. Hum Mol Genet 10, 1393-1401.

McDonald, L.E., Paterson, C.A., and Kay, G.F. (1998). Bisulfite genomic sequencing-derived methylation profile of the *Xist* gene throughout early mouse development. Genomics 54, 379-386.

McGrath, J., and Solter, D. (1984). Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. Science 226, 1317-1319.

McMahon, A., and Monk, M. (1983). X chromosome activity in female mouse embryos heterozygous for Pgk-1 and Searle's translocation, T(X; 16) 16H. Genet Res 41, 69-83.

Melvin M. Grumbach, A.M., and J. Herbert Taylor (1963). Human sex-chromosome abnormalities in relation to DNA replication and heterochromatinization. Proc Natl Acad Sci U S A 49, 581–589.

Migeon, B. (2007). Females are mosaics. book Oxford university press.

Migeon, B.R., Luo, S., Jani, M., and Jeppesen, P. (1994). The severe phenotype of females with tiny ring X chromosomes is associated with inability of these chromosomes to undergo X inactivation. Am J Hum Genet 55, 497-504.

Migeon, B.R., Luo, S., Stasiowski, B.A., Jani, M., Axelman, J., Van Dyke, D.L., Weiss, L., Jacobs, P.A., Yang-Feng, T.L., and Wiley, J.E. (1993). Deficient transcription of *XIST* from tiny ring X chromosomes in females with severe phenotypes. Proc Natl Acad Sci U S A 90, 12025-12029.

Minks, J., Robinson, W.P., and Brown, C.J. (2008). A skewed view of X chromosome inactivation. The Journal of clinical Investigation 118, 20-23.

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

Monk, M., and Harper, M.I. (1979). Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. Nature 281, 311-313.

Nguyen, D.K., and Disteche, C.M. (2006). Dosage compensation of the active X chromosome in mammals. Nat Genet 38, 47-53.

Nicodemi, M., and Prisco, A. (2007). Symmetry-breaking model for X chromosome inactivation. Phys Rev Lett 98, 108104.

Norris, D.P., Patel, D., Kay, G.F., Penny, G.D., Brockdorff, N., Sheardown, S.A., and Rastan, S. (1994). Evidence that random and imprinted *Xist* expression is controlled by preemptive methylation. Cell 77, 41-51.

Ogawa, Y., and Lee, J.T. (2003). *Xite,* X-inactivation intergenic transcription elements that regulate the probability of choice. Mol Cell 11, 731-743.

Ohno, S., and Hauschka, T.S. (1960). Allocycly of the X chromosome in tumors and normal tissues. Cancer Res 20, 541-545.

Okamoto, I., Arnaud, D., Le Baccon, P., Otte, A.P., Disteche, C.M., Avner, P., and Heard, E. (2005). Evidence for de novo imprinted X chromosome inactivation independent of meiotic inactivation in mice. Nature 438, 369-373.

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

Okamoto, I., Tan, S., and Takagi, N. (2000). X chromosome inactivation in XX androgenetic mouse embryos surviving implantation. Development 127, 4137-4145.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247-257.

Panning, B., Dausman, J., and Jaenisch, R. (1997). X chromosome inactivation is mediated by *Xist* RNA stabilization. Cell 90, 907-916.

Panning, B., and Jaenisch, R. (1996). DNA hypomethylation can activate *Xist* expression and silence X-linked genes. Genes Dev 10, 1991-2002.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. Nature 379, 131-137.

Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science 300, 131-135.

Prissette, M., El-Maarri, O., Arnaud, D., Walter, J., and Avner, P. (2001). Methylation profiles of DXPas34 during the onset of X-inactivation. Hum Mol Genet 10, 31-38.

Rastan, S. (1982). Primary non-random X-inactivation caused by controlling elements in the mouse demonstrated at the cellular level. Genet Res 40, 139-147.

Rastan, S. (1983). Non-random X chromosome inactivation in mouse X-autosome translocation embryos--location of the inactivation centre. J Embryol Exp Morphol 78, 1-22.

Rastan, S., and Robertson, E.J. (1985). X chromosome deletions in embryo-derived (EK) cell lines associated with lack of X chromosome inactivation. J Embryol Exp Morphol 90, 379-388.

Russell, L. (1963). Mammalian X chromosome action: inactivation limited in spread and region of origin. Science 140, 976-978.

Russell, L.B., and Montgomery, C.S. (1969). Comparative studies on X-autosome translocations in the mouse. I. Origin, viability, fertility, and weight of five T(X;1)'S. Genetics 63, 103-120.

Russell, L.B., and Montgomery, C.S. (1970). Comparative studies on X-autosome translocations in the mouse. II. Inactivation of autosomal loci, segregation, and mapping of autosomal breakpoints in five T (X;1) S. Genetics 64, 281-312.

Sado, T., Hoki, Y., and Sasaki, H. (2006). *Tsix* defective in splicing is competent to establish *Xist* silencing. Development 133, 4925-4931.

Sado, T., Okano, M., Li, E., and Sasaki, H. (2004). De novo DNA methylation is dispensable for the initiation and propagation of X chromosome inactivation. Development 131, 975-982.

Sado, T., Tada, T., and Takagi, N. (1996). Mosaic methylation of *Xist* gene before chromosome inactivation in undifferentiated female mouse embryonic stem and embryonic germ cells. Dev Dyn 205, 421-434.

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

Schoeftner, S., Sengupta, A.K., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., Jenuwein, T., and Wutz, A. (2006). Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. Embo J 25, 3110-3122.

Shibata, S., and Lee, J.T. (2003). Characterization and quantitation of differential *Tsix* transcripts: implications for *Tsix* function. Hum Mol Genet 12, 125-136.

Speirs, S., Cross, J.M., and Kaufman, M.H. (1990). The pattern of X chromosome inactivation in the embryonic and extra-embryonic tissues of post-implantation digynic triploid LT/Sv strain mouse embryos. Genet Res 56, 107-114.

Sun, B.K., Deaton, A.M., and Lee, J.T. (2006). A transient heterochromatic state in *Xist* preempts X inactivation choice without RNA stabilization. Mol Cell 21, 617-628.

Surani, M.A., Barton, S.C., and Norris, M.L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature 308, 548-550.

Tada, T., Obata, Y., Tada, M., Goto, Y., Nakatsuji, N., Tan, S., Kono, T., and Takagi, N. (2000). Imprint switching for non-random X chromosome inactivation during mouse oocyte growth. Development 127, 3101-3105.

Takagi, N. (1976). Stability of X chromosome differentiation in mouse embryos. Reversal may not be responsible for the extreme X-inactivation mosaicism in extraembryonic membranes. Hum Genet 34, 207-211.

Takagi, N. (1980). Primary and secondary nonrandom X chromosome inactivation in early female mouse embryos carrying Searle's translocation T(X; 16)16H. Chromosoma 81, 439-459.

Takagi, N. (1993). Variable X chromosome inactivation patterns in near-tetraploid murine EC x somatic cell hybrid cells differentiated in vitro. Genetica 88, 107-117.

Takagi, N. (2003). Imprinted X chromosome inactivation: enlightenment from embryos in vivo. Semin Cell Dev Biol 14, 319-329.

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

Vigneau, S., Augui, S., Navarro, P., Avner, P., and Clerc, P. (2006). An essential role for the DXPas34 tandem repeat and *Tsix* transcription in the counting process of X chromosome inactivation. Proc Natl Acad Sci U S A 103, 7390-7395.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. Nature 431, 873-878.

Wang, J., Mager, J., Schnedier, E., and Magnuson, T. (2002). The mouse PcG gene eed is required for Hox gene repression and extraembryonic development. Mamm Genome 13, 493-503.

Webb, S., de Vries, T.J., and Kaufman, M.H. (1992). The differential staining pattern of the X chromosome in the embryonic and extraembryonic tissues of post-implantation homozygous tetraploid mouse embryos. Genet Res 59, 205-214.

West, J.D., Frels, W.I., Chapman, V.M., and Papaioannou, V.E. (1977). Preferential expression of the maternally derived X chromosome in the mouse yolk sac. Cell 12, 873-882.

Wolff, D.J., Brown, C.J., Schwartz, S., Duncan, A.M., Surti, U., and Willard, H.F. (1994). Small marker X chromosomes lack the X inactivation center: implications for karyotype/phenotype correlations. Am J Hum Genet 55, 87-95.

Wutz, A., and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol Cell 5, 695-705.

Wutz, A., Rasmussen, T.P., and Jaenisch, R. (2002). Chromosomal silencing and localization are mediated by different domains of *Xist* RNA. Nat Genet 30, 167-174.

Xu, N., Tsai, C.L., and Lee, J.T. (2006). Transient homologous chromosome pairing marks the onset of X inactivation. Science 311, 1149-1152.

Zuccotti, M., Boiani, M., Ponce, R., Guizzardi, S., Scandroglio, R., Garagna, S., and Redi, C.A. (2002). Mouse *Xist* expression begins at zygotic genome activation and is timed by a zygotic clock. Mol Reprod Dev 61, 14-20.

Zuccotti, M., and Monk, M. (1995). Methylation of the mouse *Xist* gene in sperm and eggs correlates with imprinted *Xist* expression and paternal X-inactivation. Nat Genet 9, 316-320.

Chapter 2

X Chromosome Choice occurs Independently of Asynchronous Replication Timing

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Chapter 2

X Chromosome Choice occurs Independently of Asynchronous Replication Timing

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In mammals, dosage compensation is achieved by X chromosome inactivation in female cells. *Xist* is required and sufficient for X inactivation, and *Xist* gene deletions result in complete skewed X inactivation. In this study, we analyzed skewing of X inactivation in mice with an *Xist* deletion encompassing sequence 5 KB upstream of the promoter through exon 3. We found that this mutation results in primary non-random X inactivation, in which the wild-type X chromosome is always chosen for inactivation. To understand the molecular mechanisms that affect choice, we analyzed the role of replication timing in X inactivation choice. We found that the two *Xist* alleles and all regions tested on the X chromosome replicate asynchronously prior to the start of X inactivation. However, analysis of replication timing in cell lines with skewed X inactivation showed no preference for one of the two *Xist* alleles to replicate early in S-phase before the onset of X inactivation, indicating that asynchronous replication timing does not play a role in skewing of X inactivation.

INTRODUCTION

In mammalian cells, dosage compensation of X-linked genes is achieved by X chromosome inactivation in female cells. X inactivation is a complex process that is regulated by integrating a number of mechanisms. Genetic studies have revealed that X chromosome silencing is initiated from one location on the X chromosome, the X inactivation center (*Xic*) (Rastan, 1983). The *Xist* gene, which lies within the *Xic,* encodes a large untranslated RNA that is required and sufficient for X inactivation (Penny et al., 1996). At the onset of X inactivation, one of the X chromosomes is chosen to remain active and *Xist* expression is stabilized on the future inactive X (Xi) and accumulates *in cis* (Brockdorff et al., 1991; Brown et al., 1991). The *Xist* RNA associating with the X chromosome recruits different chromatin modifying complexes eventually rendering the chromosome transcriptionally inactive (Silva et al., 2003).

The number of X chromosomes that will be inactivated is determined relative to the ploidy of the cell (Rastan, 1983; Rastan and Robertson, 1985). X inactivation is initiated when the number of X chromosomes exceeds one in a diploid nucleus. Each cell then makes the epigenetic choice to keep one X chromosome active (Xa) and to inactivate all supernumerary X chromosomes. These observations have led to a model that suggests the presence of a blocking factor in limited quantities that can protect only one X chromosome from inactivation per diploid set of chromosomes (Lyon, 1996).

X chromosome choice is random in the embryonic lineage of the mouse. In contrast, X inactivation in metatherian mammals such as

kangaroos (Cooper et al., 1971) and in the extra-embryonic tissues of some eutherian mammals including mice (Takagi and Sasaki, 1975) is imprinted and the paternal X chromosome always undergoes inactivation. Genetic studies have revealed that X chromosome choice is influenced by the X controlling element (*Xce*) such that X inactivation is skewed towards the chromosome possessing the weaker *Xce* allele (Simmler et al., 1993). At least four *Xce* alleles exist in mice: Xce^{a}, Xce^{b}, Xce^{c}, and Xce^{d}, with Xce^{a} being the weakest and Xce^{d} being the strongest allele.

Deletion of the *Xist* gene also leads to non-random X inactivation in embryonic tissues in mice (Csankovszki et al., 1999; Marahrens et al., 1998; Marahrens et al., 1997; Penny et al., 1996). However, it is controversial whether this non-random X-activation is achieved by primary non-random X inactivation or by random inactivation followed by post-choice selection (also referred to as secondary non-random X inactivation). Deletion of the *Xist* promoter and exon 1 ($Xist^{\Delta prom-1}$) resulted in post-choice selection in which cells that choose to inactivate the mutated X retain two active X chromosomes and die (Penny et al., 1996). In contrast, a deletion extending from part of exon 1 to exon 5 ($Xist^{\Delta 1-5}$) leaves the *Xist* promoter intact and results in primary non-random X inactivation where the wild type allele is always chosen to be inactivated (Marahrens et al., 1998; Marahrens et al., 1997; Nesterova et al., 2003).

The choice process itself is poorly understood, and it is unclear whether epigenetic differences between the two alleles prior to the onset of X inactivation are sufficient to mediate choice. Interestingly, a clear parallel has been found between genes on the X chromosome and autosomal mono-allelically expressed genes. Both display epigenetic characteristics that are already present before the choice is made which allele will be expressed. For instance, it has been shown that X-linked and imprinted genes reveal high levels of di-methylated histone H3 Lysine 4 methylation, which is restricted to the promoter region (Rougeulle et al., 2003). In contrast, bi-allelically expressed genes display equal levels of di-methylated histone H3 Lys 4 methylation in both promoter and genic regions. In addition, asynchronous replication timing has been found to correlate with loci that display mono-allelic expression including imprinted genes, olfactory receptor genes and immunoglobulin gene loci (Chess et al., 1994; Kitsberg et al., 1993; Mostoslavsky et al., 2001). A characteristic of imprinted as well as random X inactivation is that the Xi replicates late in S-phase (Takagi, 1974).Recently, asynchronous replication timing of the immunoglobulin κ-light chain locus was found to correlate with choice during VJC rearrangement, with the functional recombined allele being replicated prior to the unrecombined allele (Mostoslavsky et al., 2001). At this locus, asynchronous replication timing was present before one allele was chosen for recombination and may therefore play a role in determining choice (Mostoslavsky et al., 2001). For the X chromosomes in female cells, however, it is unknown whether asynchronous replication timing is present prior to X inactivation and whether it has an effect on determining choice.

In this study we analyzed the nature of non-random X inactivation in mice carrying different *Xist* deletions by monitoring X-linked GFP expression during early embryonic development. We then examined the role of replication timing in X chromosome choice by analyzing replication timing of the X chromosome before the onset of X inactivation in embryonic stem (ES) cell lines that show skewed X inactivation.

RESULTS

Primary non-random X inactivation in cells with an *Xist* deletion.

At the onset of X inactivation, one X chromosome is chosen for inactivation. Loss of *Xist* RNA expression leads to non-random X inactivation which results in the inactivation of the wild type X chromosome (Csankovszki et

al., 1999; Marahrens et al., 1998; Marahrens et al., 1997; Penny et al., 1996). Two different *Xist* deletions that cover either exon 1 through to exon 5 leaving the *Xist* promoter intact (*Xist*$^{\Delta 1\text{-}5}$) or delete the *Xist* promoter and most of exon 1 have separated primary non-random X inactivation from post-choice selection (Figure 1A). These results suggest that a region between exon 1 and 5 or the presence of residual transcription are required for choice.

To further define the sequence important for choice we analyzed X inactivation *in vivo* in embryos heterozygous for the previously characterized *Xist*$^{\Delta 1\text{-}5}$ allele and the *Xist*$^{\Delta lox}$ allele, which deletes 18 kb of the *Xist* locus including the promoter region and extends into intron 3 (Figure 1A) (Csankovszki et al., 1999). The wild type X in these mice was marked with an X-linked GFP allele that is subject to X inactivation (Hadjantonakis et al., 1998). We used fluorescence activated cell sorting (FACS) to monitor X inactivation as detected by a change in the number of cells expressing GFP. Based on control samples of mouse embryonic fibroblasts (MEFs) heterozygous for the X-linked GFP (Figure 1B, left panel) and wild type MEFs that do not express GFP (not shown), GFP fluorescence was arbitrarily gated into GFP-positive (Figure 1B, M3) GFP-negative (Figure 1B, M1), and intermediate GFP-fluorescence (Figure 1B, M2). Gate M2 included cells of intermediate fluorescence that were not brightly positive or

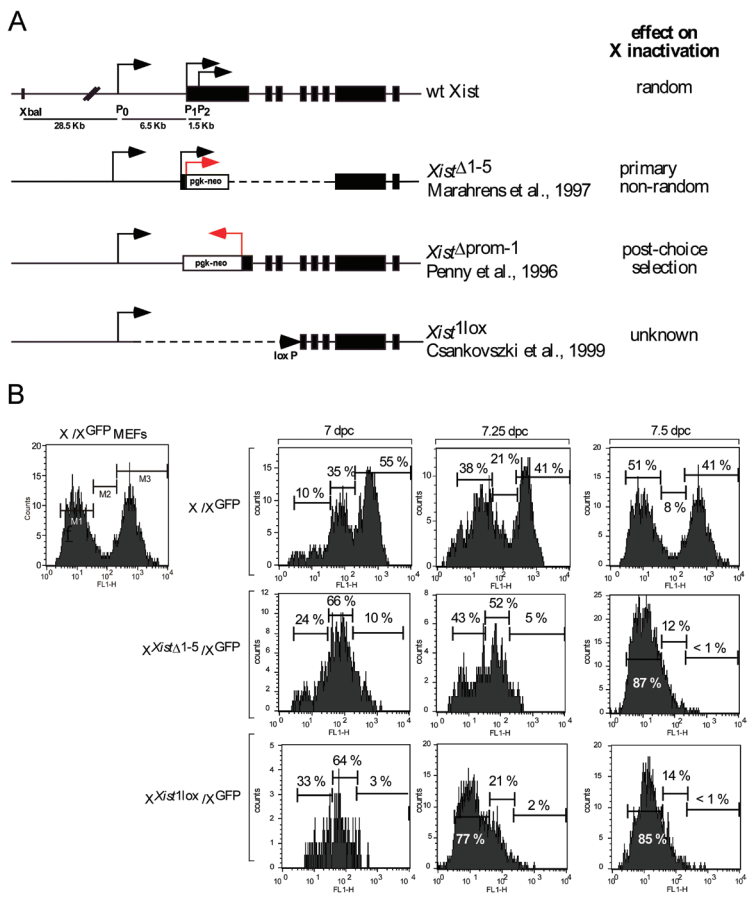


Figure 1. **Primary non-random X inactivation in the presence of different *Xist* deletions.** (A) Maps of the different *Xist* deletions, and the effect of the deletion on X inactivation choice. (B) The absence of a functional *Xist* allele leads to primary non-random X chromosome choice. Left panel: FACS analysis of control MEFs heterozygous for the X-linked GFP used to determine cut-offs for GFP-fluorescence (see text for details). Top row: FACS analysis of cells isolated from wild type embryos heterozygous for X-linked GFP at E7, E7.25 and E7.5. Middle row: FACS analysis of cells isolated from X$^{Xist\ 1\text{-}5}$/X^{GFP} embryos at E7, E7.25 and E7.5. Bottom row: FACS analysis with cells isolated from X^{Xist1lox}/X^{GFP} embryos at E7, E7.25 and E7.5.

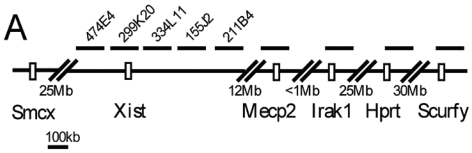
only slightly GFP positive, which represented cells that had turned off GFP expression but had not lost GFP-fluorescence completely due to the half life of the protein. All samples were compared with these exact cut-offs in order to evaluate relative fluorescence. In the embryo proper, random X inactivation begins at around E5.5 and is complete by E7.5. However, it has been shown that cells that are partially disomic for X-linked genes do not die rapidly, but that cell death continues over days until it is complete by E10 (Takagi and Abe, 1990). We isolated wild type and mutant embryos between E7.0 and E7.5, removed extraembryonic tissues for genotyping, and dissociated the embryo proper for analysis. In wild type embryos, starting at E7.0 we observed that about 41% of cells stayed GFP positive. The remaining cells gradually lost GFP fluorescence consistent with random X inactivation that was complete by E7.5 (Figure 1B, top panel). Embryos heterozygous for either *Xist* deletion invariably chose the wild type chromosome for inactivation, which resulted in the loss of GFP expression in all cells over time (Figure 1B, middle and bottom panels). Heterozygous $Xist^{\Delta 1\text{-}5}$ embryos have been shown previously to undergo primary non-random X inactivation (Marahrens et al., 1998). In agreement with this finding we observed that during X inactivation all cells synchronously became GFP negative, suggesting that in every cell the wild type chromosome was chosen to be inactivated (Figure 1B, middle panel). In $X^{Xist1lox}X^{GFP}$ embryos the dynamics of X inactivation was indistinguishable from the $Xist^{\Delta 1\text{-}5}$ deletion (Figure 1B, bottom panel). These results indicate that both *Xist* deletions cause primary non-random X inactivation.

Asynchronous replication of X-linked genes prior to X inactivation.

One marker associated with X inactivation is late replication of the Xi (Takagi, 1974). For many mono-allelically expressed loci, asynchronous replication timing is present prior to gene expression and may be involved in determining which locus will be expressed. Although the X chromosomes in female cells replicate asynchronously after X inactivation it is unknown whether the X chromosomes also replicate asynchronously prior to X inactivation. To determine whether X-linked gene loci replicate asynchronously before the onset of X inactivation, we tested replication timing of several loci along the X chromosome in undifferentiated female ES cells which have two transcriptionally active X chromosomes (Figure 2A). Undifferentiated ES cells were BrdU pulse labeled and methanol/acetic acid fixed. In contrast to formaldehyde fixation, which retains the nuclear architecture and only resolves cohesion differences, methanol/acetic acid fixation destroys the nuclear structure which allows replication timing analysis (Azuara et al., 2003). BrdU incorporation was detected in conjunction with DNA fluorescent in situ hybridization (FISH) using plasmid and BAC probes. In this analysis three different types of BrdU positive nuclei can readily be distinguished: 1) nuclei with two single signals (single-single, SS) which indicates that both loci have not replicated yet. 2) Nuclei with two double signals (double-double, DD) which indicates that both loci have replicated, and 3) nuclei with one single signal and one double signal (single-double, SD) which indicates that only one locus has been replicated and the other one not. For bi-allelically expressed gene loci that replicate synchronously the relative amount of the SD nuclei is low and varies between 10 and 20% depending on the cell type and target sequence. Asynchronously replicated gene loci display much higher relative numbers of SD nuclei in the BrdU positive population of cells that ranges from 25 to 50% (Chess et al., 1994; Kitsberg et al., 1993; Mostoslavsky et al., 2001). We used this method to analyze replication timing of the *Xic* locus including the *Xist* gene. We found that the whole *Xic* region replicated asynchronously prior to X inactivation. Analysis of replication timing of the *Xist* locus with a cDNA probe and probes covering either exon 1 or 7 gave similar results (data not

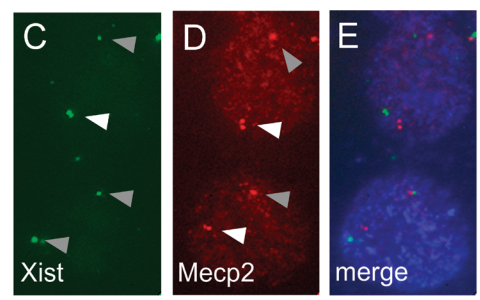
shown). The high proportion of DD nuclei (39%) relative to SS nuclei (22%) indicates that both *Xist* alleles replicate relatively early in S-phase. Interestingly, we found that different X-linked loci across the X chromosome, including *Mecp2, Irak1, Hprt* and *Scurfy* all exhibit asynchronous replication timing in undifferentiated ES cells (Figure 2B). In comparison, the bi-allelically expressed autosomal loci α-*globin* and *L23mrp* showed relatively low numbers of SD nuclei (13 -17 %), which indicates that these loci replicated synchronously in S-phase.

Besides asynchronous replication timing, promoter restricted high levels of H3 Lys 4 di-methylation has been found to be a specific mark for X-linked genes and imprinted genes (Rougeulle et al., 2003). Interestingly, *Smcx*, one of the very few genes that escape X inactivation in the mouse, does not show promoter restricted high levels of H3 Lys 4 di-methylation. To determine whether there is a correlation between H3 Lys 4 di-methylation levels and asynchronous replication timing we tested replication timing of the *Smcx* gene. We found that *Smcx* replicated asynchronously (Figure 2B), indicating that promoter restricted high levels of H3 Lys 4 di-methylation do not correlate with asynchronous replication timing. In mice, X inactivation is imprinted in extra embryonic tissues but random in the epiblast. To answer the question whether asynchronous replication timing in ES cells is due to imprinting we analyzed replication timing of *Xist* in parthenogenetic ES cells, which have two maternal chromosome sets. We found that replication timing in these cells was also asynchronous (41% SD nuclei compared to 39% in wild type ES cells) indicating that replication timing of *Xist* is not imprinted in undifferentiated ES cells (Figure 2B, *Xist* (parth.)).



B

	SS	SD	DD
Undifferentiated ES cells:			
Xist	22%	39%	39%
Xist (Parth.)	31%	41%	28%
BAC474E4	20%	43%	37%
BAC155J2	15%	38%	47%
BAC334L11	11%	41%	48%
BAC211B4	10%	43%	47%
Mecp2	22%	33%	44%
Irak	17%	41%	42%
Scurfy	38%	39%	23%
Smcx	17%	38%	45%
α globin	20%	17%	63%
L23mrp	37%	13%	50%



F

Undifferentiated ES cells:			
Xist-Irak	(n = 32)	77%	23%
Xist-Mecp2	(n = 36)	83%	17%
Xist-BAC474E4	(n = 36)	86%	14%
Xist-BAC211B4	(n = 28)	93%	7%

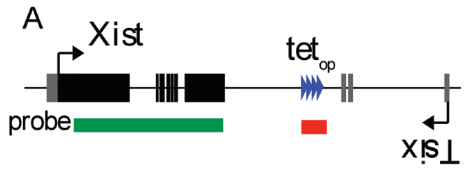
Figure 2. **X-linked genes replicate asynchronously before X inactivation.**
(A) Map indicating the location of the X chromosome specific BAC and plasmid probes used for the replication timing analysis. (B) Replication timing analysis of different X-linked loci in undifferentiated ES cells. (C, D and E) Double label DNA FISH detecting *Xist* and MeCP2. *Xist* is shown in green (C), MeCP2 in red (D) and BrdU in blue (E); white triangle = replicated, gray triangle = not replicated. (F) Fraction of nuclei with coordinated versus non-coordinated SD nuclei for different combinations of target sequences.

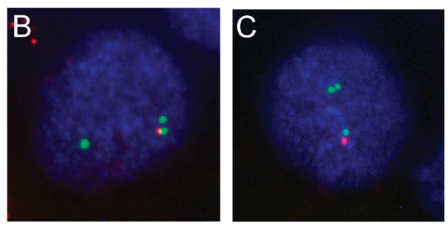
Asynchronous replication timing of non-imprinted autosomal mono-allelically expressed genes is coordinated along the chromosome (Singh et al., 2003). To test whether the same coordination is present on the X chromosome in undifferentiated ES cells, we performed double label FISH for two different loci in conjunction with BrdU staining and analyzed coordination of replication timing in nuclei with SD signals. We tested several probe combinations, including probes for *Xist* and *Mecp2*, which are separated by 12 Megabases, probes for *Xist* and *Irak1*, which are 13 Megabases apart, and probes for *Xist* and two different BACs covering the 5′ and 3′ end of the *Xic*. (Figure 2A). For all probe combinations we found that in 77 to 93% of cells the SD signals were detectable on the same allele which indicates that the replication timing along the chromosome is coordinated (Figure 2C-F). Analysis of probe combinations covering a greater distance was inconclusive because the signals were too far apart in the nucleus.

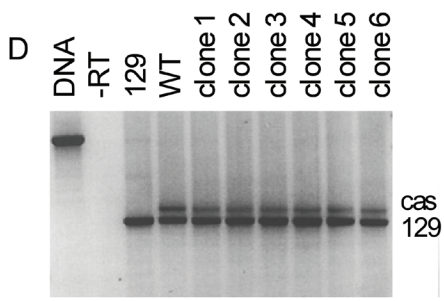
These results indicate that the X chromosomes in female cells replicate asynchronously prior to initiation of X inactivation and replication timing seems to be coordinated along the X chromosome over relatively large distances around the *Xist* locus.

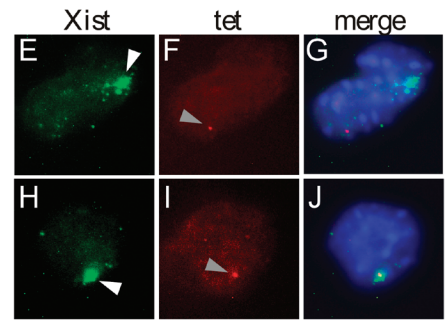
Replication timing and skewed X inactivation.

Random X inactivation is affected by the *Xce* locus and X inactivation is skewed in cells carrying *Xce* alleles of different strength. We assessed replication timing in F1 *Mus musculus* (129) / *Mus castaneus* (cas) ES cells, which preferentially inactivate the 129 X chromosome that carries the weaker Xce^{a} allele (relative to the Xce^{c} allele of the cas X). To be able to distinguish the two alleles by DNA FISH analysis we introduced 56 *tet* operator repeats downstream of the 129 *Xist* locus and removed the neomycin resistance cassette (Figure 3A - C). We picked six independent sub-clones and induced X inactivation by differentiating the ES cells with retinoic acid (RA). RT-PCR analysis with primers that detect a length polymorphism in the cas *Xist* gene showed the expected skewing towards inactivation of the 129 X chromosome after 5 days of differentiation (Figure 3D). Skewing of X inactivation in the subclones was comparable to skewing found in wild type control samples which suggests that the tet operator sequences did not affect X inactivation. In addition, we analyzed skewing of X inactivation in individual cells by RNA-DNA FISH in two independent sub-clones after 5 days of differentiation. First *Xist* RNA was detected with a probe that consisted of the complete cDNA sequence, followed by a brief fixation step and DNA FISH analysis with a probe specific for the tet operator DNA sequences (Figure 3E-K). We determined the relative number of differentiated ES cells that contained an *Xist* signal that colocalized with the tet operator signal relative to ES cells in which the *Xist* signal did not colocalize with the tet operator signal. This experiment confirmed the RT-PCR results and showed the inactivation of the 129 X chromosome in 74 - 78% of cells. We next analyzed replication timing of the cas and 129 *Xist* alleles before ES cell differentiation in the 6 ES cell sub-clones that were used for RT-PCR analysis. Using double label DNA FISH we found no preference for either the 129 or cas allele to be replicated first in S-phase (Figure 3L) which suggests that replication timing does not correlate with skewing of X inactivation. Because all subclones were derived from one founder clone, replication timing of the *Xist* locus appeared to be dynamic and switched between alleles in undifferentiated ES cells. In contrast, replication timing of X-linked genes is highly stable in cells that have undergone X inactivation (Hansen et al., 1996; Xiong et al., 1998).









K

	Xist cloud	+tet Xist cloud	N
5day differentiated ES cells:			
subcl. 1	78%	22%	45
subcl. 2	74%	26%	41

L

	129 early	Cas early	N
Undifferentiated *ES cells:*			
subcl. 1	55%	45%	20
subcl. 2	41%	59%	20
subcl. 3	50%	50%	20
subcl. 4	50%	50%	20
subcl. 5	60%	40%	20
subcl. 6	55%	45%	20

Figure 3. **Replication timing of the *Xist* locus in ES cells with different Xce alleles.**
(A) Schematic representation of the integration site of the *tet* operator repeat and the location of the DNA FISH probes used to distinguish the two *Xist* alleles. (B, C) Double label DNA FISH using an *Xist* (FITC) and tet probe (rhodamine red) shows that the tet signal co-localizes only with one of the two *Xist* signals. The 129 allele of a polymorphic ES line was targeted with the tetop repeat sequence. D) Individual subclones of the tetop targeted ES cell line were differentiated for 5 days. Cas and 129 *Xist* RNA levels were determined with RT-PCR detecting a length polymorphism in exon 7. (E-J) Combined RNA-DNA FISH with cells differentiated for 5 days with two individual clones detecting the *Xist* RNA (FITC, white triangle) and tet DNA (rhodamine red, gray triangle, DAPI in blue) showed cells with an inactivated *castaneus* X chromosome (E, F and G) or an inactivated 129 X chromosome (H, I and J). (K) Quantification of RNA-DNA FISH experiment with two different subclones. (L) Replication timing analysis of individual undifferentiated ES cell subclones.

Replication timing and complete primary non-random X inactivation.

We have shown that replication timing before X inactivation does not correlate with skewing of X inactivation. We next investigated whether replication is correlated with complete primary non-random X inactivation. We generated several *Xist*$^{1lox/+}$ heterozygous ES cell lines and analyzed replication timing of the wild type and mutant alleles in undifferentiated ES cells before the onset of X inactivation using two different probes. One probe covered exon 1 and therefore only detected the wild type *Xist* allele, and a second probe covered exon 7 and detected both the wild type and mutant alleles (Figure 4A). Double label DNA FISH in conjunction with BrdU staining showed no preference for either the wild type or mutant allele to be replicated first in S-phase which suggests that asynchronous replication timing has no causal relation to primary non-random X inactivation (Figure 4B- E).

Next, we tested whether the *Xist* gene is required for chromosome wide coordination of replication timing. We analyzed coordination of replication timing of *Xist* with *Irak1* and *Xist* with *Mecp2* in *Xist*$^{1lox/+}$ ES cell lines and detected coordinated SD nuclei in around 80% of the cells, similar to our findings with wild type ES cells (Figure 4F,G and H). These results indicate that the *Xist* gene is not required for the coordination of replication timing along the X chromosome.

DISCUSSION

In this study, we investigated several aspects of the X inactivation choice process. We analyzed the effect of an *Xist* deletion that spans 5 KB upstream of the *Xist* promoter to intron 3, on choice and found that this mutation results in primary non-random X inactivation. This result is consistent with previous reports that describe primary non-random X inactivation for a different deletion that encompasses part of exon 1 to exon 5. It therefore appears that an X chromosome can only be chosen for inactivation if *Xist* is intact. In addition, we studied the role of replication timing and found that asynchronous replication timing is present prior to X inactivation at all tested regions on the X chromosome. After completion of X inactivation, asynchronous replication timing of the two X chromosomes is stable and propagated through many cell divisions. In contrast, we found that early replication timing switches between alleles before the initiation of X inactivation. Analysis of asynchronous replication timing in ES cell lines with severe or complete skewing of X chromosome choice showed that replication timing does not correlate with choice, indicating that replication timing does not mediate skewing of the choice process.

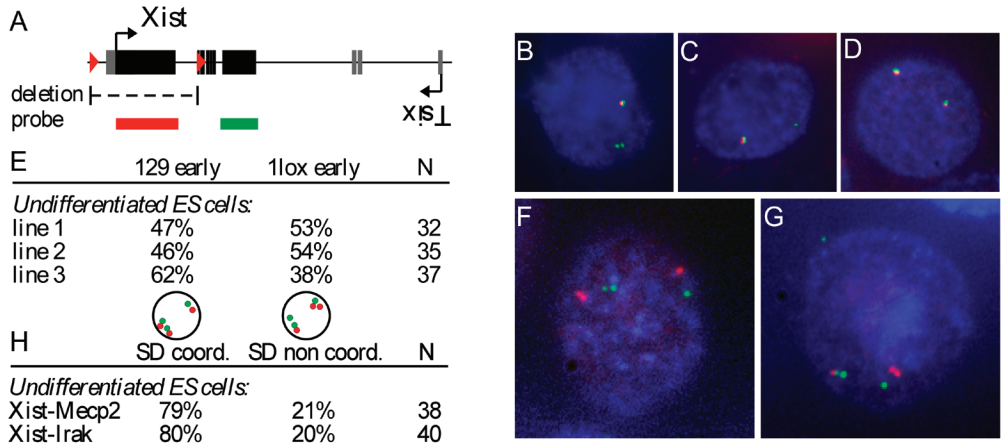


Figure 4. **Replication timing of the *Xist* locus in conditional *Xist* knockout ES cells.**
(A) Map of the *Xist* gene and location of the probes used for DNA FISH analysis. (B, C, D) BrdU detection in combination with double label DNA FISH using an exon1 probe (rhodamine red) and an exon7 probe (FITC) reveals nuclei with the mutant allele being replicated before (B) or after (C) the wild type allele. (D) Control cells show two co-localizing signals. (E) Replication timing analysis with three independent *Xist*1lox/+ undifferentiated ES cell lines. (F, G) BrdU detection and double label DNA FISH detecting the *Xist* (FITC) and Mecp2 (rhodamine red) loci, reveals cells that have coordinated replication timing (F) and non-coordinated replication timing (G). (H) Relative amount of nuclei with coordinated replication timing versus non-coordinated replication timing.

Primary non-random X inactivation or post-choice selection?

In mammalian ES cell and embryonic tissues, cells randomly designate one X as the Xa and inactivate any remaining Xs. The most parsimonious model proposes the presence of an autosomally encoded blocking factor that marks the future active X and is present in limited quantities such that only one X can remain active per diploid set of autosomes (Brockdorff, 1998). A number of *cis*-acting elements have been identified that lead to skewing of X chromosome choice, presumably by affecting the likelihood that the blocking factor binds. Evidence suggests that *Xist* and its negative regulator, the antisense RNA *Tsix*, play an important role in choice. In female cells heterozygous for and *Xist* deletion, only the wild type chromosome is inactivated (Csankovszki et al., 1999; Marahrens et al., 1998; Marahrens et al., 1997; Penny et al., 1996). While loss of a functional *Xist* can increase the chance of the mutant X to be chosen as the Xa (Marahrens et al., 1997), a chromosome with elevated sense transcription across *Xist* is more likely to be inactivated (Nesterova et al., 2003; Newall et al., 2001). Loss of *Tsix* transcription also leads to skewed choice with the mutated X being the inactive chromosome (Lee and Lu, 1999; Luikenhuis et al., 2001; Sado et al., 2001), and *Tsix* transcription negatively regulates *Xist* RNA steady-state levels *in cis* (Lee and Lu, 1999; Sado et al., 2001). Another genetic element implicated in choice is the X choosing element, *Xce*, which lies 3′ of *Xist* and beyond *Tsix* (Simmler et al., 1993). Crossing divergent mouse strains heterozygous for the *Xce* leads to skewing of X inactivation in the F1 female offspring such that the X chromosome bearing the stronger *Xce* allele is chosen more frequently as the Xa. Evidence suggests that the *Xce* might affect choice by modulating *Tsix* expression and in turn *Xist* RNA levels (Brockdorff et al., 1991). Recently, *Xite*, a *cis*-acting element that harbors intergenic transcription start sites and DNaseI hypersensitive sites, has been identified as a candidate locus for the *Xce* (Ogawa and Lee, 2003). These findings together suggest that choice is determined by a complex interplay of a variety of superimposed mechanisms.

Non-random X inactivation due to an *Xist* mutation can be achieved by either primary non-random X inactivation or random choice followed by post-choice selection. Primary non-random X inactivation which leads to the inactivation of the wild type X in a heterozygous embryo was observed in cells carrying a deletion extending from part of exon 1 through to exon 5, $Xist^{\Delta 1\text{-}5}$ (Marahrens et al., 1998; Marahrens et al., 1997) as well as in the longer deletion ($Xist^{1lox}$) (Csankovszki et al., 1999) described here. In contrast, Penny et al. (Penny et al., 1996) concluded that random X inactivation was followed by post-choice selection in a cell line carrying a deletion of the transcriptional start site of *Xist* and part of exon 1. This is similar to previous results in cells that inherit both products of the Searle's X-autosome translocation (T(X;16)16H) (McMahon and Monk, 1983). In these embryos, random choice results in either the inactivation of the wild type X chromosome, which results in balanced cells, or in the inactivation of the translocation product that bears the *Xic*, which results in unbalanced cells. The cells that inactivated the translocation product are then progressively lost from the embryo with about 75% being lost between E7 and E8 (McMahon and Monk, 1983). Embryos that carry the Searle's translocation are only partially disomic for the X chromosome. We cannot exclude that embryos that are disomic for the complete X show a more severe phenotype which may result in earlier lethality. In this case our analysis starting at E7 may have missed cells that carry two active X chromosomes. However, the Searle's translocation results in massive cell loss, and newborn pups are 20% smaller than their wild type littermates (Lyon et al., 1964). In contrast, pups heterozygous for the $Xist^{\Delta 1\text{-}5}$ deletion are indistinguishable from their wild type littermates (Marahrens et al., 1998). Because the two *Xist* mutations that do affect choice (Csankovszki et al., 1999; Marahrens et al., 1998; Marahrens et al., 1997) encompass all

sequences deleted in the Penny et al. mutation (Figure 1A), it is difficult to define genetic elements that regulate this process. We consider the following possibilities to reconcile these differences: (i) It is possible that incomplete differentiation of the ES cells or the stability of the gene products measured by Penny et al. affected the outcome of the experiment. (ii) It also cannot be excluded that the presence of the selectable marker in the antisense orientation and of exons 2 and 3 in the mutant allele somehow abrogated the role of *Xist* in choice.

Replication timing does not correlate with choice.

Most mono-allelically expressed genes display asynchronous replication timing, which, for all tested loci, is already present before expression. Interestingly, replication timing studies of the kappa light chain locus have indicated that replication timing may play a role in choosing which of the two kappa alleles will be recombined and subsequently expressed (Mostoslavsky et al., 2001). This prompted us to determine the role of replication timing in X inactivation choice. We have applied 2D DNA FISH to analyze replication timing of sequences covering the *Xic* in ES cells. Although we cannot fully exclude the possibility that this method detects asynchronous chromatid separation rather than asynchronous replication timing, we and others have previously shown that 2D DNA FISH analysis gives results similar to cell cycle fractionation analysis which does detect differences in replication timing (Simon et al., 1999; Gribnau et al., 2003; Singh et al., 2003; Azuara et al., 2003).We found that the *Xic* region replicated asynchronously prior to X inactivation, and that replication timing along the entire *Xic* is coordinated. Therefore, the possibility that replication timing of a small region within the *Xic* is not coordinated is unlikely. In addition, we found that all other X-linked loci tested replicated asynchronously, including the *Smcx* gene, which escapes X inactivation. In contrast, bi-allelically expressed autosomal genes that are scattered in between coordinated asynchronously replicating mono-allelically expressed genes replicate synchronously (Singh et al., 2003) which suggests that the mechanism that governs replication timing of the X may be different. Earlier studies have shown that imprinted genes and X-linked genes display promoter restricted high levels of di-methylation of H3 Lys 4. *Smcx* and *Xist* were the only X-linked genes without promoter restricted high levels of H3 Lys 4 di-methylation. Our studies suggest that there is no correlation between specific levels of H3 Lys di-methylation and asynchronous replication timing. The finding that the bi-allelically expressed *Smcx* gene replicated asynchronously could be explained by the fact that *Smcx* is inactivated on the Xi in the early stages of X inactivation and that bi-allelic expression is the consequence of relaxation of silencing (Lingenfelter et al., 1998). In addition, the region that escapes X inactivation in mouse is very small, roughly 30 kb. It encompasses only the *Smcx* gene and may lack an origin of replication (Tsuchiya 2004). In contrast to promoter restricted di-methylation of H3 Lys 4, hyperacetylation of core histones, hyper(di)methylation of H3 Lys 4, and hypo(di) methylation of Lys 9 have been found to be specific for all X linked genes in female ES cells prior to X inactivation, including *Smcx* and *Xist* (O'Neill et al., 2003). The function of these specific histone modifications remains unknown, however, they correlate well with the pattern of asynchronous replication timing we have determined for X linked genes.

Our results show that prior to X inactivation the proportion of asynchronously replicating (SD) X chromosomes is comparable to that of imprinted genes and non-imprinted mono-allelically expressed genes (Gribnau et al., 2003; Simon et al., 1999; Singh et al., 2003). Two characteristics distinguish asynchronous replication timing before and after X inactivation. Asynchronous replication timing prior to X inactivation switches between alleles and is not affected by the *Xce* allele carried on the respective X. In contrast, in somatic cells, asynchronous

replication timing of the Xa and Xi is clonal and stable through many cell divisions (Hansen et al., 1996; Xiong et al., 1998), and is influenced by the *Xce*. Another difference is the time window between the replication of the two alleles. Prior to X inactivation we found 40% of the nuclei with an SD signal by FISH. Earlier studies that use S-phase fractionation analysis, which measures the DNA content at different stages in S-phase, have shown that this proportion of SD nuclei reflects a difference in replication timing of 1.5 to 2 hours (Gribnau et al., 2003; Simon et al., 1999; Singh et al., 2003). In contrast, after X inactivation is complete, the time window is significantly larger and extends through almost the entire S-phase (Xiong et al., 1998). We conclude that replication timing prior to X inactivation does not correlate with skewing of the X chromosome choice. The difference between asynchronous replication timing prior and after X inactivation most likely reflects the distinct chromatin states of the two X chromosomes before and after X inactivation.

Asynchronous replication timing, what does it do?

The vast majority of mono-allelically expressed gene loci have been shown to replicate asynchronously in S-phase, which suggests a direct role for replication timing in the choice processes. In this study we found that asynchronous replication timing is present throughout the X inactivation process. However, replication timing does not correlate with skewing of X inactivation choice.

Asynchronous replication timing of X-linked genes and other non-imprinted mono-allelically expressed genes is random with respect to the parental origin (Mostoslavsky et al., 2001). In contrast, asynchronous replication timing of imprinted gene loci is parent specific (Simon et al., 1999). Interestingly, loss of imprinting caused by the erasure of methylation marks in the germline or after fertilization does not result in a loss of asynchronous replication timing of imprinted gene loci (Gribnau et al., 2003). In addition, at the imprinted *Igf2-H19* locus, a 3Mb inversion, which results in the establishment of a paternally imprinted *Igf2-H19* locus in the female germline, does not change the replication timing characteristics of this locus (Cerrato et al., 2003). Therefore, gene expression and asynchronous replication timing appear to be separable mechanisms, raising the question about the significance of asynchronous replication. We found that asynchronous replication timing prior to X inactivation is random, switches between alleles, and is independent of the *Xce* and *Xist* mutations. However, we cannot exclude a role for asynchronous replication upstream of choice. It is possible that asynchronous replication timing before X inactivation reflect epigenetic differences between the two X chromosomes such as transient blocking factor binding which may be involved in the counting process upstream of choice (Brockdorff, 1998).

Asynchronous replication timing could also be the remnant of an ancient imprinting or choice mechanism. Asynchronous replication timing could have played a role in setting up and maintaining imprints or determining choice processes for random mono-allelically expressed genes. Over time, different epigenetic mechanisms, like DNA methylation or chromatin modifications, may have taken over the regulation of replication timing.

MATERIALS AND METHODS

Analysis of X inactivation in embryos

The appropriate genotypes were obtained by crossing *Xist*$^{1lox/+,}$ *Xist*$^{\Delta 1\text{-}5/+}$ or wild type females to males homozygous for the X-linked GFP (Hadjantonakis et al., 1998). Pregnant females were sacrificed at the appropriate times and embryos collected. The embryo was separated into embryonic and extraembryonic tissues. Extraembryonic tissues were used for PCR genotyping by incubating them in 20µl 1xPCR buffer (GIBCO) supplemented with 2 mM $MgCl_2$ and 1mg/

ml proteinase K for 1 h at 50˚C, followed by 10 min at 95˚C. Standard PCR was carried out by using 9 µl of the above lysate in a 20 µl reaction (30 cycles, with an annealing temperature of 52˚C). For the *Xist*^{1lox} allele the primers Xint 3R (5'-CAC TGG CAA GGT GAA TAG CA-3'), XpromL (5'-TTT CTG GTC TTT GAG GGC AC-3') and 5' Lox R (5-ACC CTT GCC TTT TCC ATT TT–3') were used which gave a 427 bp band for the wild type allele a 513 bp band for the 1lox allele. For the Xist^{KO} allele we used the primers *Xist* KO F (5'-AAC TGA GTG GGT GTT CAG GG-3'), *Xist* KO R (5'-ACC ACA AAT CAA GGC GAA TC-3') and PGK-Pr1 (5'-GGG AAC TTC CTG ACT AGG GG-3'), which gave a 200 bp band for the wild type allele and a 260 bp band for the KO allele.

Embryonic tissues were washed twice in HEPES followed by trypsinization for 5 min and dissociation by pipetting. The trypsinization reaction was stopped by the addition of a small volume of DME/10 % FCS. Cells were then pelleted and resuspended in PBS/2 % FBS supplemented with a final concentration of 1 mg/ml propidium iodine and analyzed by FACS.

Cell culture

Polymorphic *Mus musculus* / *Mus castaneus* F1-2-1 ES cells were grown on mouse embryonic fibroblasts (MEFs) in DME (GIBCO), 15 % fetal calf serum (FCS, Hyclone), and 1000 U LIF/ml (Marahrens et al., 1997). ES cells were differentiated for 5 days in ES media without LIF and MEFs in the presence of 100 nM all-trans-retinoic acid on gelatinized coverslips and the medium was changed every day.

DNA fluorescence in situ hybridization

DNA FISH was performed as in Selig et al. (Selig et al., 1992) with minor modifications. Briefly medium of exponentially growing cells was supplemented with 10 µM BrdU and incubated for 45 minutes. Cells were trypsinized, washed with Hepes and resuspended in 0.75 M KCl. After trypsinization ES cells were incubated for 10 min on ice, all other cell types for 10 min at 37˚C. Cells were fixed for 10 min in ice cold methanol:acetic acid solution (3:1 ratio), washed three times with methanol:acetic acid and stored at 4˚C or spotted onto polylysine coated slides.

Slides were treated with RNase (100 µg/ml, 2 x SSC) for 30 min at 37˚C and washed 3 x 5 min in 2 x SSC and dehydrated in 70 %, 90 % and 100 % ethanol. Target sequences were denatured by applying 100 µl of 70 % formamide, 10 mM phosphate buffer in 2 x SSC under a coverslip and incubated for 3 min on a hotplate (75˚C). After removal of the coverslip, slides were washed in 2 x SSC (5 min; 4˚C), in 70 % ethanol (5 min; -20˚C), and through 90 % and 100 % ethanol for 3 min. Meanwhile nick translated BAC and cosmid probe sequences were dissolved in a hybridization mixture containing 50 % formamide, 2 x SSC, 50 mM phosphate buffer pH 7.0, 10 mg/ml salmon sperm DNA, 10 % dextrane sulfate and 100 ng/µl mouse Cot DNA to a final concentration of 2 ng/µl. The probe mix was denatured for 5 min, prehybridized for a minimum of 45 min, and then applied onto the slide. Slides were incubated overnight in a humidified chamber at 37˚C.

BAC probes covering the *Xic* have been sequenced and described before (Chureau et al., 2002). The BAC probes for *Scurfy* and *Irak1* have been described and were BAC 196K10 (Brunkow et al., 2001) and BAC 228O4 (Reichwald et al., 2000) respectively. The BAC probe for *Hprt* was RPCI23 173F3 (Gen Bank acc. Nr BX649621). All BACs were acquired from Research Genetics Inc. *Alpha globin* and *L23mrp* cosmid probes have been described before (Gribnau et al., 2003). The *Mecp2* probe was a 11 KB *Kpn*I fragment covering part of the *Mecp*2 gene. The *Smcx* probe was a 15 KB *Xho*I fragment subcloned from BAC 330G24 (Tsuchiya 2004). All probes were digoxygenin labeled by nick translation (Roche) or biotin labeled with a random prime labeling kit (Invitrogen), purified over G50 columns, precipitated and resuspended in hybridization mix.

After hybridization slides were washed in 2 x SSC (5 min; 37˚C), in 50 % formamide, 2 x SSC (3 x 10 min; 37˚C) and in 0.1 M Tris, 0.15 M NaCl, 0.05 % Tween 20 (2 x 5 min; RT), then incubated in 2 mg/ml BSA in 0.1 M Tris, 0.15 M NaCl in a humidified chamber (30 min; RT). Detection was with subsequent incubation steps with anti-digoxygenin (Boehringer), anti-sheep (FITC, Jackson Labs, only when necessary), anti-BrdU (DAKO), anti-mouse (Rhodamine Red, Jackson Labs), antibodies in 0.1 M Tris, 0.15 M NaCl (30 min; RT). For double label DNA FISH, detection of biotin was with

anti-biotin (Roche), and anti mouse (Rhodamine Red, Jackson Labs) and detection of BrdU was with anti-BrdU (Abcam) and anti-rat (AMCA, Jackson Labs). Slides were washed twice in between each detection step with 0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20 and mounted with Vectashield (Vector Labs) and stored at 4°C. Fluorescence was detected by epifluorescence/CCD. For replication timing coordination studies more than 20 BrdU positive cells with two SD signals were counted per cell line. Images were acquired using a Nikon E800 microscope equipped with a 100x DIC H oil immersion lens with 1.4 n/a. The camera was a Princeton Instruments, inc., model RTE/CCD 1317=k/2 with a Kodak KAF-1400 1317 x 1035 chip. As acquisition software we used Openlab2.2 (Improvision).

RNA-DNA FISH analysis

RNA FISH analysis was performed as described (Panning and Jaenisch, 1996). Differentiated ES cells were grown on coverslips, extracted with cytoskeletal buffer and fixed in 4 % paraformaldehyde in PBS. The *Xist* probe was a cDNA sequence (Wutz and Jaenisch, 2000), which was digoxygenin labeled by nick translation (Roche). After overnight hybridization slides were washed in 2 x SSC (5 min; 37˚C), in 50 % formamide, 2 x SSC (3 x 10min; 37˚C) and fixed for 15 min in 4 % paraformaldehyde/PBS at RT. Slides were washed twice with PBS, dehydrated and target sequences were denatured. DNA FISH was as described in the previous paragraph. The tet operator probe was a 500 bp fragment containing 7 direct tet repeats which was biotin labeled with a random prime labeling kit (Invitrogen). Image acquisition was performed as described in the previous paragraph.

RT-PCR analysis

RNA was isolated with Trizol (Invitrogen), and 5 µg RNA was DNase treated and reverse transcribed with Superscript II (Invitrogen). *Xist* RNA was amplified with primer pair, *Xist*-forward 5'-TTCCCATGTTTCTCCTGCAT-3', *Xist*-reverse 5'-GGAGACATGCAAAGGAAGGA-3'. These primers amplify a length polymorphism in exon 7 of *Xist* and amplification results in a 790 bp *Mus musculus* specific product and a 820 bp *Mus castaneus* specific product, which were resolved on a 1.5 % agarose gel.

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REFERENCES

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

Azuara, V., Brown, K.E., Williams, R.R., Webb, N., Dillon, N., Festenstein, R., Buckle, V., Merkenschlager, M., and Fisher, A.G. (2003). Heritable gene silencing in lymphocytes delays chromatid resolution without affecting the timing of DNA replication. Nat Cell Biol *5*, 668-674.

Brockdorff, N. (1998). The role of *Xist* in X-inactivation. Curr Opin Genet Dev *8*, 328-333.

Brockdorff, N., Ashworth, A., Kay, G.F., Cooper, P., Smith, S., McCabe, V.M., Norris, D.P., Penny, G.D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. Nature *351*, 329-331.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafreniere, R.G., Grompe, M., Tonlorenzi, R., and Willard, H.F. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature *349,* 38-44.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet *27,* 68-73.

Cerrato, F., Dean, W., Davies, K., Kagotani, K., Mitsuya, K., Okumura, K., Riccio, A., and Reik, W. (2003). Paternal imprints can be established on the maternal Igf2-H19 locus without altering replication timing of DNA. Hum Mol Genet *12*, 3123-3132.

Chess, A., Simon, I., Cedar, H., and Axel, R. (1994). Allelic inactivation regulates olfactory receptor gene expression. Cell *78*, 823-834.

Chureau, C., Prissette, M., Bourdet, A., Barbe, V., Cattolico, L., Jones, L., Eggen, A., Avner, P., and Duret, L. (2002). Comparative sequence analysis of the X-inactivation center region in mouse, human, and bovine. Genome Res *12*, 894-908.

Cooper, D.W., VandeBerg, J.L., Sharman, G.B., and Poole, W.E. (1971). Phosphoglycerate kinase polymorphism in kangaroos provides further evidence for paternal X inactivation. Nat New Biol *230*, 155-157.

Csankovszki, G., Panning, B., Bates, B., Pehrson, J.R., and Jaenisch, R. (1999). Conditional deletion of *Xist* disrupts histone macroH2A localization but not maintenance of X inactivation. Nat Genet *22*, 323-324.

Gribnau, J., Hochedlinger, K., Hata, K., Li, E., and Jaenisch, R. (2003). Asynchronous replication timing of imprinted loci is independent of DNA methylation, but consistent with differential subnuclear localization. Genes Dev *17*, 759-773.

Hadjantonakis, A.K., Gertsenstein, M., Ikawa, M., Okabe, M., and Nagy, A. (1998). Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech Dev *76*, 79-90.

Hansen, R.S., Canfield, T.K., Fjeld, A.D., and Gartler, S.M. (1996). Role of late replication timing in the silencing of X-linked genes. Hum Mol Genet *5*, 1345-1353.

Kitsberg, D., Selig, S., Brandeis, M., Simon, I., Keshet, I., Driscoll, D.J., Nicholls, R.D., and Cedar, H. (1993). Allele-specific replication timing of imprinted gene regions. Nature *364*, 459-463.

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

Lingenfelter, P.A., Adler, D.A., Poslinski, D., Thomas, S., Elliott, R.W., Chapman, V.M., and Disteche, C.M. (1998). Escape from X inactivation of Smcx is preceded by silencing during mouse development. Nat Genet *18*, 212-213.

Luikenhuis, S., Wutz, A., and Jaenisch, R. (2001). Antisense transcription through the *Xist* locus mediates *Tsix* function in embryonic stem cells. Mol Cell Biol *21*, 8512-8520.

Lyon, M.F. (1996). X chromosome inactivation. Pinpointing the centre. Nature *379*, 116-117.

Lyon, M.F., Searle, A.G., Ford, C.E., and Ohno, S. (1964). A Mouse Translocation Suppressing Sex-Linked Variegation. Cytogenetics *15*, 306-323.

Marahrens, Y., Loring, J., and Jaenisch, R. (1998). Role of the *Xist* gene in X chromosome choosing. Cell *92*, 657-664.

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

McMahon, A., and Monk, M. (1983). X chromosome activity in female mouse embryos heterozygous for Pgk-1 and Searle's translocation, T(X; 16) 16H. Genet Res *41*, 69-83.

Mostoslavsky, R., Singh, N., Tenzen, T., Goldmit, M., Gabay, C., Elizur, S., Qi, P., Reubinoff, B.E., Chess, A., Cedar, H., *et al.* (2001). Asynchronous replication and allelic exclusion in the immune system. Nature *414*, 221-225.

Nesterova, T.B., Johnston, C.M., Appanah, R., Newall, A.E., Godwin, J., Alexiou, M., and Brockdorff, N. (2003). Skewing X chromosome choice by modulating sense transcription across the *Xist* locus. Genes Dev *17*, 2177-2190.

Newall, A.E., Duthie, S., Formstone, E., Nesterova, T., Alexiou, M., Johnston, C., Caparros, M.L., and Brockdorff, N. (2001). Primary non-random X inactivation associated with disruption of *Xist* promoter regulation. Hum Mol Genet *10*, 581-589.

O'Neill, L.P., Randall, T.E., Lavender, J., Spotswood, H.T., Lee, J.T., and Turner, B.M. (2003). X-linked genes in female embryonic stem cells carry an epigenetic mark prior to the onset of X inactivation. Hum Mol Genet *12*, 1783-1790.

Ogawa, Y., and Lee, J.T. (2003). *Xite*, X-inactivation intergenic transcription elements that regulate the probability of choice. Mol Cell *11*, 731-743.

Panning, B., and Jaenisch, R. (1996). DNA hypomethylation can activate *Xist* expression and silence X-linked genes. Genes Dev *10*, 1991-2002.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. Nature *379*, 131-137.

Rastan, S. (1983). Non-random X chromosome inactivation in mouse X-autosome translocation embryos-location of the inactivation centre. J Embryol Exp Morphol *78*, 1-22.

Rastan, S., and Robertson, E.J. (1985). X chromosome deletions in embryo-derived (EK) cell lines associated with lack of X chromosome inactivation. J Embryol Exp Morphol *90*, 379-388.

Reichwald, K., Thiesen, J., Wiehe, T., Weitzel, J., Poustka, W.A., Rosenthal, A., Platzer, M., Stratling, W.H., and Kioschis, P. (2000). Comparative sequence analysis of the MECP2-locus in human and mouse reveals new transcribed regions. Mamm Genome *11*, 182-190.

Rougeulle, C., Navarro, P., and Avner, P. (2003). Promoter-restricted H3 Lys 4 di-methylation is an epigenetic mark for monoallelic expression. Hum Mol Genet *12*, 3343-3348.

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

Selig, S., Okumura, K., Ward, D.C., and Cedar, H. (1992). Delineation of DNA replication time zones by fluorescence in situ hybridization. Embo J *11*, 1217-1225.

Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H., Jenuwein, T., Otte, A.P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev Cell *4*, 481-495.

Simmler, M.C., Cattanach, B.M., Rasberry, C., Rougeulle, C., and Avner, P. (1993). Mapping the murine Xce locus with (CA)n repeats. Mamm Genome *4*, 523-530.

Simon, I., Tenzen, T., Reubinoff, B.E., Hillman, D., McCarrey, J.R., and Cedar, H. (1999). Asynchronous replication of imprinted genes is established in the gametes and maintained during development. Nature *401*, 929-932.

Singh, N., Ebrahimi, F.A., Gimelbrant, A.A., Ensminger, A.W., Tackett, M.R., Qi, P., Gribnau, J., and Chess, A. (2003). Coordination of the random asynchronous replication of autosomal loci. Nat Genet *33*, 339-341.

Takagi, N. (1974). Differentiation of X chromosomes in early female mouse embryos. Exp Cell Res *86*, 127-135.

Takagi, N., and Abe, K. (1990). Detrimental effects of two active X chromosomes on early mouse development. Development *109*, 189-201.

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

Wutz, A., and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol Cell *5*, 695-705.

Xiong, Z., Tsark, W., Singer-Sam, J., and Riggs, A.D. (1998). Differential replication timing of X-linked genes measured by a novel method using single-nucleotide primer extension. Nucleic Acids Res *26*, 684-686.

Chapter 3

X Inactivation Counting and Choice is a Stochastic Process: Evidence for Involvement of an X-Linked Activator

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Chapter 3

X Inactivation Counting and Choice is a Stochastic Process: Evidence for Involvement of an X-Linked Activator

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Female mammalian cells achieve dosage compensation of X-encoded genes by X-chromosome inactivation (XCI). This process is thought to involve X chromosome counting and choice. To explore how this process is initiated we analyzed XCI in tetraploid XXXX, XXXY and XXYY embryonic stem cells and find that every X chromosome within a single nucleus has an independent probability to initiate XCI. These findings suggest a stochastic mechanism directing XCI counting and choice. The probability is directly proportional to the X chromosome:ploïdy ratio, indicating the presence of an X-encoded activator of XCI, that itself is inactivated by the XCI process. Deletion of a region including *Xist, Tsix* and *Xite* still results in XCI on the remaining wild type X chromosome in female cells. This result supports a stochastic model in which each X chromosome in a nucleus initiates XCI independently, and positions a X-encoded trans-acting XCI-activator outside the deleted region.

Introduction

In placental mammals, gene dosage of X chromosomal genes is equalized between sexes by inactivation of one of the two X chromosomes in female cells (Lyon, 1961). In mouse and human embryos, XCI is initiated early in development and is random with respect to the parental origin of the X chromosome. Three X-linked non-coding genes, *Xist*, *Tsix*, and *Xite*, located within the X inactivation center (Xic), play a crucial role in the XCI process (Lee and Lu, 1999; Marahrens et al., 1997; Ogawa and Lee, 2003; Penny et al., 1996). At the start of XCI, the cell determines the number of X chromosomes and elects the future inactive and active X chromosomes. Next, *Xist* RNA accumulates in cis on the future inactive X chromosome (Xi), followed by several epigenetic changes that 'lock in' and maintain the inactive state through many cell divisions (Brockdorff et al., 1992; Brown et al., 1992). *Tsix* and *Xite* play an essential role in down-regulation of *Xist* RNA before and during the XCI process

Despite the progress made over the last decades in understanding XCI, the mechanism underlying the counting and choice process remains unclear. X:autosome translocations suggested the presence of a blocking factor that protects one X chromosome from inactivation per diploid nucleus (Rastan, 1983). Different deletions 3' of the *Xist* gene resulted in XCI in male cells thus suggesting the location of the blocking factor binding site (Clerc and Avner, 1998; Morey et al., 2004). Other elements are also involved in the counting and choice process. For example, in female cells with a heterozygous *Xist* mutation (that abolishes *Xist* function), the mutant allele is never chosen to be inactivated (Gribnau et al., 2005; Marahrens et al., 1997). In agreement with this, heterozygous mutations that abolish *Tsix* transcription result in preferential inactivation of

the mutant X chromosome (Clerc and Avner, 1998; Lee and Lu, 1999; Luikenhuis et al., 2001). To explain the finding that initiation of XCI is absent in a *Tsix* mutant male cells, an X-linked competence factor was introduced (two copies are required for initiation of XCI) (Lee and Lu, 1999). Interestingly, a homozygous mutation of the *Tsix* promoter revealed a 'chaotic' choice mechanism, in which zero, one or two X chromosomes were chosen for inactivation (Lee, 2005). However, only cells with a single Xi were capable of contributing to a developing embryo (Lee, 2002).

Besides a blocking factor model, other models have been put forward to explain XCI counting and choice. One model predicts that the fate of an X chromosome is determined prior to the start of the XCI process and is based on differences in sister chromatid cohesion in female ES cells (Mlynarczyk-Evans et al., 2006). A different model explains counting and choice through transient cross communication between X chromosomes (Marahrens, 1999), This is supported by two studies showing that the X chromosomes in female cells transiently move closer during the initiation phase of XCI in a subset of cells. However, it is currently unclear how this model can explain observations made in diploid XXXX and tetraploid XXXX cells, that inactivate three and two X chromosomes respectively, and the XX 65kb deletion line that does not show a counting defect despite the absence of transvection (Bacher et al., 2006; Xu et al., 2006) (Brown et al., 1992; Webb et al., 1992).

Stochastic model systems have been postulated to explain a variety of cellular choice processes, including lineage specification in the haematopoietic system (Till et al., 1964), VDJ recombination (Cohn and Langman, 1990), olfactory receptor choice (Shykind, 2005), and the retinal mosaic for color vision (Wernet et al., 2006). Here we propose a stochastic model for XCI, in which each X chromosome has a probability to be inactivated within a certain time span. To validate such a model, we have analyzed XCI in differentiating diploid and tetraploid ES cells, the latter providing a much wider spectrum of possible outcomes of the XCI process. In addition, we have analyzed XCI in female ES cells with a deletion encompassing all elements that have previously been shown to be involved in XCI counting, including *Xist*, *Tsix* and *Xite*. Our results support a stochastic model for XCI counting and choice, and indicate the presence of a novel X-encoded factor, XCI activator, involved in initiation of XCI.

RESULTS

Diploid female ES cells with two *Xist* clouds

In differentiating diploid female ES cell cultures we observed a reproducible proportion of cells with two *Xist* RNA FISH clouds in one nucleus (Figure 1A). In ICM-derived cells that were allowed to differentiate in vitro (Figure 1B) we also noticed female cells with two *Xist* clouds. These observations suggest that some of the female cells attempt to inactivate both X chromosomes. Three independent EB differentiation experiments with female ES cells showed that the relative number of cells with two *Xist* clouds, as determined by RNA FISH, is low but consistent throughout differentiation. The proportion of these double cloud cells seemed to increase up to day 5 of differentiation and then decreased subsequently (Figure 1C). The presence of cells with two *Xist* clouds could be attributed to leakiness of the XCI mechanism, resulting in inactivation of the future Xa, and would predict the presence of a comparable percentage of *Xist* clouds in male cells during ES cell differentiation. Analysis of three different male cell lines showed almost no cells initiating XCI on their single X chromosome, indicating that the presence of the female cells with two Xi's cannot be explained by sporadic leakiness of the XCI mechanism (Figure 1D). To exclude the possibility that cells with two *Xist* clouds were aneuploid, we performed RNA/DNA FISH on day 3, 5 and 7 EB differentiated cells, and in vitro

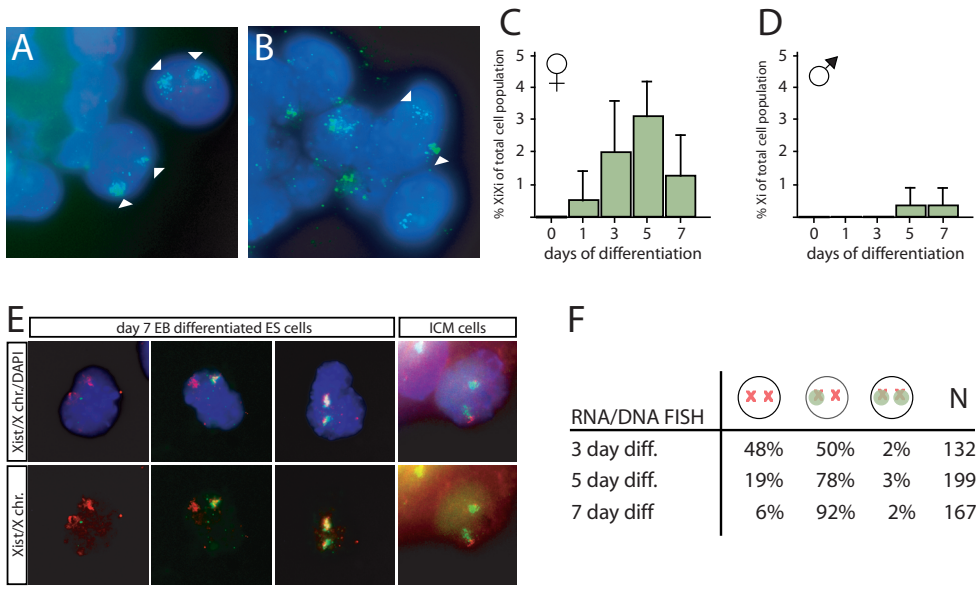

Figure 1. **Double *Xist* clouds in diploid female cells**
(A,B) *Xist* RNA FISH (FITC) on 3 day differentiated F1 2-1 female diploid ES cells (A) and differentiating ICM cells (B) revealed cells with two *Xist* clouds (indicated with triangles). (C,D) Three independent experiments with standard deviation of the relative number of double clouds in F1 2-1 (C) and single clouds in male (J1, V6.5 and E1) (D) diploid ES cells at different time points after differentiation, determined by RNA FISH. (E) RNA/DNA FISH with an *Xist* probe (FITC) and an X chromosome paint probe (Cy3, DNA is DAPI stained, blue), on day 7 differentiated ES cells. Left panels: cells with no and one *Xist* cloud, right panels: ES and ICM cells with two *Xist* clouds. (F) Quantification of *Xist* clouds in day 3, 5 and 7 differentiated ES cells by RNA/DNA FISH.

differentiated ICM cells. The ploidy status of cells with two *Xist* clouds was confirmed to be diploid (Figure 1E and F). Thus, cells with two *Xist* RNA clouds are present in early differentiating ES and ICM cell populations.

Generation of tetraploid ES cell lines

To investigate this observation in more detail, we generated tetraploid ES cell lines. Different combinations of neomycin and puromycin resistant female and male diploid F1 hybrid ES cell lines were fused to generate tetraploid XXXX, XXXY and XXYY cell lines (Figure 2A). Clones were grown under continuous double selection. Withdrawal of double selection or the use of inbred ES cells resulted in accelerated chromosome loss. FACS analysis of different tetraploid ES cell lines showed a doubling in DNA content compared to diploid XX ES cells and male mouse embryonic fibroblasts (MEFs, Figure 2B). The 2n peak in our tetraploid samples decreased after one hour of pre-plating on non-gelatinized dishes, indicating that this peak represents male MEFs used for culturing our ES cells. DNA FISH analysis using different autosomal and sex-chromosomal probes confirmed tetraploidy of the cell lines (Figure 2C and D). In addition, extensive karyotyping of two XXXX (8 and 10) lines and two XXXY (5 and 11) lines showed that the chromosome number in all the lines was around 80 chromosomes, with the majority of cells retaining 80 chromosomes (Figure 2E, F and G). With DNA FISH analysis using an X paint probe, we found that around 94% of the XXXX cells retain four X chromosomes, and more than 94% of the XXXY cells retain three X chromosomes (Figure

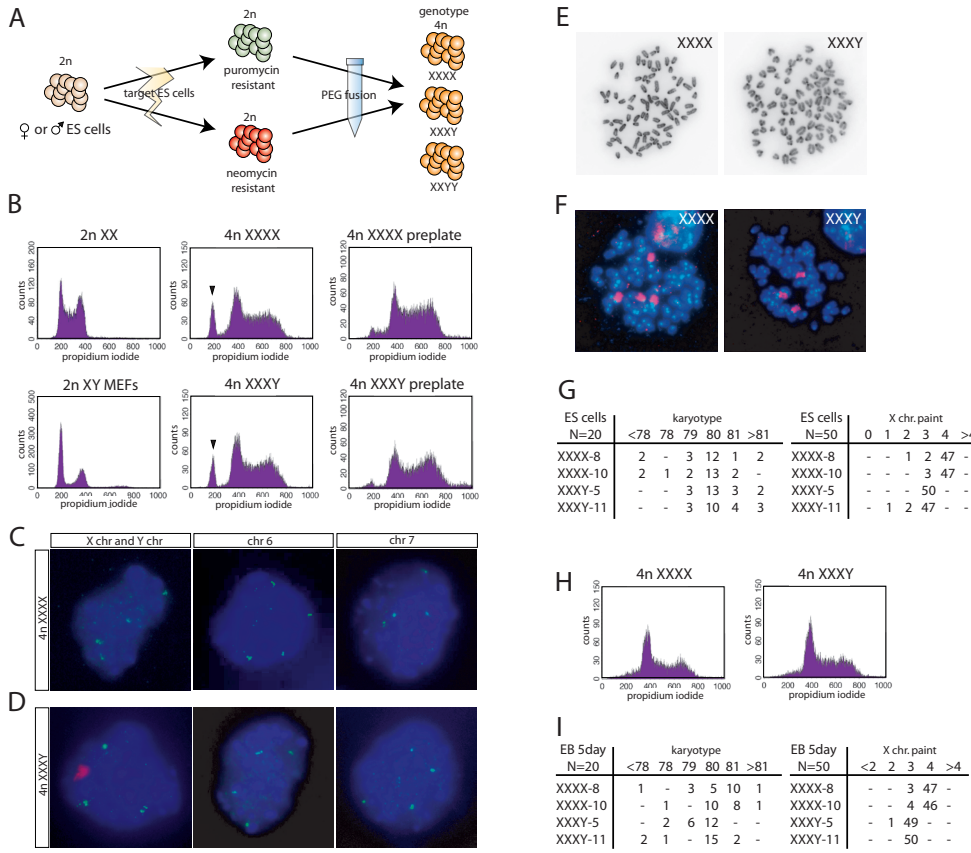

Figure 2. **Generation of tetraploid ES cell lines**
(A) Neomycin or puromycin resistant male and female diploid ES cells were targeted and fused in different combinations to generate tetraploid ES cells. (B) FACS analysis of tetraploid XXXX-8 and XXXY-5 lines shows doubling of DNA content compared to diploid XX ES lines and XY MEFs. Note the decrease diploid MEFs after 1 hour preplating (arrowheads). (C and D) DNA FISH: X chr., chr. 6 and 7 in FITC (green), Y chr. in Rhodamine (red), DNA is DAPI stained, blue, performed on XXXX-1 (C) and XXXY-1 (D) ES cells.(E) Metaphase spreads of XXXX-8 and XXXY-5 cells (inverted Dapi image).(F) X paint analysis of XXXX-8 and XXXY-5 cells (X paint in Cy3, DNA is DAPI stained).(G) Left panel, shows the number of chromosomes determined in 20 metaphase spreads of undifferentiated tetraploid cell lines. Right panel, shows the number of X chromosomes of 50 metaphase spreads using X chromosome paint. (H) FACS analysis of XXXX-8 and XXXY-5 tetraploid ES lines after 5 days of differentiation.(I) Same as G only in day 5 differentiated cells

2E, F and G). We did not find XXXX cells with more than four X chromosomes, or XXXY cells with more than three X chromosomes.

To test whether tetraploid ES cells have a stable karyotype throughout embryoid body (EB) differentiation, we repeated the FACS analysis and karyotyping with day 5 EB differentiated cells from two different XXXX and XXXY ES cell lines. We found that our ES cells maintain a stable karyotype throughout the differentiation process (Figure 2H and I). More importantly, X-paint DNA FISH analysis indicated that a small gain in chromosome number could not be attributed to an increase in the number of X chromosomes. Taken together, these results show that the generated tetraploid ES cell lines have a stable karyotype throughout EB differentiation.

Analysis of X chromosome inactivation in tetraploid cells

To study the XCI process in different XXXX, XXXY and XXYY tetraploid ES cell lines, we differentiated and fixed the cells at day 3, 5, 7 and 10 of differentiation. Interestingly, RNA FISH analysis with an *Xist* probe on XXXX ES cells indicated the presence of cells with zero to four Xi's (Figure 3A). The populations of cells with different numbers of Xi's during EB differentiation changed over time (Figure 3B). At day 3 of differentiation most of the cells had no *Xist* cloud while fewer cells have one, two and three clouds. Later in differentiation (day 5, 7 and 10) cells with two clouds represented the largest population, and this increased over time. Other populations with no, one, or three clouds, declined over time.

In addition to the karyotyping (Figure 2I), we performed RNA/DNA FISH on XXXX line 8 at different time points during differentiation to further exclude the possibility that the analyzed cell lines were aneuploid. We first selected cells with 4 X chromosomes as judged by X chromosome paint signal (day 7 and day 10 samples) or an X chromosome specific BAC signal (day 3 and day 5 samples) and subsequently counted the number of *Xist* RNA clouds in the nucleus (Figure 3C). We compared these results to the RNA FISH data obtained at different time points and confirmed the presence of cells with 3 and 4 *Xist* clouds (Figure 3D). We also found a relative increase in the percentage of cells with two clouds and a relative decrease in the percentage of cells with one cloud in day 5, 7 and 10 differentiated ES cells. These differences can be explained by the fact that RNA/DNA FISH

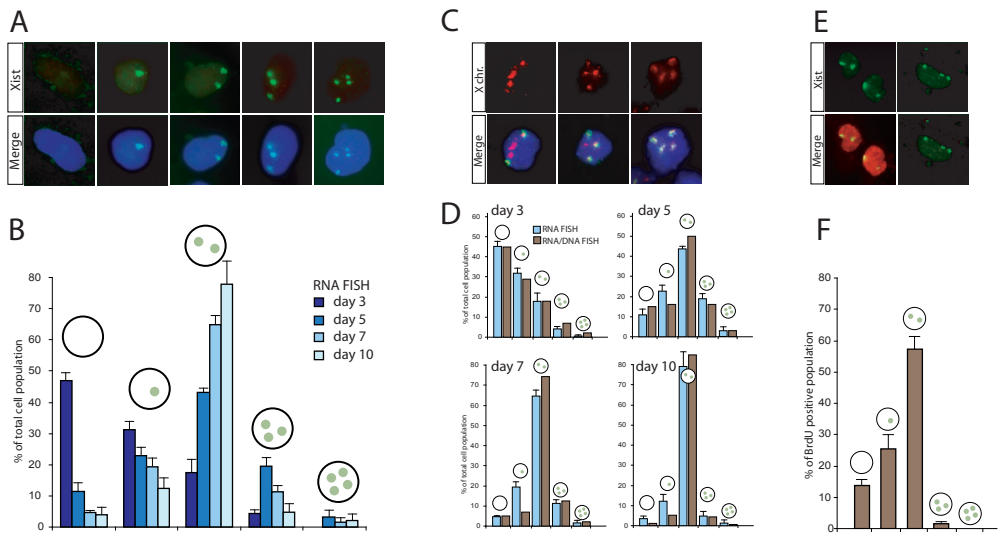

Figure 3. **XCI in tetraploid XXXX ES cells**
(A) *Xist* RNA FISH (top panels *Xist* in FITC, bottom panel *Xist* in FITC and DNA stained with DAPI) for day 5 differentiated XXXX ES cells. (B) Average distribution and standard deviation of sub-populations of cells with different numbers of Xi's at different time points after start of differentiation for four different XXXX (1, 2, 8 and 10) ES cell lines, determined by RNA FISH. (C) RNA/DNA FISH with an *Xist* probe (FITC) and X chromosome paint (Cy3, DNA is DAPI stained, blue) on 7 day differentiated cells. From left two right panels show cells with two, three and four *Xist* clouds. (D) Quantification of cells with different numbers of Xi's determined by RNA/DNA FISH, plotted next to the distribution found by RNA FISH alone. (E) Immuno-RNA FISH detecting *Xist* RNA (FITC) and BrdU (Rhodamine red) for day 7 differentiated XXXX ES cells. (F) Distribution and standard deviation of Xi's in BrdU positive day 7 differentiated XXXX (1, 2 and 8) ES cells.

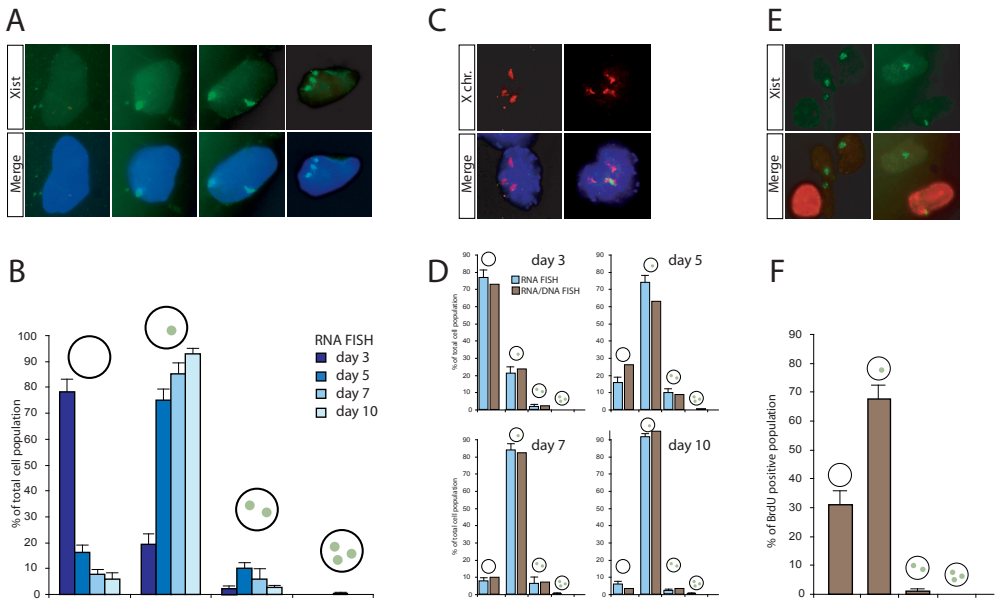

Figure 4. **XCI in tetraploid XXXY ES cells**
(A) *Xist* RNA FISH (top panels *Xist* in FITC, bottom panel *Xist* in FITC and DNA stained with DAPI) for day 5 differentiated XXXY ES cells. (B) Average distribution and standard deviation of sub-populations of cells with different numbers of Xi's at different time points after start of differentiation for four different XXXY (1, 2, 5 and 11) ES cell lines, determined by RNA FISH. C) RNA/DNA FISH with an *Xist* probe (FITC) and an X chromosome paint probe (Cy3, DNA is DAPI stained, blue). From left two right panels show cells with no, and one *Xist* cloud. (D) Quantification of cells with different numbers of Xi's determined by RNA/DNA FISH, plotted next to the distribution found by RNA FISH alone. (E) Immuno-RNA FISH detecting *Xist* RNA (FITC) and BrdU (Rhodamine red) for day 7 differentiated XXXY ES cells. (F) Distribution and standard deviation of Xi's in BrdU positive day 7 differentiated XXXX (1, 2 and 11) ES cells.

analysis results in the exclusion of XXXO cells (Figure 2I) that show preferential inactivation of one X chromosome (see below).

The distribution of cells over time suggests that cells with the correct Xa:ploïdy ratio, are selected for during differentiation. Indeed, BrdU analysis of ES cells at day 7 of differentiation showed that BrdU positive cells with three and four Xi's are severely reduced compared to the total population of cells, indicating that these cells stop dividing (Figure 3E and F). In contrast, the BrdU positive population consists almost entirely of cells with no, one or two Xi's indicating that this part of the population remains proliferative (Figure 3F). The small number of BrdU positive cells with three Xi's may indicate that these cells can still divide, albeit at a slower rate when compared to cells with two or less Xi's, or perhaps more likely, they represent cells that were BrdU labeled prior to XCI. Interestingly, cells with none or one Xi disappeared during the differentiation process, suggesting that these cells initiate XCI on the remaining Xa's.

XXXY cells preferably inactivate one X chromosome (Figure 4A, and B) Initiation of XCI was delayed as demonstrated by the percentage of cells with no clouds at day 3 of differentiation, as compared to day 3 differentiated XXXX ES cells (Figure 3B, and 4B). RNA/DNA FISH on XXXY ES line 11, at different time points after differentiation revealed a similar distribution when compared to RNA FISH alone (Figure 4C and D). BrdU incorporation analysis

of cells at day 7 of differentiation showed that cells with more than one Xi stop dividing. Cells with no Xi are a large proportion of the BrdU positive cells, but disappear over time, again indicating that these cells are still initiating XCI (Figure 4E and F). XXYY cell lines only sporadically initiate XCI at day 3 and day 7 of differentiation similar to diploid XY cells (<0.3%, data not shown).

The above experiments with tetraploid ES cells show that all cell lines preferably keep one Xa per diploid genome. Nevertheless, a significant number of cells do inactivate an aberrant number of X chromosomes during the XCI process. Cells with less than one Xa per diploid genome stop dividing, and are lost in time in the proliferating cell population.

Calculating a probability to initiate XCI

The existence of cells with an unexpected number of *Xist* clouds in differentiated ES cells led us to hypothesize that each X chromosome within a cell has a certain probability to initiate XCI. Indeed, for the XXXX cell lines the distribution of cells with different numbers of Xi's (day 3 of differentiation) can be explained by assuming a 27% probability for each X chromosome to initiate XCI, after subtraction of cells without an *Xist* cloud (Figure 5A). We excluded this fraction because we cannot distinguish between the cell population that, has not yet initiated XCI and that which has initiated XCI but has not chosen an X chromosome for inactivation. Also, this population is not required to calculate the probability. Day 3 differentiated samples were used to avoid potential influences of cell selection or multiple rounds of inactivation in our calculation. Nonetheless, the calculated probability remains a cumulative probability over the first 3 days of differentiation. For the XXXY cells, a probability of 8% would generate a distribution that matches our experimental observation at day 3 of differentiation (Figure 5B). Based on these calculations, we conclude that the distribution of the different populations could be explained by assuming a probability for each X chromosome in a cell to initiate XCI. In addition, the probability increased with the X:ploïdy ratio, indicating the presence of an X linked activator of XCI.

Initiation of XCI in female cells despite deletion of *Xist Tsix* and *Xite*

Our results suggest an independent probability for each X chromosome to initiate XCI, followed by selection of cells with the correct number of Xa's. Therefore, deletion of the cis acting genes (*Xist*, *Tsix* and *Xite*) and all known elements involved in the counting process should have no effect on the probability to initiate inactivation on the remaining wild type

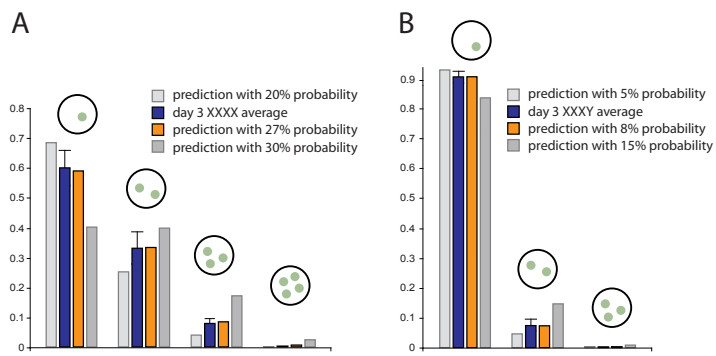

Figure 5. **Comparison of the experimental and predicted distributions**
(A) Distribution of subpopulations of cells with different numbers of Xi's at day 3. The average of four XXXX cell lines (blue bars), and predicted distribution after one choice round based on a 20%, and 30% probability (gray bars) and a 27% probability per X chromosome to be inactivated (orange bar). (B) Similar to (A) but for the four XXXY cell lines (blue bars), and the predicted distributions based on a 5%, and 15% probability (gray bars) and 8% probability per X chromosome to be inactivated (orange bar).

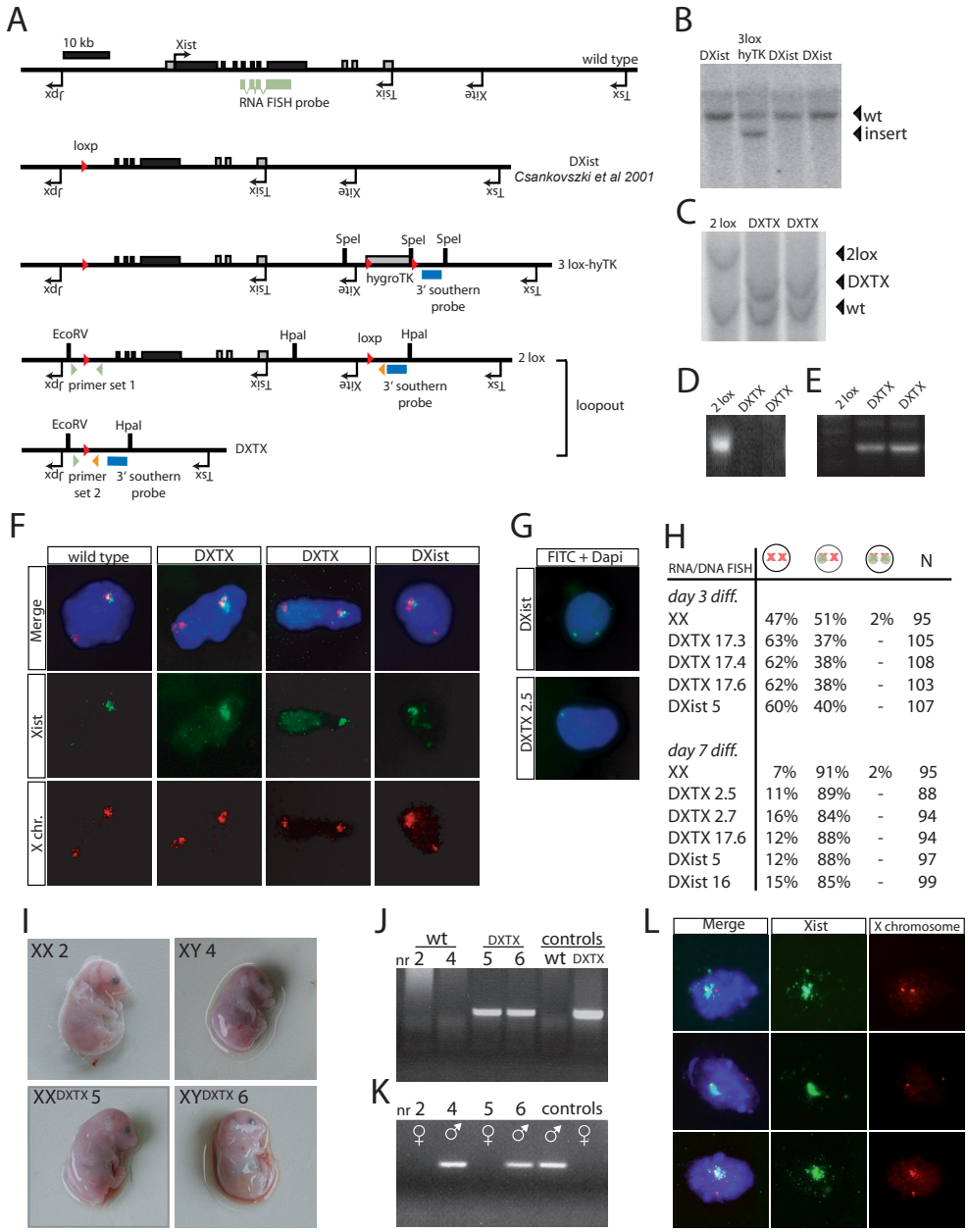

Figure 6. **Analysis of XCI in XTX deletion cell lines**
(A) Schematic representation of the generation of the XTX deletion ES cell lines. (B) Southern blot analysis with a 3' external probe on SpeI digested DNA of different targeted clones. (C) Southern analysis with DNA of different clones after transient Cre expression. DNA was digested with HpaI and EcoRV, and the 3' external probe was used for hybridization. (D,E) Correct loopout was confirmed with PCR analysis using primer set 1 amplifying the original Δ*Xist* deletion (D) and primer set 2 amplifying the correct loopout (ΔXTX, E). (F) RNA/DNA FISH analysis with an *Xist* probe (FITC) and X-chromosome paint (Cy3, DNA stained with Dapi) on 7 day differentiated wild type (XX) and mutant ΔXTX and Δ*Xist* cells. (G) *Xist* RNA FISH analysis (FITC, DNA in DAPI) on Δ*Xist* and ΔXTX 2.5 cells showing pinpoint signals in undifferentiated ES cells. (H) Quantification of cells

X chromosome in a diploid female cell. To test this hypothesis, we integrated a floxed hygroTK cassette between *Tsix* and *Xite* in a previously described (1lox) Δ*Xist* ES cell line with a conditional *Xist* deletion (Csankovszki et al., 1999; Gribnau et al., 2005) (Figure 6A). Cre recombinase mediated deletion of the remaining part of *Xist*, and *Tsix* and *Xite*, designated Δ*Xist-Tsix-Xite* (ΔXTX), was confirmed by Southern and PCR analysis (Figure 6B-E). RNA FISH analysis of undifferentiated $XX^{\Delta XTX}$ cell lines with an *Xist* probe detecting *Tsix* transcription showed a single pinpoint signal (N=100), also confirming that the remaining part of *Tsix* has been deleted from the Δ*Xist* allele (Figure 6F).

To explore the pattern of XCI in differentiating $XX^{\Delta XTX}$ ES cell lines, we subjected EB differentiated cells to DNA/RNA FISH using an *Xist* RNA probe in combination with an X chromosome specific Bac probe (day 3) or an X chromosome paint probe (day 7). Since the ΔXTX and Δ*Xist* cell lines had a tendency to become XO, (reported to occur for many inbred lines) cells with two X chromosomes were selected prior to examination for an *Xist* signal. Interestingly, ~38% of the cells at day 3 of differentiation and ~87% of the cells at day 7 of differentiation showed an *Xist* RNA cloud, similar to the percentages obtained with the Δ*Xist* cell lines. Both at day 7 and, more pronounced at day 3, the percentages of cells which initiated XCI were lower for the ΔXTX and Δ*Xist* lines than to the wild type control. We attribute this difference to the fact that in both the ΔXTX and Δ*Xist* cells only one X chromosome has a probability to initiate XCI while wild type female cells have a probability to initiate XCI in two X chromosomes.

To test our findings in vivo, we injected the $XX^{\Delta XTX}$ ES cells into blastocysts and generated chimeras. In the second litter of the female founder, two out of nine embryos contained an $X^{\Delta XTX}$ chromosome (Figure 6I and J). This was verified by Southern blotting (data not shown). PCR analysis with Sry specific primers indicated that one ΔXTX embryo was male and one female (figure 6K). The $XX^{\Delta XTX}$ and $X^{\Delta XTX}Y$ embryos did not show any structural abnormalities or growth retardation (Figure 6I). MEFs were derived from all embryos and subjected to DNA/RNA FISH (*Xist* probe and two BAC probes). We observed *Xist* RNA clouds in 99% of the $XX^{\Delta XTX}$ MEFs and 98% of the wild type MEFs, confirming our finding with the $XX^{\Delta XTX}$ ES cell lines (Figure 6L).

These data demonstrate that female diploid cells show XCI despite the XTX-deletion, and confirmed that the probability to initiate XCI is determined independently by each X chromosome. Since XO and XY ES cells do not initiate XCI, these results also indicate the presence of an, as yet unidentified, X-encoded trans-acting factor located outside the deleted region that is required for XCI.

Truncation of *Tsix* leads to earlier onset of XCI

What factors determine the probability for an X chromosome to inactivate? Studies with *Xist* promoter driven transgenes show a clear difference in expression between differentiating male and female ES cells, indicating the presence of a sex-linked transcription factor driving *Xist* expression (Sun et al., 2006). In addition, more abundant *Xist* expression has been correlated with a stronger Xce allele in mice (Brockdorff et al., 1991), suggesting that the amount of *Xist* expression could be a positive parameter correlating with XCI-probability. An additional parameter is *Tsix*, known as a negative regulator of *Xist*. Previous studies have shown that introduction of a stop cassette in

with no, one or two *Xist* clouds at day 3 and day 7 of differentiation using wild type, ΔXTX cells and Δ*Xist* cells, determined by RNA/DNA FISH. (I) Wild type and ΔXTX mutant female and male littermates. (J) PCR analysis of the mice shown in (I) with primer set 2 amplifying the ΔXTX loopout. (K) PCR analysis of the mice shown in (I) with a primer set amplifying the Sry gene. (L) *Xist* RNA/DNA FISH analysis (FITC) and X chromosome specific BAC probes (Rhodamine red, DNA is DAPI stained, blue) on MEFs isolated from the ΔXTX mutant female. The panels show three representative cells.

Tsix prematurely abrogates *Tsix* transcription and results in almost exclusive inactivation of the mutated X chromosome (Luikenhuis et al., 2001). Conversely, if *Tsix* expression persists upon differentiation, the wild type allele is always selected for inactivation (Luikenhuis et al., 2001). These observations suggest that *Tsix* expression reduces the probability by inhibiting *Xist* expression. Therefore, abolishing *Tsix* expression greatly increases the probability of that allele to initiate XCI, and implies that XCI initiation should occur faster on the *Tsix*-stop allele compared to the wild type allele.

To test this hypothesis we analyzed *Xist* cloud formation by RNA FISH in the heterozygous *Tsix*-stop female ES cell line (Luikenhuis et al., 2001) at different time points of differentiation. Indeed, analysis of XCI in wild type and *Tsix*-stop cells showed that the *Tsix*-stop cells initiate XCI much faster compared to wild type cells (Figure 7A-C). Also, the number of cells with two *Xist* clouds is significantly reduced compared to wild type cells at all time points measured (Figure 7C). Because XCI in *Tsix*-stop cells is skewed towards inactivation of the mutated allele, we conclude that initiation of XCI is initiated faster on the mutated allele than the wild type allele. The reduced number of cells with two Xi's is most likely due to the fact that the mutated X chromosome generates a probability to initiate XCI well before the wild type allele does, and as a consequence only in a few cells will both X chromosomes have a probability to initiate XCI. Our results showing accelerated initiation of XCI in *Tsix* stop cells, is consistent with the finding that *Xist* promoter methylation is detected much earlier in cells with a heterozygous ΔCpG *Tsix* deletion compared to wild type cells, indicating that the XCI process is accelerated on the mutant X chromosome (Sun et al., 2006).

DISCUSSION

We find that in the course of the XCI process a significant proportion of cells do not comply with the 1Xa/diploid genome rule and have less or more than the expected number of Xi's.

This finding suggests that XCI is a stochastic process with an independent probability for each X chromosome to initiate XCI and that this probability is directly proportional to the X:ploïdy ratio. These results also suggest the presence of an X-encoded probability-promoting factor, which is located outside the region we deleted in our female ΔXTX lines.

Comparing different tetraploid studies

Our findings that 10 day differentiated XXXX tetraploid ES cells preferably inactivate two X chromosomes, are in agreement with Webb et al. 1992. Similar to our findings in differentiating ES cells, Webb et al. reported a significant number of cells with zero, one or three Xi's in 10-day-old tetraploid XXXX embryos, indicating that cells with an aberrant Xi number are also present in vivo (Webb, 1992). A different study analyzing 9 to 12 day differentiated tetraploid XXXX lines, generated by fusion of EC cells with lymphocytes, showed variable results (Takagi, 1993). We attribute the different findings presented in this study to the method used to detect Xi's; i.e. BrdU incorporation in metaphase spreads, a technique that does not allow detection of cells that stop dividing. Also, ES cell x somatic cell fusion lines have been reported to be karyotypically unstable (Matveeva et al., 2005) in contrast to our ES x ES cell fusion lines, that retain a stable karyotype for more than 20 passages.

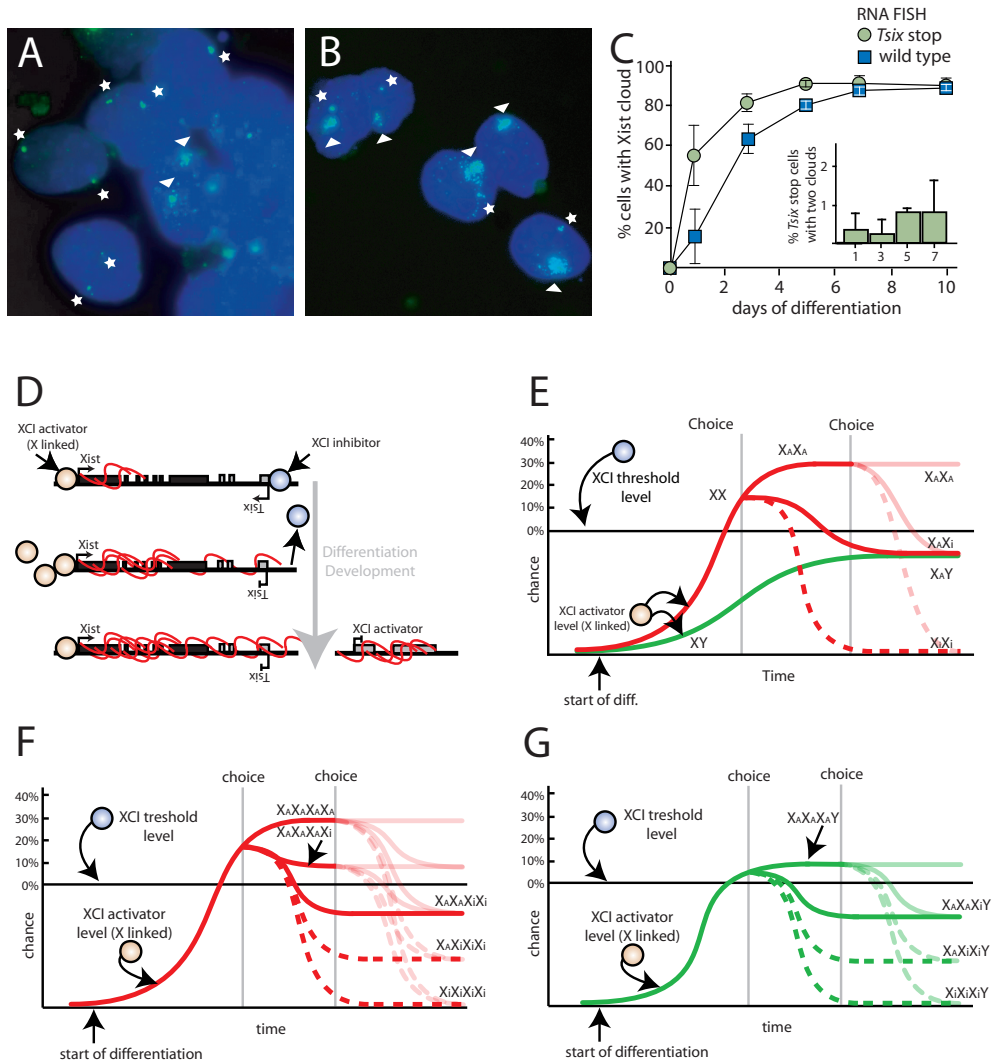

Figure 7. **Analysis of the *Tsix* stop mutation, and a stochastic model for XCI counting and choice**
(A,B) *Xist* RNA FISH analysis on day 1 EB differentiated wild type ES cells (A) and cells heterozygous for the *Tsix*-stop insertion (B). Triangles: *Xist* clouds, stars: *Tsix* pinpoints. (C) Quantification of cells with an *Xist* cloud (line graph), and cells with two *Xist* clouds (bar graph) at different time points of EB differentiation. (D) Before differentiation *Tsix* transcription represses *Xist* expression, and the level of XCI-activator is insufficient to overcome *Tsix* repression. Upon differentiation, the XCI-activator level rises and in female cells reaches a level sufficient to overcome *Tsix* repression with a certain probability. After *Tsix* is silenced, *Xist* accumulates and silences the XCI-activator gene in cis, preventing inactivation of the second X chromosome. (E) Schematic representation of D: after start of differentiation the XCI-activator level will rise and, in female cells (red lines), exceed the level required to generate a chance to initiate XCI. After XCI is initiated, cells have either two Xa's, one Xa and one Xi or two Xi's. Cells with two Xi's stop dividing and disappear from the population (dashed lines). In cells with a Xa and a Xi the XCI-activator level drops below the level required to generate a probability. Cells with two Xa's continue with XCI (faint red lines). (F) In XXXX ES cells the XCI-activator concentration increases during differentiation generating a probability to initiate XCI for each X chromosome. After the choice is made there are five different outcomes. Cells with less than 2 Xa's stop dividing and disappear from the population (dashed lines), cells with two Xi's and two Xa's stop the XCI process, and cells with no or one Xi continue the

Each X chromosome has a probability to initiate XCI

Different models have been proposed explaining the XCI counting and choice process. One model explains XCI counting and choice through the presence of a single autosomally encoded blocking factor, which prevents inactivation of one X chromosome per diploid genome. XCI counting and choice could also be explained through transient spatial cross communication between the different X chromosomes, or pre-determined Xic's prior to the start of XCI (Bacher et al., 2006; Rastan, 1983; Xu et al., 2006). These deterministic models predict a tightly regulated XCI counting and choice process and do not explain the presence of XXXX cells with three or four Xi's. Aberrant numbers of Xi's in XXXY and XXXX ES cells could be an artifact introduced by tetraploidization or an instable karyotype. However, our tetraploid cells maintain a stable karyotype and the expected number of X chromosomes throughout differentiation. Hence, our finding that XCI is properly regulated in XXYY tetraploid cells argues against this possibility. Instead, the distribution of cells with different numbers of Xi's can be explained by assuming a stochastic model, in which each X chromosome has a certain probability to be inactivated.

A stochastic model predicts the presence of diploid female cells with two Xi's. We did observed 2 Xi's in some differentiating ES and ICM cells and others reported the presence of diploid cells with two Xi's (Lee, 2005). Nonetheless, the number of cells with two Xi's is lower than would be expected based on a 27% probability we calculated for each X chromosome in the tetraploid XXXX line (same X:ploïdy ratio as a diploid cell). Although the differences could be due to potentially different cell volumes, cell division or differentiation characteristics of diploid and tetraploid cells, this discrepancy is most likely based on the fact that there is a strong selection against cells with all X chromosomes inactivated.

Examination of cell division kinetics by BrdU incorporation analysis indicates that cells with one or more Xa per diploid genome keep dividing. Nonetheless, cells with more than one Xa per diploid genome decrease in time, suggesting that these cells keep initiating XCI, or are eliminated due to aberrant dosage compensation, although the latter may only play a role at later stages during differentiation. Cells with less than one Xa per diploid genome stop dividing. Therefore, continued proliferation of the other cells within the population will result in a relative decrease of cells with less than one Xa per diploid genome. Currently, we do not know whether these cells remain in the population, are actively selected against or disassemble their *Xist* cloud(s) and rejoin the pool of dividing cells.

In vivo evidence supporting cell loss as a consequence of the XCI process comes from studies that show a significant size difference between female and male early implantation diploid embryos before hormonal cues start to influence growth (Burgoyne et al., 1995). Interestingly, female mouse XO embryos did not differ in size compared to XY male embryos. The size difference between male and normal female embryos is most pronounced around the time of XCI and decreases later during development. Thus, similar to our in vitro findings with differentiating tetraploid ES, gender specific size differences could very well be related to the loss of XiXi and XaXa cells during development.

XCI process (faint red lines). Note that in cells with one Xi the probability will drop. (G) For XXXY cells there are four possible outcomes, and the probability to initiate XCI is lower when compared to the XXXX line because of a lower level of XCI-activator. Cells with two or three Xi's stop dividing and disappear from the population (dashed lines). Cells with one Xi stop the XCI process, and cells that have not initiated XCI will continue the inactivation process (faint green lines.

Evidence against a single blocking factor

If the probability to initiate XCI is an independent property of the deleted *Xist-Tsix-Xite* region, and dependent on trans-acting factors located elsewhere in the genome, deletion of this region in female cells should have no effect on the probability of the wild type allele to initiate XCI. Our results with the ΔXTX ES cell lines and mice confirm this hypothesis and show that the deleted area is not required for the counting process in female cells. We find initiation of XCI as expected for a single allele in ΔXTX cells, indicating that our results are not the consequence of initiation in a few cells followed by a selection process.

These and other findings indicate that a single blocking factor may not be present at all, because of the following arguments. XCI is initiated in male ES cells with a 65kb deletion, a 20 kb bipartite deletion and a smaller 1.2 kb DXPas34 deletion, all located 3' of *Xist* (Clerc and Avner, 1998; Morey et al., 2004; Vigneau et al., 2006), placing the putative blocking factor binding site inside this region. Nevertheless, we find robust initiation of XCI despite the deletion of this entire region. This does not exclude the possibility that the blocking factor binding site is located outside the deleted region. However, if this were true an increased number *Xist* double clouds would be expected in female cells heterozygous for the 65kb deletion or the *Tsix* stop insertion, because in both cell lines half of the wild type X chromosomes are unprotected from XCI. Remained presence of a blocking factor binding site on the mutated allele would therefore have resulted in an increased number of cells with two Xi's. Neither we nor others have found this (Clerc and Avner, 1998). We therefore conclude that our findings preclude a blocking factor model.

Evidence for an X-encoded factor involved in promoting XCI

Our findings that XXΔXTX cells initiate XCI in contrast to XY cells, provides evidence for the presence of an unidentified X-linked gene encoding a trans-acting factor that is involved in promoting initiation of XCI (XCI-activator). Analysis of the XCI initiation frequency at day 3 of differentiation shows that the XXXX ES cells initiate XCI much faster than XXXY ES cells. In addition, the calculated probability to initiate XCI is much lower for XXXY cells than the XXXX cells. Both observations indicate the presence of an X-encoded probability-determining factor that is located outside our XTX-deletion but resides in the genetically defined Xic. The presence of a XCI-activator is supported by studies with *Xist* promoter driven transgenes that show a clear difference in expression between differentiating male and female ES cells, also indicating the presence of a sex-linked transcription factor (Sun et al., 2006).

Interestingly, a previous study, which analyzed XCI in a male cell line with a 450kb transgene, encompassing *Xist* and flanking regions, showed initiation of XCI on the single X chromosome, indicating that the transgene may harbor the gene encoding this XCI activator (Lee et al., 1996). A different study showed that introduction of a BAC sequence located 5' to *Xist*, not including *Xist* itself, into ES cells also results in initiation of XCI on the single X chromosome in male cells, and on both X chromosomes in female cells (Augui et al., 2007). This result was attributed to ectopic pairing between the transgene and the Xic. We think that this study indicates that the sequence encoding the XCI-activator may be located within the transgene. The reported transient pairing could be the consequence of the differentiation process, and related to changes in the expression of genes located within the Xic, resulting in transient changes in the nuclear positioning of these genes.

A stochastic model for XCI counting and choice

This study indicates that XCI is a stochastic process, in which each X chromosome has a probability to be inactivated. The outcome of the XCI process is the resultant of; 1) an equal probability for each individual X chromosome to be inactivated (in the same genetic back ground), 2) the probability to initiate XCI is directly proportional to the X:ploïdy ratio, 3) selection in favor of cells retaining one Xa per diploid genome.

What factors determine the probability for an X chromosome to be inactivated? Cell line studies indicate that the *Xist*, *Tsix*, and *Xite* genes play a key role in determining the probability of an X chromosome to initiate XCI. Although the molecular factors involved in the regulation of these genes remain elusive so far, studies with *Xist* promoter driven transgenes indicate the presence of a sex-linked transcription factor, which is supported by our observations (Sun et al., 2006). *Tsix* is transcribed in both male and female cells before the onset of XCI indicating the presence of a, most likely autosomal, factor that drives *Tsix* transcription in both male and female cells (Lee et al., 1999). These observations suggest that the probability to initiate XCI is the resultant of the balance between an X-encoded *Xist* activator (XCI-activator), that itself is inactivated by XCI, and a *Tsix* activator (XCI-inhibitor). Upon differentiation, the concentration of the XCI-activator rises (Figure 7D and E). In contrast, the XCI-inhibitor concentration remains stable or may even decrease in time, providing a stable threshold level throughout early differentiation or development, which has to be overcome to generate a probability to initiate XCI. In male cells the maximum XCI-activator concentration is not sufficient to overcome the XCI threshold level. In female cells the concentration of the XCI-activator will be twice as high and sufficient to induce *Xist* mediated silencing of *Tsix* with a certain probability in a particular time frame, e.g. one cell division. Because both X chromosomes generate a certain probability, a proportion of the differentiating cells will inactivate two X chromosomes. After XCI has been initiated, the X-encoded XCI-activator gene will be silenced in cis. This results in a drop in the XCI-activator level to a level equal to that found in male cells, preventing inactivation of the second X chromosome. Cells that have not initiated XCI will start another round of inactivation. *Xist* expression on the future Xi persists because less XCI activator is required as a result of a lack of *Tsix* inhibition in cis. In addition, chromatin modifications or cis interactions may fix the *Xist* active state on the future Xi.

For tetraploid cells this model becomes more complicated because of the increased number of possibilities (Figure 7F and G). XXXY tetraploid ES cell lines will have less XCI-activator when compared to the XXXX lines. This explains the decreased probability that was calculated for the XXXY line (8%) compared to the XXXX line (27%).

Important prerequisites for this model are the rapid down-regulation of the XCI-activator level after the initiation of XCI, and *Xist* mediated silencing of *Tsix*. Indeed, studies with cell lines with an inducible *Xist* cDNA transgene showed that inactivation of flanking genes occurs within several hours after *Xist* up-regulation (Wutz and Jaenisch, 2000). To date, it is unclear whether *Tsix* is silenced by *Xist*, or whether *Xist* up-regulation is due to autonomous silencing of *Tsix* by developmental cues. Constitutive or inducible expression of *Tsix* shows that persistent expression of *Tsix* results in preferential inactivation of the (other) wild type X chromosome. Conversely, elevated transcription through *Xist*, as a consequence of an integration of a selection cassette upstream of the *Xist* promoter, results in preferential inactivation of the mutated X chromosome (Nesterova et al., 2003). Although we cannot exclude a model in which the probability is solely dependent on autonomous down-regulation of *Tsix*, these findings indicate the

presence of a transcriptional balance between *Xist* and *Tsix*, in which both genes have mutual inhibiting properties.

Interpretation of the stochastic model

A stochastic model for XCI predicts that SNPs, mutations or deletions of binding sites for the XCI-activator or -inhibitor will change the probability to inactivate the respective X chromosome and will result in skewed XCI. According to this model, a truncation of *Tsix* or deletions that lead to severe down-regulation of *Tsix* expression, will result in a reduced level of XCI-activator required for initiation of XCI, and would explain ectopic XCI observed in mutant male cells (Clerc and Avner, 1998). Similarly, *Xist* transgenes lacking *Tsix* repression will require less XCI-activator, which may lead to ectopic XCI in male cells (Herzing et al., 1997). In addition, a homozygous mutation of the *Tsix* major promoter that abolishes *Tsix* expression in female cells will lead to increased probabilities for both X chromosomes explaining the high frequency of differentiating cells with two Xi's (Lee, 2005)

A stochastic model would also explain the sex-ratio distortion found for *Tsix* double mutant offspring as a consequence of expression differences of the XCI-activator between male and female cells (Lee, 2002).These observations indicate that *Tsix* may not be required for proper XCI to occur, as proposed for humans. As long as the probability to initiate XCI on an X chromosome remains low. This could be accomplished by down-regulation of the XCI-activator activity or *Xist* promoter activity. Although the factors driving the XCI process remain as yet elusive, the ΔXTX deletion described here locates the X-encoded XCI-activator gene outside the deleted region but within the region delineated by the Searle's translocation and the HD3 truncation, which originally defined the Xic (Lyon et al., 1964; Rastan and Robertson, 1985). Future identification of the XCI-activator and -inhibitor will be crucial for our understanding of the XCI process.

METHODS

Generation of tetraploid ES cell lines

M. cast / 129/Sv F1 female (F1 2-1) and male (F1 2-3) ES cell lines and a male C57Bl6 / 129/Sv (V6.5) ES cells were targeted with neomycin and puromycin resistance cassettes. PEG 1500 (Roche Cat. No. 783 641) fusion was performed according to manufacturers instructions. Tetraploid ES cell lines were grown on male MEFs, under continuous double selection and have not been frozen before analysis.

DNA and RNA FISH

Pre-plated ES cells were transferred to non gelatinized bacterial dishes to start EB differentiation, in IMDM + Glutamax (Gibco), 15% FCS, Asc. Acid 50 µg/µl, NEAA, PenStrep (PS), Monothioglycerol (97%) 37,8 µl/l. One day prior to fixation, EBs were trypsinized and transferred to dishes with gelatin coated cover slips. BrdU (20µM) was added 16 hours (less then one cell division) prior to harvesting.

For RNA FISH experiments on differentiating ICM cells E3.5 blastocysts were flushed out of the uterus, and allowed to attach for two days in DMEM, 15% FCS, NEAA, PS, β-mercapthoeth. 8µl/l. Expanded ICMs were micro dissected with a mouth pipette and plated on gelatinized slides. ICM cells were allowed to attach and proliferate for two more days before fixation.

DNA and RNA FISH were performed as described (Gribnau et al., 2005), *Xist* RNA and chr. 6, 7 and Y specific probes have been described (Geijsen et al., 2004; Gribnau et al., 2005). Criteria for scoring the *Xist* clouds: first in Dapi a non-overlaid intact nucleus was selected, then in FITC, the number of clouds was counted. Every *Xist* cloud that was counted was clearly distinguishable from neighboring clouds. If not specifically indicated more than 100 cells were counted per cell line per time point. For combined DNA/RNA FISH, slides were pretreated for 4 min with 0.2% pepsin in 10mM HCl at 37°C, post fixed for 5 minutes in 4% PFA/PBS, washed twice with

PBS and dehydrated prior to denaturation. Probes for hybridization were *Xist*, a Cy3 labeled X paint probe (Cambio), or a combination of two biotin labeled BACs (CT7-155J2 and CT7-474E4). Slides were washed at 42°C in 2xSSC and 3 washes of 2xSSC / 50% formamide. Detection was as described (Gribnau et al., 2005). For DNA/RNA FISH diploid cells with two and tetraploid cells with four X chromosomes were selected in the red channel, then *Xist* clouds were counted in the FITC channel.

Karyotyping

Cells were treated with colcemid for 1h, fixed and hybridized with an X paint probe (Cambio). Criteria for scoring painted X chromosomes: first in Dapi a metaphase spread was selected, next in the red channel the number of X chromosomes was counted.

Generation of the ΔXTX ES cell line

An 8.2 kb XhoI-ClaI fragment from BAC 299K20 was subcloned into pBluescript KS, followed by the insertion of a PGK-DTA cassette into the ClaI site. Next, a floxed hygro-TK cassette was cloned into an EcoRV site. The resulting pXite-DTA-hygroTK was targeted to a heterozygous Δ*Xist* 1lox ES cell line (Gribnau et al., 2005). After transient Cre expression, positive clones were identified by southern analysis (SpeI digest) with a 3' external probe, a 565 bp PCR product (AAGCTTGGGTCCTCCTCTGT and CCACTCAGACATCCCCAGAT). Cre mediated excision was confirmed by PCR analysis using primer set 1 (A, TTTCTGGTCTTTGAGGGCAC x B, CACTGGCAAGGTGAATAGCA) detecting the original Δ*Xist* 1lox allele and primer set 2 (A x C, GGACATTTTGTCCTGGCAGT) detecting the ΔXTX allele.

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REFERENCES

Augui, S., Filion, G.J., Huart, S., Nora, E., Guggiari, M., Maresca, M., Stewart, A.F., and Heard, E. (2007). Sensing X chromosome pairs before X inactivation via a novel X-pairing region of the Xic. Science 318, 1632-1636.

Bacher, C.P., Guggiari, M., Brors, B., Augui, S., Clerc, P., Avner, P., Eils, R., and Heard, E. (2006). Transient colocalization of X-inactivation centres accompanies the initiation of X inactivation. Nat Cell Biol 8, 293-299.

Brockdorff, N., Ashworth, A., Kay, G.F., Cooper, P., Smith, S., McCabe, V.M., Norris, D.P., Penny, G.D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. Nature 351, 329-331.

Brockdorff, N., Ashworth, A., Kay, G.F., McCabe, V.M., Norris, D.P., Cooper, P.J., Swift, S., and Rastan, S. (1992). The product of the mouse *Xist* gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell 71, 515-526.

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

Burgoyne, P.S., Thornhill, A.R., Boudrean, S.K., Darling, S.M., Bishop, C.E., and Evans, E.P. (1995). The genetic basis of XX-XY differences present before gonadal sex differentiation in the mouse. Philos Trans R Soc Lond B Biol Sci 350, 253-260 discussion 260-251.

Clerc, P., and Avner, P. (1998). Role of the region 3' to *Xist* exon 6 in the counting process of X-chromosome inactivation. Nat Genet 19, 249-253.

Cohn, M., and Langman, R.E. (1990). The protection: the unit of humoral immunity selected by evolution. Immunol Rev 115, 11-147.

Csankovszki, G., Panning, B., Bates, B., Pehrson, J.R., and Jaenisch, R. (1999). Conditional deletion of *Xist* disrupts histone macroH2A localization but not maintenance of X inactivation. Nat Genet 22, 323-324.

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., and Daley, G.Q. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 427, 148-154.

Gribnau, J., Luikenhuis, S., Hochedlinger, K., Monkhorst, K., and Jaenisch, R. (2005). X chromosome choice occurs independently of asynchronous replication timing. J Cell Biol 168, 365-373.

Herzing, L.B., Romer, J.T., Horn, J.M., and Ashworth, A. (1997). *Xist* has properties of the X-chromosome inactivation centre. Nature 386, 272-275.

Lee, J.T. (2002). Homozygous *Tsix* mutant mice reveal a sex-ratio distortion and revert to random X-inactivation. Nat Genet 32, 195-200.

Lee, J.T. (2005). Regulation of X-chromosome counting by *Tsix* and *Xite* sequences. Science 309, 768-771.

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

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

Lee, J.T., Strauss, W.M., Dausman, J.A., and Jaenisch, R. (1996). A 450 kb transgene displays properties of the mammalian X-inactivation center. Cell 86, 83-94.

Luikenhuis, S., Wutz, A., and Jaenisch, R. (2001). Antisense transcription through the *Xist* locus mediates *Tsix* function in embryonic stem cells. Mol Cell Biol 21, 8512-8520.

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

Lyon, M.F., Searle, A.G., Ford, C.E., and Ohno, S. (1964). A Mouse Translocation Suppressing Sex-Linked Variegation. Cytogenetics 15, 306-323.

Marahrens, Y. (1999). X-inactivation by chromosomal pairing events. Genes Dev 13, 2624-2632.

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

Matveeva, N.M., Pristyazhnyuk, I.E., Temirova, S.A., Menzorov, A.G., Vasilkova, A., Shilov, A.G., Smith, A., and Serov, O.L. (2005). Unequal segregation of parental chromosomes in embryonic stem cell hybrids. Mol Reprod Dev 71, 305-314.

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

Morey, C., Navarro, P., Debrand, E., Avner, P., Rougeulle, C., and Clerc, P. (2004). The region 3' to *Xist* mediates X chromosome counting and H3 Lys-4 dimethylation within the *Xist* gene. Embo J 23, 594-604.

Nesterova, T.B., Johnston, C.M., Appanah, R., Newall, A.E., Godwin, J., Alexiou, M., and Brockdorff, N. (2003). Skewing X chromosome choice by modulating sense transcription across the *Xist* locus. Genes Dev 17, 2177-2190.

Ogawa, Y., and Lee, J.T. (2003). *Xite*, X-inactivation intergenic transcription elements that regulate the probability of choice. Mol Cell 11, 731-743.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. Nature 379, 131-137.

Rastan, S. (1983). Non-random X-chromosome inactivation in mouse X-autosome translocation embryos--location of the inactivation centre. J Embryol Exp Morphol 78, 1-22.

Rastan, S., and Robertson, E.J. (1985). X-chromosome deletions in embryo-derived (EK) cell lines associated with lack of X-chromosome inactivation. J Embryol Exp Morphol 90, 379-388.

Shykind, B.M. (2005). Regulation of odorant receptors: one allele at a time. Hum Mol Genet 14 Spec No 1, R33-39.

Sun, B.K., Deaton, A.M., and Lee, J.T. (2006). A transient heterochromatic state in *Xist* preempts X inactivation choice without RNA stabilization. Mol Cell 21, 617-628.

Takagi, N. (1993). Variable X chromosome inactivation patterns in near-tetraploid murine EC x somatic cell hybrid cells differentiated in vitro. Genetica 88, 107-117.

Till, J.E., McCulloch, E.A., and Siminovitch, L. (1964). A Stochastic Model of Stem Cell Proliferation, Based on the Growth of Spleen Colony-Forming Cells. Proc Natl Acad Sci U S A 51, 29-36.

Vigneau, S., Augui, S., Navarro, P., Avner, P., and Clerc, P. (2006). An essential role for the DXPas34 tandem repeat and *Tsix* transcription in the counting process of X chromosome inactivation. Proc Natl Acad Sci U S A 103, 7390-7395.

Webb, S., de Vries, T.J., and Kaufman, M.H. (1992). The differential staining pattern of the X chromosome in the embryonic and extraembryonic tissues of postimplantation homozygous tetraploid mouse embryos. Genet Res 59, 205-214.

Wernet, M.F., Mazzoni, E.O., Celik, A., Duncan, D.M., Duncan, I., and Desplan, C. (2006). Stochastic spineless expression creates the retinal mosaic for colour vision. Nature 440, 174-180.

Wutz, A., and Jaenisch, R. (2000). A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. Mol Cell 5, 695-705.

Xu, N., Tsai, C.L., and Lee, J.T. (2006). Transient homologous chromosome pairing marks the onset of X inactivation. Science 311, 1149-1152.

Chapter 4

Probability to Initiate X Chromosome Inactivation is Determined by X-autosomal Ratio and Xce Strength

Bas de Hoon, Kim Monkhorst, Iris Jonkers, Jos Hoogerbrugge, Eveline Rentmeester, Anton Grootegoed and Joost Gribnau

In prep

Chapter 4

Probability to Initiate X Chromosome Inactivation is Determined by X:Autosomal Ratio and Xce Strength

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In eutherian female cells, one X chromosome is transcriptionally inactivated to equalize the dosage of X chromosomal genes. In the embryo proper, X chromosome inactivation (XCI) is random, and is the result of a stochastic XCI process in which each X chromosome has a certain probability to be inactivated. Three genes that have been reported to play a role in XCI, *Xist*, *Tsix* and *Xite*, are not required for counting the number of X chromosomes, indicating the presence of a trans acting X encoded activator of XCI. Here we have analyzed XCI in triploid XXY cells and in cells with different X:autosomal ratio's. We found that the initiation frequency of XCI is very low in triploid XXY cells, resulting in a mixed population of XaXiY and XaXaY cells. This result indicates that the XCI-activator concentration is just above the threshold level required to initiate XCI and supports the hypothesis that the probability for any X chromosome to be inactivated is determined by the X:autosomal ratio. Analysis of skewing of XCI in F1 hybrid ES cell lines with different Xce's indicates that skewing of XCI correlates with skewed *Xist* expression prior to XCI, suggesting a direct role for differential *Xist* expression in the XCI choice process.

INTRODUCTION

In mammals, dosage compensation of X-encoded genes is achieved by inactivation of one of the two X chromosomes in female cells (Lyon, 1961). X chromosome inactivation (XCI) is initiated early during female development, and results in a transcriptionally inactive X chromosome (Xi), which is clonally propagated through many cell divisions. At the onset of XCI the X-linked non-coding *Xist* gene is transcriptionally up regulated on the future Xi, and coats the Xi in cis (Borsani et al., 1991; Brockdorff et al., 1992; Brown et al., 1991; Brown et al., 1992). *Xist* is required for XCI and most likely attracts chromatin modifying enzymes involved in the silencing process. *Tsix* and *Xite* play a crucial role in suppression of *Xist* accumulation during the early stages of XCI (Lee and Lu, 1999; Ogawa and Lee, 2003). Both *Tsix* and *Xite* are non-coding genes that overlap with, but are transcribed anti-sense to *Xist*.

The first phase of XCI comprises the counting and choice process. In mouse and human, the outcome of the XCI process is random with respect to the parental origin of the X chromosome. Nevertheless, in mice and humans XCI can be skewed towards one of the parental chromosomes. Skewing of XCI has been attributed to differences in the X controlling element (Xce), which overlaps and extends 3' of *Xist* (Cattanach and Isaacson, 1967; Chadwick et al., 2006). In cells were two X chromosomes are present with different Xce alleles, a strong Xce has been associated with a lower probability to be inactivated compared to the X chromosome harboring the weaker Xce.

We have recently shown that XCI counting and choice is a stochastic process, in which every X chromosome in a nucleus has a probability to initiate XCI within a certain time-span (Monkhorst et al., 2008). The probability to inactivate an X chromosome is determined by the X:autosome ratio, and is most likely dependent on two factors that act through *Xist* and *Tsix*; an X-encoded XCI-activator directs *Xist* expression, and is itself inactivated by the XCI process, and an autosomally encoded XCI-inhibitor that suppresses *Xist* by acting on *Tsix*. Early in mouse development or upon differentiation of ES cells the XCI-activator concentration in a cell increases and in female cells will break through a threshold level (set by the XCI-inhibitor) required to generate a probability to initiate XCI. In male cells the XCI-activator concentration is too low to break through the threshold level and therefore these cells only sporadically induce XCI.

Evidence for the presence of the X-linked XCI-activator are the observation that female cells with a heterozygous deletion including *Xist*, *Tsix* and *Xite* still results in initiation of XCI on the wild type X chromosome, indicating a trans acting activator which is low or absent in male cells (Monkhorst et al., 2008). The presence of an activator is supported by the finding that XXXX cells initiate XCI significantly faster than XXXY cells (Monkhorst et al., 2008). An X-linked competence factor required for XCI has previously been proposed to explain the absence of XCI in *Tsix* mutant male cells (Lee and Lu, 1999). Furthermore, studies with stably integrated *Xist* transgenes in differentiating ES cell lines, show significantly more *Xist* expression in female cells compared to male cells, supporting the presence of an X-encoded XCI-activator (Sun et al., 2006).

Triploid cells provide a unique situation for studying the mechanism of XCI counting and choice. In diploid and tetraploid cells, one X chromosome will remain active per diploid genome. In triploid cells this ratio of one active X chromosome per diploid autosomal set cannot be achieved. In attempts to determine the pattern of inactivation in XXY and XXX triploid embryos several studies have been conducted on both human and mouse triploid embryos and embryo-derived cell lines. The majority of cell lines derived from human live born triploids predominantly show two active X chromosomes (Hendriksson et al., 1974; Leisti et al., 1970; Willard and Breg, 1977), except for one report, in which all cultured cells of a human XXX triploid embryo examined had two late-replicating X chromosomes (Fryns et al., 1977). Because all these experiments examined cultured cells interpretation of the data is complicated by the fact that selection processes may have affected the outcome of the XCI process (Gartler et al., 2006). Studies with mouse triploid embryos show that these embryos prefer one active X chromosome (Speirs et al., 1990). Analysis of 10-day-old XXY and XXX triploid embryos showed that most cells had one active X chromosome. In contrast to studies with human cell lines this study describes the direct analysis of embryonic cells. However, also in this study one cannot discriminate between initiation of XCI and cell selection processes.

To explore the influence of the X:autosome ratio on the initiation of XCI, and to dissect the mechanism determining the probability of an X chromosome to be inactivated, we have generated XXY triploid ES cells. Differentiation of these ES cells allows both analysis of initiation of XCI, and cell selection processes. In addition, we have studied skewing of XCI, and analyzed the XCI process on individual X chromosomes in female ES cells with X chromosomes with different Xce's.

RESULTS

Generation of triploid ES cells

Previous studies with tetraploid XXXX, XXXY and XXYY ES cell lines have indicated that the probability for an X chromosome to be inactivated is directly related to the X:autosome ratio. To further explore this finding we generated

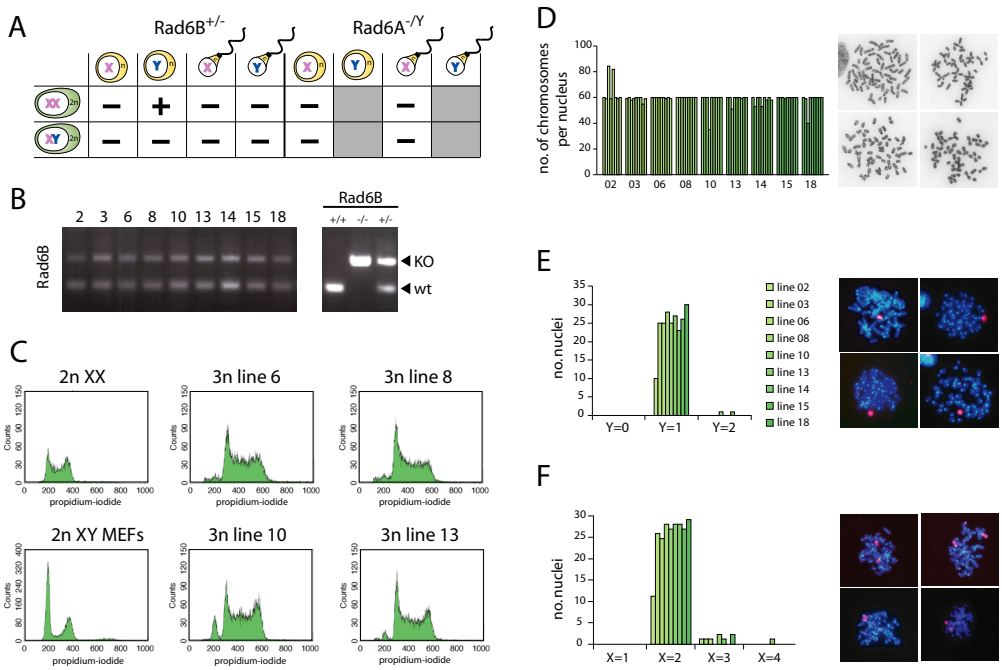

Figure 1. **Generation of triploid ES cells**
A) The different fusion experiments performed, (-) no clones present, (+) clones present which could be picked and expanded. Gray boxes indicate that these clones could not be selected for because of the X linked selection marker. B) PCR on genomic DNA detecting the wild type and mutated Rad6B allele. C) FACS analysis detecting the DNA content of diploid ES cells and feeders, and four different triploid ES cell lines. D) Karyotyping of 9 triploid ES cell lines; shown are chromosome counts of 9 individual methaphase spreads. Right panels show representative examples of metaphase spreads. E) Y chromosome paint analysis; shown is the number of metaphase spreads with 0, 1 and 2 Y chromosomes. F) X chromosome paint analysis; shown is the number of metaphase spreads with 1, 2, 3 and 4 X chromosomes.

triploid ES cell lines because two (XYY and XXY) karyotypes have an X:autosome ratio for which XCI has not been studied before. To generate triploid ES cell lines we fused puromycin resistant female and male ES cells with haploid round spermatids and spermatozoa containing a neomycin resistant gene located in the autosomally linked Rad6B gene or the X-linked Rad6A gene. Both Rad6A and Rad6B encode ubiquitin-conjugating enzymes involved in replicative damage bypass. Fusion of ES cells with round spermatids isolated by staput purification, lacking a functional copy of one of these genes, should have no effect on the fusion or on the hybrid cells thereafter, since only one functional copy of Rad6A or Rad6B is required to generate viable diploid mice. Also, spermatogenesis is not dysregulated in Rad6A homozygous knockout and Rad6B heterozygous knockout mice (Roest et al., 2004).

All PEG mediated fusion experiments were conducted twice. Fusion of the X-encoded Rad6A-neo resistant round spermatids and spermatozoa with female and male ES cells did not result in double resistant colonies (Figure 1A). A similar result was obtained with fusions of Rad6B-neo resistant round spermatids with male ES cells and Rad6B-neo resistant spermatozoa with both female and male ES cells. In contrast, fusion of Rad6B-neo resistant round spermatids with female ES cells resulted in double resistant colonies, which were picked

and expanded for further analysis. PCR analysis of genomic DNA indicated the presence of the mutated Rad6B allele in all the ES clones picked, confirming the fusion of a round spermatid with the Rad6B mutation (Figure 1B). FACS analysis, using propidium iodide, indicated that all our lines were triploid. The small 2n peak we attribute to feeder contamination (Figure 1C). Karyotyping also indicated that the majority of cells have 60 chromosomes in all our cell lines, which are stably maintained through many passages (Figure 1D, and data not shown). Interestingly, X and Y chromosome paint analysis showed that all our cell lines had an XXY 3n karyotype (N=18) although both Y and X containing round spermatids were used for fusion, suggesting that introduction of an X chromosome through a round spermatid leads to an non-viable triploid ES cell.

Our results also indicate that spermatozoa cannot be used for fusion, most likely because of chromatin changes, like protamine incorporation, that cannot be reversed by ES cells. The fact that we do not obtain XYY ES cell lines may indicate that these cells die because of X or Y chromosomal dosage problems. Our finding that triploid ES cells with a XXY karyotype can be generated by PEG mediated fusion suggested that the absence of triploid XXY ES clones after fusion of XY ES cells with mutated Rad6A and Rad6B round spermatids may be due to the presence of an inactive X chromosome. During spermatogenesis the unpaired sex chromosomes are inactivated by a process called meiotic sex chromosome inactivation (MSCI), leading to the transcriptional shut down of X-encoded genes (Turner, 2007). To test whether reactivation of the MSCI mediated Xi was perturbed, we fused round spermatids of males containing an X-linked GFP transgene with male and female ES cells (Hadjantonakis et al., 2001). Analysis of diploid ES cells containing the X-linked GFP transgene shows robust GFP expression, indicating that the transgene is properly expressed in ES cells. In contrast, after fusion of round spermatids with this transgene we did not see reactivation of the transgene (data not shown). Selection for reactivation of the transgene by applying puromycin selection, at day 0, 3 and 5 after fusion did not result in clones resistant to both selection reagents, indicating that ES cells are incapable of reactivating the Xi inactivated by MSCI.

We conclude that triploid ES cells are viable. Nevertheless, we were only able to generate triploid XXY ES cell lines which received an Y chromosome from the fused round spermatid. The absence of ES lines with a XYY or XXX sex chromosome constitution is most likely due to dosage related lethality of triploid XYY ES cells and lethality of fusion lines that received an MSCI inactivated X chromosome respectively.

X chromosome inactivation in triploid ES cells

To study XCI in XXY triploid ES cells we differentiated 9 different triploid XXY ES cell lines into embryoid bodies (EB). Cells were fixed and subjected to RNA-FISH after 3, 5, 7 and 10 days of differentiation, using a *Xist*-specific probe to stain the *Xist*-coated Xi's. After a three-day differentiation period of XXY triploid ES cells we preferentially found cells with zero and one inactive X chromosome, indicating that XCI is induced in XXY triploid cells (Figure 2A). Over time the relative number of cells with a certain inactivation pattern changed. At day 3 of differentiation only 2.5% of cells had one Xi (XaXiY) and sporadically found cells with two Xi's (XiXiY, <0.1%). The distribution changed during differentiation with an increase in the relative number of cells with one Xi, compared to cells with two Xa's (Figure 2B).

To exclude the possibility that the triploid ES cells lost or gained X chromosomes, we performed DNA-FISH with an X chromosome specific BAC probe on triploid ES cells differentiated for eight days. To obtain a reliable measurement, at least 100 nuclei were scored for every cell line. We found that over 93% of the cells analyzed still contained two

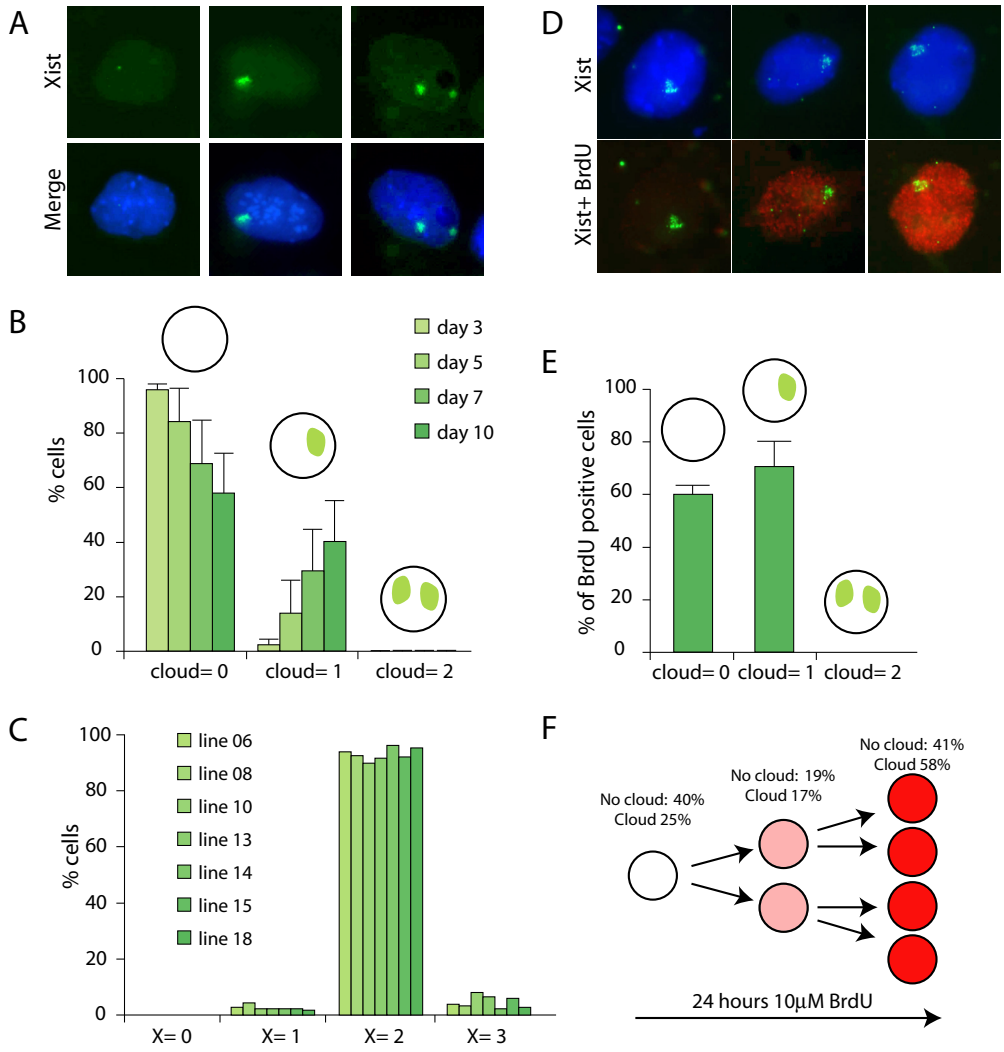

Figure 2. **Analysis of XCI in differentiating triploid ES cells**
A) RNA FISH analysis with an *Xist* specific probe (FITC, DNA in DAPI blue) on day 3 differentiated triploid ES cells, shows cells with no (left panels), one (middle panels) and two (right panels) *Xist* clouds. B) The distribution of cells with different numbers of *Xist* clouds throughout differentiation. C) DNA FISH analysis on day 7 differentiated triploid ES cells; shown is the relative number of cells with 0, 1, 2 and 3 X chromosomes. D) Combined *Xist*-BrdU detection (*Xist* in FITC, BrdU in Rhodamine red, DNA in DAPI blue), indicating the presence of cells with no, intermediate and intense BrdU staining (from left to right). E) Quantification of the *Xist*-BrdU detection; shown are the relative number of BrdU positive cells (intermediate +intense staining cells) with no and 1 *Xist* cloud. F) The relative percentages of BrdU negative, intermediate positive, and intense positive cells with or without *Xist* cloud.

X chromosomes, indicating that a vast majority of cells have maintained the expected number of X chromosomes (Figure 2C). The percentage (~40%) of XaXiY cells at day 7 can therefore not be explained by a gain in the number of X chromosomes (average 3%).

We further examined whether the increase of XiXaY cells in time was the consequence of a cell selection process. We therefore added BrdU 24 hours prior to cell fixation of day 8 differentiated ES cells, and performed immuno/RNA FISH, detecting BrdU positive cells and *Xist* RNA. Our BrdU staining resulted in three different cells, cells without staining, cells staining positive for BrdU, and cells which stained twice as intense as the BrdU positive cells (Figure 2D). We concluded that these double positive cells were cells that went through Sphase twice. Comparison of BrdU positive cells with one or no *Xist* cloud shows that there are significantly more cells with one cloud, indicating a proliferative advantage for these cells.

Our analysis of XCI in differentiating triploid XXY ES cells shows that the probability to initiate XCI on the two X chromosomes is very low. Our data also indicates that XaXiY cells have a growth advantage over XaXaY cells, which explains the continuous increase of XaXiY cells in the population.

Comparison between XCI initiation rate and X:autosome ratio

Previously, we have shown that the probability of an X chromosome to be inactivated is directly proportional to the X:autosome ratio (Monkhorst et al., 2008). To further explore this finding we compared the probability to start XCI of our XXY triploid ES cell lines, with an X:autosome ratio of 0.66 with the probability in cells with different X:autosome ratios (4n XXXX X:A=1, 4n XXXY X:A=0.75, 4n XXYY X:A=0.5, 2n XX X:A=1 and 2n XY X:A=0.5).

To compare the probability for individual X chromosomes to be inactivated in cells with a different number of X chromosomes or X:autosome ratio's we determined the relative

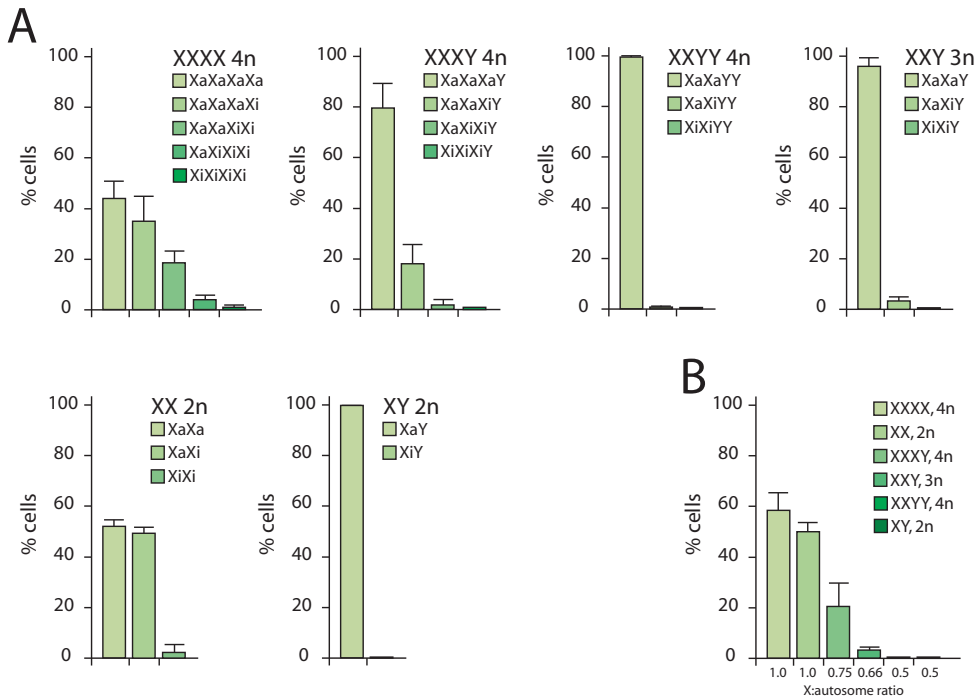

Figure 3. **Comparison of XCI in tetraploid, triploid and diploid cell lines**
A) Determination of the number of Xi's in tetraploid XXXX, XXXY, XXYY (4n), triploid XXY (3n) and diploid XX, XY (2n) ES cell lines after three days of differentiation, as judged by the number of *Xist* clouds. B) The relative number of cells that have initiated XCI, per cell line at day 3 of differentiation

number of cells that initiated XCI at day 3 of differentiation. ES cell lines were differentiated through EB differentiation and subjected to RNA FISH detecting *Xist* RNA. For each line with a different X:autosome ratio, or a different ploidy number we did three independent differentiation experiments.

Our results confirm our previous findings that at day 3 of differentiation XXXX cells have initiated XCI in significantly more cells (58%) than XXXY cells (20%). XXYY cells initiated XCI in less than 0.3% of the cells (Figure 3A). Interestingly, we found that 3-4% of the triploid XXY cells had initiated XCI. This percentage falls between that found for XXXY and XXYY cells, supporting our hypothesis that the probability to initiate XCI depends on the X:autosome ratio (Figure 3B).

Xce strength and initiation of XCI

Although X chromosome inactivation is random with respect to the parental origin of the X chromosome, in many cases XCI is skewed in mouse and human towards one of the parental alleles (Amos-Landgraf et al., 2006; Cattanach and Williams, 1972). In mice, genetic studies have indicated that skewing of XCI is directed by the X-linked X controlling element (Xce). A stochastic model for XCI counting and choice predicts that skewing is caused by allele specific thresholds to the X encoded XCI-activator. We therefore reasoned that alleles with a weak Xce (low threshold) initiate XCI faster than alleles with a strong Xce (higher threshold). To test this we analyzed XCI in differentiating female ES cells with strong Xce^{c} (*Mus. castaneus*) and intermediate weak Xce^{b} (*Mus musculus C57/B6*) alleles, or ES cells with strong Xce^{c} and weak

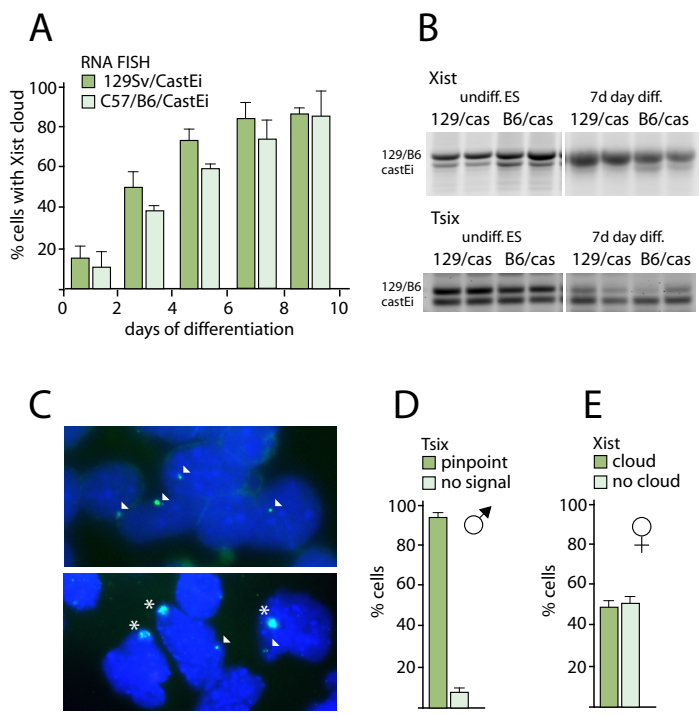

Figure 4. **Xce strength correlates with the probability to initiate XCI**
(A) Two different female F1 129Sv/CastEi and C57/B6/CastEi ES cell lines were differentiated and subjected to *Xist* RNA FISH. The number of cells with *Xist* clouds was determined per time point and the average and standard deviation of the two different lines were plotted. (B) RT-PCR analysis with *Xist* (top) and *Tsix* (bottom) specific primer sets with cDNA isolated from undifferentiated (left panel) and day 7 differentiated (right panel) ES cells. The *Xist* primer set detects a length polymorphism in exon 7, the *Tsix* primer set amplifies a MnlI restriction polymorphism present on the castEi allele. (C) *Xist* RNA FISH (stars) on day three differentiated male (top panel) and female (bottom panel) ES cells. *Tsix* pinpoint indicated by arrowheads. (D) Quantification of the number of *Tsix* signals in three independent male ES cells (J1, V6.5 and E1) at day three of differentiation. (E) Quantification of the number of cells with *Xist* clouds in three independent differentiation experiments of female F1 2-1 ES cells at day three of differentiation.

Xce^a (*Mus musculus 129Sv*) alleles. Indeed, our analysis shows that Xce^c/Xce^b ES cells initiate XCI with a lower velocity than Xce^c/Xce^a ES cells, indicating that the Xce acts through affecting the XCI initiation rate of an allele (Figure 4A).

The difference in strength of Xce alleles could be the result of a different response of the *Xist* promoter to the XCI-activator, the the *Tsix* promoter to the XCI-inhibitor or a combination of both. A previous study has shown that Xce strength is correlated with *Xist* expression after XCI (Brockdorff et al., 1991). Interestingly, RT-PCR analysis with an *Xist* RNA specific primer set indicates that already in undifferentiated Xce^c/Xce^a and Xce^c/Xce^b ES cells *Xist* expression is already skewed towards the weak Xce (Figure 4B). To exclude contamination of differentiating ES cells, we have cultured the ES cells on male feeders in the presence of Mek1 inhibitor and 10x LIF. *Xist* RNA FISH revealed the absence of cells with an *Xist* cloud, confirming that *Xist* expression is already skewed prior to XCI. We found that skewing is more pronounced after differentiation. We attribute this change in skewing of *Xist* expression to the fact that prior to XCI all cells express *Xist* from both alleles. After XCI most cells express *Xist* from the X chromosome with the weak Xce, however expression from this allele is higher than *Xist* expression from a strong Xce, and therefore will enhance skewing of *Xist* expression. In contrast, *Tsix* expression only becomes skewed after the start of XCI, indicating that *Xist* expression and not *Tsix* expression is directly correlated with the probability to be inactivated.

Our finding that *Xist* expression is correlated with skewing of XCI suggests that *Xist* up-regulation results in *Tsix* repression, rather than *Xist* up-regulation being the consequence of autonomous repression of *Tsix*. To test this hypothesis we determined the number of *Tsix* signals in three independent day three differentiated male ES cells and found that 7% of the cells have no *Tsix* signal (Figure 4C and D). In contrast, for female cells 49% of the cells have one X chromosome inactivated. If *Xist* up-regulation would be the consequence of autonomous silencing of *Tsix,* a much lower percentage of 14% of female cells with an *Xist* cloud would be expected (Figure 4C and E). We therefore conclude that *Xist* up-regulation cannot be the sole consequence of down regulation of *Tsix* expression on one X chromosome.

DISCUSSION

We have analyzed XCI in differentiating triploid ES cells and found that triploid ES cells initiate XCI less frequently compared to cells with a higher X:autosome ratio. Nevertheless, cells that do initiate XCI (XaXiY) proliferate faster than XaXaY cells, and slowly accumulate in time. Our studies with female ES cell lines harboring different Xce alleles, indicates that weak Xce's initiate XCI earlier during differentiation than strong Xce's, which explains skewing of XCI as a difference in the probability of individual X chromosomes to start XCI.

Natural and fused triploid ES cells

In this study we have generated triploid ES cells by PEG mediated fusion of diploid ES cells with haploid round spermatids. At present there are no reports mentioning murine triploid ES cell lines, although human triploid ES cells have been described before (Baharvand et al., 2006). Previous attempts to fuse somatic cells with spermatozoa to create somatic triploid hybrid cells to study reactivation of the spermatozoic nucleus, showed only sporadic decondensation of spermatozoic chromatin, indicating that spermatozoa are not suitable as donor of an haploid genome (van Meel and Pearson, 1979). Our study showed that ES cells are also not proficient in reprogramming the haploid genome of spermatozoa. We did generate triploid ES cell lines by fusion of round spermatids, which represent an earlier stage of differentiation, suggesting that reprogramming of the spermatozoan nucleus is most likely hampered by the condensed chromatin and the presence of protamines.

Interestingly, we could only generate triploid ES cells with a XXY karyotype, in which the Y chromosome was donated by the round spermatid. The fact that we could not generate an ES cell line with the same karyotype by fusion of a male ES cell with a round spermatid donating an X chromosome indicates that the donation of a paternal X chromosome results in a triploid cell that is not viable. Although we do not know whether these triploid cells are not viable because of epigenetic modifications that cannot be reversed, fusion experiments of round spermatids with an X-linked GFP transgene, indicate that the paternal X chromosome cannot be reactivated by the ES cell. Because fusion of ES cells with somatic XaXi diploid cells results in proper reactivation of the inactive X chromosome (Hochedlinger Jaenisch Cell Stem Cell 2007) we attribute the absence of reactivation in our triploid ES cells to epigenetic differences between the two X chromosomes laid down during the MSCI and XCI processes. Failure to reprogram the X chromosome may also explain the absence of XXX ES cells after fusion, because previous studies have shown that XXX mouse embryos, similar to XXY embryos, are viable up to E10 (Speirs et al., 1990). The fact that the Y chromosome is also subject to MSCI indicates that this conclusion should be taken with care.

The absence of triploid XYY cells we attribute to the fact that the sex chromosome composition of the resulting hybrid cells is associated with low viability. Although XYY triploid embryos have been observed to occur in nature spontaneously in mouse and human, the observed frequency is much lower than what could be expected (Iliopoulos et al., 2005; Uchida and Freeman, 1985). Why XYY triploidy is associated with early or immediate lethality remains unclear but could be due to X chromosome dosage problems. Interestingly, in differentiating mouse ES cells there is a preference for cells with a single Xa, indicating that this dosage problem is specific for ES cells or early embryonic development.

The need for speed

In a differentiating population of XXY triploid ES cells we found an increasing number of XaXiY cells, which made up 41.0% of the total cell population after 10 days of EB differentiation. The observed increase in XaXiY cells during differentiation indicates that this is the preferred inactivation pattern. This observation is supported by previous in vivo experiments, examining XCI in 10 day old mouse XXY triploid embryos, which showed that 82.9% of the cells were XaXiY (Speirs et al., 1990). Therefore, we conclude that during XCI, mouse triploid cells preferably keep only one of their X chromosomes active.

Previous studies have indicated that the probability to inactivate an X chromosome is directly proportional to the X:autosome ratio (Monkhorst et al., 2008). Based on this observation we have proposed the presence of an X-encoded XCI-activator. The gene encoding this activator itself is inactivated during the XCI process. During differentiation or development the concentration of this XCI-activator will increase and eventually break through a threshold level required to generate a probability (Figure 5A). In cells with a relative high X:autosome ratio, the XCI-activator concentration will break through the threshold level at an earlier time point and will plateau at a higher level (and therefore generate a higher probability) compared to cells with a lower X:autosome ratio. Indeed, experiments with XXYY, XXXY and XXXX tetraploid ES cells showed a significant difference in the number of cells that initiated XCI after three days of differentiation. Despite a similar XCI-activator concentration (taking into account nuclear volume differences between cell with different ploidies; see Chapter 3) less diploid XX than tetraploid XXXX cells initiated XCI after three days of differentiation. We attribute this difference to the different number of X chromosomes that have a probability to initiate XCI. Our observation that after three days of ES cell differentiation 3-4% of XXY triploid ES cells have started XCI provides additional evidence for the hypothesis

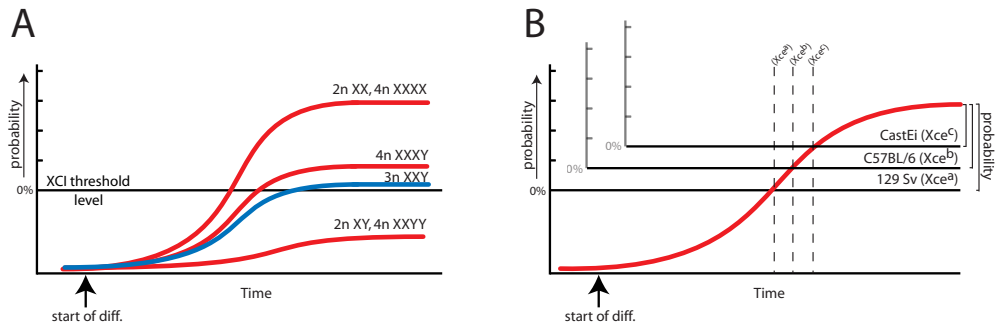

Figure 5. **Changing the threshold level or XCI-activator concentration**
A) The XCI-activator concentration increases after induction of differentiation. In 4n XXYY, and 2n XY cells the concentration is not sufficient to break the threshold level and initiate XCI. In the other cell lines the concentration is high enough to generate a probability. Because the XCI-activator is X-encoded the height of the probability is directly proportional to the X:autosome level. Note that the XCI-activator concentration breaks through the threshold at an earlier time point with an increasing X:autosome ratio. B) In cells with two different Xce's, the XCI-activator concentration will accumulate after induction of differentiation. However, the threshold level that has to be overcome to induce XCI is lower for weak Xce alleles, compared to stronger Xce alleles. Therefore, XCI will be initiated at an earlier time point and with a higher probability on the X chromosome harboring a weak Xce compared to X chromosomes containing stronger Xce's.

that the X:autosome ratio indeed determines the probability to initiate XCI. Our studies also show that the initiation rate for the differentiating XXY triploid ES cells is too low to allow all cells to inactivate one X chromosome, within the time span were XCI can be initiated.

Xist expression correlates with skewing of XCI

Skewing of XCI is attributed to differences in strengths between different Xce alleles, of which four have been described in mice ($Xce^{a,b,c,d}$) (Cattanach et al., 1969; Johnston and Cattanach, 1981; Simmler et al., 1993; West and Chapman, 1978). In F1 hybrid mice the Xce^{a} allele is the weakest allele with the highest probability to be inactivated, whereas Xce^{d} is the strongest allele with the lowest probability to be inactivated. Analysis of F1 ES cell lines with combinations of different Xce alleles, showed that the velocity with which XCI is initiated is directly related to the Xce strength. We attribute this difference in initiation of XCI to the different probabilities associated with different Xce's. Although within a cell the same concentration of XCI-activator is present different Xce alleles have different threshold levels required to initiate XCI and therefore will have different probabilities per allele (Figure 5B). For instance, Xce^{c} alleles will require more XCI-activator than Xce^{a} and Xce^{b} alleles, and will therefore break through the threshold at a later time point and initiate XCI with a lower probability.

A recent study indicated that the 1.8 Mb minimum Xce interval includes both *Xist* and *Tsix* (Chadwick 2006). Interestingly, *Xist* expression levels in adult inbred mice have previously shown to be directly proportional to Xce strength (Brockdorff 1991). Our findings show that this is true already before the onset of XCI, in contrast to *Tsix* expression, indicating that *Tsix* provides a stable threshold level and supports our hypothesis that the XCI-activator acts through *Xist*. We cannot exclude a possible role for *Tsix* in skewing of XCI since *Tsix* deletions or persistent expression of *Tsix* result in completely skewed XCI (Lee 1999, Luikenhuis 2001). Nevertheless, in wild type mice the Xce effect seems to act through *Xist* rather than *Tsix*. We conclude that the strength of the Xce

is the resultant of the strength of the *Xist* and *Tsix* promoters. SNPs or mutations that affect expression of either gene will therefore result in a stronger or weaker Xce.

Models for XCI counting and choice

Recently, we have proposed a stochastic model for XCI counting and choice, in which every X chromosome has a probability to be inactivated within a given time span (Monkhorst et al., 2008). This model predicts the presence of an X-encoded XCI-activator and -inhibitor. The XCI-activator promotes *Xist* expression and its transcription itself is inhibited by XCI, whereas the XCI-inhibitor promotes *Tsix* expression thereby generating a threshold, which has to be overcome by *Xist*. In the past, several other models have been proposed to explain XCI counting and choice. A blocking factor model predicts the presence of an autosomally encoded factor or nuclear entity of which one is present in the diploid nucleus, preventing inactivation of one X chromosome (Rastan 1983). The symmetry-breaking model postulates that the blocking factor is self-assembled out of diffusible molecules (Nicodemi 2007). XCI counting and choice has also been explained by transient interactions between the X chromosomes (Bacher 2006, Xu 2006 and 2007, Augui 2007). For this model different outcomes of XCI in XXXX diploid cells (XiXiXiXa) and XXXX tetraploid cells (XiXiXaXa) suggest the presence an autosomal component involved in blocking XCI.

Our results after a 3-day differentiation period of the different tetraploid cell lines show that XCI counting and choice works properly in XXYY cells. Nevertheless, for the XXXY and XXXX cell lines we find many cells that initiated XCI on the wrong number of X chromosomes. A blocking factor model cannot explain these results because the two blocking factors that properly silence both X chromosomes in XXYY cells should have done the same in XXXY and XXXX cells. Nevertheless, these results could be explained if the blocking factor is assembled out of a limiting amount of molecules as predicted by the symmetry-breaking model, which are titrated out with an increasing number of X chromosomes. Comparison of XCI in diploid XX cells with triploid XXY cells argues against this possibility. Despite the fact that the concentration of the molecules making up the blocking factor is the same in both cell lines, we found a much lower number of triploid XXY cells that initiated XCI at day 3 compared to diploid XX cells.

A stochastic model explains the difference in frequency of initiation of XCI at day 3 of differentiation between cell lines with different X:autosome ratios, as a consequence of different probabilities to initiate XCI. In this model the probability is determined by the X:autosome ratio. The X:autosome ratio of 0.66 for our XXY triploid cells is lower than the 0.75 for XXXY, and 1.0 for XXXX tetraploid cells, which explains the low frequency of cells that initiate XCI. Our results with the triploid XXY lines therefore confirm previous findings that the probability is directly proportional to the X:autosome ratio, and further support the presence of an X-encoded XCI-activator. Characterization of these probability determining factors will be essential for understanding the XCI counting and choice process.

MATERIALS AND METHODS

Culture and differentiation of ES cells

ES cells were cultured with 15% heat inactivated foetal calf serum, 100 U ml-1 penicillin, 100 mg ml-1 streptomycin, non-essential amino acids, 1000 U/ml leukaemia inhibitory factor (LIF) and 0,1mM β-mercaptoethanol. ES cells were grown on a layer of male mouse embryonic fibroblast (MEF) feeder cells. To induce differentiation into EBs, ES cells were pre-plated for 60 minutes and non-adherent ES cells were transferred to non-gelatinized bacterial culture dishes without feeder cells in differentiation medium, IMDM Glutamax, 15% heat inactivated foetal calf serum, 50 µg/ml ascorbic acid, 100 U ml-1 penicillin, 100 mg ml-1 streptomycin, 37.8 µl/l monothioglycerol.

Staput isolation of round spermatids

Testes from 2 homozygous Rad6A mutant mice and 2 Rad6B heterozygous mutant mice were excised and decapsulated to remove the tunica albuginea. Decapsulated testes were pooled in 20ml PBS (140 mM NaCL, 3mM KCl, 1.5 mM KH2PO, 8 mM NaH2PO4) / 1.1 mM Ca2+/ 0.5 mM Mg2+/ 12 mM lactate (Sigma-Aldrich) of 34°C, containing 10mg hyaluronidase (from ovine testes, Roche-Diagnostics), 20mg trypsin (from bovine pancreas, Roche-Diagnostics) and 20 mg collagenase A (Roche-Diagnostics). Testes were shaken for 20 minutes at 90 rpm with 10 mm amplitude to release seminiferous tubuli from interstitial cells. Tubuli were collected by centrifugation for 3 minutes at 2000 rpm and resuspended in 34°C PBS/ 12mM lactate. After shaking 10 minutes at 120 rpm with 10 mm amplitude to release germinal cells from the tubuli, tubuli remnants were removed. Germinal cells were collected by centrifugation and resuspended in 34°C PBS/ 1.1 mM Ca2+/ 0.5 mM Mg2+/ 12mM lactate. The cell suspension was filtrated using a 60 µm filtration cloth. Germinal cells were collected by centrifugation and resuspended in 50 ml PBS/ 1.1 mM Ca2+/ 0.5 mM Mg2+/ 12mM lactate/ 0.5% w/v BSA. Cells were separated by sedimentation velocity at unit gravity in a 1-4% w/v BSA gradient at room temperature. First 20 ml PBS/ 1,1 mM Ca2+/ 0.5 mM Mg2+/ 12mM lactate was bottom-loaded in a chamber, followed by 50 ml cell suspension. A BSA gradient was created by loading a total of 500 ml of 1%, 2% and 4% w/v BSA in PBS. Cells were allowed to sediment for 2 hours. Chamber was emptied in 8 ml fractions using a fraction collector. Fractions were identified using a 340 nm UV light source. Fractions containing round spermatids were pooled, collected by centrifugation and resuspended in PBS/ 1.1 mM Ca2+/ 0.5 mM Mg2+/ 12mM lactate.

Purity of cell fractions derived by this procedure were previously shown to be >90% as determined by microscopic analysis of an aliquot of purified cells fixed in Bouins' fixative on glass slides (Baarends et al., 2003).

Fusion experiments

Mus musculus castaneus/ 129/Sv F1 (F1-2 1) female and C57Bl6/ 129/Sv (V6.5) male ES cell-lines were separated from MEF feeder cells by trypsinizing and preplating for 45 minutes on uncoated culture dishes. PEG1500 fusion was performed according to the manufacturer's instructions (Invitrogen). Briefly, 4•106 cells were combined briefly with 4•106 round spermatids in DMEM. After centrifugation cells were resuspended in 300µl 50% PEG 1500 and incubated for 2 minutes at 37°C under continuous stirring. The mixture was gradually diluted with serum containing medium and plated on drug-resistant MEF feeder cells. After 24 hours medium was replaced with medium containing 0.3 µg/ml neomycin and 2 µg/ ml puromycin. After nine days, individual ES cell colonies were picked, trypsinized and plated on individual culture dishes in neomycin and puromycin containing medium.

Karyotyping

ES cells were blocked in metaphase by incubation in medium containing 0.12 µg/ml karyomax/colcemid for 1 hour. Cells were trypsinized and resuspended in 5 ml 0.075 M KCl at 37°C, collected and resuspended in 0.0625M KCl/ 12.5% methanol/ 4.17% acetic acid. Cells were fixed by washing three times in 75% methanol/ 25% acetic acid and stored in 200µl at 4°C. The fixed cell suspension was spotted on ethanol cleaned slides and air dried. For determining the total number of chromosomes slides were mounted with 20 µl Dapi vectashield. For determining the number of X chromosomes slides were denatured by incubating three minutes at 80°C in 100µl 50% formamide/ 2x SSC/ 10mM phosphate buffer. Subsequently slides were dehydrated, and hybridised overnight at 37°C with a Cy3 labelled X-paint probe (Cambio). After hybridisation slides were washed once with 2xSSC at 45°C, three times with 2xSSC/ 50% formamide at 45°C and two times with PBS. Slides were air-dried and mounted with 20 µl dapi vectashield. For determining the number of Y chromosomes, Y-chromosome paint (Cambio) was applied, following the same protocol as for X chromosome paint.

RNA FISH analysis

One day prior to fixation, non-adherent EBs were trypsinized and differentiated ES cells were grown on gelatin-coated cover slips. Cells were rinsed once with PBS and permeabilized by successive incubation in cytoskeletal buffer (100 mM NaCl, 300 mM sucrose, 3 µM MgCl2, 10 mM PIPES pH 6.8 in H2O) for 30 seconds, cytoskeletal buffer containing detergent (0.5% triton X-100, 100 mM NaCl, 300 mM sucrose, 3 µM MgCl2, 10 mM PIPES pH 6.8 in H2O) for 2 minutes and cytoskeletal buffer for 30 seconds. Cells were fixed in 4% paraformaldehyde/PBS for 10 minutes, rinsed three times with 70% ethanol and stored in 70% ethanol at 4°C.

The *Xist* probe was a digoxygenin labelled 5.5 kb cDNA sequence. To suppress repetitive sequences 25 µg/ml mouse Cot1 DNA was added and probe mixture was incubated at 95°C for 5 minutes and at 37°C for 45 minutes. After overnight hybridisation at 37°C, slides were washed in 2xSSC at 37°C for 5 minutes, and three times in 50% formamide/ 2xSSC at 37°C for 10 minutes. Probe detection was performed at room temperature. Detection was with a sheep anti-digoxigenin antibody (Roche diagnosics), followed by a FITC labelled rabbit anti-sheep antibody (Jackson labs) and a FITC labelled goat anti-rabbit antibody (Jackson labs), each for 30 minutes, in 100 mM Tris pH 7.5/ saline/ Tween, BSA. After detection cover slips were dehydrated and mounted on a slide in Vectashield and DAPI to counter stain DNA.
For determining the number of inactive X chromosomes in a cell, first a non-overlapped intact nucleus was selected in DAPI, and then in FITC the number of *Xist* clouds were scored. For early timepoints, cells were scored negative for a cloud only if two distinct pinpoint signals could be observed.

BrdU analysis

For BrdU analysis, differentiated ES cells of trypsinized non-adherent EBs were grown on gelatin-coated cover slips in the presence of 20 µM BrdU, and fixed as described in the RNA FISH section. Cover slips were dehydrated, air-dried and denatured in 70% formamid/ 2x SSC/ 50mM phosphate for 3 minutes at 85°C. Coverslips were washed in ice cold 70% ethanol and through 70%, 90% and 100% ethanol washes and air dried after which the *Xist* probe was applied. Detection of *Xist* RNA was as described in the previous section, detection of BrdU was with a mouse monoclonal BrdU antibody (DAKO), followed by a rhodamin labelled donkey anti-mouse antibody (Jackson labs), 30 minutes incubation each.

To determine the number of BrdU labelled cells for the XaXaY and XaXiY cell populations, first in FITC a microscope field was selected containing one or more intact nuclei with a *Xist* cloud. Then, for the number of cells containing a *Xist* cloud with negative, intermediate and highly positive BrdU staining was determined. Subsequently this was also done for all cells without a *Xist* cloud in the same microscopic field.

DNA FISH analysis

For DNA-FISH cells were fixed as for RNA FISH, and pretreated for 4 min with 0.5% pepsin in 10mM HCl at 37°C, post fixed for 5 minutes in 4% paraformaldehyde/PBS, washed twice with PBS and dehydrated prior to denaturation. Denaturation of target sequences was as described in the BrdU analysis section. Cover slips were incubated with a combination of two biotin-labelled BACs (CT7-155J2 and CT7-474E4) at 37°C overnight. BACs were detected using mouse anti-biotin (Roche diagnostics) and donkey anti-mouse (Jackson labs) as described for RNA-FISH. For determining the number of X chromosomes first in dapi non-overlapping nuclei were selected and then in rhodamin red the number of FISH spots was determined.

REFERENCES

Amos-Landgraf, J. M., Cottle, A., Plenge, R. M., Friez, M., Schwartz, C. E., Longshore, J., and Willard, H. F. (2006). X chromosome-inactivation patterns of 1,005 phenotypically unaffected females. Am J Hum Genet 79, 493-499.

Baarends, W. M., Wassenaar, E., Hoogerbrugge, J. W., van Cappellen, G., Roest, H. P., Vreeburg, J., Ooms, M., Hoeijmakers, J. H., and Grootegoed, J. A. (2003). Loss of HR6B ubiquitin-conjugating activity results in damaged synaptonemal complex structure and increased crossing-over frequency during the male meiotic prophase. Mol Cell Biol 23, 1151-1162.

Baharvand, H., Ashtiani, S. K., Taee, A., Massumi, M., Valojerdi, M. R., Yazdi, P. E., Moradi, S. Z., and Farrokhi, A. (2006). Generation of new human embryonic stem cell lines with diploid and triploid karyotypes. Dev Growth Differ 48, 117-128.

Borsani, G., Tonlorenzi, R., Simmler, M. C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., and et al. (1991). Characterization of a murine gene expressed from the inactive X chromosome. Nature 351, 325-329.

Brockdorff, N., Ashworth, A., Kay, G. F., Cooper, P., Smith, S., McCabe, V. M., Norris, D. P., Penny, G. D., Patel, D., and Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. Nature 351, 329-331.

Brockdorff, N., Ashworth, A., Kay, G. F., McCabe, V. M., Norris, D. P., Cooper, P. J., Swift, S., and Rastan, S. (1992). The product of the mouse *Xist* gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell 71, 515-526.

Brown, C. J., Ballabio, A., Rupert, J. L., Lafreniere, R. G., Grompe, M., Tonlorenzi, R., and Willard, H. F. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. Nature 349, 38-44.

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

Cattanach, B. M., and Isaacson, J. H. (1967). Controlling elements in the mouse X chromosome. Genetics 57, 331-346.

Cattanach, B. M., Pollard, C. E., and Perez, J. N. (1969). Controlling elements in the mouse X-chromosome. I. Interaction with the X-linked genes. Genet Res 14, 223-235.

Cattanach, B. M., and Williams, C. E. (1972). Evidence of non-random X chromosome activity in the mouse. Genet Res 19, 229-240.

Chadwick, L. H., Pertz, L. M., Broman, K. W., Bartolomei, M. S., and Willard, H. F. (2006). Genetic control of X chromosome inactivation in mice: definition of the Xce candidate interval. Genetics 173, 2103-2110.

Fryns, J. P., van de Kerckhove, A., Goddeeris, P., and van den Berghe, H. (1977). Unusually long survival in a case of full triploidy of maternal origin. Hum Genet 38, 147-155.

Gartler, S. M., Varadarajan, K. R., Luo, P., Norwood, T. H., Canfield, T. K., and Hansen, R. S. (2006). Abnormal X: autosome ratio, but normal X chromosome inactivation in human triploid cultures. BMC Genet 7, 41.

Hadjantonakis, A. K., Cox, L. L., Tam, P. P., and Nagy, A. (2001). An X-linked GFP transgene reveals unexpected paternal X-chromosome activity in trophoblastic giant cells of the mouse placenta. Genesis 29, 133-140.

Hendriksson, P., Hakansson, L., and Sandahl, B. (1974). A live-born triploid infant. Acta Paediatr Scand, 447-449.

Iliopoulos, D., Vassiliou, G., Sekerli, E., Sidiropoulou, V., Tsiga, A., Dimopoulou, D., and Voyiatzis, N. (2005). Long survival in a 69,XXX triploid infant in Greece. Genet Mol Res 4, 755-759.

Johnston, P. G., and Cattanach, B. M. (1981). Controlling elements in the mouse. IV. Evidence of non-random X-inactivation. Genet Res 37, 151-160.

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

Leisti, J., Raivo, K. O., Rapola, J., Saksela, E., and Aula, P. (1970). Triploidy (69, XXY) in a newborn infant: a new syndrome in man? Scand J Clin Lab Invest 25, 60.

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

Monkhorst, K., Jonkers, I., Rentmeester, E., Grosveld, F., Gribnau, J. (2008). X inactivation counting and choice is a stochastic process: evidence for involvement of an X-linked activator. Cell 132, 410-421.

Ogawa, Y., and Lee, J. T. (2003). *Xite*, X-inactivation intergenic transcription elements that regulate the probability of choice. Mol Cell 11, 731-743.

Roest, H. P., Baarends, W. M., de Wit, J., van Klaveren, J. W., Wassenaar, E., Hoogerbrugge, J. W., van Cappellen, W. A., Hoeijmakers, J. H., and Grootegoed, J. A. (2004). The ubiquitin-conjugating DNA repair

enzyme HR6A is a maternal factor essential for early embryonic development in mice. Mol Cell Biol 24, 5485-5495.

Simmler, M. C., Cattanach, B. M., Rasberry, C., Rougeulle, C., and Avner, P. (1993). Mapping the murine Xce locus with (CA)n repeats. Mamm Genome 4, 523-530.

Speirs, S., Cross, J. M., and Kaufman, M. H. (1990). The pattern of X-chromosome inactivation in the embryonic and extra-embryonic tissues of post-implantation digynic triploid LT/Sv strain mouse embryos. Genet Res 56, 107-114.

Sun, B. K., Deaton, A. M., and Lee, J. T. (2006). A transient heterochromatic state in *Xist* preempts X inactivation choice without RNA stabilization. Mol Cell 21, 617-628.

Turner, J. M. (2007). Meiotic sex chromosome inactivation. Development 134, 1823-1831.

Uchida, I. A., and Freeman, V. C. (1985). Triploidy and chromosomes. Am J Obstet Gynecol 151, 65-69.

van Meel, F. C., and Pearson, P. L. (1979). Do human spermatozoa reactivate in the cytoplasm of somatic cells? J Cell Sci 35, 105-122.

West, J. D., and Chapman, V. M. (1978). Variation for X chromosome expression in mice detected by electrophoresis of phosphoglycerate kinase. Genet Res 32, 91-102.

Willard, H. F., and Breg, W. R. (1977). Two highly synchronous, early-replicating X chromosomes in fibroblasts from a live-born 69, XXY triploid infant. Am J Hum Genet 29.

Chapter 5

Simulated X Chromosome Inactivation

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Chapter 5

Simulated X chromosome Inactivation

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INTRODUCTION

A stochastic model explains XCI counting and choice by attributing a probability to each X chromosome in a nucleus to be inactivated. Cell line studies indicate that the *Xist*, *Tsix*, and *Xite* genes play a key role in determining this probability to initiate XCI.

Data presented in Chapter 3 suggests that the probability to initiate XCI on an X chromosome is the resultant of the balance between an X-encoded *Xist* activator (that gene itself is inactivated by XCI) and a *Tsix* activator (XCI-inhibitor). Upon differentiation, the concentration of the XCI-activator rises. In contrast, the XCI-inhibitor concentration remains stable or may even decrease in time, providing a stable threshold level throughout early differentiation or development, which has to be overcome to generate a probability to initiate XCI. In male cells the maximum XCI-activator concentration is not sufficient to overcome the XCI threshold level. In female cells the concentration of the XCI-activator will be twice as high and sufficient to induce *Xist* mediated silencing of *Tsix* with a certain probability in a particular time frame. Because both X chromosomes generate a certain probability a proportion of the differentiating cells will inactivate two X chromosomes. After XCI has been initiated, the X-linked XCI-activator gene will be silenced in cis. This results in a drop in the XCI-activator level to a level equal to that found in male cells, preventing inactivation of the second X chromosome. Cells that have not initiated XCI will start another round of inactivation.

This model predicts the emergence of three different outcomes of the process in female diploid cells; cells with two active X's, cells with one active and one inactive chromosome, and cells with two inactive X chromosomes. The latter die because they are functionally nullisomic for the X chromosome and are thus selected against. For diploid XX cells it is imaginable how the XCI process would take effect in time, although it is already hard to envisage how the distribution of different cell states (XaXa's, XiXa's and XiXi's) will take shape after several rounds of XCI and cell selection. Matters become even more complicated if one tries to implement skewing of XCI as is found in many F1 hybrids or tetraploid cells to these calculations. Therefore computer simulated XCI would be very helpful, so that different probabilities can be explored (or even probability curves) for different cell ploidies without having to calculate manually. It will help to test whether a stochastic model is feasible and applicable to different types of cells (XX, XY, XXY, XXXY and XXXX) with the same or mixed genetic background and even in cells with reported XCI related mutations. We can do simulations for a wide number of parameters and pertubations and we can make predictions that can be tested experimentally to corroborate our hypothesis.

PARAMETERS OF THE MODEL

Basically there are three important parameters for a stochastic model; cell division characeristics, the 'time frame' that harbors a defined probability and the probability itself.

Cell division

Does the length of the cell cycle matter in simulating the XCI process? In relation to the distribution of the different outcomes of the XCI process, it does. Since cells with two inactive X chromosomes do not divide, the relative amount of cells with two inactive X chromosomes is dependent on how often the cells that are still proliferative, divide. Every division of XaXa and XiXa cells will cause the XiXi cells to become a smaller fraction of the total population. The cell division rates of XaXa and XiXa cells do not chance. Unfortunately we do not know exactly how fast differentiating ES cells divide but counts of cell numbers led us estimate it to be around once per day on average.

The probability time frame

The probability time frame, also determines the amount of double clouds. A 10% probability to initiate XCI in a certain time frame will result in 1% double clouds. So, two time frames will result in 2% double clouds. In contrast, if two time frames are taken together, a 20% probability will result in 4% double clouds. If an X chromosome has been inactivated in a particular time frame, the levels of the X-linked XCI activator should adjust to the new X active:ploidy ratio before a new time frame starts. So, the length of the time frame is determined by the turnover of the XCI activator. Unfortunately, the activator remains to be identified and we do not know the stability of the RNA encoding the activator or the stability of the protein. However, experiments with inducible *Xist* transgenes flanked by a puro resistance gene indicated that puro sensitivity emerged around 22 hours in undifferentiated ES cells (Wutz and Jaenisch 2000). Since doxycyline induction of *Xist* also requires several hours it is reasonable to assume that it takes less than a day for the XCI activator to be down regulated by XCI and we assume the time frame of one 'round of XCI' to be around 1 day.

The probability

The probability to initiate XCI is an independent probability for each X chromosome and can thus be different between two X chromosomes within one female XX cell. The XCI activator does not induce XCI in male cells because its concentration is too low to overcome a certain threshold. In female cells the concentration is sufficient to overcome this threshold and causes XCI initiation. The probability therefore can be interpreted as an XCI activator signal that has to overcome a critical threshold. The difference between the XCI activator signal and the threshold determines the probability (Figure 2 upper right panel)

Can the cell cycle and the time frame be synchronous?

The XCI time frame is determined by the speed the nuclear/cellular XCI activator concentration is degraded. Passage through S-phase may result in a drop in the concentration of the XCI activator because the volume of a G2 nucleus is twice that of a G1 nucleus. Since the XCI activator acts dosage dependent, in a dividing XiXa cell the activator level will drop to levels comparable to XY cells (within one cell division). So cell division could provide the end of a 'round of XCI' and is a candidate for the time frame in which one round of XCI takes place. We therefore synchronized the cell cycle and the time frame in our computer simulation and set them both to 1 day. Identification of the XCI activator and its turnover characteristics together with data investigating the cell division characteristics in differentiating cells will shed more light on this issue in the future.

LETTING THE COMPUTER DO THE CALCULATIONS

Each allele has an independent probability to initiate XCI. One can calculate the outcome of every round of XCI but this does not truly represent a biological process. When X chromosomes are inactivated with a certain independent probability this results in three subpopulations of cells. The relative distribution of these subpopulations will vary depending on the initial number of cells. More cells will result in less variation during the XCI process. To simulate this natural variation the computer randomly assigns a state ('on' or 'off') to an X chromosome, with a certain probability. The outcome will be slightly different every time you run the simulation. The more cells are in the simulation, the less variability in the distribution (like in in-vivo experiments). In our computer model we use 100 cells because, first, it mimics the number of cells in the embryo proper around the time of initiation of XCI and second because the variability of the outcome of our simulations tends to stabilize around this number.

The way a randomizer 'randomizes' a population in different subpopulations is as follows. The computer generates a very large amount of white and red balls in specified distribution e.g. 15% are red and 85% is white. Then 100 balls are blindly picked. The more balls are picked, the more the distribution of the cells picked will resemble the original distribution of the balls. From now on 'the original distribution' (in this case 15%) is called the probability of an X chromosome to inactivate.

If applied to the two X chromosomes in 100 cells (so 200 balls are picked), one will end up with populations of cells with XaXa, XiXa and XiXi. The distribution of those populations is dependent on the probability specified for an X chromosome to inactivate. A low probability will result in low amounts of XiXi cells; a high probability will result in more XiXi cells.

RESULTS & DISCUSSION

Simulating XCI

Figure 1 demonstrates how our model for XCI works with a calculated 10% probability (non randomized) per day in a differentiating population of 100 cells for 3 rounds of XCI. Every X chromosome will have a 10% probability to become inactivated. There are two X chromosomes in every cell so there will be 0.9 * 0.9 cells that have no inactivated X chromosome (XaXa), 0.1 * 0.1 cells will have two inactive X chromosomes (XiXi) and 2*(0.9 * 0.1) will have one active and one inactivated X chromosome (XiXa). XiXi cells will die. XiXa cells will stop initiating XCI because of the drop in concentration of the XCI activator. XaXa cells will initiate XCI in the next round of XCI. The cells divide every day (Figure 1). If we run the simulation for 5%, 10%, 20%, 30% 40% and 50% with randomized probability per round of XCI and compare it to our experimental data it becomes clear that the probability must be between 10% and 20% per X chromosome in a diploid XX cell. (Figure 2)

Our experimental data however, suggests that the probability at the start of XCI is lower than in later rounds (compare 10% and 20% probability with experimental data in Figure 2). As expected if the probability is proportional to an increase in the concentration of the activator of this process, we made use of a probability curve to make the simulated data fit the experimental data. For a diploid XX cell the following probabilities in our simulation did fit the experimental data: 6%, 11%, 15%, 17% and 18% at day 1, 2, 3, 4, and 5-10 respectively (Figure 3). We picked these particular percentages because they follow a curve. Taken together, a curve representing the probability can simulate experimental XCI data in female diploid cells. Figure 3 displays this curve with a certain threshold, which is the same for both alleles. The probability is read from the graph by subtracting the threshold from the probability curve.

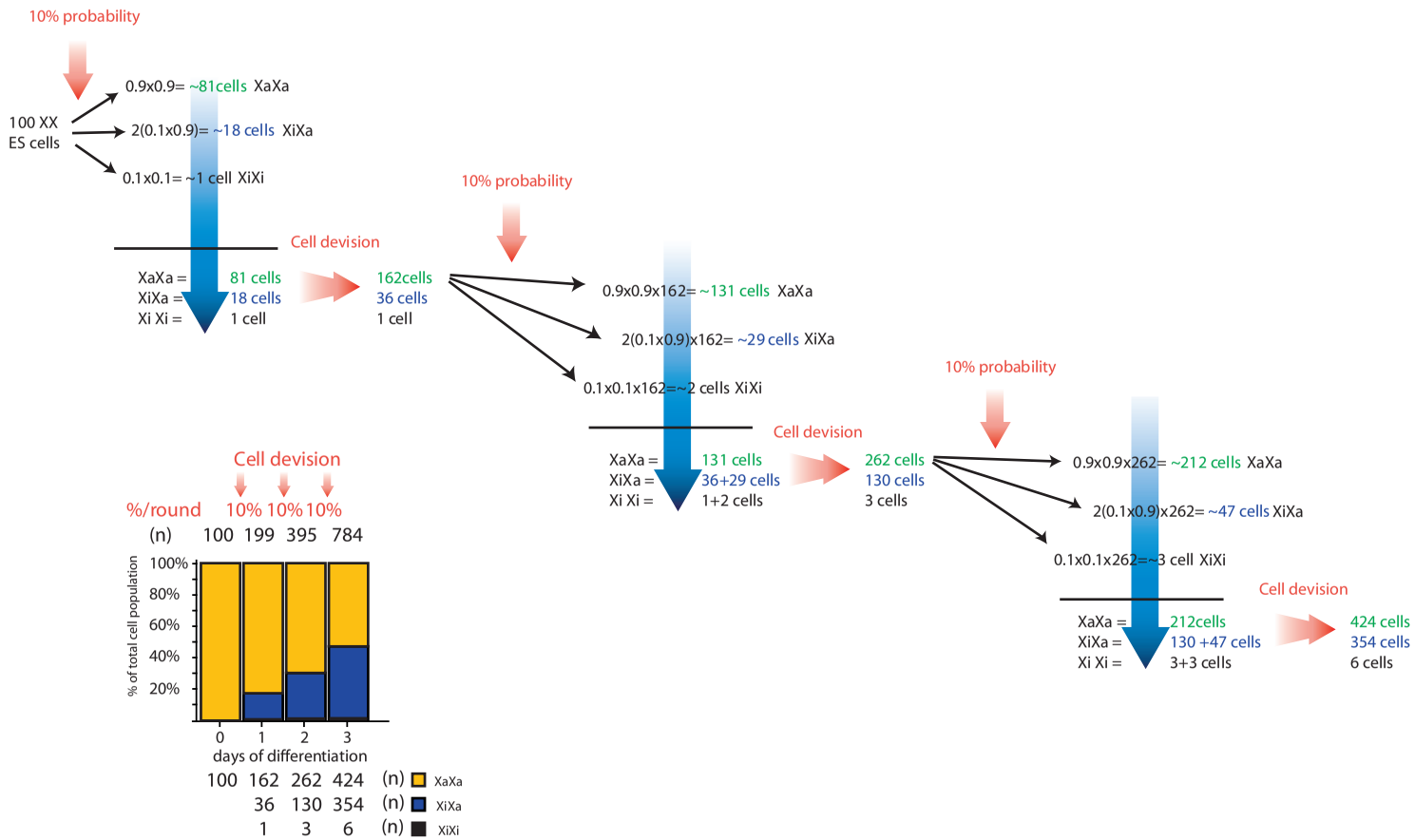


Figure 1. **Flow chart of the stochastic model fot three rounds of XCI**
100 female cells start XCI with a 10% probability per X chromosome to initiate XCI. The 81 XaXa cells will procede to the next round and the 18 XiXa cells stop initiating XCI. All cells divide after the first round of XCI except the XiXi cells. The relative distributions of the different outcomes of the XCI process (XaXa, XiXa, and XiXi cells) are shown in the lower left corner in a bar-graph.

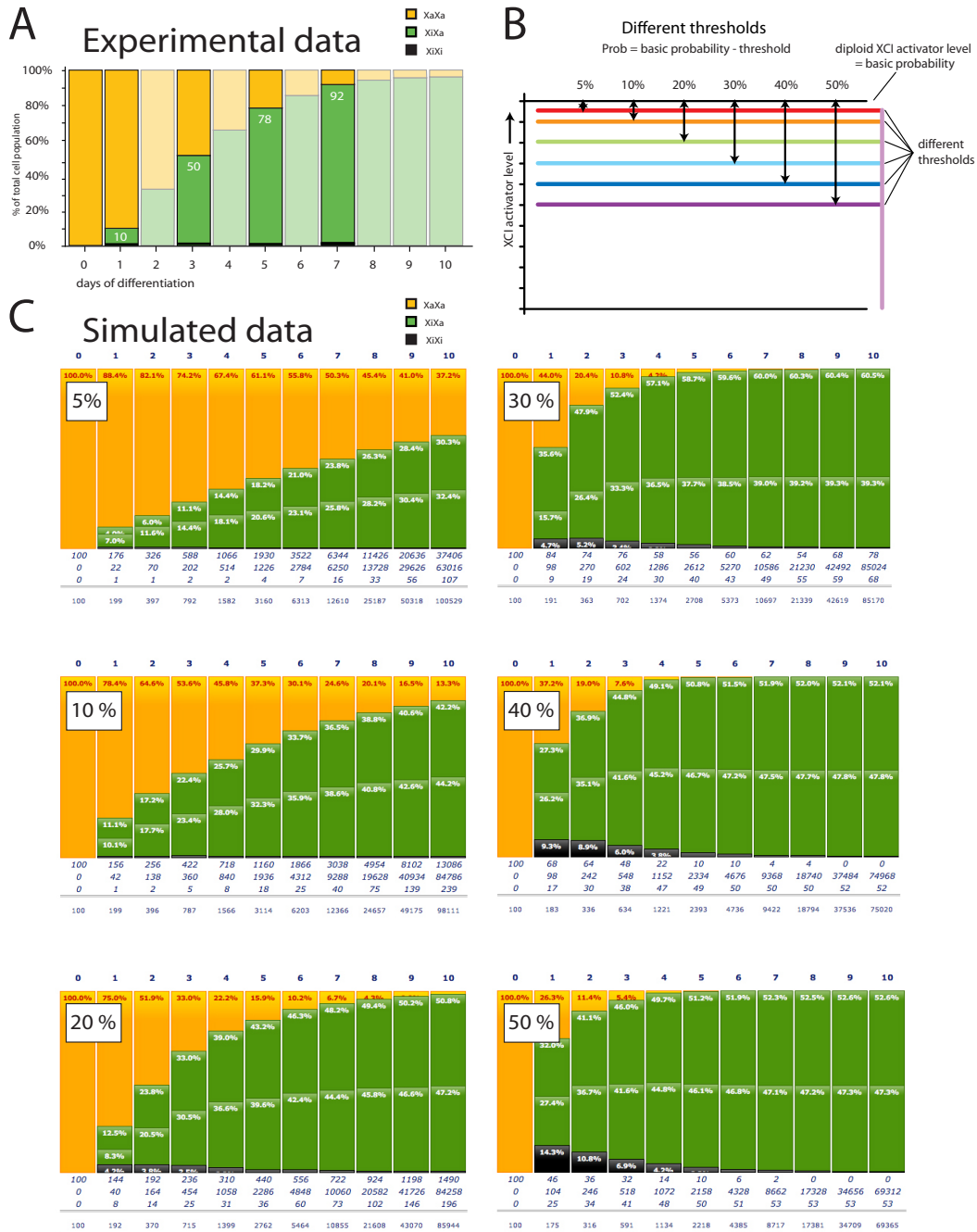


Figure 2. **XCI simulation with 5%, 10%, 20%, 30%, 40% and 50% probability per X chromosome**
The different bar-graphs show the relative distribution of the three different cells (XaXa, XiXa, XiXi) that result from the XCI process. (A) Experimental data from differentiated female XX ES cells. (B) Different probabilities are presented as different threshold for the same level of XCI activator. (C) Simulated data indicates that the probability must be between 10% and 20%. Numbers above the bar graphs indicate days of differentiation (1-10)

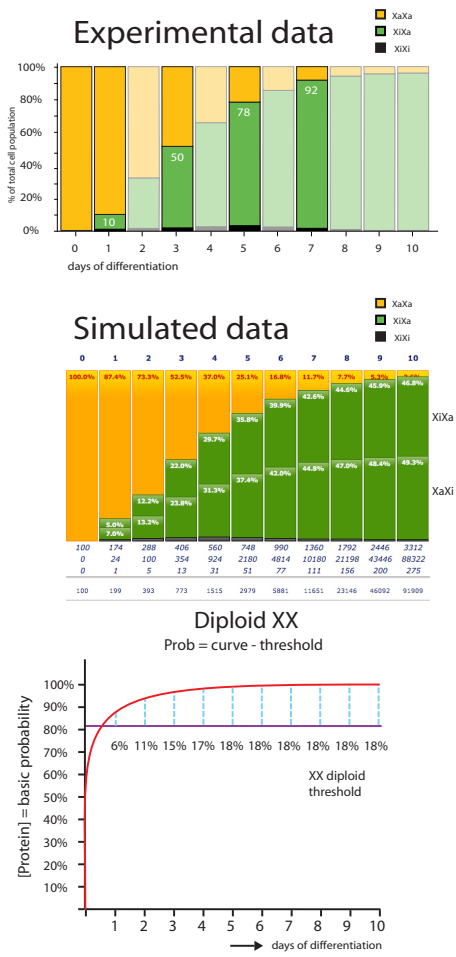


Figure 3. **A probability curve simulates the experimental data**
Experimental and simulated data for female XX cells. The experimental data is most accurately simulated by a probability curve. The two X chromosomes in these cells have the same probabilities. This is reflected by the 50/50 distribution of XiXa and XaXi cells (green part of the bars).

The probabilities for initiation of XCI are likely to form a curve in time

The evidence presented in the previous chapters strongly indicates an X-linked XCI activator. Because this is most likely a protein that is developmentally regulated, the activator concentration will rise in time and reach steady state levels depending on the protein's kinetics. Furthermore, protein-effect relations often follow a sigmoid curve (Hill coefficient is > 1). So, at low concentrations the protein-effect relation is not linear. If we want to apply probabilities to Xic's on X chromosomes, it is very well possible that the probabilities will follow a curve in time for a particular cell type and not just one probability. The overall probability will be lower in the beginning of XCI initiation, and will rise to a steady state level.

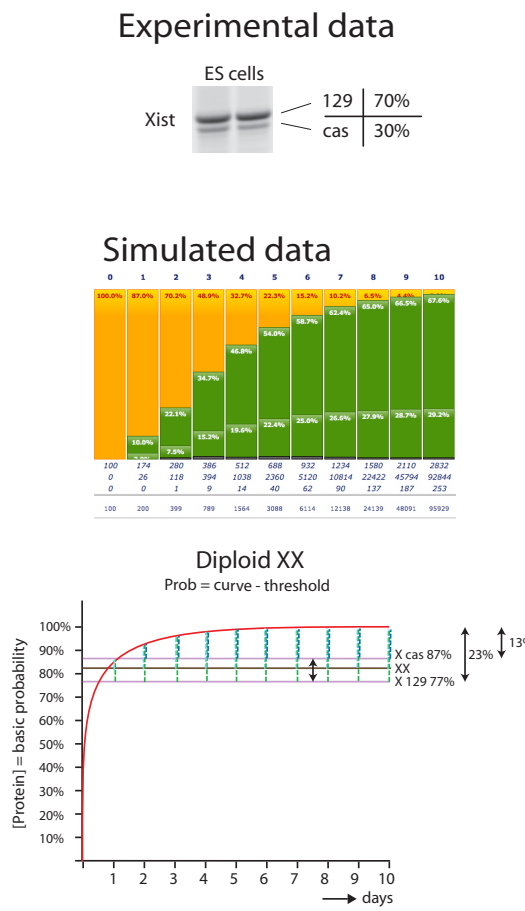


Figure 4. **Skewing in female F1-XX cells**
XCI skewing can be simulated by attributing different probabilities to the two X chromosomes in female XX cells. To simulate the 30/70 skewing observed in *castaneus*-129 F1 hybrids (upper pannel) a 10% (87%-77%) difference in probability is needed.

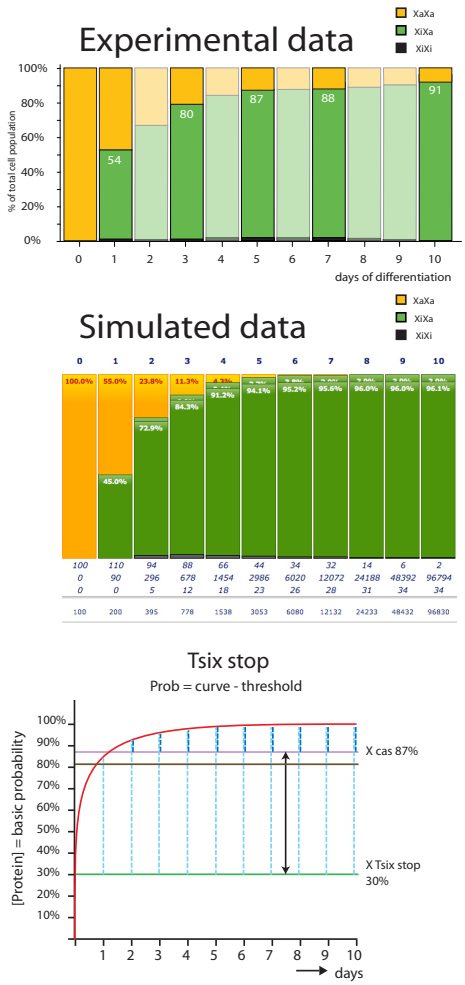


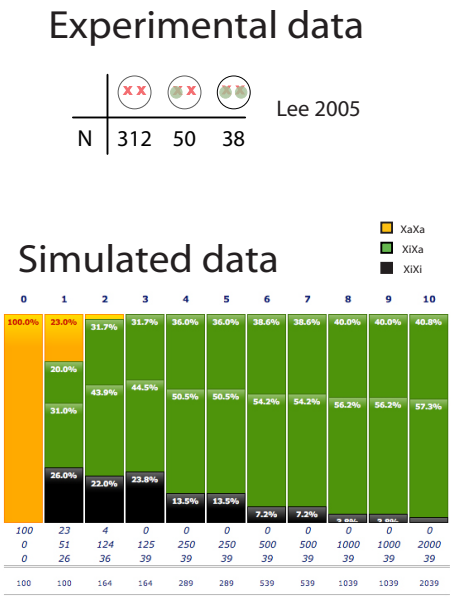
Figure 5. **Almost complete skewing with a *Tsix*-stop allele**
To accomplish almost complete skewing, the *Tsix* stop threshold had to be lowered to almost 60% difference relative to the wild type allele. Note that these cells initiate XCI faster than wild type XX cells (Figure 3 and 4)

The allele specific threshold for the XCI activator

A stochastic model implies that different alleles can have different probabilities to inactivate because they behave independent from each other. With ES cells from inbred mice the alleles are identical and will result in two populations of XiXa cells with one of the parental X chromosome inactivated that are evenly distributed. However, with F1 hybrid mice the probability for the two alleles can be different. Some F1 hybrids demonstrate skewed XCI patterns in somatic tissues, which could reflect different probabilities for each allele. Another way to look at these differences in probabilities is to see them as different thresholds levels for the XCI activator. An allele that is more sensitive for the XCI activator will have a higher probability in a cell with a certain XCI activator concentration than a less sensitive allele. So one XCI activator concentration can result in different probabilities for different alleles in the same cell nucleus. Figure 4 shows that the 30/70 skewing ratio observed experimentally in day 7 differentiated 129sv/j ES cells can be simulated by adjusting the thresholds for the different alleles. The 129 allele, which is inactivated in 70% of the cells, has a higher probability than the *castaneus* allele that is inactivated in 30% of the cells. The 129 allele therefore has a lower threshold compared to the *castaneus* allele that has a higher threshold. This result indicates that allele specific differences like skewing can be simulated by adjusting the XCI threshold to the XCI activator.

Tsix stop mutation

Tsix is a negative regulator of *Xist*. Deletion of the *Tsix* promoter or a transcriptional stop site integrated in the *Tsix* gene results in (almost) complete skewing (Luikenhuis et al., 2001). The mutated allele, which has no *Tsix* transcription, will always be inactivated. Furthermore, these cells initiate XCI much faster (compare experimental data Figures 3 and 5). The stochastic model can explain this observation by an increased probability of the mutated allele (by lowering the threshold). *Tsix* stop ES cells harbor the mutation on the 129 allele. These F1 hybrid ES cells thus have one *castaneus* allele and one *Tsix* stop allele. In the simulation we therefore used the threshold we found for the *castaneus* alleles in the F1 hybrid (129/cas) experiments



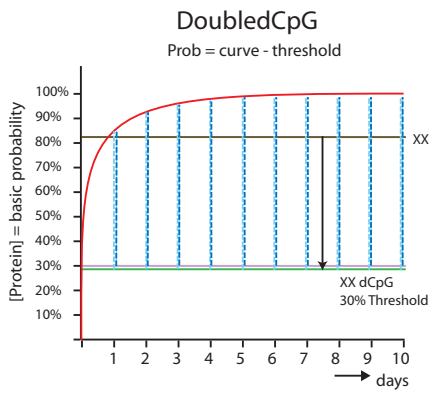


Figure 6. **A homozygous *Tsix* mutant (dCpG)**
A homozygous *Tsix* mutant (dCpG) can be simulated by lowering the thresholds for both alleles. Compare the experimental data (upper panel XiXa : XiXi cells) to the simulated data.

(Figure 4), that is 87% (max. 13% probability). Next, we lowered the threshold of the *Tsix* stop allele and found a threshold of 30% (max. 70% probability) to be sufficient to match the experimental data (Figure 5). Another research group generated a double *Tsix* mutant ES cell line and found that one third of the cells that initiated XCI had two *Xist* clouds (Lee 2005). Interestingly,

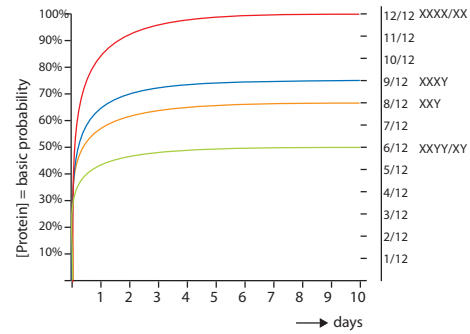


Figure 7. **XCI probability curves**
Predicted probability curves for different X:ploidy ratios when the protein-effect curve is linear.

we can simulate this finding by applying the threshold found in the previous experiment (30%) to both alleles (Figure 6)

Different X:ploidy ratios give different probability curves

Since the relationship between the XCI-activator concentration and the probability is unclear we decided to study XCI in tetraploid and triploid cell lines with different X:ploidy ratio's. XXXY cells have a ¾ concentration of the XCI activator compared to XXXX cells and XXY cells a ⅔ concentration. Analysis of XCI of these cell lines demonstrated what effect these concentrations have on the XCI process (Chapters3 and 4). Simulation of these cell lines

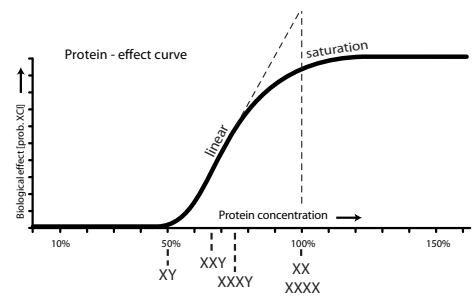


Figure 8. **Protein effect curve**
At higher concentrations of the XCI activator the relation may not be linear.

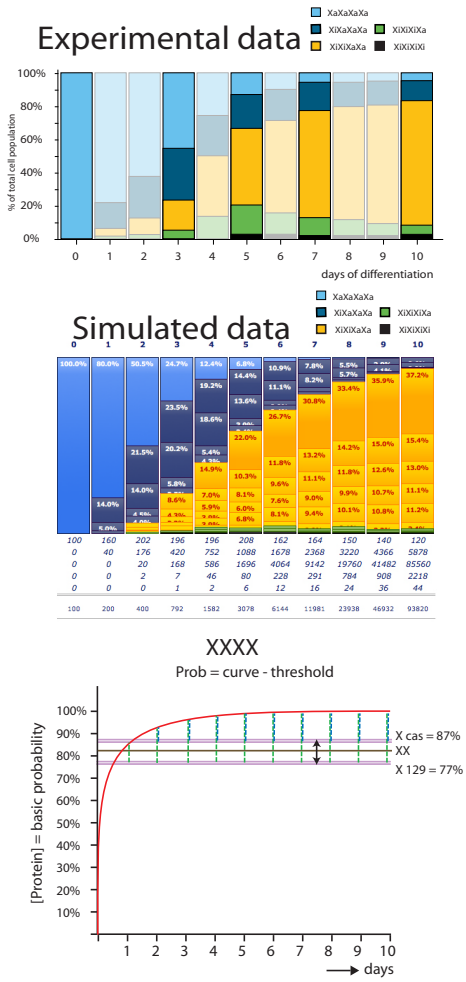


Figure 9. **XCI in XXXX tetraploid cells**
XCI patterns can be simulated by taking the same probability curve and thresholds as for XX cells. (now 4 probabilities instead of 2) Compare with figure 4.

will provide more insight in the XCI activator concentration – probability relation and help us to corroborate our stochastic model.

In all differentiating cells there is at least one X chromosome that harbors the XCI activator gene. Every cell thus builds up a particular concentration of XCI-activator depending on the X:ploidy ratio according to the kinetics of the protein. The trend of the protein curve does not chance between different cell lines (XX, XXXX, XY XXY and XXXY) with the same genetic background, but only the steady state level of it (Figure 7). If the relation between the XCI activator and its effect (XCI) was linear, we could use these curves to represent the probabilities in the XCI process. However, the XCI activator-effect curve is likely to have a sigmoid shape like most protein protein/affinity curves. The sigmoid shape of the protein-effect relation has implications for higher concentrations of the XCI activator (Figure 8). At high concentrations of the XCI activator the effect may not be linear. Increases in concentration will not result in a proportionally higher probability (dashed lines) but instead will result in a probability that is lower than expected. On a molecular level this can be explained by *Xist* promoter saturation. Increased transcription factor concentration cannot cause more transcription of *Xist* because the promoter is saturated. We next tried to simulate the XCI process in polyploid cells to test these theoretically defined probability curves.

Simulation of XXXX tetraploid cells

Since the XXXX cells are fused F1(129$^{sv/j}$/*Castaneus*) hybrids, we simulated XCI in XXXX tetraploid cells by taking the thresholds we found with the F1 hybrid (129/cas) simulation (Figure 4) together with the same probability curve (X:ploidy ratio = 1). In Chapter 3 we describe that XiXiXiXa cells divide slower or not at all, probably because of X chromosome dosage problems (see chapter 3, BrdU experiments). We therefore set the cell division rate for this population to 2 days instead of 1 day. Figure 9 shows the comparison of the experimental data and the simulated data. Predominantly two X chromosomes are inactivated. This data shows that the same probability curves and allele specific thresholds can be used as in 2n XX F1 hybrids to simulate XCI in 4n XXXX cells. We do find less cells with XiXiXiXa than expected. This difference may be attributed to XCI activator kinetics and will be discussed later in this chapter.

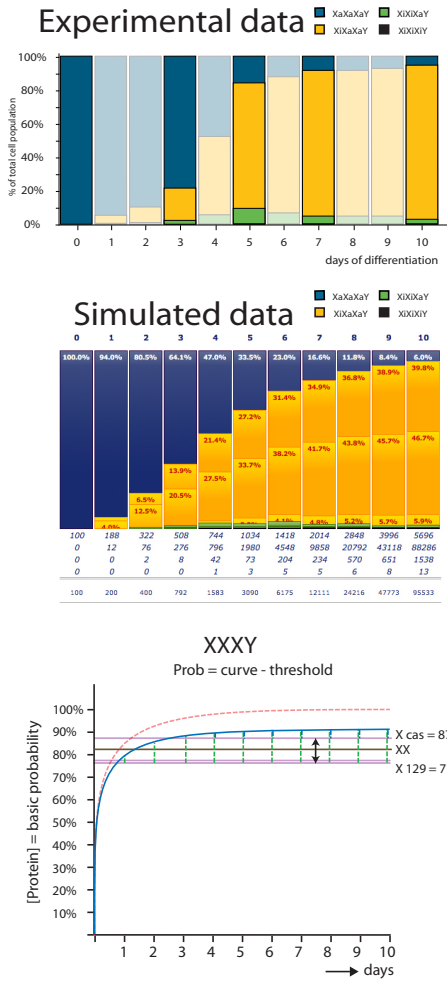


Figure 10. **XCI in XXXY tetraploid cells**
For XXXY cells (X:ploidy ratio of ¾) the probability curve is lower than for XXXX and XX cells (X:ploidy ratio of ½). The XXXY cells have two 129 chromosomes and one *castaneus* chromosome.

Simulation of XXXY tetraploid cells

The tetraploid XXXY cells contain two 129 X chromosomes and one *castaneus* X chromosome. We therefore used the thresholds for these alleles that we found in previous simulations (F1 hybrids Figure 4). The concentration in these cells of the XCI activator is lower than in XXXX cells, because the XCI activator is X encoded. To simulate the experimental data we had to drop the probability curve that theoretically should be applicable to XXXY cells (Figure 7). Cells with an XiXiXaY XCI pattern divide once in two days as used for the XXXX simulation. Figure 10 shows that we can simulate the XCI process in XXXY cells and that predominantly one X chromosome is inactivated. Our results are comparable to the experimental

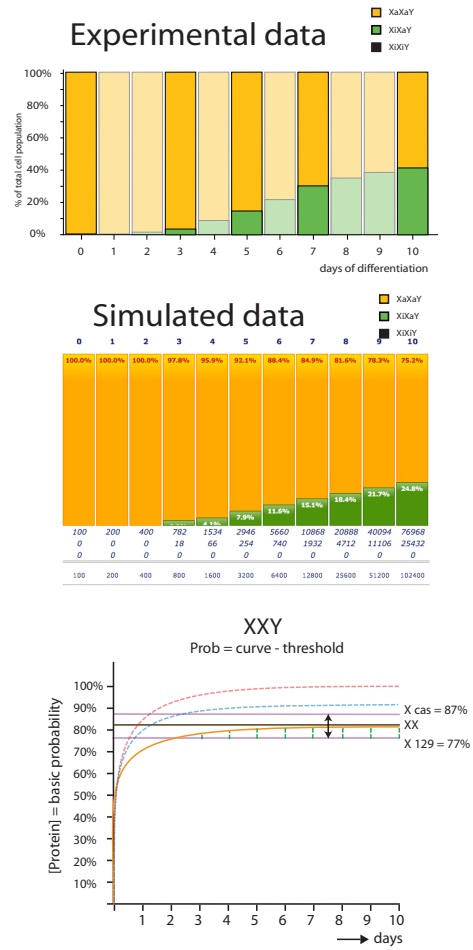


Figure 11. **XCI in XXY triploid cells**
For XXY cells (X:ploidy ratio of ⅔) the probabililty curve is lower than for XXXY cells. Note that only one X chromosome (129 X chromosome) can initiate XCI because the *castaneus* threshold is too high for this level of the XCI activator. Simulation results in less XiXa cells (green bars) compared to the experimental data. (see text for details).

data, although similar to the XXXX simulation we found less XiXiXaY cells in our simulation. This will be discussed later in this chapter.

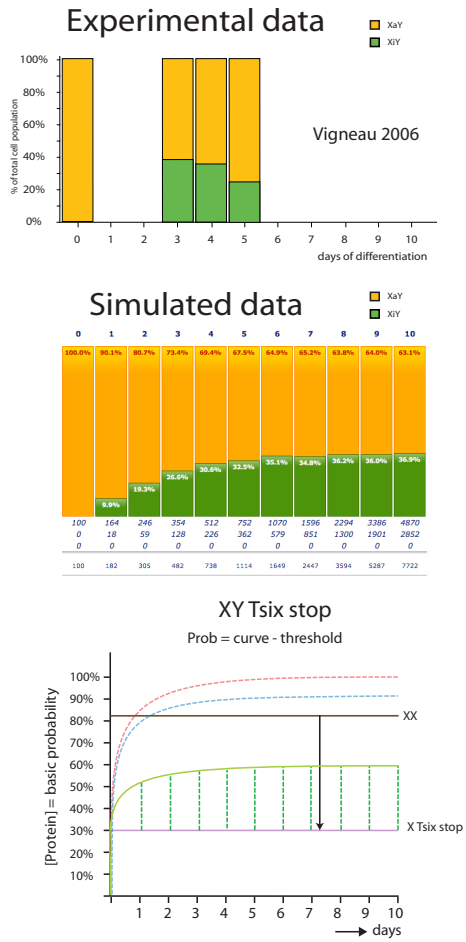


Figure 12. **XCI in XY cells with a *Tsix* mutation**
The single X chromosome in XY cells with a *Tsix* mutation has a threshold that is low enough to be reached by male XCI activator levels (green line). These cells will die because of X chromosome nullisomy.

Simulation of XXY triploid cells

The triploid XXY cells are F1 *castaneus* / 129 ES cells fused with Y round spermatids. For XCI simulation we took the *castaneus* and the 129 thresholds. The concentration of the XCI activator in these triploid ES cells is ⅔ compared to cells with an X:ploidy ratio of one, like 2n XX or 4n XXXX cells (Figure 7). The simulation of the XCI process that fits best our experimental data shows lower numbers of cells compared to the experimental data. We explain this difference by a growth advantage of the XiXaY cells relative to XaXaY cells, as shown in Chapter 4 (Figure 11). Our simulation also suggests that only the *castaneus* X chromosome will never be inactivated in thes cells.

Simulation of *Tsix* stop XY cells

In our *Tsix* stop XX cells simulation we attributed a 30% threshold to the *Tsix* stop allele (Figure 5). This threshold can also simulate the data in double *Tsix* defective cells (dCpG Figure 6). In XY cells the XCI activator concentration will be ½ compared to wild type cells (Figure 7). Simulation with the XY probability curve together with the *Tsix* stop threshold (30%) can simulate published data regarding a *Tsix* stop allele and a *Tsix* deletion in male cells (Figure 12) (Vigneau et al., 2006). This data should be taken with caution because of cell selection processes.

Our simulations predict less 4n XXXX cells with three and four Xi's and 4n XXXY cells with two and three Xi's when compared to the experimental data (Figures 9 and 10). This difference can be explained by the following arguments. In the computer model cells such as 2n XiXa cells and 4n XiXiXaXa cells stop initiating XCI because the XCI activator level drops to a concentration that can not induce XCI on the remaining alleles. This however, might not truly reflect the biological situation. The time it takes for the XCI activator to drop is designated 'the probability time frame' earlier in this chapter. During this time frame the XCI activator

concentration drops slowly and not instantly. This slow descent will generate a probability on the remaining Xa's, roughly half the probability of the last time frame. Although this is a relative small population, it will increase the number of XiXi's and the number of XiXiXiXa's approximately twice, and may explain the relative low numbers of these cells in our simulations. Furthermore, in XiXaXaXa cells the XCI activator will drop to XXXY (¾) levels. So in our simulation we should adjust the probability for these particular cells. The XXXY probability curve is approximately 10% lower than the XXXX curve (Figure 13). So XiXaXaXa cells will drop to this lower probability during one time frame. To simulate the different cell ploidies we adjusted the relative height of the curves in Figure 7 indicating that the protein-effect curve of the XCI activator is not linear as expected. Close examination of the relative distributions of the 4 curves (X:ploidy = 1, ¾ , ⅔ and ½) revealed that we could match the curves as predicted with a linear protein-effect curve to the curves we found by simulating the XCI process by scaling down the curve for an X:ploidy ratio of 1 (XX and XXXX) cells. This makes sense if one realizes that the sigmoid shape of the protein-effect curve can explain this down scaling.

Taken together, we can simulate all observed XCI patterns in cell lines with a different ploidy or sex chromosome composition. We can combine allele specific thresholds (*castaneus* and 129), mutation specific thresholds (*Tsix* stop or *Tsix* deletion) with probability curves for different ploidies and X:ploidy ratio's. The simulations therefore confirm and extend our hypothesis (Chapter 3) that a stochastic model can explain XCI counting and choice.

REFERENCES

Lee, J. T. (2005). "Regulation of X-chromosome counting by *Tsix* and *Xite* sequences." Science 309(5735): 768-71.

Wutz, A. and R. Jaenisch (2000). "A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation." Mol Cell 5(4): 695-705.

Luikenhuis, S., Wutz, A., and Jaenisch, R. (2001). Antisense transcription through the *Xist* locus mediates *Tsix* function in embryonic stem cells. Mol Cell Biol 21, 8512-8520.

Vigneau, S., Augui, S., Navarro, P., Avner, P., and Clerc, P. (2006). An essential role for the DXPas34 tandem repeat and *Tsix* transcription in the counting process of X chromosome inactivation. Proc Natl Acad Sci U S A 103, 7390-7395.

Chapter 6

Discussion

Discussion

XCI counting and choice, a stochastic model

At day 5.5 of embryonic development around gastrulation, random XCI initiates in cells derived from the inner cell mass of the blastocyst. ES cells are thought to represent the inner cell mass before initiation of XCI and thus, female ES cells have two active X chromosomes. At this stage, RNA FISH detects two *Tsix* pinpoint signals. *Xist* can only be demonstrated in very low amounts using RT PCR (as expected since XCI is not yet initiated). Nevertheless, doxycyclin inducible *Xist* transgenes can induce silencing in ES cells. *Tsix* is involved in repressing *Xist* and *Tsix* deletions result in increased *Xist* expression. The level of XCI induction after removal of *Tsix* is not sufficient to start XCI in ES cells. Taken together, in ES cells *Xist* is repressed by *Tsix*. However, even in the absence of *Tsix*, *Xist* expression is too low to induce XCI (Figure 1).

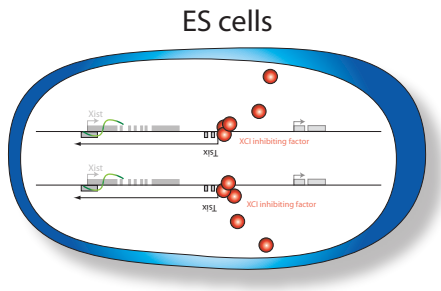


Figure 1. **Undifferentiated ES cells**
Tsix suppresses (leaky) *Xist* transcription. Both X chromosomes are active.

When female ES cells are differentiated XCI is initiated. A developmentally regulated X-linked XCI activator becomes expressed from both X chromosomes. This activator stimulates

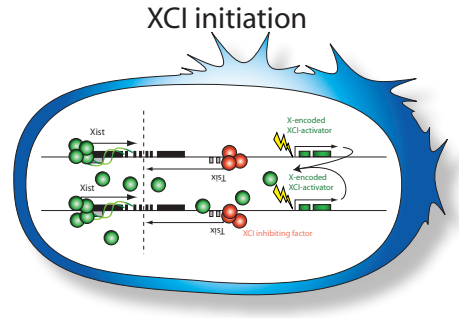


Figure 2. **XCI initiation**
The XCI activator is developmentally upregulated and causes *Xist* up regulation.

Xist transcription stochastically, meaning that induction of *Xist* expression is probabilistic. The XCI activator can be a protein or a non-coding RNA that is X linked (Figure 2).

Depending on the strength of the *Xist* and *Tsix* promoter, a certain probability to initiate inactivation emanates. *Tsix* expression is not down regulated in male cells at the time of XCI in female cells and *Tsix* RNA is only temporarily seen in 10% of *Xist* foci of cells that started XCI. Moreover, *Tsix* defective alleles have a higher probability to initiate XCI. We therefore hypothesize that *Xist* expression somehow terminates *Tsix* expression. By doing that, less of the XCI activator is required for constitutive expression of *Xist* (Figure 3).

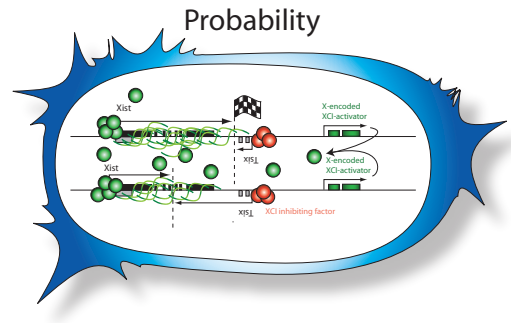


Figure 3. **XCI probability**
Xist upregulation creates a certain probability to silence *Tsix* expression; the negative regulator of *Xist*.

Xist RNA coats the X chromosome and induces inactivation by an unknown mechanism. In XX female cells, three populations of cells emerge: cells with two Xa's, cells with one Xa and one Xi, and cells with two Xi's. The distribution of these populations is dependent on the probabilities for the two alleles. Cells with two Xa's will continue with the process. In the XaXi cells, the XCI activator gene that is located on an inactivated X chromosome will be silenced and the concentration of the activator protein or RNA will subsequently drop to a level comparable to male cells. This is not sufficient to induce XCI on the remaining allele where *Tsix* expression persists. It is however sufficient to induce *Xist* expression on the Xi because the lack of inhibition by *Tsix* raises the sensitivity of the allele to the activator (Figure 4).

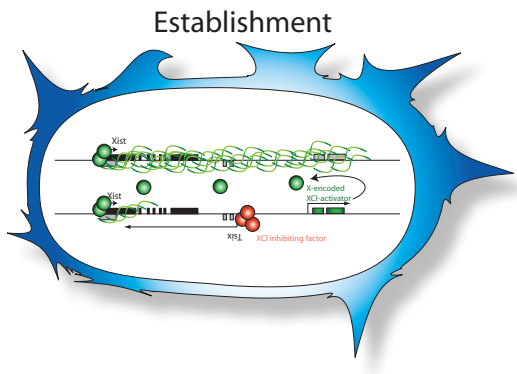


Figure 4. **XCI Establishment**
Xist expression abolishes X chromosome transcription by an unknown mechanism. The XCI activator gene on an X chromosome that is silenced will also cease expression. Epigenetic modifications are detectable.

Intuitively, the drop in the level of the XCI activator should go fast because a slow decrease in concentration will result in initiation of XCI on the other allele. In our simulations (Chapter 5) we found that 15% probability is typical for a XX diploid ES cell with one choice round per cell cycle (= 1 day). If it takes a day to drop to male levels the other allele would get ½ a (day) chance to still inactivate. If the initial probability was 15% for both alleles, 7,5% probability will remain for the Xa allele over a day. This implies that 1 out of 13 XiXa cells will become a XiXi cell and with 100 cells with 15% probability, 2 out of 25 XiXa become XiXi. This calculation indicates that the very fast drop in concentration of the XCI activator is not an urgent requirement for a stochastic model. Cell death due to XCI in male cells with an inducible *Xist* gene can be detected after 32 hours. *Xist* transgenes cause puromycin sensitivity in cis caused by inactivation of a cointegrated puromycin gene, only after 22 hours of *Xist* induction. (Wutz and Jaenisch 2000). This indicates that XCI is capable of down regulating X encoded genes within one day.

Xist expression is locked in on the X chromosome from which it is expressed and its promoter is methylated on X chromosomes from which it is not. DXPas34, the *Tsix* enhancer,

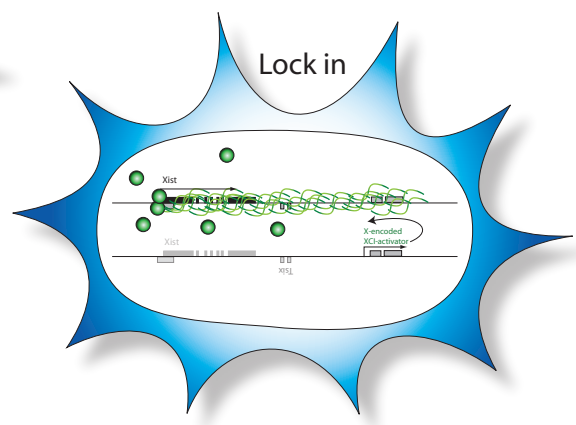


Figure 5. **XCI Lock in**
Epigenetic modifications lock in *Xist* and *Tsix* expression. *Xist* is expressed only from the Xi. *Tsix* is not expressed.

becomes methylated on the Xi. Cells are now subjected to selection for the proper X chromosome dosage. (Figure 5)

Interpretation of the model

Skewing of XCI has been extensively studied in mice. In mice, skewing becomes evident when two inbred strain are crossed to form F1 hybrids. The region responsible for skewing of XCI is the 'X controlling element' (Xce) which has been mapped to a region including the *Xist*, *Tsix* and *Xite* genes (Chadwick, Pertz et al. 2006). Although some argue that the Xce is located outside the Xic, recent studies indicate that it is more likely that it resides within. In mice, *Xist* expression levels after XCI completion are correlated with Xce strength (amount of skewing) (Brockdorff, Ashworth et al. 1991). Our studies show that differential *Xist* expression is already present prior to XCI, indicating a role for *Xist* expression in the skewing of XCI. In humans a single point mutation in the *Xist* promoter causes a more than 95% skewing (Plenge, Hendrich et al. 1997). Therefore it is reasonable to speculate that the Xce represents the sensitivity of the Xic to the XCI activator. It would therefore be interesting to determine the *Xist* and *Tsix* promoter sequences and correlate these with Xce strength.

A deletion of the *Xist* gene from one allele in a female cell will disable that allele to initiate XCI. Since the other allele has an independent probability it will still initiate inactivation. This will result in a slightly slower onset of the XCI process (See Chapter 3). Indeed this is what we observed for the XTX deletion.

How does *Tsix* negatively regulate *Xist*?

Deletion studies indicated that *Tsix* expression inhibits *Xist* expression. These studies include ectopic *Xist* accumulation in male cells after differentiation (Sado, Li et al. 2002). Experiments on *Tsix* double KO female cells demonstrate that half the cells contain double clouds (Lee 2005). Analysis of extraembryonic tissues also demonstrates that *Tsix* is a negative regulator. Deletions inherited through the maternal germ line give ectopic *Xist* upregulation in male and female embryos (Sado, Hoki et al. 2005). Analysis of an ES cell line harboring a *Tsix* stop cassette traps all transcripts and abolishes *Tsix* expression, revealed that transcription rather then a DNA element caused this inhibition (Luikenhuis, Wutz et al. 2001). Furthermore, *Tsix* is not dependent on splicing for its function. A splicing defective *Tsix* gene stably suppresses *Xist* expression and the repressive chromatin configuration of the *Xist* promoter is properly established (Sado, Hoki et al. 2006). On *Tsix* defective alleles *Xist* is not silenced and remains active. The promoter region of that *Xist* gene shows an open chromatin structure with hypersensitive sites and hypomethylation. If this is the cause of persistent *Xist* transcription or the result of a lack of *Tsix* transcription remains an open question (Sado, Hoki et al. 2005). RNA Chip experiments showed that Dnmt3A is pulled down with *Tsix* RNA in d0 ES cells suggesting that *Tsix* regulates *Xist* by attracting chromatin modification proteins or complexes (Sun, Deaton et al. 2006). Interestingly, in yeast an antisense transcription dependent silencing mechanism was recently reported to act by attracting HDAC's. Similar to *Tsix*, an antisense stop cassette abolished antisense transcription and suppression (Camblong, Iglesias et al. 2007). Thus, *Tsix* could act by attracting chromatin modifyers to the *Xist* promoter. However, *Tsix* also shows striking similarities with intergenic transcription. It is non-coding, its function is independent of splicing and it is initiated at more cryptic promoters. Intergenic transcripts may play a regulatory role (Gribnau, Diderich et al. 2000) and have been shown to be required for gene repression in yeast via transcription interference (Martens, Laprade et al. 2004). Moreover, a transcription interference based mechanism has been found to regulate cell fate by

modulating *IME4* expression in yeast (Hongay, Grisafi et al. 2006). Thus, *Tsix* could also act via transcription interference.

Some reports claim that regulation of *Tsix* transcription orchestrates *Xist* upregulation in female ES cells. However, because *Tsix* levels are not down regulated in day 3 differentiating male ES cells this seems unlikely. Luciferase assays with the *Tsix* major promoter also showed that *Tsix* expression in the absence of *Xist* did not recapitulate the developmental dynamics of wild type expression. In contrast it remained constitutively active, so shut down of *Tsix* transcription is likely to be the consequence of something else than a developmentally regulated transcription factor (Stavropoulos, Rowntree et al. 2005). Also, *Tsix* expression is almost solely detected at alleles that do not show *Xist* expression in female cells suggesting that *Xist* is a negative regulator of *Tsix*. It is tempting to speculate that *Xist* silences *Tsix* as it would silence (almost) all genes on the inactive X chromosome. For proper silencing the 5' A-repeat in *Xist* is required. That implies that *Tsix* silencing is also dependent on the A-repeat of *Xist* RNA. Unfortunately, the A –repeat deletion has only been introduced in *Xist* cDNA transgenes and not in the original locus and this question will have to be addressed in the future.

The window of opportunity and a stochastic model

Xist has to be expressed within 24-48h after start of differentiation. Further, expression must persist for 72 hours to cause irreversible silencing (Wutz and Jaenisch 2000). How do we interpret these results in the light of our stochastic model in which it takes the XCI process 10 days to reach an end state comparable to what we find in somatic tissues (almost all cells XiXa)? We have shown that XCI initiation is not a synchronous process but is stochastically variable between cells. The window of opportunity in vivo probably has a bell shaped curve and is not exactly limited to the time frame Wutz found with an inducible Xist transgene. Furthermore, after day 7 of differentiation, XCI initiation may be finished and cell selection may influence different cell distributions.

Role of Methylation in Xic Probability

The promoter of *Xist* is extensively methylated on the active X chromosome, indicating a role for methylation in the maintenance of *Xist* silencing. Could differential DNA methylation of the *Xist* and *Tsix* promoters play a role in the probability to initiate XCI? DNA methyltransferase knockout studies have shed some light on the role of DNA methylation in the initiation phase of XCI. Dnmt3A/3B (denovo DNA methyltransferases) double homozygous knockout male ES cells show low percentages of *Xist* clouds (~10%) at day 5 of differentiation. However, XCI levels are not comparable to female levels of XCI initiation at this timepoint (80%). In addition Dnmt1 (maintenance methylase) knockout ES cell also show XCI initiation, but levels rise later in differentiation compared to the Dnmt3A/3B knockout ES cells. Although this suggest a role for methylation in the initial probability for an X chromosome to inactivate (methylation lowers the probability), the observation that the Dnmt3A/3B knockout male ES cells show XCI levels up to 70% of the cells at day 12 of differentiation points more to a defect in the maintenance of *Xist* silencing (Sado, Okano et al. 2004). Indeed, despite the presence of *Xist* clouds, silencing of the X chromosome could not be detected. Most likely because XCI was initiated after the window of opportunity.

XCI initiation curves in the very first days of differentiation of Dnmt3A/3B knockout ES cells might show the altered probability of these alleles. Nonetheless, we do not predict a major role for methylation, since its abolishment does not show robust XCI in males.

Furthermore, it would be interesting to analyze XCI initiation rates in male cells with no functional Dnmt3A and 3B and a *Tsix* stop allele on their X chromosome. Robust initiation will reveal synergistic properties of both players.

Imprinted XCI and the stochastic model

In the mouse, XCI starts early during development around the 2-4 cell stage. Because human XCI is not imprinted in the extraembryonic tissues it is of particular interest that human XCI occurs later in development. In contrast, marsupials, that show imprinted XCI, display an earlier onset of XCI relative to mouse development. Moreover parthenogenetic mouse embryos initiate XCI later in development than androgenetic embryos, indicating that maternal Xic alleles initiate XCI at a later timepoint in development than male alleles. These observations make it tempting to speculate about the mechanism of imprinted XCI as follows. Extrapolating our stochastic model for XCI we explain imprinted XCI by assigning particular probabilities to the X chromosomes inherited through male or female gametogenisis. If these probabilities are skewed enough, the result will be primary total non-random skewing. The male germ line and the female germ line both lay down heritable marks to male and female Xic's. In the male germ line, meiotic sex chromosome inactivation (MSCI) could provide a preemptive repressive mark. Other features that are specific to the male haploid genome, like the replacement of protamines or the male specific active DNA demethylation in the zygote could provide a male specific mark as well (Oswald, Engemann et al. 2000). Nuclear transfer studies showed that the Xi in somatic cell nuclei will become the Xi in mouse extraembryonic tissues after nuclear reprogramming. This suggests that the repressive state of an Xi resembles the repressive state of an Xp that has been subjected to MSCI (Eggan and Jaenisch 2003). Our fusion experiments to generate triploid ES cells even indicate that MSCI provides a more repressive chromatin state than somatic XCI. However, a role for MSCI in imprinted XCI has been disputed by others (Okamoto et al., 2005). Studies with parthenogenetic embryos show that a maternal imprint on the X chromosome is set during oocyte maturation between the non-grown and full-grown oocyte stage (Tada, Obata et al. 2000). Interestingly, between these two stages of oogenesis the imprinting marks of imprinted genes are also established. However, the enzymes Dnmt3A/B, responsible for imprinted gene expression, are not required for imprinted XCI. Furthermore, imprinted XCI is different from normal imprinting in that it is erased in the embryo proper.

Based on a stochastic model one can speculate that if low concentrations of XCI activator are present in the 2-4 cell stage, this concentration is sufficient to initiate XCI on an X chromosome that has a threshold that is low enough to be overcome by *Xist*. The model implies that androgenetic and parthenogenetic embryos have similar probabilities to initiate XCI within a cell as in random XCI and predict the presence of double clouds in both, in contrast to imprinted XCI in wild type cells. Indeed XpXp androgenetic embryos initially show XCI on both X chromosomes and also in parthenogenetic embryos double clouds have been observed. Moreover in triploid male digynic embryos (XmXmY) the distribution of inactivation of the two Xm-s is random (Okamoto, Tan et al. 2000; Matsui, Goto et al. 2001). If, however, the XCI activator is not expressed at the early embryonic stage or too low (below the threshold), imprinted XCI will not start. The latter would represent mammals that exclusively show random XCI, like humans. If the XCI activator is upregulated by a developmental cue, at a time that the male Xic is equivalent to the female, random XCI will occur. The difference between imprinted and random XCI is now explained by temporal en quantitative properties of the level of the XCI activator, and the erasure of gamete specific properties of both genomes. The observation that *Tsix* is expressed from the blastocyst stage onward, and not at the time that the maternal alleles are repressed during imprinted XCI, indicates that *Tsix* does not play a role in imprinted XCI on the maternal allele. It supports the hypothesis that the maternal allele is not sensitive enough for inactivation.

Xist transgenes and counting

Contradictory results have been published regarding *Xist* transgenes and counting. This is not surprising when one realizes that counting and choice are the result of a stochastic process that is dependent on multiple factors (i.e. XCI activator concentration, *Xist* and *Tsix*). Analysis of fragmented transgenes will therefore result in confusing data. An *Xist* transgene for example, including flanking regions but not *Tsix*, is expressed and accumulates *in cis* on an autosome in male differentiated ES cells (Herzing, Romer et al. 1997). This result could be explained through the presence of two Xics. In contrast, a stochastic model predicts that with no inhibition by *Tsix*, the *Xist* transgene is sensitive enough for half the level of XCI activator present in male cells, comparable to male cells with a defective *Tsix* allele (Luikenhuis, Wutz et al. 2001; Sado, Wang et al. 2001; Vigneau, Augui et al. 2006).

Multicopy Xic transgenes in male cells resulted in *endogenous Xist* expression from the single X chromosome. Surprisingly, this expression was abolished when DXPas34, that is a known negative regulator of *Xist*, was deleted form the transgenic YACs. (Debrand, Chureau et al. 1999; Heard, Mongelard et al. 1999). In light of a stochastic model, if these YAC transgenes harbor the XCI activator, the deletion of DXPas34 will result in a faster rate of XCI on the transgene (comparable to *Tsix* stop allele) because it is not longer repressed. Subsequently, the XCI activator concentration will drop because it is silenced on the transgene, and the endogenous locus will not initiate XCI.

All Xic transgenes only express their *Xist* gene in multicopy arrays and not as a single copy integrant (Heard, Mongelard et al. 1999). This may be explained by the observation that multicopy transgenes may lack cis regulatory sequences (like *Xist* missing *Tsix*). Otherwise, as mentioned above, the XCI activator may be located on one of these YACs and cause endogenous *Xist* expression depending on variegated expression of the XCI activator and the sensitivity of that Xic for the XCI activator. Since these factors are largely unknown it is very difficult to interpret these results correctly.

MODELS

The blocking factor is not likely to exist

The blocking factor model was posed to explain results obtained by translocation studies. Different translocations indicated that a particular part of the X chromosome had to be present at least twice to induce X chromosome inactivation. In addition, the presence of three of these regions in one cell results in two inactivated chromosomes per cell. This let Rastan to introduce the blocking factor that could block only one of the regions mentioned above and only one blocking factor would be present per diploid nucleus (Rastan and Robertson 1985). The region necessary for this counting phenomenon and XCI to occur was called 'the Xic', and harbors the *Xist*, *Tsix* and *Xite* genes. Deletions of the, (at the time unknown) *Tsix*/*Xite* region induced ectopic X chromosome inactivation in male cells and suggested the location of the blocking factor binding site. Nevertheless this deletion also removed the negative regulator of *Xist* which makes unequivocal interpretation of the data difficult (Clerc and Avner 1998). Our *Xist-Tsix-Xite* (XTX) deletion demonstrates the lack of a BF binding site in this region because it shows robust XCI, comparable to a *Xist* deletion i.e. one allele is fully functional. The hypothetical BF binding site in the XTX deletion predicts, in strong contrast to our results, no induction of XCI at all (Figure 6).

The BF could also bind outside the XTX deletion and could be impaired to fulfill its function because of requirements of *cis* components that have been deleted. Thus, in the case of the *Tsix* stop allele, the BF cannot block because *Tsix* cannot fulfill its function. A *Tsix* stop mutation in female cells would therefore result in half the cells with two inactive X chromosomes, since in half the cells with

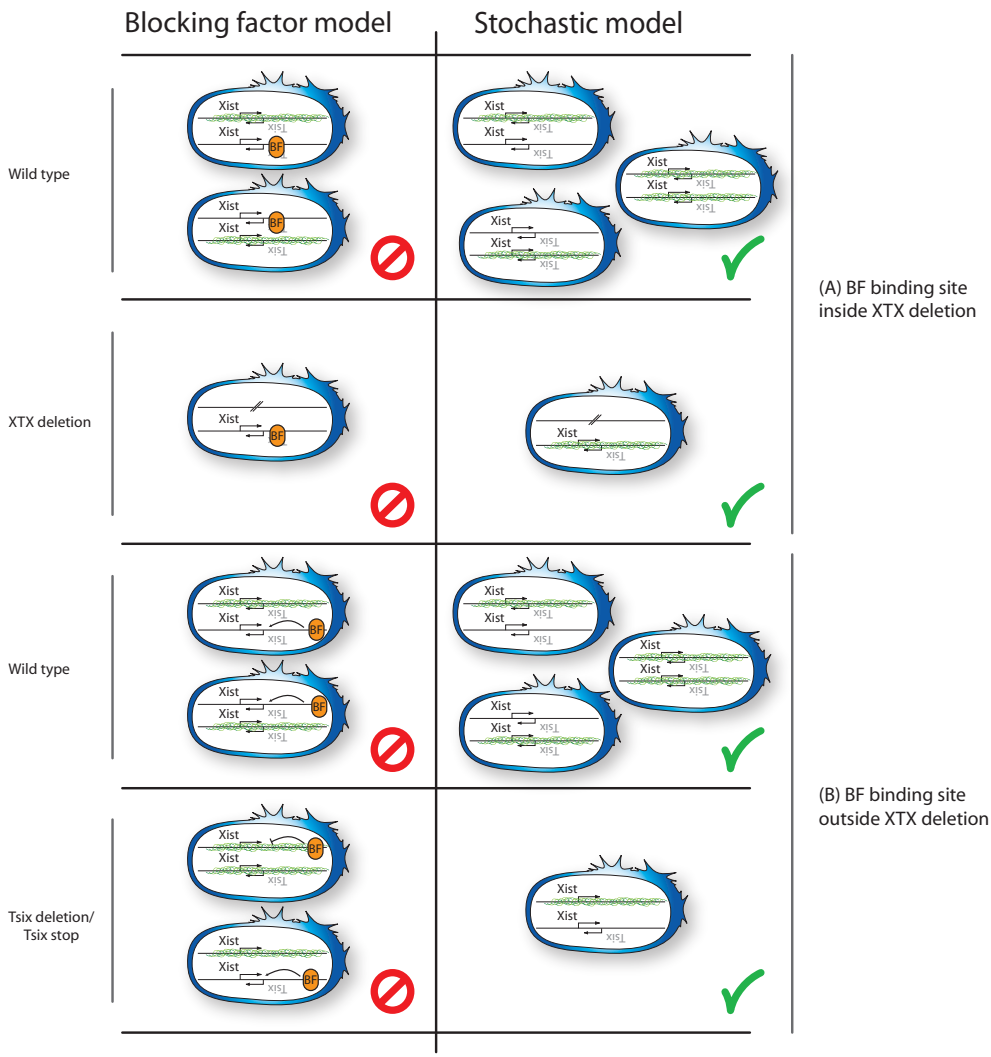


Figure 6. **The blocking factor versus a stochastic model**
(A) Blocking factor binding within the XTX deletion region predicts; no double clouds in wild type cells and no XCI in heterozygous XXdXTX cells. (B) blocking factor binding outside the XTX deletion region predicts: no double clouds in wild type cells and double clouds in 50% of the cells in heterozygous *Tsix* mutants. A stochastic model accurately predicts all observed phenotypes.

such a mutation, the blocking factor binds to the mutated allele leaving the wild type allele unprotected. Because the mutated allele is not blocked since the mutations prevent that, both alleles will initiate XCI. These double cloud cells however have never been reported nor have we detected them in our experiments (Figure 6). We therefore conclude that a BF is highly unlikely and moreover, is not necessary to explain XCI counting and choice.

Transvection

If counting and choice would be regulated by transient crosstalk between Xic's this would predict a counting defect in a cell line that has lost this association. Since the 65kb deletion (Clerc and Avner 1998) shows no counting defect in a XX cell (i.e. these cells show induction of XCI on the wild type chromosome), the supposed function of this spatial approximation remains to be determined. Moreover, these studies did not score colocalization, but distances less than 0.3-2μm. This is, in the context of a nucleus, a significant distance and furthermore, it is unclear how this proximity would orchestrate XCI counting and choice (Bacher, Guggiari et al. 2006; Xu, Tsai et al. 2006). Recent 4C data (chromatin confirmation capture on chip) technique that suggests that the position of chromosomes is probabilistic and that chromatin folds according to self-organizing principles (de Laat and Grosveld, 2007). The position of a chromosome in the nucleus seems to depend on the properties of its flanking chromatin segments, neighboring genes and repetitive sequences. It is therefore hard to imagine how one locus can position a whole chromosome through the nucleus to make crosstalk possible. The non random distribution could very well be the result of gene activation in the vicinity of the *Xist* gene or of the *Xist* gene itself. Gene activation could drag its flanking chromatin in the direction of a particular gene transcription factory that can be non-randomly distributed themselves. Interestingly, sequences that neighbor sequences that show pairing, display a random distribution in the nucleus. Because, the distance between these genomic loci can maximally be 1.5μm because of the tertiary structure of the chromatin, this finding remains puzzling (Augui, Filion et al. 2007). If transvection would really regulate XCI it is hard to envisage how this is possible. Therefore, further research is needed to clarify the meaning of this spatial non-random approximation (Simonis, Klous et al. 2006; de Laat and Grosveld 2007).

A role for replication timing in stochastic mechanisms?

Olfactory receptors are mono-allelically expressed in sensory neurons. The chosen receptor is transcribed only from one allele; the other allele is silent and late replicating. Surprisingly this asynchronous replication timing is detectable in all tissues and not only in the neuronal cells where they are expressed, and is random with respect to parental origin. This can be due to two reasons. First, late replication is established early in development and clonally propagated. Second asynchronous replication timing can be a feature of this particular locus and is randomly established every time the cells enter Sphase.

The replication timing of the X chromosome in ES cells is not clonally propagated because clones display a random distribution. Because imprinted genes and immunoglobin loci also display asynchronous replication timing, this suggests that asynchronous replication timing is a property of loci that are characterized by monoallelic expression. If replication timing has a functional role in the mechanism that establishes monoallelic expression remains an open question.

However, one can speculate that the probability of an X chromosome to inactivate upon differentiation is correlated with asynchronous replication timing. If early replication occurs randomly per locus with a certain probability, this could result in chromosomes with different chromatin configurations because different chromatin modifiers have been shown to be active at different stages in Sphase. The differential chromatin composition could influence the expression of *Xist* i.e. the probability that *Xist* accomplishes silencing. Effects like skewing can act downstream of this and exert their effect by modifying *Xist* expression. This would explain why we did not find a relation between skewing and replication timing prior to XCI. Nonetheless, since replication timing is coupled to the cell cycle it would be interesting to investigate if the probability of a Xic to

inactivate its X chromosome is influenced by manipulation cell cycle parameters e.g. duplication times of differentiating ES cells.

STOCHASTIC GENE EXPRESSION

There is an increasing body of evidence that the initiation of transcription from a gene is not a simple matter of 'on or off'. The fact that a gene is active and transcribed in a population of cells does not imply that all the cells in that population actually transcribe that gene. Support for this hypothesis comes from different lines of research and indicates a stochastic basis for transcriptional activation.

Cook et al. (1998) showed with a transcription initiation simulation model that particular transcription initiation kinetics causes genes to express in an intermittent manner. The rate of transcription initiation versus no transcription initiation is dependent on the activation-rate of a particular promoter combined with a deactivation-rate and results in varying types of intermittent transcription. The activation rate is dependent on promoter strength, transcription factors and enhancing sequences. If there is no deactivation, no intermittent transcription can occur which could represent the stochastic binding of factors or cis sequences to the promoter region. The gene switches randomly between inactive and active, and the (stochastic) fluctuation of the actual protein level is inversely correlated to the speed of this switching. So this can explain the molecular basis for stochastic initiation of transcription but also stochastic fluctuation in protein concentration. The amount of variation is dependent on the kinetics of transcription initiation and deactivation (e.g. transcription initiation complex binding properties) (Cook, Gerber et al. 1998).

Genes can be on and off within one nucleus

Evidence that a gene can show intermittent expression in vivo comes form the analysis of the human β globin locus. FISH data revealed that the expression of different globin genes alternate from a single locus. With primary transcripts of one gene (γ globin) detectable in the nucleus, the cytoplasm of that same cell shows the mRNA of another gene (e.g. β globin) while the locus is only capable of transcribing one gene at the time (Wijgerde, Grosveld et al. 1995). Interestingly, enhancers do not increase the initiation frequency per template but increase the probability that it is active. So, increased expression in a cell population is not accomplished by increasing polymerase density on a

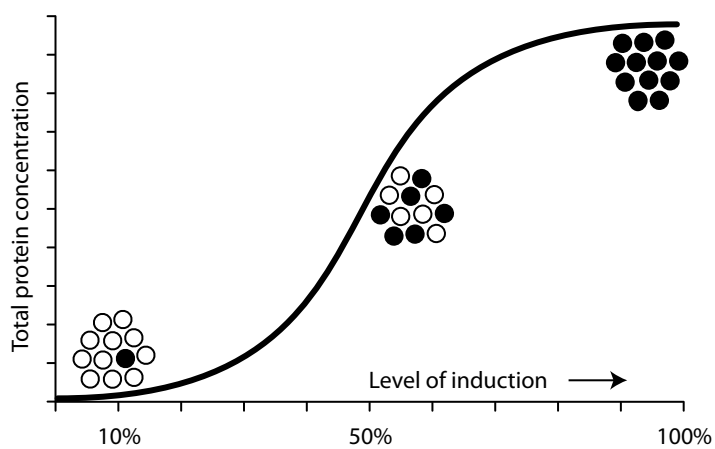


Figure 7. **Dosage dependent stochastic expression**
More induction does not increase the expression rate per cell but causes more cells to express.

gene but by increasing the absolute number of cells in that population that express the gene (Weintraub 1988; Walters, Fiering et al. 1995).

Stochastic gene activation can account for dose dependent protein levels

When genes are 'activator dosage dependent' the observation that an intermediate level of this activator does not induce half the level of transcription initiation in these cells but instead, induces expression of the gene in only half of the cells supports a stochastic initiation model for gene expression. The relative amount of cells that expresses the gene now achieves the 'dosage effect' and not the initiation rate of the gene per allele (Figure 7).

This also predicts that cell number instead of transcription rates can regulate protein levels. Indeed, stimulation of T-cells that carry a LacZ gene downstream of the stimulated T-cell receptor pathway show that activation of the LacZ gene is dosage dependent on the intensity of stimulation. It is however not the expression rate of LacZ that is increased but the amount of cells that express LacZ. Over a 100-fold β-gal activity is demonstrated upon stimulation whereas the binding properties of the responsible transcription factor NF-AT only shows a 5-fold induction. The authors explain these results by assuming that LacZ expression is dependent on NF-AT above a certain threshold. Stochastic variation in the level of NF-AT is then responsible for the bimodal expression pattern of LacZ (Fiering, Northrop et al. 1990). Observations on cells harboring a dosage dependent glucocorticoid-inducible gene also showed that cell numbers and not expression rates per cell were responsible for the dose-response relationship (Ko, Nakauchi et al. 1990). Liver enzymes, that are induced to express by adding glucocorticoids to the medium, show intercellular heterogeneity regarding protein expression. This heterogeneity is inversely correlated to the half-life of the mRNA and protein and persists at steady state levels of induction. These observations indicate intermittent transcription upon stimulation and support a stochastic mechanism for transcription initiation (van Roon, Aten et al. 1989; Dingemanse, de Boer et al. 1994).

OTHER STOCHASTIC REGULATED PROCESSES

Ly49 receptor family in Natural Killer cells

The murine Ly49 receptor family in Natural Killer (NK) cells is monoallelically expressed in only a subset of the cells. The family consists of 14 genes and a cell can display a selection of available Ly49 receptors clonally. These can be co-expressed from the same or the opposite chromosome randomly (Held, Kunz et al. 1999). The mechanism driving the choice process which receptor to express remains unclear but a stochastic model seems the most likely explanation. A small probability for each allele will result in a random mixed expression patterns from both chromosomes, and sometimes a receptor will be expressed from both alleles. Indeed, this has been reported for the Ly49 receptor family (Held and Raulet 1997). Interestingly, a dosage dependent activator driving Ly49 receptor expression has been identified, the transcription factor T-cell factor 1 (TCF-1). The concentration of TCF-1 determines the amount of cells that express a Ly49 receptor and the relative usage of an allele coding for a Ly49 receptor. In concordance with this, the amount of biallelically expressed receptors is also dosage dependent (Ioannidis, Kunz et al. 2003).

Stochastic patterns in globin gene expression

During differentiation tissue specific genes are activated and, at some point, locked in by an epigenetic memory and clonally propagated. If the initial activation of a gene is stochastic, the 'lock in' of these different outcomes

should result in a population of cells with different patterns that are heritable. Evidence for this hypothesis is provided by FISH analysis of the β globin locus. The expression of α and β globin is the main function of erythroid precursor cells. The ratio between these two proteins is ideally 1:1 because they form a tetrameric complex consisting of two heterodimers. Interestingly, not all the cells accomplish this ratio and about one quarter of the cells show imbalanced expression of the α and β genes. In these cells not all four loci are expressed and the particular expression pattern per cell is clonally propagated. Moreover the decision whether an allele is expressed is made for a whole locus and not per promoter, suggesting that the choice is made prior to transcriptional activation. Interestingly, the particular distribution of different expression patterns can be explained by assuming different probabilities to transcribe for the α and the β locus. The α locus has a slightly higher probability then the β locus explaining the existence of cell expressing only the α locus, and the almost absence of cells with only β expression (de Krom, van de Corput et al. 2002).

Stochastic Olfactory Receptor gene regulation

The monoallelically expressed Olfactory Receptor (OR) gene family in mice contains more than 1000 OR genes that are clustered throughout the genome in ~50 loci on many different chromosomes. The one receptor-one neuron hypothesis states that only one OR is expressed in a neuron and from only one of the two alleles. An enhancer that regulates one of the OR-clusters (with 3 genes) that is relatively over expressed (10% of the olfactory neurons) is called 'the H enhancer'. Intriguingly, additional H enhancers allow the expression of more than one OR gene, but only one is functional. Cells that express a non-functional OR gene, like a pseudo gene, switch to another gene until they express a functional OR gene (Serizawa, Miyamichi et al. 2003; Lomvardas, Barnea et al. 2006). A feedback mechanism appears to be initiated as soon as a functional receptor is expressed and commits the cell to one functional receptor. So far, the one receptor-one neuron rule is far from being proven and the mechanism of OR gene regulation remains elusive. Because of the high number of possibilities when combining 1000 genes, it is hard to disprove that not one but a few genes are expressed in a single neuron (Mombaerts 2004; Shykind 2005).

Interestingly, similar to what we have proposed for XCI, a stochastic mechanism can very well explain OR gene regulation. Low probabilities for OR genes to stochastically express a single (or maybe a few) OR genes long enough to allow a feedback mechanism to lock in the chosen repertoire can explain the one receptor-one neuron hypothesis. Indeed, simulation assuming low (1/1000th) probabilities for a receptor gene to be activated in cells with 500 genes (1000 alleles) show that in most cells zero, one or two genes will be expressed (Figure 8). If the OR choice is made early in development

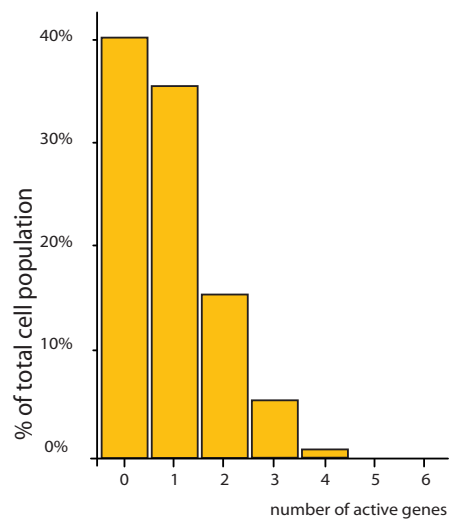


Figure 8. **Olfactory receptor expression simulation**
Applying 1/1000th probability to 1000 genes in 1000 cells results in the above distribution. Almost all cells have zero, one, two or three expressing genes.

a clonal propagation after stochastic activation can account for the axonal convergence to the glomeruli in the olfactory bulbs.

Stochastic gene expression can regulate cell fate decisions

If cell fate decisions are dependent on the level of a protein (or RNA in the case of *Xist*) that needs to exceed a certain threshold (protein concentration or *Tsix* mediated suppression) one can imagine that cell fate decisions can be regulated in a dosage dependent manner at a population level but not on a single cell level. To explain this in more detail, a certain concentration of a gene activator will give a particular intermittent transcription pattern (of a cell fate decision protein) in a cell. Depending on the kinetics mentioned above together with the RNA and protein half-lives, a particular protein concentration (of the cell fate decision protein) is present that varies stochastically per cell. In some cells the cell fate threshold will be reached but in other cell it will not. This will result in a particular distribution of cells that have different cell fates. The distribution is activator dosage dependent. The all-or-none cell fate switch in Xenopus oocytes illustrates this mechanism. Progesterone stimulation determines cell fate by activating the MAPK cascade in an all or nothing fashion. An intermediate dose of progesterone does not result intermediate MAPK phosphorylation per cell but in a population in which half the oocytes activated the pathway and the other half did not. (Ferrell and Machleder 1998). Furthermore, in *drosophila*, the pulsed expression of a protein called spineless, regulates the entire retinal mosaic required for color vision. The fly retina contains two types of ommatidia, called 'pale' and 'yellow' in the retina. In a dosage dependent fashion, the distribution of them is regulated by the stochastically expressed transcription factor *spineless*. *Spineless* is detected in the same percentage of cells as the final percentage of yellow ommatidia indicating that the choice to turn yellow is directed by it. So, a stochastically expressed protein directs cell fate (Wernet, Mazzoni et al. 2006).

Stochastic *Xist* expression can explain XCI patterns

Our data indicate the presence of an XCI activator that is developmentally regulated and induces *Xist* expression. Our model for XCI counting and choice predicts that initiation of XCI is stochastic, meaning that the *Xist* gene will be in an 'on' or 'off' state. The rate and length at which the *Xist* gene is 'on' depends on the

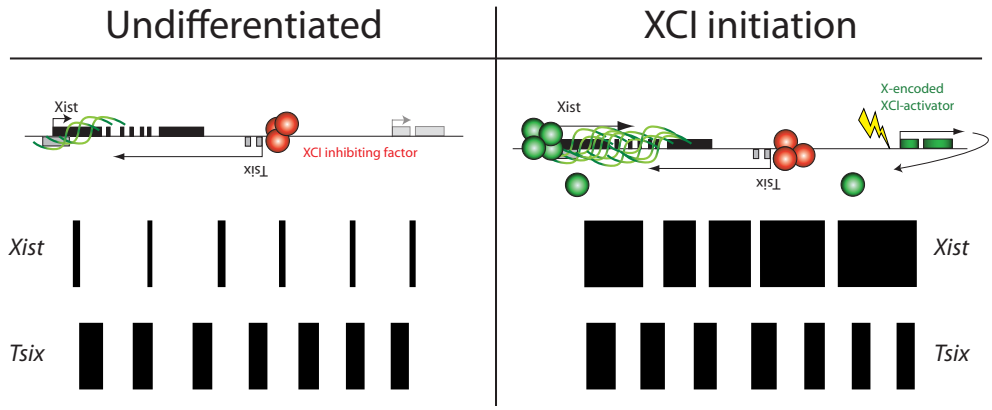


Figure 9. **Stochastic expression of *Xist* and *Tsix***
The Xist and Tsix genes are in an 'on or off' state. The expression rate is XCI activator dependent.

level of the activator and correlates with the probability to initiate XCI. The exact relation between the XCI activator and XCI initiation remains speculative (Figure 9). If the activator is an *Xist* transcription factor, the protein will first rise to steady state levels and with that the probability. Indeed in our simulations we need a probability curve to explain our experimental data supporting this hypothesis. Transcription factor regulated initiation also implies that the *Xist* promoter can be saturated. In that case, it predicts that the relation between the activator and XCI initiation will not be linear at higher concentrations of the activator. Indeed, this is what we observe when we simulate XCI in different ploidy cell types with different concentrations of the activator. Isolation of the activator will provide the experimental tools to prove this hypothesis.

What is the molecular basis for a Xic probability?

First, one should realize that in our stochastic model a probability is an average of different probabilities that characterizes a cell population. In individual cells this may vary.

Second, what determines the probability? The XCI activator is a likely candidate. Stochastic expression of it can cause an all or nothing effect in individual cells with 10% of the cells expressing the activator above threshold levels. This however implies that the created probability is cell specific and not Xic specific; 10% of the cells will end up with two Xi's. Asynchronous replication of the loci may for most of the cells allow only one Xic to inactivate resulting in an average 10% probability per Xic. Thus, stochastic XCI activator expression together with asynchronous replication timing can provide a molecular basis for a Xic probability.

The XCI activator concentration may also be invariable between cells and induce all Xic's evenly to initiate XCI. Variable resistance to this stimulus can now explain probability. This variability could be provided by the stochastic expression of Xist. In 10% of the cells Xist reaches threshold levels and causes initiation of XCI. The threshold represents the Xist RNA silencing its own negative regulator Tsix. Tsix is a negative regulator of Xist and Tsix mutants cause ectopic upregulation of Xist expression (Luikenhuis, Wutz et al. 2001; Sado, Wang et al. 2001). Furthermore, inducible constitutive expression of Tsix from an allele causes it to never inactivate (Luikenhuis, Wutz et al. 2001). Thus, by taking away its own inhibitor Xist becomes fully expressed. The created probability is not cell specific but Xic specific. The stochastic expression of Xist implies that it is expressed in an on or off fashion. This is indeed what we observe in RNA FISH experiments on differentiating ES cells. We therefore advocate the latter hypothesis regarding the molecular basis for Xic probability.

REFERENCES

Augui, S., G. J. Filion, et al. (2007). "Sensing X chromosome pairs before X inactivation via a novel X-pairing region of the Xic." Science **318**(5856): 1632-6.

Bacher, C. P., M. Guggiari, et al. (2006). "Transient colocalization of X-inactivation centres accompanies the initiation of X inactivation." Nat Cell Biol **8**(3): 293-9.

Brockdorff, N., A. Ashworth, et al. (1991). "Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome." Nature **351**(6324): 329-31.

Camblong, J., N. Iglesias, et al. (2007). "Antisense RNA Stabilization Induces Transcriptional Gene Silencing via Histone Deacetylation in S. cerevisiae." Cell **131**(4): 706-17.

Chadwick, L. H., L. M. Pertz, et al. (2006). "Genetic control of X chromosome inactivation in mice: definition of the Xce candidate interval." Genetics **173**(4): 2103-10.

Clerc, P. and P. Avner (1998). "Role of the region 3' to *Xist* exon 6 in the counting process of X-chromosome inactivation." Nat Genet **19**(3): 249-53.

Cook, D. L., A. N. Gerber, et al. (1998). "Modeling stochastic gene expression: implications for haploinsufficiency." Proc Natl Acad Sci U S A **95**(26): 15641-6.

de Krom, M., M. van de Corput, et al. (2002). "Stochastic patterns in globin gene expression are established prior to transcriptional activation and are clonally inherited." Mol Cell **9**(6): 1319-26.

de Laat, W. and F. Grosveld (2007). "Inter-chromosomal gene regulation in the mammalian cell nucleus." Curr Opin Genet Dev.

Debrand, E., C. Chureau, et al. (1999). "Functional analysis of the DXPas34 locus, a 3' regulator of *Xist* expression." Mol Cell Biol **19**(12): 8513-25.

Dingemanse, M. A., P. A. de Boer, et al. (1994). "The expression of liver-specific genes within rat embryonic hepatocytes is a discontinuous process." Differentiation **56**(3): 153-62.

Eggan, K. and R. Jaenisch (2003). "Micromanipulating dosage compensation: understanding X-chromosome inactivation through nuclear transplantation." Semin Cell Dev Biol **14**(6): 349-58.

Ferrell, J. E., Jr. and E. M. Machleder (1998). "The biochemical basis of an all-or-none cell fate switch in Xenopus oocytes." Science **280**(5365): 895-8.

Fiering, S., J. P. Northrop, et al. (1990). "Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor." Genes Dev **4**(10): 1823-34.

Gribnau, J., K. Diderich, et al. (2000). "Intergenic transcription and developmental remodeling of chromatin subdomains in the human beta-globin locus." Mol Cell **5**(2): 377-86.

Heard, E., F. Mongelard, et al. (1999). "*Xist* yeast artificial chromosome transgenes function as X-inactivation centers only in multicopy arrays and not as single copies." Mol Cell Biol **19**(4): 3156-66.

Held, W., B. Kunz, et al. (1999). "Mono-allelic Ly49 NK cell receptor expression." Semin Immunol **11**(5): 349-55.

Held, W. and D. H. Raulet (1997). "Expression of the Ly49A gene in murine natural killer cell clones is predominantly but not exclusively mono-allelic." Eur J Immunol **27**(11): 2876-84.

Herzing, L. B., J. T. Romer, et al. (1997). "*Xist* has properties of the X-chromosome inactivation centre." Nature **386**(6622): 272-5.

Hongay, C. F., P. L. Grisafi, et al. (2006). "Antisense transcription controls cell fate in Saccharomyces cerevisiae." Cell **127**(4): 735-45.

Ioannidis, V., B. Kunz, et al. (2003). "Initiation and limitation of Ly-49A NK cell receptor acquisition by T cell factor-1." J Immunol **171**(2): 769-75.

Ko, M. S., H. Nakauchi, et al. (1990). "The dose dependence of glucocorticoid-inducible gene expression results from changes in the number of transcriptionally active templates." Embo J **9**(9): 2835-42.

Lee, J. T. (2005). "Regulation of X-chromosome counting by *Tsix* and *Xite* sequences." Science **309**(5735): 768-71.

Lomvardas, S., G. Barnea, et al. (2006). "Interchromosomal interactions and olfactory receptor choice." Cell **126**(2): 403-13.

Luikenhuis, S., A. Wutz, et al. (2001). "Antisense transcription through the *Xist* locus mediates *Tsix* function in embryonic stem cells." Mol Cell Biol **21**(24): 8512-20.

Martens, J. A., L. Laprade, et al. (2004). "Intergenic transcription is required to repress the Saccharomyces cerevisiae SER3 gene." Nature **429**(6991): 571-4.

Matsui, J., Y. Goto, et al. (2001). "Control of *Xist* expression for imprinted and random X chromosome inactivation in mice." Hum Mol Genet **10**(13): 1393-401.

Mombaerts, P. (2004). "Odorant receptor gene choice in olfactory sensory neurons: the one receptor-one neuron hypothesis revisited." Curr Opin Neurobiol **14**(1): 31-6.

Okamoto I, Arnaud D, Le Baccon P, Otte AP, Disteche CM, Avner P, Heard E. (2005). Evidence for de novo imprinted X-chromosome inactivation independent of meiotic inactivation in mice. Nature. 438(7066):297-8

Okamoto, I., S. Tan, et al. (2000). "X-chromosome inactivation in XX androgenetic mouse embryos surviving implantation." Development **127**(19): 4137-45.

Oswald, J., S. Engemann, et al. (2000). "Active demethylation of the paternal genome in the mouse zygote." Curr Biol **10**(8): 475-8.

Plenge, R. M., B. D. Hendrich, et al. (1997). "A promoter mutation in the *Xist* gene in two unrelated families with skewed X-chromosome inactivation." Nat Genet **17**(3): 353-6.

Rastan, S. and E. J. Robertson (1985). "X-chromosome deletions in embryo-derived (EK) cell lines associated with lack of X-chromosome inactivation." J Embryol Exp Morphol **90**: 379-88.

Sado, T., Y. Hoki, et al. (2005). "*Tsix* silences *Xist* through modification of chromatin structure." Dev Cell **9**(1): 159-65.

Sado, T., Y. Hoki, et al. (2006). "*Tsix* defective in splicing is competent to establish *Xist* silencing." Development **133**(24): 4925-31.

Sado, T., E. Li, et al. (2002). "Effect of *Tsix* disruption on *Xist* expression in male ES cells." Cytogenet Genome Res **99**(1-4): 115-8.

Sado, T., M. Okano, et al. (2004). "De novo DNA methylation is dispensable for the initiation and propagation of X chromosome inactivation." Development **131**(5): 975-82.

Sado, T., Z. Wang, et al. (2001). "Regulation of imprinted X-chromosome inactivation in mice by *Tsix*." Development **128**(8): 1275-86.

Serizawa, S., K. Miyamichi, et al. (2003). "Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse." Science **302**(5653): 2088-94.

Shykind, B. M. (2005). "Regulation of odorant receptors: one allele at a time." Hum Mol Genet **14 Spec No 1**: R33-9.

Simonis, M., P. Klous, et al. (2006). "Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C)." Nat Genet **38**(11): 1348-54.

Stavropoulos, N., R. K. Rowntree, et al. (2005). "Identification of developmentally specific enhancers for *Tsix* in the regulation of X chromosome inactivation." Mol Cell Biol **25**(7): 2757-69.

Sun, B. K., A. M. Deaton, et al. (2006). "A transient heterochromatic state in *Xist* preempts X inactivation choice without RNA stabilization." Mol Cell **21**(5): 617-28.

Tada, T., Y. Obata, et al. (2000). "Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth." Development **127**(14): 3101-5.

van Roon, M. A., J. A. Aten, et al. (1989). "The initiation of hepatocyte-specific gene expression within embryonic hepatocytes is a stochastic event." Dev Biol **136**(2): 508-16.

Vigneau, S., S. Augui, et al. (2006). "An essential role for the DXPas34 tandem repeat and *Tsix* transcription in the counting process of X chromosome inactivation." Proc Natl Acad Sci U S A **103**(19): 7390-5.

Walters, M. C., S. Fiering, et al. (1995). "Enhancers increase the probability but not the level of gene expression." Proc Natl Acad Sci U S A **92**(15): 7125-9.

Weintraub, H. (1988). "Formation of stable transcription complexes as assayed by analysis of individual templates." Proc Natl Acad Sci U S A **85**(16): 5819-23.

Wernet, M. F., E. O. Mazzoni, et al. (2006). "Stochastic spineless expression creates the retinal mosaic for colour vision." Nature **440**(7081): 174-80.

Wijgerde, M., F. Grosveld, et al. (1995). "Transcription complex stability and chromatin dynamics in vivo." Nature **377**(6546): 209-13.

Wutz, A. and R. Jaenisch (2000). "A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation." Mol Cell **5**(4): 695-705.

Xu, N., C. L. Tsai, et al. (2006). "Transient homologous chromosome pairing marks the onset of X inactivation." Science **311**(5764): 1149-52.

Summary
Samenvatting

Summary

X CHROMOSOME INACTIVATION COUNTING AND CHOICE

Placental mammalian female cells have two X chromosomes. One of these chromosomes is randomly inactivated in each nucleus so that females are functionally mosaic for genes expressed from their X chromosomes. The evolutionary basis for this phenomenon is based on the fact that females would have twice the number of X-linked gene product compared to their male counterpart. This unequal distribution of X-linked genes requires gene dosage compensation. Species that have distinguishable sex chromosomes have evolved different ways to prevent a difference in dosage of the sex chromosome-encoded proteins between the two sexes. In female mammals one X chromosome is transcriptionally inactivated in female somatic cells by a process called X chromosome inactivation (XCI).

The initiation phase of this process is characterized by a counting and choice mechanism that determines the number of X chromosomes per nucleus. Subsequently, all but one X chromosome per diploid genome are inactivated. The counting and choice process ensures that the dosage of X-encoded gene products is equal between male and female and thus regulates that the active X chromosome: autosome ratio is always 1:2. The choice which X chromosome is elected to be the future active X chromosome occurs randomly but is often skewed, so that the distribution of Xi's between the two parental X chromosomes is not equal.

A genetically defined region of 10Mb located on the X chromosome is required for XCI and is called the X chromosome inactivation center (XIC). Three genes, *Xist*, *Tsix* and *Xite*, have been mapped to the XIC and play a crucial role in XCI. All three are non-coding RNA genes but have different expression patterns. *Xist* RNA is transcribed from the inactive X chromosome at the onset of XCI and binds to or 'coats' the future inactive X chromosome. *Xist* is essential for the silencing process and an *Xist* deletion results in a XCI defective X chromosome. *Tsix* is a negative regulator of *Xist* and represses *Xist* transcription throughout the XCI process. Deletion of *Tsix* results in ectopic *Xist* expression in cis and aberrant XCI. *Xite* has a similar role as *Tsix* although its effects are milder.

On a molecular level the counting and choice mechanism can be explained by different models. The prevailing model has been the 'blocking factor model'. This model states that a limited blocking factor (BF) is present in each nucleus per diploid genome and binds only one X chromosome randomly and protects it from the inactivation process.

Despite extensive research, no blocking factor has been isolated yet, and no binding element for the blocking factor on the X chromosome has been identified. Another model is based on a temporal non-random distribution of the XICs in female cells and tries to explain counting and choice via spatial cross talk of the two X chromosomes.

Deletion of the *Xist* gene results in a XCI defective X chromosome and causes complete skewing of the XCI process in female cells. In somatic cells heterozygous for this deletion (at the end of the XCI process) the wild type X chromosome is inactivated in every cell. The mutated X chromosome is always active. Complete skewing of the XCI process can be the result of the fact that the mutated X chromosome is never chosen to inactivate (primary non-random XCI). Alternatively, the mutated X chromosome can be chosen but can not initiate XCI (because of the *Xist* deletion) resulting in a cell that has two active X chromosomes. These cells will die because of X chromosome dosage problems and the end result will be complete skewing of XCI (secondary non-random XCI). **Chapter 2** describes experiments with female *Xist* heterozygous knockout embryos that indicate that all cells choose the wild type X chromosome

to inactivate, indicating that an *Xist* deletion causes primary non-random X inactivation. A hallmark of imprinted and non-imprinted monoallically expressed genes is that they replicate asynchronously during S phase. In **Chapter 2** we show that this is also true for X chromosomal genes, including the Xic prior to XCI, which could provide a template for X chromosome inactivation choice. However, we show that asynchronous replication timing of the Xic before initiation of XCI does not correlate with skewing of differentiated F1 hybrid cas/129 cells. Nor does primary non-random XCI correlate with asynchronous replication timing. This indicates that XCI choice and asynchronous replication timing are separable mechanisms.

The occasional observation of female cells with two inactive X chromosomes present in our differentiating ES cell cultures can not be explained by the blocking factor model and led us to explore a different model with a stochastic nature. In a stochastic model each X chromosome in a nucleus has a probability per timeframe e.g. a cell cycle, to inactivate independent of any other X chromosome(s) in the same nucleus. Per time frame, 3 populations of female cells emerge, cells with two active X's, cells with one active and one inactive X, and cells with two inactive X's. Cells with two inactive X's die and are thus selected against. On a molecular level the probability per chromosome is explained by the balance between *Xist* expression and *Tsix* repression. In **Chapter 3** we show that tetraploid XXXX and XXXY cells display XCI patterns with zero to four inactive X chromosomes per nucleus. These XCI patterns can be predicted by attributing a certain probability to every Xic. BrdU analysis indicates that cells with a proper X active: ploidy ratio are selected for. Deletion of a region including *Xist*, *Tsix* and *Xite* shows that each X chromosome has an independent probability to initiate XCI. The deletion indicates the presence of an X-encoded XCI-activator driving the X chromosome inactivation counting and choice process, most likely by regulating *Xist* expression.

Chapter 4 reports the generation and analysis of triploid XXY cells. These cells show XCI initiation in only a minority of the population indicating that the XCI activator concentration is just above the threshold concentration required to initiate XCI. This also suggests that the threshold for XCI initiation must be between the XCI activator concentration in XY cells (X:ploidy ratio =1/2) and the XCI activator concentration in XXY cells (X:ploidy ratio = 2/3). BrdU analysis shows that cells with an XiXaY pattern divide faster than cells with an XaXaY pattern, indicating that these cells will outgrow the XaXaY cells in time. In that chapter we also present data showing that *Xist* expression levels prior to XCI are skewed in F1 hybrid cell lines, indicating a direct role for *Xist* expression in XCI probability.

In **Chapter 5** we provide data in which we simulate XCI and compare the outcomes with the experimentally found XCI patterns during the XCI process (from day 0 to day 10 of differentiation). For the analysis we use ES cell lines with different X:autosome ratios or *Xist* or *Tsix* mutations of different ES cell lines by attributing a probability per day to each XIC in a nucleus to inactivate. For reasons discussed in **Chapter 5** we argue that the probability of an X chromosome to start XCI is not likely to be just one fixed number throughout the differentiation process but, instead is likely to follow a curve. Moreover, the probabilities can be different per X chromosome, depending on the genetic background (Xce's) or cis mutations. Since, this makes the simulations very labor intensive we decided to program a computer-based simulation. This provides us with a powerful tool to explore how a stochastic model explains the XCI process in more detail. The simulations are based on 4 probability curves (XY = ½, XXY = 2/3, XXXY ¾ and XX/XXXX = 1) representing the different X-encoded XCI activator concentrations in these cells, and allele specific thresholds (sensitivities for the XCI

activator). Using a SQL based program we are able to simulate XCI patterns of all cell ploidies but also of XCI related mutations, like *Tsix* deletions, supporting the stochastic model posed in **Chapter 3**.

Taken together, this thesis describes analysis of the XCI counting and choice process in cells with different deletions and ploidies. We propose a new model that describes the stochastic nature of X chromosome counting and choice and points to an X linked XCI-activator.

Samenvatting

X CHROMOSOOM INACTIVATIE IS EEN STOCHASTISCH PROCES

Vrouwelijke zoogdieren bezitten twee X chromosomen in iedere cel. Een van hen wordt willekeurig geinactiveerd door een proces dat 'X chromosoom inactivatie' (XCI) wordt genoemd. Het gevolg hiervan is dat zowel in mannelijke als vrouwelijke cellen één X chromosoom actief is en dat vrouwelijke en mannelijke cellen een gelijk aantal actieve X chromosomale genen hebben.

Het X chromosoom inactivatie proces begint met het tellen van het aantal X chromomen in de celkern. Daarna wordt één van de X chromosomen gekozen om actief te blijven en worden de eventueel overige X chromosomen geinactiveerd. De keuze welk X chromosoom actief blijft is willekeurig, maar heeft vaak een voorkeursverdeling die afwijkt van een 50:50 verdeling. Samenvattend begint XCI dus met het tellen van het aantal X chromosomen en de keuze van het toekomstige actieve X chromosoom.

Een specifieke plek op het X chromosoom is nodig voor het XCI proces en wordt het X chromosoom inactivatie centrum (Xic) genoemd. Zonder dit inactivatie centrum kan een X chromosoom zichzelf niet inactiveren. In de Xic liggen drie genen, *Xist*, *Tsix* en *Xite*, die allen een rol spelen in het XCI proces. Het inactivatie proces begint met accumulatie van het *Xist* RNA dat het hele X chromosoom inpakt en op een nog onbekende manier de genen op het X chromosoom uitzet. *Tsix* en *Xite* spelen een rol bij de keuze van het actieve X chromosoom. Als *Tsix* en *Xite* afwezig zijn op een X chromosoom zal dat X chromosoom vaker worden gekozen om inactief te worden.

Op moleculair niveau wordt het voorgaande verklaard door verschillende modellen. Het meest gangbare model is het 'blocking factor' model. Een bepaalde factor die aanwezig is in zowel vrouwelijk als mannelijke cellen kan precies één X chromosoom 'blokkeren' en zodoende beschermen tegen het inactivatie proces. Deze factor bindt willekeurig aan één van de twee X chromosomen in vrouwelijke cellen en bindt altijd aan het enkele X chromosoom in mannelijke cellen. Maar, ondanks veel onderzoek dat de afgelopen 20 jaar is verricht is de 'blocking factor' nooit geidentificeerd.

Hoofdstuk 2 beschrijft de analyse van een deletie van het *Xist* gen op een van de twee X chromosomen in vrouwelijke cellen. De deletie heeft tot gevolg dat alle cellen uiteindelijk het gemuteerde X chromosoom (dus met de deletie) als het actieve X chromosoom kiezen. De data leert dat dit niet het gevolg is van een willekeurige keuze tussen de twee X chromosomen gevolgd door celdood van de cellen die het wild type X chromosoom gekozen hebben om actief te blijven (deze cellen hebben twee actieve X chromosomen, wat celdood tot gevolg heeft). Echter, dit is het gevolg van het feit dat het gemuteerde X chromosoom *altijd* wordt gekozen om actief te blijven en het andere wild type X chromosoom *altijd* wordt gekozen om geinactiveerd te worden. Dit laatste wordt 'primair niet willekeurige X chromosoom inactivatie' genoemd.

Tijdens de celdeling worden alle chromosomen verdubbeld. Sommige genen worden niet tegelijkertijd verdubbeld maar met een paar uur verschil. Dit wordt 'asynchrone replicatie' genoemd en typeert genen die maar van één chromosoom worden afgeschreven. Voor het proces van XCI begonnen is worden ook de twee X chromosomen in vrouwelijke cellen asynchroon gerepliceerd. In **hoofstuk 2** testen we de hypothese of dit correleert met de keuze welk X chromosoom wordt gekozen als toekomstig actief en inactief X chromosoom. Dit blijkt niet het geval te zijn en asynchrone replicatie heeft dus waarschijnlijk geen rol in het XCI keuze proces.

Tijdens de bestudering van het XCI proces zagen we soms vrouwelijke cellen met twee inactieve X chromosomen. Dit is interessant

omdat dit niet kan worden verklaard met het 'blocking factor' model. We besloten daarom te testen of XCI een stochastisch proces is. In een stochastisch proces heeft elk X chromosoom een bepaalde kans dat het geinactiveerd wordt en het toeval bepaalt of geen, één van beide of beide X chromosomen in vrouwelijke cellen geinactiveerd zal worden. Dit model voorspelt dus dat er tijdens het inactivatie proces vrouwelijke cellen zijn die twee inactieve X chromosomen laten zien. In cellen met 4 X chromosomen voorspelt het model cellen met 0,1,2,3 of 4 inactieve X chromosomen. Cellen met te veel inactieve X chromosomen (2 in wild type vrouwelijke XX cellen) zullen niet overleven omdat ze te weinig of geen X chromosoom genen actief hebben. Cellen met het juiste aantal inactieve X chromosomen zullen overleven en het grootste gedeelte van de populatie vormen. Onze bevindingen zijn beschreven in **hoofdstuk 3** en zijn in overeenstemming met een stochastisch model.

Een deletie van *Xist*, *Tsix* en *Xite* van één X chromosoom in vrouwelijke cellen laat nog steeds XCI zien van het wild type X chromosoom. Dit bevestigt een stochastisch model waarin iedere X een onafhankelijke kans heeft geinactiveerd te worden. Daarnaast suggereert deze vinding het bestaan van een XCI activator eiwit dat wordt gecodeerd door een gen op het X chromosoom. De concentratie van deze XCI activator in een cel bepaalt wat de kans is van de X chromosomen in deze cel om XCI te initiëren.

In **hoofdstuk 4** beschrijven we XCI in triploide XXY cellen. Analyse van deze cellen laat XCI zien in slechts een klein percentage van de cellen. Dit is in overeenstemming met een stochastich model omdat in deze cellen net genoeg van de door het X chromosoom gecodeerde XCI activator aanwezig is om XCI te initiëren. Verder laten we zien dat *Xist* expressie correleert met de kans van een X chromosoom om XCI te initiëren al voordat het XCI proces begonnen is.

Het XCI proces kan gesimuleerd worden door aan elk X chromosoom een kans toe te kennen, onafhankelijk van de aanwezigheid van andere X chromosomen in dezelfde celkern. Deze kans correleert met de X chromosoom:autosoom ratio. Hoe hoger deze ratio is hoe groter de kans per X chromosoom om te inactiveren. Mutaties in de Xic en de genetische achtergrond van het locus hebben ook invloed op deze kans. Verder is de kans niet één getal maar neemt toe in de tijd om redenen uiteengezet in **hoofdstuk 5**. De kans zal dus tijdens het XCI proces geleidelijk toenemen. Door alle voorgaande variabelen is de uitkomst van een stochastich model al snel moeilijk te berekenen. Om het proces te simuleren zijn ingewikkelde schema's nodig. We hebben daarom besloten om XCI te simuleren met behulp van een computerprogramma. In **hoofdstuk 5** beschrijven we de resultaten van computer simulaties. Samenvattend kunnen we alle observaties aangaande XCI in XY, XX, XXY, XXXY en XXXX cellen simuleren met behulp van twee parameters; de XCI activator concentratie, afhankelijk van de X chromosoom:autosoom ratio, en de X chromosoom specifieke gevoeligheid voor de XCI activator. De computersimulaties onderschrijven het in **hoofdstuk 3** voorgestelde stochastiche model.

Deze dissertatie beschrijft de analyse van het XCI proces in verschillende cellijnen en muis-embryo's. De resultaten kunnen niet verklaard worden met de huidige modellen, maar wel met het voorgestelde stochastische model dat gebaseerd is op de aanwezigheid van een door het X chromosoom gecodeerde XCI-activator.

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LIST OF PUBLICATIONS

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Gribnau, J., Luikenhuis, S., Hochedlinger, C., Monkhorst, K. and Jaenisch, R. (2005). X chromosome choice occurs independently of asynchronous replication timing. J Cell Biol. 168, 365-73.

Monkhorst, K., P.T. den Hoed. (2005). Osteosynthese van mediale collumfracturen bij patiënten jonger dan 75 jaar. Ned. Tijdschrift voor Traumatologie 13-4, 108-112.

Mc Morrow. T., van den Wijngaard, A., Wollenschlaeger, A., van den Corput, M., Monkhorst, K., Trimborn, T., Fraser, P., van Lohuizen, M., Jenuwein, T., Djabali, M., Philipsen, S., Grosveld, F. and Milot, E. (2000). Activation of the beta globin locus by transcription factors and chromatin modifiers. EMBO J. 19, 4986-96.

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