Dosage Compensation in Mammals

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SUMMARY

Many organisms show major chromosomal differences between sexes. In mammals, females have two copies of a large, gene-rich chromosome, the X, whereas males have one X and a small, gene-poor Y. The imbalance in expression of several hundred genes is lethal if not dealt with by dosage compensation. The male–female difference is addressed by silencing of genes on one female X early in development. However, both males and females now have only one active X chromosome. This is compensated by twofold up-regulation of genes on the active X. This complex system continues to provide important insights into mechanisms of epigenetic regulation.

Outline

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OVERVIEW

In mammals, as in many other organisms, there is a major chromosomal difference between the sexes. For example, humans have 22 pairs of chromosomes known as autosomes that are present in both males and females; one member of each pair is inherited from the mother and one from the father. But there are two other chromosomes, the sex chromosomes, designated X and Y, that differ between the sexes; females have two Xs, whereas males have one X and one Y. This matters because although the X is a medium-sized chromosome with more than 1000 genes, the Y is small and gene poor. A similar situation exists in other mammals, including rodents and marsupials. Chromosomal differences are linked to the mechanism by which sex is determined and seem to have evolved over many millions of years.

Sex chromosome imbalance presents the organism with a problem: The two sexes differ in the copy number of X-linked genes. This can lead to an imbalance in the amount of gene products (RNAs and proteins), which would, in turn, require differences in metabolic control and other cellular processes. To avoid this, dosage compensation mechanisms have evolved that balance the level of X-linked gene products between the sexes. There are three general methods by which this can be performed: first, a twofold up-regulation in the expression of X-linked gene in males; second, a twofold down-regulation of genes on each of the two X chromosomes in females; and finally, the complete inactivation of one of the two X chromosomes in females. The first strategy has been adopted in the fruit fly, *Drosophila* (see Lucchesi and Kuroda 2014), the second in the worm *Caenorhabditis elegans* (see Strome et al. 2014), and, it now seems, both the first and the last in mammals.

Over recent years, studies of dosage compensation in mammals have provided crucial insights into fundamental epigenetic mechanisms and how patterns of gene expression are regulated through development. It can be confidently predicted that they will continue to do so.

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1 INTRODUCTION

1.1 Sex Determination Creates a Need for Dosage Compensation

Sexual reproduction is common among eukaryotes. Even plants that can replicate perfectly well asexually by sending out shoots or runners often have an alternative sexual mode of reproduction. A possible explanation is that sexual reproduction brings an enormous increase in genetic variability on which natural selection can operate. The reshuffling of alleles that occurs with every sexual generation produces a population better able to cope with environmental shifts compared with a relatively homogeneous population derived from asexual methods of reproduction. But sex is complicated, requiring developmental pathways that lead to male and female sexual organs, as well as the physiological and biochemical apparatus required for meiosis, germ cell maturation, the attraction of partners, and mating (see Marshall Graves and Shetty 2001, and references therein, for further discussion of these issues).

Genetic mechanisms used in defining different sexes vary widely from one organism to another. The simplest system involves a single locus that is homozygous in one sex (the "homogametic" sex) and heterozygous in the other (the "heterogametic" sex; Fig. 1). This system has evolved in different ways to reach varying levels of complexity in different organisms. In some, mechanisms have been put in place that suppress meiotic recombination (crossing-over) of the sex-determining alleles in the heterogametic sex (Fig. 1), a step that helps prevent the generation of mixtures of alleles leading to intersex states. The inability to recombine, in many cases, has spread to include part or all of one chromosome, with an accompanying loss of genetic information. The evolutionary pressures that have driven this chromosome degeneration are still not understood, but the end result in many species is that the two sexes show differences, not just in alleles at one or a few loci, but in complete chromosomes. In mammals, it is the males who carry the degenerate chromosome, whereas in birds, it is the females (Marshall Graves and Shetty 2001).

Sexual differentiation is usually triggered by one or a small number of crucial genes being switched on or off during development. The products of these genes initiate a cascade of gene regulatory events that mediate progression down one or the other pathway of sex determination (see Strome et al. 2014 for details in *C. elegans*; see Lucchesi and Kuroda 2014 for details in *Drosophila*). In humans, it is the protein product of the *SRY* gene on the Y chromosome that sends the early embryo down the male pathway (reviewed by Quinn and Koopman 2012). A mechanism of this sort does not need major chromosome differences to operate successfully, so why have such differences arisen so often and in such diverse organisms, including mammals, birds, and fruit flies? It may be that they have occurred as a by-product of the suppression of crossing-over required to prevent intersex states (Fig. 1). Mathematical analysis of the factors that influence the spread of alleles through populations shows that suppression of crossing-over will inevitably lead to the gradual accumulation of deleterious mutations around the region of crossover suppression. This is largely because such mutations rarely become homozygous, which is necessary if they are to be selected against.

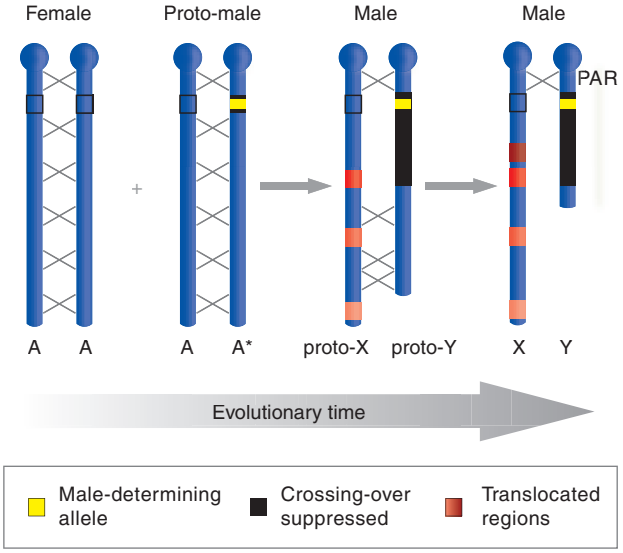

Figure 1. Evolution of the Y chromosome. Early in evolution, the two sexes may have differed at only a single, autosomal locus (marked by a black box); one sex is homozygous at this locus (female) and the other sex (male) is heterozygous (designated proto-male). The "male-determining allele" is shown in yellow. If mating requires one member of each sex, then individuals homozygous for the male-determining allele cannot arise. At this early stage, physiological differences between the sexes will be subtle, comparable to those that distinguish the two mating types in yeast. To prevent the formation of intersex states, crossing-over will be suppressed within and around the male-determining locus (dark shading). Mutations, including deletions and inversions, will accumulate and cause the degenerate region in which crossing-over is suppressed to gradually expand ("Muller's ratchet") until the chromosome has lost most of its active, functional genes. (Mutations accumulate because suppression of crossing-over reduces the probability that they will occur in homozygous form, hence, reducing the selection pressure against them.) A small, active region must remain that is homologous to the X chromosome to allow pairing and crossing-over at meiosis (indicated by a gray ×). This is the pseudoautosomal region (PAR). The autosome, originally homologous to the future X (A in the diagram), will itself evolve sometimes through translocations from other chromosomes (shown as red shaded areas), eventually forming the distinctive X chromosome. The X, like other chromosomes, is a mosaic of DNA fragments put in place at different periods through evolution; some of these are ancient and some are relatively recent. On the human X, the more recent arrivals are enriched in genes that escape X inactivation.

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Mutations, including deletions and translocations, gradually spread beyond the original suppressed region, leading to the progressive degeneration of one of the two originally homologous chromosomes (Fig. 1). This irreversible, degenerative process has been termed "Müller's ratchet," in recognition of the geneticist who first proposed it and modeled it mathematically. There is no selection for this process, it just happens as a consequence of local crossover suppression, which, in turn, was made necessary by the adoption of a two-sex strategy for reproduction (discussed in Charlesworth 1996; Charlesworth and Charlesworth 2005). But whatever the evolutionary drive behind chromosome degeneration, the fact that it has occurred (and is presumably continuing) has required the coevolution of mechanisms to cope with two contingent problems: First, there is a major chromosomal difference between members of the same species, and second, the heterogametic sex is monosomic for a large chromosome and thus monoallelic for a large number of genes. Both these issues must be addressed by mechanisms of dosage compensation.

1.2 Solving the Male Problem: Up-Regulation of X-Linked Genes

In both mammals and *Drosophila*, males have one copy of each sex chromosome, an X and a Y, whereas females have two copies of the X. In both groups of organisms, the Y is gene poor and largely heterochromatic. It contains just a few genes needed for male development or fertility. In contrast, the X is a large, gene-rich chromosome. In all organisms gene products (RNAs and proteins) are made in direct proportion to the number of copies of the gene per cell. Thus, the presence of one or two X chromosomes would cause a twofold difference in the intracellular concentrations of many gene products between the sexes. Furthermore, XY males are monoallelic for the great majority of X-linked genes (only a few genes have homologs on the Y or autosomes). This is surprising, considering higher eukaryotes are usually very intolerant of the loss of even a part of a chromosome; small chromosome deletions generally cause major deformities, whereas autosomal monosomies are always lethal. How, then, do males survive with only one X chromosome?

In attempting to answer this question, it is important to realize that cells and organisms tolerate monoallelism for individual genes rather well. For example, a heterozygous individual with an allele encoding an inactive form of an enzyme, and thus with only half the normal level of active enzyme, is usually perfectly healthy, although homozygosity for the defective allele may be lethal. However, some genes are clearly more dose sensitive than others. Recent studies have suggested that a twofold dosage change in genes encoding components of large multiprotein complexes are particularly likely to exert a phenotypic effect (Pessia et al. 2012). Also, having half the normal level of "multiple" gene products can have a cumulative effect. For example, if several components of a metabolic or signaling pathway are all reduced twofold, the end product of that pathway may be reduced severalfold, with likely phenotypic effects (Fig. 2) (Oliver 2007). Thus, problems will inevitably accumulate for the heterogametic sex as the proto-Y chromosome degenerates over evolutionary time, both through loss of X-linked genes that are individually dosage sensitive and the progressive loss of genes that are individually dosage tolerant, but collectively less so (Fig. 2).

The problem faced by males was recognized more than 40 years ago by the geneticist Susumu Ohno, who speculated that the problem could be solved by up-regulating twofold, expression of genes on the single male X chromosome (Ohno 1967). He also noted that this could occur on a gene-by-gene basis over evolutionary time. Certainly, loss of a dosage-sensitive gene (or a gene in a pathway in which some members had already been lost) would result in a strong selection pressure in favor of up-regulating (i.e., compensating) expression of the remaining gene copy. However, if the male (heterogametic, XY) sex were to adopt this strategy, it would cause problems for the female (homogametic, XX) members of the species, who would then have to cope with increasing numbers of genes whose expression had been "increased" twofold. It may be, therefore, that silencing of one of the two X chromosomes in female mammals, the subject of most of this article, is a response to this overexpression.

The general feasibility of Ohno's suggestion was established by work on dosage compensation in the fruit fly *Drosophila melanogaster* in which genetic, biochemical, and recent high-throughput gene expression studies have confirmed that dosage compensation occurs through up-regulation of X-linked genes in XY males (discussed in Lucchesi and Kuroda 2014). Crucially, the mechanism that evolved in the fly is such that up-regulation occurs only in males, circumventing the problem of overexpression in XX females. Detailed exploration of Ohno's hypothesis in mammals has had to wait for the advent of methodologies that can accurately assay the expression of large numbers of genes. The reason is that assaying transcript levels of individual genes, however accurately, cannot determine whether the gene is up-regulated or not. Genes vary enormously, one from another, in the level at which they are expressed, and even the same gene often changes expression depending on the tissue or cell type in which it is housed, the stage of development, or even over time in the same cell. How, then, can we tell whether any particular gene is twofold up-regulated? What is our baseline?

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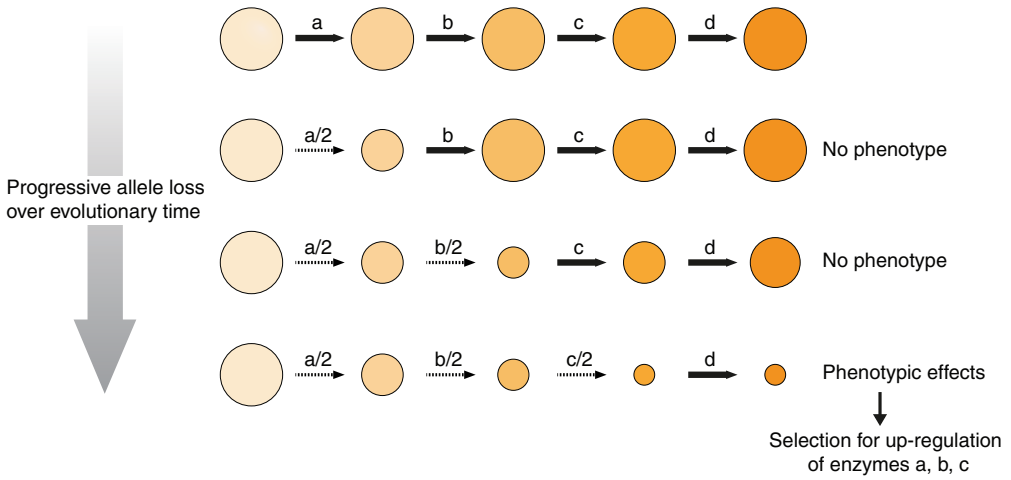

Figure 2. The effects of progressive loss of X-linked alleles on a hypothetical signaling pathway. The diagram shows successive components of the pathway as colored discs, each put in place by the actions of enzymes a, b, c, and d. The size of the discs is proportional to the amount of each component (level 1). The model proposes that enzymes a, b, and c are all encoded by genes on the X chromosome, and early in evolution were encoded by genes on the two proto-X chromosomes. Alleles are lost as the proto-X progressively degenerates, eventually forming the gene-poor Y chromosome (Fig. 1). A twofold reduction in the amount of an enzyme is likely to cause a reduction in its product, although not necessarily twofold. The cell's normal homeostatic mechanisms are likely to correct small disturbances and there is likely to be little or no effect on subsequent steps in the pathway (level 2). Even the loss of two enzymes may be corrected with no physiological effect (level 3). However, a stage will eventually be reached when the cumulative effects of enzyme (gene) depletion cause key components to decrease below a critical level and trigger an effect on phenotype (shown here on level 4). Selection pressure will be exerted to correct the phenotypic effect, most readily by up-regulating expression of one or more of the remaining, single alleles of enzymes a, b, or c.

Recent technologies, first microarrays and now high-throughput RNA sequencing, can assay the expression of large numbers of genes, allowing us to test the proposition that, in any particular tissue or cell type, the "overall" expression of X-linked genes in males (i.e., over the whole, wide range of expression levels) is higher than that of autosomal genes. If the expression ratio of X-linked to autosomal genes in males, with one X and two copies of each autosome, is 0.5, then there is no up-regulation; that is, expression levels reflect gene copy number. On the other hand, if it is 1.0, then there is complete, twofold up-regulation and Ohno's hypothesis is validated (Fig. 3).

Over the past 5 years or so, several studies using either microarray data (Nguyen and Disteche 2006; Lin et al. 2007) or, more recently, RNA sequencing (Deng et al. 2011) have addressed this issue providing strong evidence for the up-regulation of X-linked genes in mammals. However, the data requires careful interpretation. Complications arise from the fact that the X chromosome contains a higher proportion of tissue-specific genes (often involved in sexual development) than autosomes, with the result that the proportion of X-linked genes in any given tissue or cell type that is switched off (silenced) in both sexes is higher than for autosomes (Ellegren and Parsch 2007; Meisel et al. 2012). This must be taken into account. Though the weight of evidence strongly supports the up-regulated expression of X-linked genes in both male and female cells, the situation is complex, as is often the case with dosage compensation. Although the X:autosome expression ratio is consistently >0.5, it does not reach 1.0 (Deng et al. 2011; Lin et al. 2011). The reason for this may be that up-regulation is not applied to all X-linked genes. Perhaps only the more dosage-sensitive genes are up-regulated (Pessia et al. 2012), something that would fit with an evolutionary model in which up-regulation was determined on a gene-by-gene basis (Fig. 1).

There are many questions that remain to be answered concerning expression of X-linked genes, and the issues they raise are relevant to the much more intensively studied question of X-chromosome inactivation, with which the rest of this article is concerned. Perhaps most pressing is the need to decide whether genes are up-regulated by a common, chromosome-wide mechanism, whether different genes have adopted different mechanisms, or whether both factors come into play. Similar issues have been addressed in attempting to unravel the dosage compensation mechanism in *D. melanogaster*, and although the male-specific process that is used in flies is unlikely to operate in mammals, there are undoubtedly important lessons to be learned from this widely studied model organism.

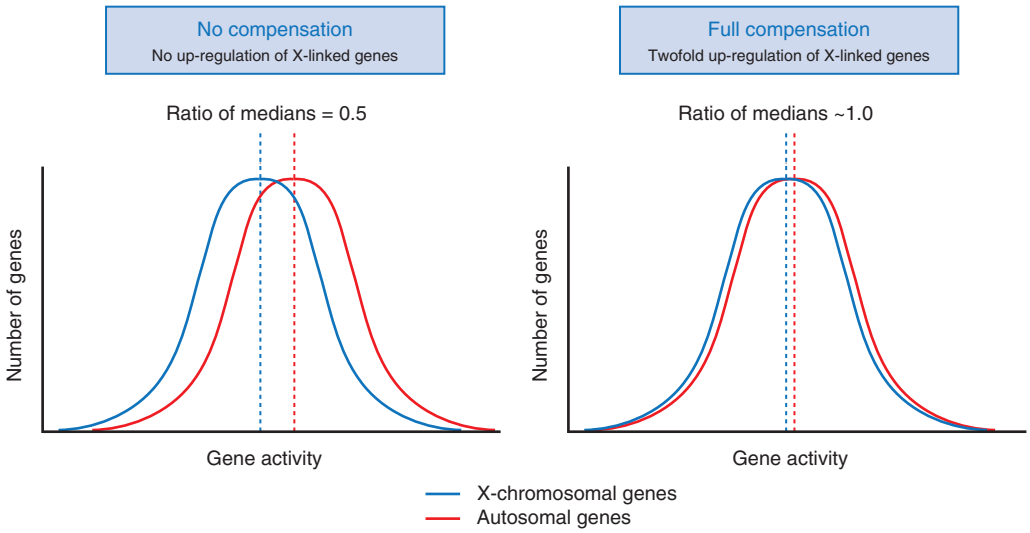

Figure 3. Shift in median expression caused by selective up-regulation of X-linked genes. Measurement of the expression of large numbers of genes on either the X chromosome (blue) or autosomes (red) by microarrays or RNA sequencing shows that transcript levels are normally distributed with a range of expression levels spread over several orders of magnitude. If expression of genes on the single male X (or the single active X in females) is not compensated, then the X:autosome ratio of median expression levels should be 0.5, reflecting the difference in (active) copies (*left* panel). Alternatively, if expression of X-linked genes is up-regulated twofold to compensate for dosage differences, then the ratio of medians should be close to 1.0 (*right* panel).

1.3 Identification of an Inactive X in Mammalian Females

In 1949, Barr and Bertram described the sex chromatin body, a structure visible under the light microscope in the nuclei of only female cells in various mammalian species. The structure proved useful in studies of sexual abnormalities, but it was not until 1959 that Ohno and colleagues showed that this structure was derived from one of the two female X chromosomes (see Ohno 1967). Shortly thereafter, in 1961, Mary Lyon described genetic experiments on the expression of X-linked coat color genes in female mice. To explain the patterns of inheritance for this variable patchwork (mosaic) of coat color in individual female mice, Lyon hypothesized that in each female cell one of the two X chromosomes is stably inactivated early in development (Lyon 1961). The sex chromatin body, now known as the Barr body, is thus the cytological manifestation of the inactive X chromosome. Elegant experiments using skin fibroblasts from females heterozygous for a polymorphism of the X-linked enzyme glucose-6-phosphate dehydrogenase, showed that only one of the two possible alleles was expressed in colonies grown from individual cells (clones), thereby demonstrating the heritability of the inactive state from one cell generation to the next (Davidson et al. 1963), and confirming the occurrence of X inactivation in human females (Beutler et al. 1962). Further studies of X inactivation in human females with multiple copies of the X (with karyotypes such as 47XXX or 48XXXX), showed that all X chromosomes in excess of one were inactivated. This has been generalized as the "n – 1 rule," which states that if an individual has n X chromosomes, then n – 1 will be inactivated (Ohno 1967). This rule explains the remarkably mild clinical symptoms associated with X-chromosome aneuploidies. The X inactivation hypothesis has continued to provide an explanation for the peculiarities of X-linked gene expression in female cells and has remained essentially unchanged since first proposed. But the past 50 years or so have been spent trying to work out the molecular mechanisms by which it operates.

2 OVERVIEW OF X INACTIVATION

2.1 X Inactivation Is Developmentally Regulated

X inactivation in female mammals is developmentally regulated. Both X chromosomes are active in the early zygote (Epstein et al. 1978), and inactivation then proceeds coincident with cellular differentiation from the pluripotent state. Normally there is an equal probability that cells will inactivate the X chromosome that is either derived maternally (Xm) or paternally (Xp). Exceptions to this are imprinted X inactivation that occurs throughout marsupials and in early preimplantation mouse embryos, in which it is always Xp that is inactivated. In the latter case,

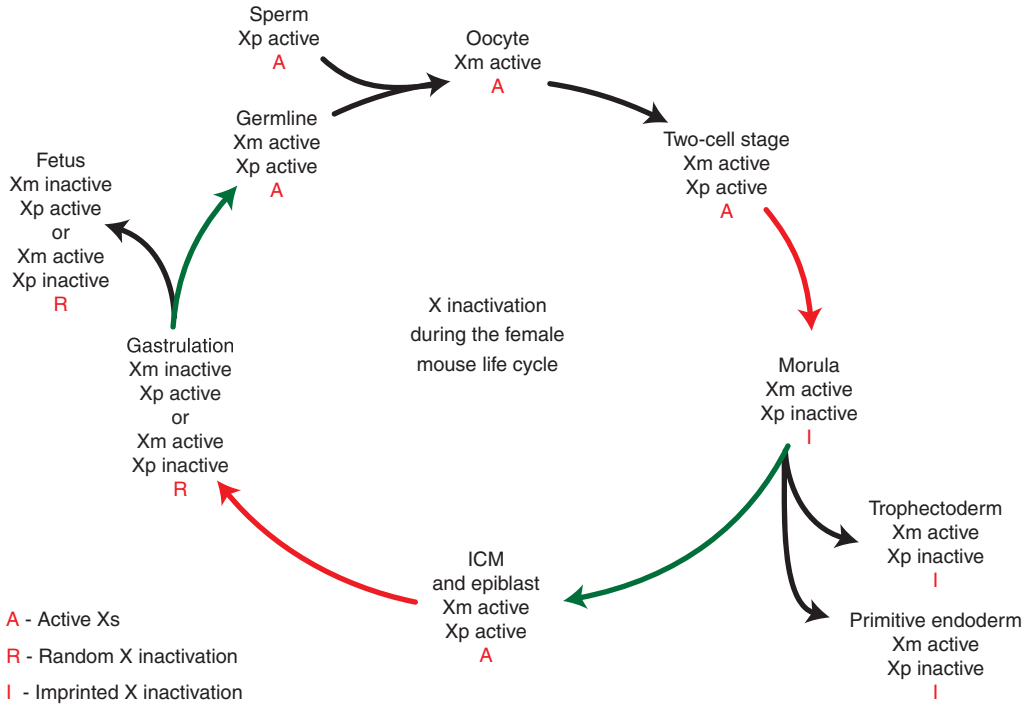

Figure 4. The cycle of X inactivation and reactivation. The X chromosome undergoes a cycle of X inactivation and X reactivation during development. Red arrows indicate X inactivation steps and green arrows indicate X reactivation steps. Inactivation first occurs in early preimplantation embryos (imprinted X inactivation) and subsequently in cells of the epiblast at the time of gastrulation (random X inactivation). The inactive X is reactivated in ICM cells when they are first allocated at the blastocyst stage, and also in the developing germ cells.

imprinted Xp inactivation is maintained in the first differentiating lineages, namely, the extraembryonic trophectoderm (TE) and primitive endoderm (PE) cells, but the inactive X is reactivated in the inner cell mass (ICM) cells that give rise to the embryo. Reversal of X inactivation also occurs in developing primordial germ cells (PGCs), ensuring the X chromosome is again active in the gamete. Figure 4 illustrates the cycle of X inactivation and reactivation in the female mouse.

2.2 Chromosome Silencing Involves Multiple Levels of Chromatin Modification

Silencing of the X chromosome is achieved at the level of chromatin structure by modification of histone tails, incorporation or exclusion of variant histones, DNA methylation of some CpG islands, and reorganization of higher-order chromatin folding, all contributing to a stable facultative heterochromatic structure. There is redundancy built into the system and not all components are essential for all aspects of silencing (e.g., Sado et al. 2004). The layers of chromatin modification are established progressively through ontogeny, as detailed in Section 4.4. Collectively, they ensure stable propagation of the inactive X through multiple rounds of cell division.

2.3 Some Genes Escape X Inactivation

X inactivation affects most of the X chromosome, but some genes escape silencing (Berletch et al. 2011). These include genes within a small region on the X chromosome that pairs with the Y chromosome during male meiosis, referred to as the PAR or XY pairing region (Fig. 1). Genes located in this region do not require dosage compensation as two copies are present in both males and females.

Other genes that escape X inactivation, both with and without Y-linked homologs, have also been characterized. They total ~15% of genes on the human X chromosome (Carrel and Willard 2005). Interestingly, many of these genes lie on the short arm of the chromosome (also referred to as the p arm), which, in evolutionary time, is a recently acquired segment of the X chromosome. Studies in mouse indicate that some escapees can be inactivated in early ontogeny, with progressive reactivation occurring during development (Sec. 4.6). In marsupials, most genes studied have been found to escape X inactiva-

tion to some extent. This may reflect a failure to maintain silencing through ontogeny, possibly related to the lack of CpG island methylation on the inactive X (Xi) in these species.

2.4 X Inactivation Is Regulated by a Master Switch Locus: The X Inactivation Center (Xic)

Classical genetic studies showed that X inactivation is mediated by a single *cis*-acting master switch locus, referred to as the X inactivation center (Xic). The Xic was shown to be required both for silencing the X chromosome in *cis*, and ensuring correct and appropriate initiation of random X inactivation. More recent studies have characterized the Xic at the molecular level. The locus produces a large noncoding RNA termed *Xist* (X-inactive-specific transcript) that has the unique property of binding in *cis* and accumulating along the entire length of the chromosome from which it is transcribed (Fig. 5) (Brown et al. 1991; Brockdorff et al. 1992; Brown et al. 1992). Coating of the chromosome with *Xist* RNA provides the trigger for X-chromosome silencing (Lee et al. 1996; Penny et al. 1996; Wutz and Jaenisch 2000). Studies to date indicate that this occurs, at least in part, through *Xist*-mediated recruitment of chromatin modifying complexes (Fig. 5A).

A second noncoding RNA, *Tsix*, is also located in the Xic region (Lee et al. 1999) and plays a key role in regulating *Xist* expression. *Tsix* overlaps with the *Xist* gene, but is transcribed in the antisense direction; hence, its name is *Xist* spelled backwards.

Phylogenetic studies have revealed that the noncoding *Xist* RNA evolved from a protein-coding transcript, *Lnx3* (Duret et al. 2006). The *Lnx3* gene has retained protein-coding capacity in other vertebrate species and also in marsupial mammals. The latter finding was unanticipated as obvious similarities in X inactivation in marsupials led to the supposition that chromosome silencing is mediated by a direct homolog of *Xist*. After many years of searching, a recent study has revealed that marsupials independently evolved a *cis*-acting noncoding RNA locus, *Rsx* (RNA on

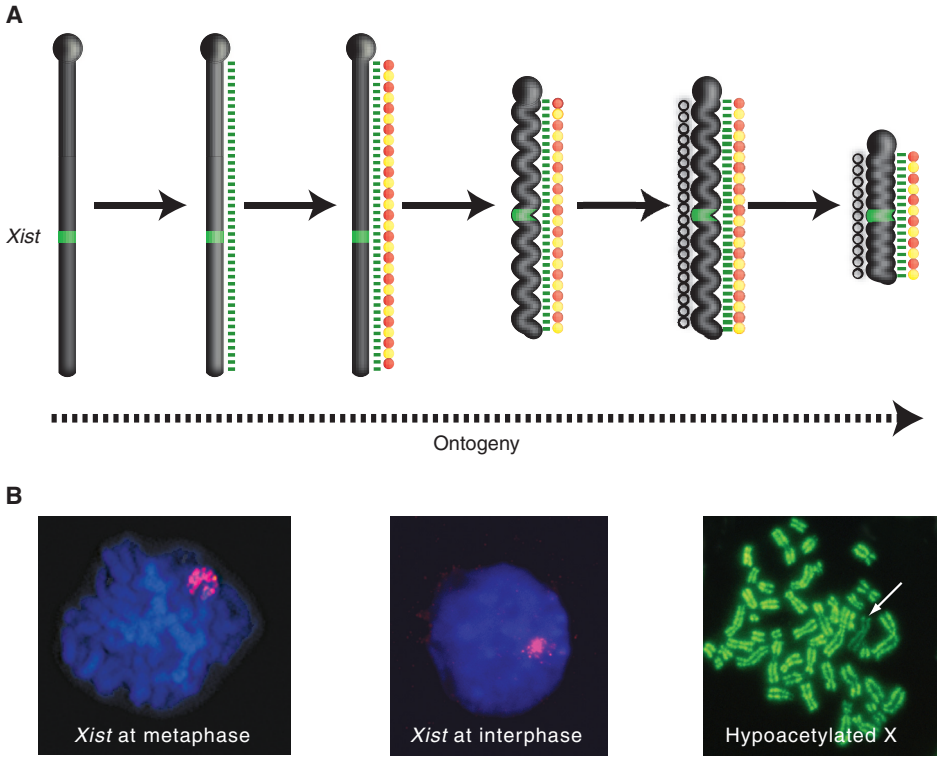

Figure 5. Progressive chromosome-wide heterochromatinization induced by *Xist* RNA. (*A*) When the *Xist* gene is expressed, the RNA binds to and coats the X chromosome from which it is transcribed (green dashed line). *Xist* RNA is thought to trigger silencing of the chromosome by recruiting chromatin modifying activities (red and yellow circles). The initial wave of silencing, in turn, leads to recruitment of additional layers of epigenetic modification (white circles), further stabilizing the heterochromatic structure. Establishment of these different levels of epigenetic silencing is achieved in a stepwise manner through development and ontogeny. (*B*) Localization of *Xist* RNA along the X chromosomes is shown by in situ hybridization in both interphase and metaphase.

the silent X), which fulfills the same function as *Xist* in marsupial species (Grant et al. 2012). Like *Xist* RNA, *Rsx* RNA is transcribed specifically from Xi and coats the length of the X chromosome in *cis*.

3 INITIATION OF X INACTIVATION

3.1 Imprinted versus Random X Inactivation

The decision to inactivate an X chromosome needs to be tightly regulated. Male cells must avoid silencing their single X chromosome, and female cells must avoid silencing both X chromosomes or keeping both X chromosomes active. Two different modes of regulation have been shown to operate. The imprinted mode of X inactivation silences the paternally derived X chromosome whereas the random mode randomly inactivates either the maternal or paternal X chromosome. Metatherian mammals (marsupials) use only the imprinted mode. Some eutherian (placental) mammals (e.g., mouse) use the imprinted mode in extraembryonic lineages and the random mode in the embryo proper (Fig. 2). Other species, notably rabbit and human, show only random X inactivation (Okamoto et al. 2011). This variation may be linked, in part, to differences in the timing of embryonic genome activation, which in mouse occurs relatively early, at the two-cell stage, compared with the four- to eight-cell stages in humans.

Important model systems for studying the initiation of X inactivation are early mouse embryos and embryonic stem (ES) cells derived from the ICM of these embryos. XX ES cells are especially useful as they recapitulate initiation of random X inactivation in vitro when cells are induced to undergo differentiation. There are currently no in vitro models that recapitulate initiation of imprinted X inactivation.

3.2 Regulation of Imprinted X Inactivation

Paternally imprinted X inactivation was first observed in a marsupial (Sharman 1971). Subsequently, imprinted X inactivation was shown to occur in the TE and PE of mouse embryos (Takagi and Sasaki 1975). In imprinted X inactivation, it is the parent of origin from which the X derives that governs its status; that is, paternal, but not maternal, X chromosomes are inactivated regardless of how many X chromosomes or chromosome sets are present. Note that the single X in XY males is always maternally derived and therefore not inactivated in imprinted tissues.

What then is the nature of the imprint? Studies of *Xist* expression indicate that there is a repressive imprint on the Xm allele from morula stage mouse embryos. This imprint prevents *Xist* expression, keeping the X chromosome active (see Fig. 4). Nuclear transfer experiments showed that the repressive *Xist* imprint is established during oocyte maturation (Tada et al. 2000). The molecular basis of the imprint is unknown, but DNA methylation is not required, contrasting with many other imprinted genes (see Barlow and Bartolomei 2014 for details on genomic imprinting).

One theory for the preferential inactivation of Xp in the zygote is that there is carryover of silencing of the XY bivalent that is established during the pachytene stage of male meiosis (meiotic sex-chromosome inactivation, MSCI; Huynh and Lee 2003). Recent studies argue against this. First, MSCI has been shown to be a distinct and *Xist*-independent mechanism that is triggered in pachytene by the presence of unpaired chromosomal regions on both sex chromosomes and autosomes (Turner et al. 2006, and references therein). Second, expression analysis of a number of X-linked genes in early zygotes has shown that Xp silencing occurs de novo in response to zygotic Xp *Xist* expression (Okamoto et al. 2005, and references therein).

The paternal *Xist* expression (and resultant Xp silencing) that begins at the onset of zygotic gene activation (at the two- to four-cell stage) indicates that the Xp *Xist* allele is poised to express (Fig. 6). A region-specific demethylation of CpG sites in the *Xist* promoter occurs during spermatogenesis (Norris et al. 1994) and is thought to be important for this.

The *Tsix* gene, an antisense regulator of *Xist*, is required for imprinted X inactivation as deletion of the major promoter results in early embryo lethality when transmitted by the maternal, but not the paternal, gamete (Lee 2000). Lethality appears to be attributable to inappropriate Xm *Xist* expression (i.e., a failure to retain an active X chromosome both in XmY and XmXp embryos). It is not known if expression of *Tsix* RNA is the primary imprint or functions only later to maintain the imprint.

3.3 Regulation of Random X Inactivation: Counting

In the random mode of X inactivation, cells use the $n - 1$ rule described in Section 1.3, in which all X chromosomes except one are inactivated per diploid chromosome set. The process that senses the number of X chromosomes is often referred to as counting. Where more than one X chromosome is present, the selection of active and inactive X chromosome—referred to as choice—is normally random. However, there are factors that can bias this decision resulting in nonrandom or skewed X inactivation. The process of choice is considered separately in Section 3.4, but this division is one of convenience as it is clear that counting and choice must be inextricably linked.

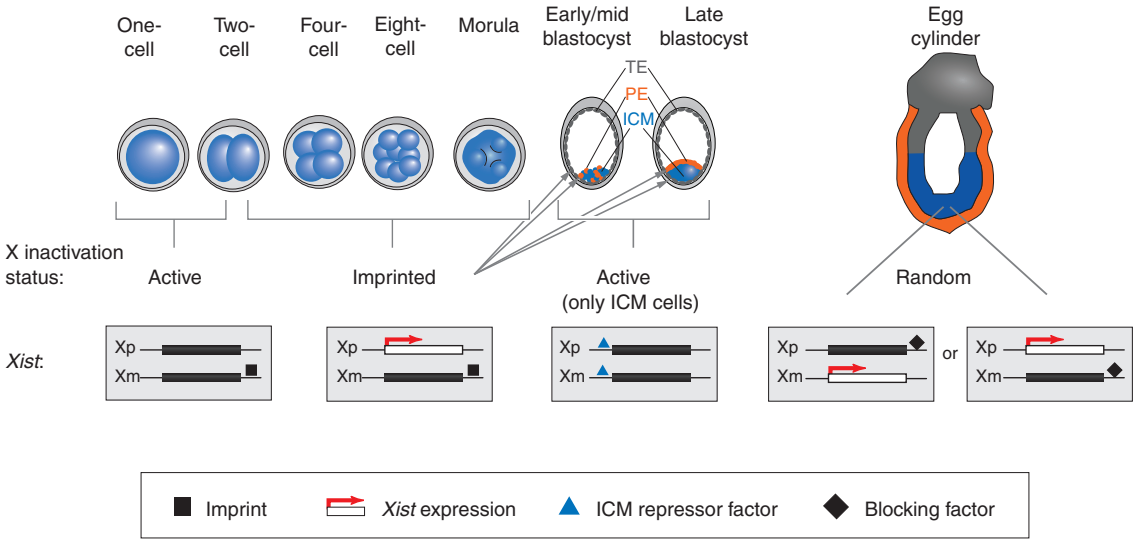

Figure 6. *Xist* gene regulation in early development. The figure illustrates current knowledge and models for imprinted and random *Xist* regulation in early XX mouse embryos. The Xm *Xist* allele arrives in the zygote with a repressive imprint possibly mediated through the antisense *Tsix* locus (black square). The Xp *Xist* allele is primed to be active and is expressed as soon as embryonic gene activation occurs at the two-cell stage. From the two- to four-cell stage up until morula stage, Xp *Xist* is expressed in all cells (expression indicated by open rectangle and arrow at 5′ end). This pattern is maintained at the early blastocyst stage and subsequently in TE and PE cells and their fully differentiated derivative (extraembryonic) tissues. In the late blastocyst, ICM *Xist* expression is extinguished, possibly by an ICM-specific repressor factor (blue triangle). *Xist* expression then commences subsequently at the time of gastrulation. Here, the blocking factor (black diamond) ensures that *Xist* expression cannot occur on one of the two alleles (counting).

A number of different models for counting and choice have been proposed (Fig. 7). Early models, developed before the discovery of *Xist*, invoked a single autosomally encoded blocking factor present in limiting quantities sufficient to bind and repress a single X inactivation center (Rastan 1983). In this model, X inactivation is a default pathway that in diploid cells is inhibited on a single X chromosome (the active X). In cells with more than one X chromosome, choice is determined by the probability of the blocking factor binding to a given X inactivation-center allele.

A related model invokes two factors: an autosomally encoded blocking factor and an X-encoded competence factor (Gartler and Riggs 1983). The blocking factor is suggested to be sufficient to disable the competence factor on a single X inactivation center. Other X inactivation centers can be bound by competence factor leading to the onset of X inactivation. Although initially developed before the discovery of *Xist*, this model has been readopted to help explain experimental observations arising from the deletion of *Tsix*, the antisense regulator of *Xist* (Lee and Lu 1999).

A third and more contemporary model invokes a stochastic process in which autosomal factors promote *Xist* repression, for example, by inducing *Tsix*, whereas the X chromosome produces *Xist* gene activators that compete with the repressors. The resultant competition could create a probability for *Xist* gene activation that is modulated as cells begin to differentiate. The model predicts a stochastic probability of maintaining a single X active in all circumstances, but also invokes checkpoint and feedback mechanisms that ensure incorrect X inactivation patterns, notably inactivation of both or neither X chromosome in XX cells, can be reset at an early stage. In part, this could be attributable to levels of X-linked activators becoming limiting once silencing has spread to the locus encoding the activator on one of the two X chromosomes (Nora and Heard 2009).

These models are not necessarily mutually exclusive and, moreover, they continue to evolve to take into account new experimental findings. As things stand, none of the models can be said to satisfactorily account for all available data, but they nevertheless provide a useful framework both for integrating current data and determining new experimental directions.

Although a complete model for *Xist* regulation in random X inactivation has not yet been found, there is a growing consensus that there is a finely balanced competition

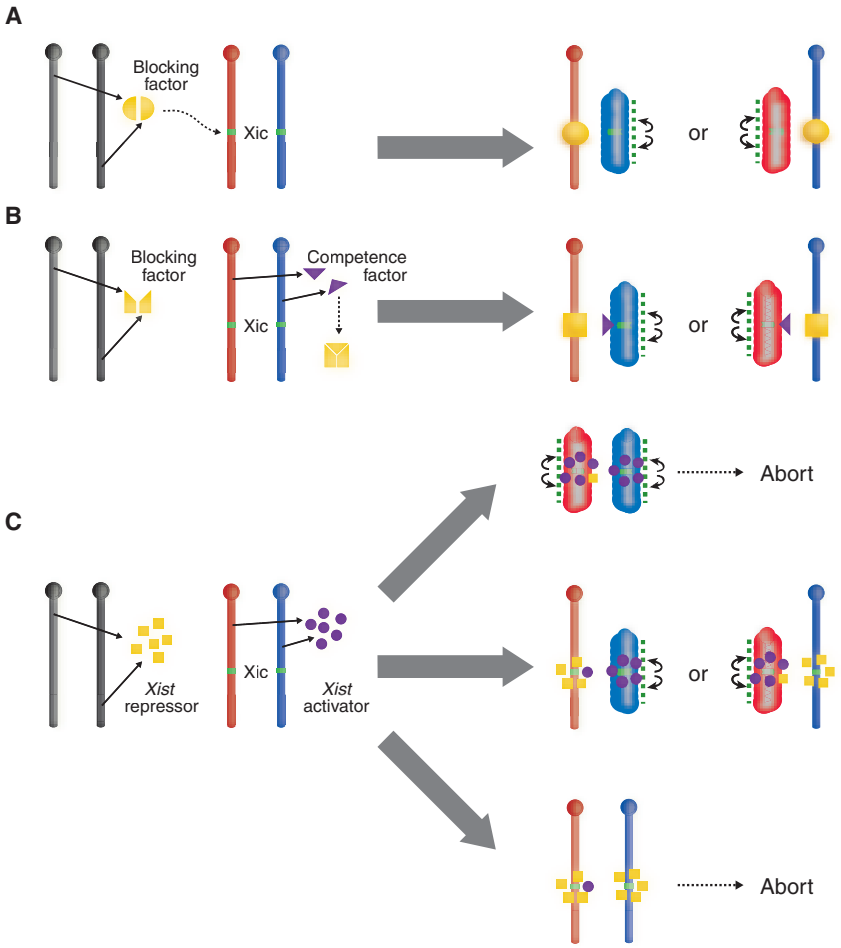

Figure 7. Models for the regulation of random X inactivation. (*A*) Autosomally encoded blocking factor (yellow shapes) is produced in sufficient quantities to occupy a single Xic. Binding of blocking factor to the Xic inhibits *Xist* transcription, thus defining a single active X chromosome. *Xist* transcription occurs on any additional X chromosomes leading to X inactivation (dark green dots). Blocking factor binds to either the maternal (Xm) or paternal (Xp) X chromosome with equal probability and in a cell-autonomous manner. (*B*) The two-factor model invokes an X-encoded competence factor (purple triangle) and an autosomally encoded blocking factor (yellow shapes). Blocking factor titrates away competence factor (purple triangle). In cells with a single X chromosome, there is insufficient available competence factor to activate *Xist*, but in cells with additional X chromosomes, competence factor can activate all X chromosomes except the single X chromosome bound by blocking factor. (*C*) The stochastic model invokes that autosomally encoded repressors (yellow circles) and X-encoded activators (purple circles) compete with one another. All *Xist* alleles have an equal probability of being activated and this is increased in cells with more than one X chromosome (higher levels of *Xist* activators). By chance, some cells with two X chromosomes will initiate inactivation of either both or no X chromosomes. This may be dealt with by checkpoint mechanisms or cell death.

between the pathways that repress *Xist* and those that activate *Xist*. The decision to initiate and then maintain *Xist* up-regulation on a given allele may then be reinforced by feedback and/or feedforward loops.

Genetic studies have shown that the antisense gene, *Tsix*, is an important *Xist* repressor. *Tsix* is transcribed on the X chromosome before and concordant with the onset of *Xist* expression in random X inactivation. *Tsix* promoters lie immediately downstream from the *Xist* locus (Fig. 8). The antisense transcript spans the entire *Xist* locus, terminating immediately upstream of the major *Xist* promoter. Antisense transcription across the *Xist* promoter region is required for *Tsix*-mediated repression (Ohhata et al. 2008). The detailed molecular mechanisms are not fully understood, but are thought to involve a switch in the histone modification state of the *Xist* promoter (Sado et al. 2005; Navarro and Avner 2010), and recruitment of the de novo DNA methyltransferase Dnmt3a (Sun et al. 2006).

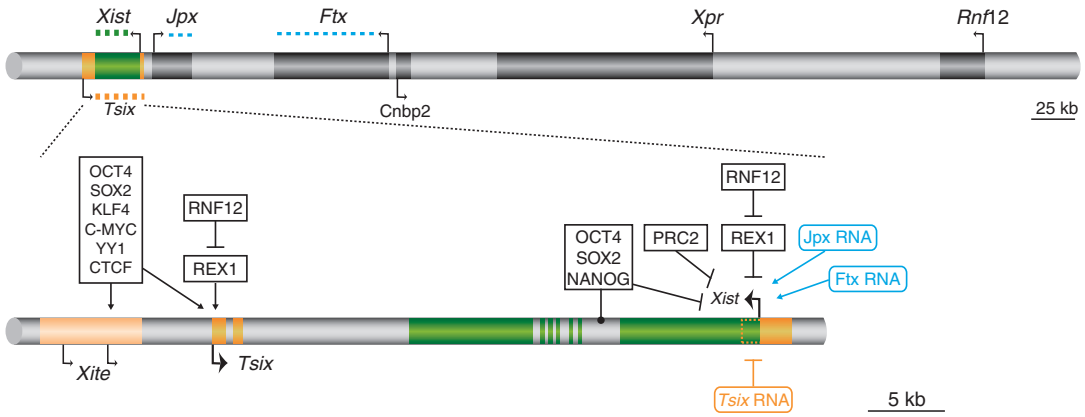

Figure 8. Genes and regulatory elements in the X inactivation-center region. The key region on the mouse X chromosome comprising known elements involved in *Xist* gene regulation is illustrated, showing noncoding RNA (ncRNA) genes and protein-coding genes. The *Xpr* region, several hundred kilobases upstream of *Xist*, has been implicated in *trans*-interaction of Xic alleles in XX cells and as such is thought to be important for initiation of X inactivation. The expanded view illustrates the intron/exon structure of the *Xist* and *Tsix* loci, including the Xite elements that function as *Tsix* enhancers. The network of protein factors (boxes) and ncRNAs (ovals) implicated in *Xist* gene regulation is shown with arrows and bars indicating repressor and activator function, respectively. Note that RNF12 mediates degradation of REX1, which functions both as a *Xist* repressor and a *Tsix* activator.

Tsix is thought to play a role in counting based on the observation that *Xist* is strongly up-regulated in *Tsix* null XY ES cells undergoing differentiation. However, not all *Tsix* null ES cell lines show this effect (the reasons for the discrepancy remain unclear) and, moreover, *Tsix* deletion leads to overt *Xist* gene up-regulation in undifferentiated XY ES cells. These observations indicate that other pathways contribute to *Xist* repression. Recent work has highlighted an important role for transcription factors linked to pluripotent cell circuitry, notably Oct4, Nanog, Sox2, and Rex1 (Donohoe et al. 2009; Navarro et al. 2010). These repressive effects are mediated by promoting *Tsix* expression, directly repressing *Xist*, and also by suppressing levels of the *Xist* activator, Rnf12 (see below). Because levels of pluripotency factors decline rapidly at the onset of differentiation, the regulatory balance at the *Xist* locus will be tipped toward activation, providing a plausible explanation for the link between the onset of *Xist* expression and cell differentiation.

Polycomb family repressors (elaborated in Secs. 4.4 and 4.5) are also implicated in *Xist* repression based on observed synergistic effects in cells with mutations affecting both *Tsix* and the PRC2 Polycomb pathway (Shibata et al. 2008). It is not yet known if this is linked to the pluripotency factor pathway or represents a third independent *Xist* repression pathway.

The existence and location of X chromosome-encoded activators of *Xist* expression was predicted through the analysis of polyploid ES cells and a deletion encompassing known *Xist* regulatory elements on a single X chromosome in XX ES cells (Monkhorst et al. 2008). This study led directly to the identification of the first known *Xist* activator, the ubiquitin E3 ligase Rnf12 (Jonkers et al. 2009). Overexpression of *Rnf12* promotes *Xist* expression in XY ES cells, whereas *Rnf12* deletion delays *Xist* up-regulation in differentiating XX ES cells. The location of the *Rnf12* gene immediately upstream of *Xist* supports the idea of a feedback loop in which the spread of X inactivation on one X chromosome represses *Rnf12*, reduces levels of Rnf12 protein, and thereby serves to reduce the probability of inappropriately inactivating remaining X chromosomes. Rnf12 functions as an E3 ligase for ubiquitin-mediated degradation of the pluripotency factor Rex1, found at the promoter regions of both *Xist* and *Tsix* genes. Thus, Rnf12-mediated degradation of Rex1 may simultaneously activate *Xist* and repress *Tsix* (Gontan et al. 2012).

Although it is clear that Rnf12 plays an important role in *Xist* gene activation, the fact that *Rnf12* null XX ES cells are able to up-regulate *Xist*, albeit with some delay, suggests the presence of additional activators, also possibly encoded on the X chromosome. Such a role has been ascribed to a ncRNA, *Jpx*, which is located immediately upstream of the *Xist* locus (Tian et al. 2010). The mechanism of Jpx RNA function is not yet fully understood although there is recent evidence that it evicts the silencing protein CTCF from the *Xist* locus (Sun et al. 2013). Similarly, another ncRNA produced by the *Ftx* locus, immediately upstream of *Jpx*, is implicated in *Xist* gene activation (Chureau et al. 2011). The loci and pathways known to participate in *Xist* repression and *Xist* activation are summarized in Figure 8.

3.4 Regulation of Random X Inactivation: Choice

In XX cells, the choice of which X chromosome to inactivate in a given cell is normally random. However, animals that are heterozygous for certain Xic alleles can show a bias toward one allele or the other. Before discussing these, it is important to first make the distinction between primary and secondary nonrandom X inactivation. Primary nonrandom X inactivation relates to a bias in the choice step that occurs at the time random X inactivation is initiated. Secondary nonrandom X inactivation, on the other hand, occurs as a consequence of selection against cells that have nominated a given X chromosome as the inactive X. This latter process does not strictly relate to choice, but occurs commonly when mutations on one X chromosome confer a selective disadvantage to cells that inactivate the wild-type allele. Examples include the secondary nonrandom X inactivation seen in carriers of serious genetic disorders such as Duchenne muscular dystrophy. The observed cell selection effects can occur at the level of the whole developing embryo or in specific tissues or cell types, depending on the function of the mutant protein.

The first documented example of primary nonrandom X inactivation came in classical genetic studies that identified variants at the "X controlling element" (*Xce*) in different mouse strains (Cattanach 1974, and references therein). *Xce* was mapped to the approximate location of the Xic on the mouse X chromosome, suggesting the two loci could be synonymous. This has been confirmed in more contemporary studies that locate *Xce* to a region immediately downstream from *Xist*. The molecular basis of the variation underlying nonrandom X inactivation, however, remains to be determined.

Primary nonrandom X inactivation has also been observed in animals heterozygous for various Xic alleles, established in gene targeting experiments aimed at the functional dissection of *Xist* and *Tsix*. A common theme emerging from these studies is that alleles that increase the probability of being selected for X inactivation are associated with mutations that either reduce levels of antisense *Tsix* transcription or increase levels of sense *Xist* transcription (Lee and Lu 1999; Nesterova et al. 2003). There is also recent evidence that choice is linked to an *Xist* RNA splicing switch (Royce-Tolland et al. 2010). In the context of models for regulation of random X inactivation, biased choice can be viewed as tipping the balance between *Xist* repressors and *Xist* activators on a given allele.

What then is the symmetry-breaking event that results in the counting/choice machinery ultimately associating with only a single allele when there is equivalent access to both alleles? One idea that has emerged is that this may involve *trans*-interactions between Xic alleles at the time X inactivation is initiated. Specifically, three-dimensional (3D) fluorescence in situ hybridization (FISH; Xu et al. 2006; Augui et al. 2007) and locus tagging experiments (Masui et al. 2011) have shown frequent associations of Xic alleles mediated by contacts close to the *Tsix* promoter and a region located several hundred kilobases upstream of *Xist*, termed *Xpr*. Current models posit that *Xpr* is required to establish initial *trans*-interactions, thus facilitating contacts in the *Tsix* region. These *trans*-interactions have been proposed to mediate exchange of factors from one allele to another, providing an opportunity to break symmetry (see Fig. 7 of Dekker and Misteli 2014).

3.5 Switching Modes of X Inactivation Regulation in Early Embryogenesis

How do early mouse embryos instigate the switch from the imprinted to the random mode of regulation (see Fig. 4)? Until recently, it was thought that the initiation of imprinted and random X inactivation were both linked to cellular differentiation (Monk and Harper 1979). Thus, trophectoderm and primitive endoderm lineages were thought to inactivate their Xp in response to the parental imprints on *Xist* when they first differentiate. The three germ lineages that give rise to the embryo proper by gastrulation were thought to first erase the *Xist* imprint and then undergo random X inactivation (Fig. 6). More recent data, however, show that Xp inactivation occurs before the onset of cellular differentiation in cleavage stage embryos, and it occurs in all cells, including the precursors of the ICM (Fig. 6) (Mak et al. 2004; Okamoto et al. 2004). Thus, imprinted X inactivation in trophectoderm and primitive endoderm is a relic of the X inactivation pattern established in early cleavage embryos. ICM cells must instigate a program to reverse this initial wave of imprinted Xp inactivation, thereby setting the scene for subsequent random X inactivation. The basis for the reversal of Xp inactivation is unknown, but may involve an ICM-specific program that represses Xp *Xist* expression (see Sec. 5.1).

3.6 Evolutionary Variation in X Inactivation Regulatory Mechanisms

To date, the laboratory mouse has been the primary model system for studies on the initiation of X inactivation. However, recent work suggests that the lessons drawn from such studies may not be as broadly applicable as once thought (Okamoto et al. 2011). Analysis of human preimplantation embryos has shown that *XIST* is up-regulated from the X chromosome in male embryos and both X chromosomes in female embryos during early preimplantation development. This pattern is resolved in late blastocysts, in which

males extinguish *XIST* expression and females express it from only one X chromosome. The early *XIST* expression in human embryos is not linked to chromosome silencing, consistent with the requirement for expression of X-linked genes. A further variation was revealed through experiments with rabbits, in which female preimplantation embryos expressed *Xist* from both alleles in ~25% of cells. In this case, *Xist* expression is linked to chromosome silencing, suggesting either a checkpoint mechanism that can reverse and correct inappropriate biallelic *Xist* expression, or cell selection events resulting in the loss of XX cells that silence both X chromosomes (Okamoto et al. 2011). An important goal for the future will be to determine if there are conserved features in these apparently diverse systems as these would likely point to the fundamental underlying mechanisms.

4 PROPAGATION AND MAINTENANCE OF THE INACTIVE STATE

4.1 *Xist* RNA, Gene Silencing, and Heterochromatin Assembly

There is strong evidence that the *Xist* gene and its RNA product provide both the switch that initiates X inactivation in *cis* and the means by which silencing spreads across the chromosome. The evidence comes from experiments, indicating that (1) *Xist* is unique in being expressed only from Xi, (2) *Xist* RNA levels increase dramatically in preimplantation embryos at the time of X inactivation, (3) *Xist* up-regulation precedes X inactivation and appears to be an absolute requirement for it to occur, (4) *Xist* RNA colocalizes with Xi in interphase nuclei and is distributed along one of the two metaphase X chromosomes (see Fig. 5B), and (5) *Xist*-containing transgenes, when inserted into autosomes, can coat the autosome in *cis* with *Xist* RNA and initiate the adoption of a heterochromatin-like, transcriptionally silent chromatin structure. These findings suggest that *Xist* RNA is both necessary and sufficient to trigger heterochromatin formation and transcriptional silencing. However, continuing *Xist* expression is not required for the "maintenance" of X inactivation. For example, silencing of human X-linked genes is maintained in human:rodent somatic cell hybrids, in which *Xist* expression is lost on the retained human Xi chromosome (Brown and Willard 1994). This issue is discussed further in Section 5.

The mechanism(s) by which *Xist* RNA associates with and spreads along the Xi in *cis* and the mechanisms that bring about changes in chromatin structure and gene silencing are still not understood in detail. We do know that different regions of the *Xist* RNA molecule are responsible for gene silencing and spreading along the X chromosome. Experiments with an inducible *Xist* expression system in mouse ES cells, in which the functions of Xist RNA molecules carrying defined deletions could be tested, showed that silencing can be attributed to a conserved repeat sequence, the A-repeat, located at the 5′ end of the molecule, whereas coating of the X is mediated by sequences scattered throughout the rest of the molecule (Wutz et al. 2002). These observations show that *Xist* RNA spreading and chromosome-silencing functions are mechanistically separable.

4.2 Spreading of *Xist* RNA and Chromosome Silencing

The association of *Xist* RNA with Xi is selective. It is not found along the PAR, which remains active and euchromatic, or at constitutive (centric) heterochromatin. Moreover, analysis of metaphase chromosomes shows a banded localization that appears to correlate with gene-rich G-light bands (Duthie et al. 1999). In dividing cells, *Xist* RNA association with Xi is lost when cells enter anaphase. It is then rapidly resynthesized in daughter cells at the onset of G_1. *Xist* RNA is tightly associated with the nuclear matrix in the interphase nucleus and localization is retained following removal of chromatin by micrococcal nuclease, suggesting that *Xist* RNA does not contact underlying DNA sequences directly (Clemson et al. 1996).

There are approximately 2×10^3 molecules of *Xist* RNA in a female somatic cell (Buzin et al. 1994). The transcript is relatively stable with an estimated half-life of 6–8 h in dividing cells, a figure that is consistent with turnover occurring predominantly as a consequence of dissociation through mitosis. However, it may be that *Xist* RNA turnover is inhibited in the presence of transcriptional inhibitors, as a recent study using GFP-tagged *Xist* transcripts in living cells determined a relatively fast turnover rate for *Xist* RNA (Ng et al. 2011).

What then is known of the factors regulating Xist RNA localization? One interesting link has come from analysis of the protein SAF-A/hnRNPU. Originally identified as an hnRNP protein and a major component of the insoluble nuclear scaffold, SAF-A/hnRNPU was first linked to X inactivation through cell imaging studies demonstrating enrichment of the protein over the interphase Xi territory in XX somatic cells (Pullirsch et al. 2010). This observation is consistent with the association of *Xist* RNA with the nuclear matrix. More recent data suggest that genetic disruption of SAF-A/hnRNPU causes dispersal of *Xist* RNA through the nucleoplasm, indicating a role in *Xist* RNA localization (Hasegawa and Nakagawa 2011). Moreover, biochemical analysis suggests that the RNA binding domain of hnRNPU/SAFA interacts directly with *Xist* RNA (Fig. 9A).

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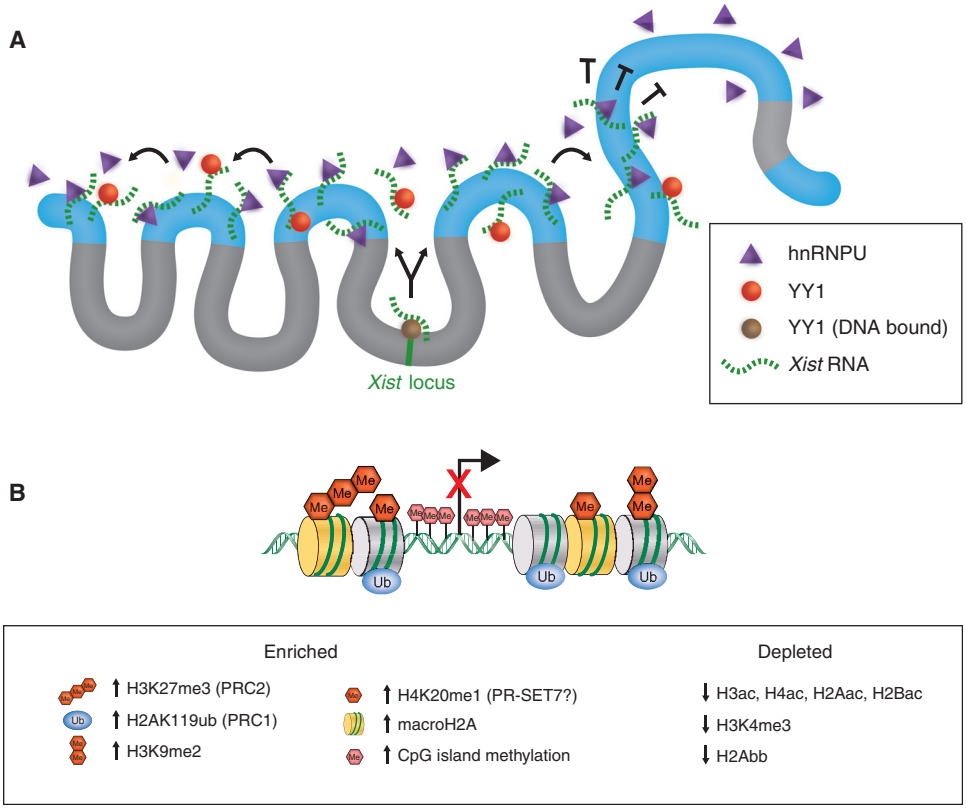

Figure 9. Factors involved in X inactivation spread. (*A*) The organization of LINE-1-rich (gray-shaded) and gene-rich (blue-shaded) domains is important in defining the extent of *Xist* RNA spreading as illustrated by the observation that large gene-rich domains attenuate *Xist* RNA spreading (barred arrows). YY1 is implicated in tethering *Xist* RNA (green wavy line) at the site of synthesis (brown circle) and interacting with *Xist* RNA to facilitate spreading (red circle). hnRNPU/SAFA also plays a role in localization of *Xist* RNA in *cis*, binding to *Xist* RNA directly. (*B*) Summary of characterized DNA methylation, histone modification, and histone changes at a silent gene on Xi.

A second factor implicated in *Xist* RNA localization is the transcription factor YY1, which is proposed to be a bifunctional protein capable of binding both DNA elements at the Xic and *Xist* RNA (Fig. 9A) (Jeon and Lee 2011; Thorvaldsen et al. 2011). Binding elements in the *Xist* gene are suggested to function as nucleation sites that mediate docking of *Xist* RNA particles with Xi.

4.3 Booster Elements Facilitate Spreading of X Inactivation

Although *Xist* RNA transgenes function on autosomes, autosomal silencing is less efficient than silencing of the X chromosome. The relative insensitivity of autosomes to *Xist*-mediated silencing was first reported in classical genetic studies that analyzed gene silencing in X:autosome translocations. Specifically the spreading of silent chromatin along the autosomal chromosome arm was found to be variable between translocations and limited in extent. This is attributable to autosomes resisting the initial spreading of *Xist* RNA and associated gene silencing, at least in some cases (Popova et al. 2006). In other cases, limited silencing appears to result from aberrant long term maintenance of silencing on autosomes following efficient spreading at the onset of X inactivation (Cattanach 1974).

Attenuated silencing on autosomes led to the idea that there are sequences on the X chromosome, originally referred to as "way stations" or "booster elements" (described in Gartler and Riggs 1983), that serve to amplify or enhance the spread/maintenance of X inactivation. More recently, it has been proposed that a common dispersed repeat family, the L1 long-interspersed repeats (LINE-1), are good candidates for the "way station" elements (Lyon 2003). These repeat sequences are common in the human and mouse genomes, but are particularly frequent along the X chromosome. Further, LINE-1 elements are most common in the more condensed, gene-poor, G-banded regions of the human and mouse genomes, suggesting that they may, in some way, favor a chromatin conformation associated with transcriptional silencing. In support of this idea, analysis of

gene silencing meditated by *Xist* transgenes located on autosomes and in X autosome translocations indicates that large gene-rich LINE-1 depleted chromosomal domains are relatively refractory to the spreading of X inactivation (Popova et al. 2006; Chow et al. 2010; Tang et al. 2010).

A common idea is that way station elements are synonymous with *Xist* RNA-binding sites. In the case of L1 LINE elements, this seems improbable as *Xist* RNA is concentrated in gene-rich chromosomal domains, reciprocal to L1 LINE distribution. Interestingly, the high density of L1 LINEs on the X chromosome has fragmented gene-rich domains such that they are, on average, much smaller than on autosomes. Thus, one idea is that L1 LINE domains influence the higher-order topological folding of the chromosome in such a way as to favor efficient in *cis* spreading of *Xist* RNA within gene-rich domains (Popova et al. 2006; Tang et al. 2010). It is important to remember that the efficiency of *Xist* RNA spreading from its site of synthesis (i.e., the *Xist* locus) through the X-chromosome territory will depend on both the overall configuration of that territory in three dimensions and how the *Xist* locus is positioned within the territory.

RNA FISH analysis has shown that there is a major burst of L1 LINE transcription maintained specifically on the inactive X chromosome at the time that X inactivation is first established (Chow et al. 2010). L1 transcription has been directly linked to silencing through a mechanism involving overlapping transcripts and the production of short RNAs, but may also play a role in facilitating spread of X inactivation in *cis*.

Knowledge of *Xist* RNA binding sites beyond that provided by cytological analysis is at present lacking, and the development of a method to map binding sites at near nucleotide resolution is an important goal for the future.

4.4 Heterochromatic Structure of the Inactive X: The Link with Chromatin Modifications

Since the very earliest light microscopical studies, it has been realized that Xi shares properties with heterochromatin. Like the constitutive heterochromatin found at and around centromeres, Xi remains visible and apparently condensed throughout interphase (as the Barr body), and its DNA is usually replicated late in S-phase. Xi is said to consist of "facultative" heterochromatin.

Further parallels between Xi and constitutive heterochromatin have come from the use of indirect immunofluorescence microscopy to study the distribution of histone modifications and variants both along metaphase chromosomes and in interphase nuclei. The facultative heterochromatin of the inactive X chromosome in both human and mouse cells is depleted in acetylated histone H4 (Jeppesen and Turner 1993), and in this way resembles constitutive, centric heterochromatin. This was the first demonstration that the inactive X chromosome was marked by a specific type of histone modification. (Histone modifications and their functions are described in Allis et al. 2014.) Subsequent experiments in several laboratories confirmed these observations and further showed that acetylated isoforms of all four core histones (H2A, H2B, H3, and H4) were depleted in both constitutive and facultative heterochromatin in interphase and metaphase cells (Fig. 5B). In particular, both centric heterochromatin and Xi are depleted in H3 di- and trimethylated at lysine 4 (H3K4me2 and H3K4me3; O'Neill et al. 2008). Like acetylation, H3K4me2/me3 are generally thought to be markers of transcriptionally active, or potentially active, chromatin.

Other chromatin modifications that are enriched on Xi include the histone modifications H3 trimethylated at lysine 27 (H3K27me3) and H2A monoubiquitinated at lysine 119 (H2AK119ub1; Fig. 9B). These modifications are catalyzed by Polycomb repressor complexes (see Grossniklaus and Paro 2014 for further discussion). There is also enrichment of H3K9me3, normally associated with constitutive pericentric heterochromatin, in humans and some other species. In mouse, H3K9me2, and not H3K9me3, enrichment has been observed. Because distinct histone lysine methyltransferases (KMTs) are associated with these modifications, this appears to represent a fundamental difference in Xi heterochromatin in the different species. Elevated H4K20me1, a histone modification associated with chromosome condensation, has also been observed on Xi (Kohlmaier et al. 2004). Finally, in addition to specific histone modifications, a variant histone, macroH2A, is enriched in Xi heterochromatin (Costanzi and Pehrson 1998), and conversely a different variant, H2A.bbd, is specifically depleted on Xi (Chadwick and Willard 2001).

A careful analysis of the distributions of histone modifications across Xi in human cultured cells has provided insights into the complexity of the system (Chadwick and Willard 2003). H3K9me3 and H3K27me3/H2AK119ub1 are enriched at defined, but nonoverlapping, regions across Xi. Thus, unlike loss of histone acetylation, enrichment in these modifications is a regional, not an overall, property of Xi. Intriguingly, those regions enriched in H3K27me3 are also enriched in *Xist* RNA and the variant histone macroH2A1.2. Conversely, those regions of Xi that are enriched in H3K9me3 also show enhanced levels of heterochromatin protein HP1 (known to bind to methylated H3K9) and H4K20me3 (also a mark associated with constitutive, centric heterochromatin). Importantly, immunostaining of the Barr body in interphase and the inactive X in metaphase

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cells showed the same costaining patterns, suggesting that the different domains are retained through the cell cycle.

Constitutive centric heterochromatin is enriched in methylated DNA, primarily 5′-methylcytosine at CpG dinucleotides (see Li and Zhang 2014). This is consistent with its low level of transcriptional activity. Perhaps surprisingly, the level of CpG methylation on Xi is not, overall, significantly higher than the rest of the genome. However, CpG islands associated with silenced genes are highly methylated and experimental evidence suggests that DNA methylation plays an important role in the stabilization of the inactive state. The overall reduction in CpG methylation on Xi is apparently attributable to reduced methylation in introns, intergenic regions, and possibly at common repeat elements.

In most cases, chromatin features of the inactive X chromosome have been identified by immunofluorescence analysis of either metaphase chromosomes or the Barr body in interphase cells. However, localized changes in histone modifications may also play important roles in the various stages of the X inactivation process. Such changes can be identified by high-resolution microscopy, or by chromatin immunoprecipitation (ChIP) approaches. ChIP analysis of chromatin surrounding the *Xist* locus has identified a region extending >340 kb 5′ of the *Xist* gene that is characterized by enrichment in methylated H3K9 and H3K27 in undifferentiated ES cells (Rougeulle et al. 2004). Hypermethylation diminishes as the cells differentiate and X inactivation proceeds. Sites within this region are enriched in acetylated H3 and H4 (O'Neill et al. 1999). Ongoing investigations will reveal to what extent these localized histone modifications in the Xic region are early, causative events driving the X inactivation process, or downstream events that are (possibly essential) components of an ongoing chromatin remodeling process.

An important recent innovation—high-throughput sequencing applied to chromatin fragments obtained by ChIP (ChIP-seq)—has been coupled with the use of single-nucleotide polymorphisms (SNPs) in interspecific mouse crosses to determine high-resolution maps of specific modifications/factors on the inactive (Xi) relative to the active (Xa) X chromosome. Using this approach it was shown that H3K27me3 on Xi occurs in large blocks, distributed broadly over promoters, gene bodies, and intergenic regions (Marks et al. 2009).

4.5 The Enzymology of Chromatin Modifications on Xi

The enzymes responsible for the deacetylation of core histones (HDACs) during X inactivation or the demethylation of H3K4 are as yet unknown. We know more about the enzymes responsible for putting histone modifications in place. H2AK119u1 and H3K27me3 are deposited on Xi by the Polycomb repressive complexes PRC1 and PRC2, respectively (Silva et al. 2003; de Napoles et al. 2004; detailed in Grossniklaus and Paro 2014). Recruitment of PRC2 to Xi is *Xist* dependent and it has been suggested that this is mediated by direct interaction of PRC2 components with the A-repeat element of *Xist* RNA (Zhao et al. 2008). Direct interaction of PRC2 with A-repeat elements is unlikely, however, to be the whole story as PcG recruitment to Xi does not occur during early mouse preimplantation development (Okamoto et al. 2004), and transgenic *Xist* RNA from which the A-repeat element is deleted can still recruit PRC2, albeit inefficiently (Kohlmaier et al. 2004).

Recruitment of the PRC1 complex to Xi is, in part, attributable to the interaction of the chromodomain of the core protein CBX2/4/7 with H3K27me3, a histone modification put in place by the PRC2 complex (see Fig. 5 of Grossniklaus and Paro 2014). There is also a PRC2-independent pathway that recruits variant RYBP-PRC1 complexes in which CBX proteins are replaced by the RYBP subunit (Tavares et al. 2012).

Specific KMT enzymes catalyzing H3K9me3 and H3K9me2 on Xi in human and mouse, respectively, have not yet been formally identified, although the enzyme systems that establish these modifications at other sites in the genome are well described (Cheng 2014). DNA methylation of CpG islands on Xi requires the de novo methyltransferase Dnmt3b, whereas Dnmt3a and the accessory protein, Dnmt3L, are dispensable (Gendrel et al. 2012). An additional factor required for DNA methylation at many Xi CpG islands is the protein Smchd1, an atypical member of the SMC (structural maintenance of chromosomes) superfamily that includes components of the condensin and cohesin complexes with roles in chromosome organization and dynamics (Blewitt et al. 2008). Smchd1 protein is enriched on Xi and homozygous mutant mice show female-specific embryo lethality attributable to incomplete silencing of X-linked genes. However, the mechanism by which Smchd1 influences CpG island methylation and silencing remains to be determined.

4.6 Higher-Order Chromatin Structure on Xi

Although Xi chromatin is often described as "condensed," careful microscopic analysis and 3D reconstruction of Xa and Xi chromosomes labeled with X-specific DNA probes suggests that the difference between them is more a matter of shape than the amount of chromatin per unit volume (Eils et al. 1996; Splinter et al. 2011). The position of Xi

relative to other nuclear structures may also be important. For example, it has often been observed that the Barr body localizes to the nuclear periphery and/or the periphery of the nucleolus.

Further insights have come from analysis of the 3D organization of genes on the Xi chromosome relative to *Xist* RNA territories in the interphase nucleus (Chaumeil et al. 2006). *Xist* RNA is found to describe a territory that includes common repeat sequences on the X chromosome and in which RNA polymerase II (Pol II) is depleted. During the establishment of X inactivation and in line with gene silencing, X-linked genes are recruited from positions external to the *Xist* RNA territory to sites either at the periphery or within the territory. Silencing deficient *Xist* RNA transgenes in which the A-repeat region is deleted can form an *Xist* RNA territory but fail to recruit genes. That the *Xist* RNA territory has been found to correspond to the location of common repeat elements on the X chromosome is somewhat paradoxical given that on metaphase chromosomes *Xist* RNA shows a reciprocal localization relative to LINE-1 elements (Sec. 4.3). Further studies are needed to resolve this point.

An important role for higher-order chromosome organization in X inactivation is also suggested by the identification of proteins potentially involved in chromosome architecture, notably Smchd1 (see Sec. 4.5) and hnRNPU/SAFA (Sec. 4.2). An additional nuclear scaffold factor, SATB1, has also been implicated in conferring competence for *Xist*-mediated silencing (Agrelo et al. 2009), although its role has recently been questioned because of the lack of an X inactivation defect in SATB1 knockout mice (Nechanitzky et al. 2012).

New methodologies to analyze 3D chromosome topology are shedding further light on higher-order chromosome organization on Xi. The 4C method described in Dekker and Misteli (2014) and illustrated in their Fig. 5, which quantifies the frequency of contact between defined positions on the chromosome, has been used in conjunction with SNPs to discriminate Xi and Xa alleles in XX somatic cells demonstrating that preferred long-range contacts involving loci on Xa are lost on Xi (Splinter et al. 2011). These interactions are partially restored by deletion of the *Xist* locus, despite the fact that *Xist* deletion does not result in reactivation of X-linked genes (see Sec. 4.1). Similarly, the 5C method has been applied to study the regulatory landscape of the Xic during the onset of X inactivation demonstrating that *Xist* and *Tsix* lie within separate topological domains (Nora et al. 2012; see Fig. 7 of Dekker and Misteli 2014). The application of these new methodologies will facilitate important advances in our understanding of Xi structure, in particular, when coupled to advanced microscopy approaches such as 3D structured illumination microscopy that are extending the resolution limit of conventional fluorescence microscopy (Schermelleh et al. 2008).

4.7 The Order of Events That Leads to X Inactivation

Differentiating XX ES cells have provided an invaluable model system for studying the dynamics of X-chromosome inactivation. In undifferentiated cells both X chromosomes are active and *Xist* and *Tsix* are expressed at low levels. Increased levels of *Xist* RNA and its coating of one X chromosome are first detected in a high proportion of cells after 1–2 d of differentiation. This is followed by rapid depletion of RNA Pol II within *Xist* RNA territories and then depletion of H3K4me3 (O'Neill et al. 2008). Recruitment of PcG proteins with associated methylation of H3K27 and monoubiquitination of H2A occur in a similar timeframe, along with deacetylation of H3K9 and loss of H3K4 methylation (Silva et al. 2003; de Napoles et al. 2004; Rougeulle et al. 2004; O'Neill et al. 2008). Global histone deacetylation and the accumulation of H3K9me2 on Xi are established with some delay, occurring at days 3–5 in the majority of cells (Fig. 10) (Keohane et al. 1996). The delayed appearance of some modifications implies that they are likely to be involved in the maintenance/stability of the inactive state, rather than its establishment. This interpretation assumes that patterns of acetylation and methylation at the promoters of individual genes undergoing inactivation reflect those determined by immunofluorescence analysis of the whole chromosome, or large domains. Initial studies by ChIP suggest that this is indeed the case (O'Neill et al. 2008), but further experimentation, using ChIP-seq, for example, is necessary.

Accumulation of the variant macroH2A1.2 histone on Xi occurs much later during XX ES cell differentiation (Mermoud et al. 1999). This variant histone has more than 200 additional amino acids in its carboxy-terminal tail and several amino acid substitutions throughout the molecule. Interestingly, *Xist* RNA expression is required to retain macroH2A on Xi in somatic cells (Csankovszki et al. 1999), but is not sufficient to recruit macroH2A in early differentiation stages (Mermoud et al. 1999; Wutz et al. 2002).

The late recruitment of three other factors to Xi has now been reported: hnRNPU/SAFA, Ash2l (Pullirsch et al. 2010), and Smchd1 (Gendrel et al. 2012). These observations indicate that the establishment of silent chromatin on Xi occurs in a sequential manner with at least two clearly separated phases (Fig. 10).

Selective DNA methylation of Xi CpG islands in ES cells accumulates slowly during differentiation (Gendrel et al. 2012). This is consistent with early studies showing that

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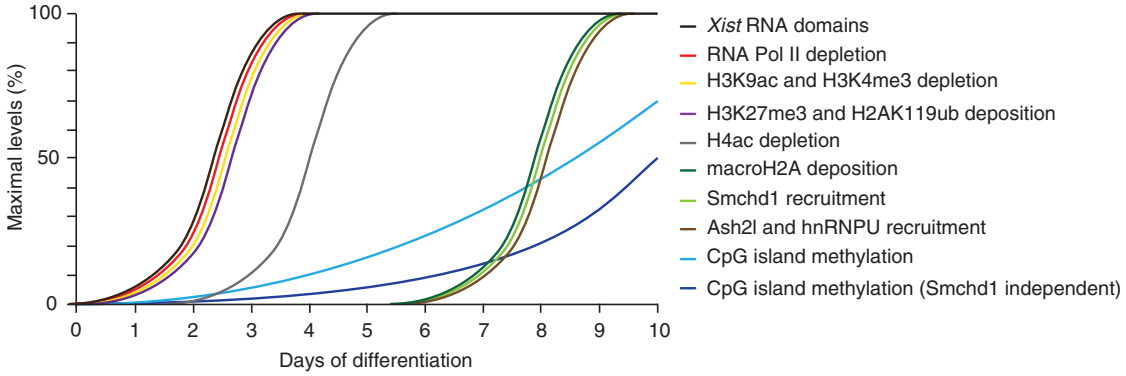

Figure 10. The order of events in differentiating XX ES cells. The diagram summarizes the order in which different silencing pathways are integrated during establishment of X inactivation in differentiating XX ES cells. Early events, depletion of RNA Pol II, loss of H3K4me3/H3K9Ac, and deposition of Polycomb-associated modifications occur coincident with the onset of *Xist* RNA expression. H4 hypoacetylation and a transition to late replication in S-phase occur slightly later. Enrichment for macroH2A, Smchd1 Ash2l, and hnRNPU/SAF-A occur in a defined temporal window relatively late in the differentiation time course. Accumulation of DNA methylation over CpG island occurs slowly following recruitment of Smchd1, although a subset of CpG islands acquire DNA methylation more rapidly and in a Smchd1-independent manner.

methylation of the *Hprt* promoter on Xi occurs relatively late in the developing embryo (Lock et al. 1987), a finding that led to the idea that DNA methylation is responsible for stabilization, or locking, of the inactive state rather than in initiation and spreading. In differentiating XX ES cells, a large proportion of CpG islands acquire little or no DNA methylation before day 7 of differentiation, attributable to the absence of Smchd1 on Xi before this time (see Sec. 4.5). A proportion of CpG islands acquire methylation earlier and at a faster rate, and in these cases methylation is Smchd1 independent (Gendrel et al. 2012). Thus, the picture that emerges is of a coordinated and carefully regulated sequence of events by which chromatin changes on the Xi are put in place as development proceeds (summarized in Fig. 10). It is remarkable that some of these changes, such as histone deacetylation and DNA methylation, take place after the cells have started to progress down various different pathways of differentiation. It seems that the program responsible for the completion of X inactivation proceeds independently of other cell differentiation programs. However, it is important to note that some aspects of random X inactivation can proceed only after differentiation has begun. For example, switching on expression of *Xist* transgenes in "undifferentiated" ES cells triggers various histone modifications associated with heterochromatinization, and also the transition to replication in late S-phase (Wutz and Jaenisch 2000), but there is no detectable incorporation of macroH2A; only after the cells have been induced to differentiate does macroH2A colocalize with *Xist* RNA on the chromosome containing the *Xist* transgene (Rasmussen et al. 2001). Association of macroH2A with *Xist*-coated chromatin is dependent on the continued presence of *Xist* RNA (Csankovszki et al. 1999), but does not require transcriptional silencing, as it is seen also in chromosomes coated with a mutant *Xist* RNA lacking regions necessary for silencing (Wutz et al. 2002). Thus, X inactivation can be seen as the end result of a series of parallel processes, only some of which are interdependent.

It should also be noted that a different order of events may occur during the establishment of imprinted X inactivation in preimplantation embryos. Notably enrichment of H3K27me3 is not detected until the 16-cell stage, considerably later than the onset of *Xist* expression (two- to four-cell stage; Mak et al. 2004; Okamoto et al. 2004). This may indicate a requirement for specific developmentally regulated cofactors to recruit the PRC2 PcG complex to Xi.

The relationship between the various chromosome-wide modifications and gene silencing that occur on the inactivating X in female ES cells is by no means clear. Recent data using microarrays (Lin et al. 2007) or RNA sequencing (Deng et al. 2011) to measure expression of X-linked genes shows that individual genes are inactivated at various times during ES cell differentiation, with some genes escaping inactivation altogether. It seems that, for most X-linked genes, silencing is triggered by conditions that occur at differing stages of ES cell differentiation.

4.8 *Xist*-Mediated Silencing: "Belts and Braces"

Accumulating evidence illustrates multiple pathways contributing to the establishment of gene silencing on the Xi. At the nucleosomal level there is a gain and loss of specific

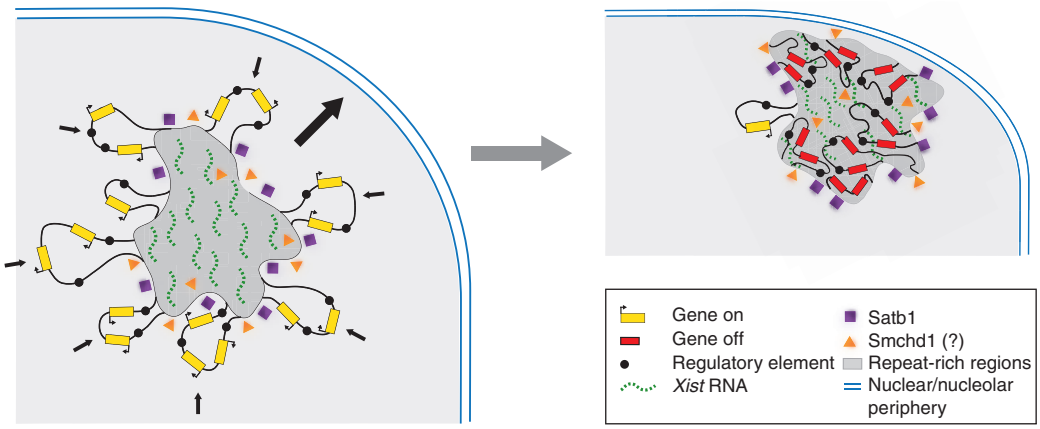

Figure 11. Factors involved in *Xist*-mediated silencing. Depiction of changes in higher-order chromosome architecture during the establishment of X inactivation. *Xist* RNA initially coats repeat rich chromosomal domains; genes and other regulatory elements occupy an external position. As X inactivation proceeds, genes are internalized within the *Xist* territory with consequent restriction in the mobility of chromosome loops. Establishment of X inactivation is also linked to positioning of the chromosome on the nuclear and/or nucleolar periphery. Nuclear scaffold factors (SATB1) and chromosome structure factors (Smchd1) may play a role in the reorganization of chromosome architecture on Xi.

posttranslational modifications, incorporation of different histone variants, and DNA methylation at CpG islands (Fig. 9B). At a higher-order level there are changes in the architecture of chromatin loops and the reorganization of chromosome domains and chromosome position in the nucleus, potentially mediated by chromosomal proteins such as Smchd1, SATB1, and SAFA/hnRNPU (Fig. 11). As such, Xi can be viewed as a "belts and braces" system in which different pathways play overlapping or redundant roles. Within this framework it is likely that different pathways are more or less important at specific times in development, an idea that is supported by the observation that, in contrast to somatic cells, chromosome silencing in cells of the early embryo is dependent on ongoing *Xist* expression. Finally, it is becoming apparent that different pathways contribute differently to silencing of specific genes or subsets of genes on Xi. For example, female Smchd1 null embryos that fail in mid-gestation, show up-regulation of only a small proportion of loci on Xi (Blewitt et al. 2008). In this regard, it is important to note that there is significant variability in the time that individual Xi genes are silenced following the onset of *Xist* expression. Thus, to some degree it may be necessary to consider the contribution of different X inactivation pathways on a gene-by-gene basis.

Although significant progress has been made toward identifying pathways involved in *Xist*-mediated silencing, it is likely that key factors remain to be found. Notably, we do not yet know the critical factors that interact with the A-repeat region of *Xist* RNA to initiate the silencing process. Thus, it remains a possibility that the known modifications of Xi and associated pathways are secondary, occurring in response to silencing established by an as yet uncharacterized primary mechanism.

5 X-CHROMOSOME REACTIVATION AND REPROGRAMMING

5.1 X Reactivation in Normal Development

Multiple layers of epigenetic modification contribute to the silencing of the inactive X chromosome. As a result, the repressed state is generally highly stable and attempts to reverse it experimentally have been consistently unsuccessful. However, there are circumstances in the course of normal development in which the entire X chromosome is reactivated. The best studied example is reversal of X inactivation in developing PGCs. In mouse, PGCs are specified at about 7–8 days of development, shortly after gastrulation. At this time, cells of the embryo have already undergone random X inactivation. Subsequently, the developing PGCs migrate along the hindgut region of the embryo and arrive at the genital ridges, the structures that give rise to the adult gonads. It is at this time that XX PGCs reactivate their Xi (Monk and McLaren 1981). This event occurs coincident with a more general epigenetic reprogramming that includes erasure of parental imprints and genome-wide DNA demethylation (see Fig. 5 of Barlow and Bartolomei 2014; see also Reik and Surani 2014 for more detail).

X-chromosome reactivation in PGCs may indicate a specialized mechanism for reversing the multilayered heterochromatic structure. Extinction of *Xist* RNA expression

has been seen to correlate with X reactivation, but given that silencing is *Xist* independent in XX somatic cells, it is not certain that this is causative. It is possible that PGCs fail to establish all of the marks associated with silencing and are, therefore, more susceptible to reactivation. Consistent with this is the evidence that CpG island methylation does not occur on the Xi in developing PGCs in mouse (Grant et al. 1992).

A second example of X reactivation is the reversal of imprinted Xp inactivation in the ICM lineage of blastocyst stage embryos, discussed in Section 3.5, which again is associated with wider genome reprogramming events. This reactivation also correlates with extinction of *Xist* RNA and a loss of epigenetic marks associated with silencing. Again, it is possible that pre-ICM cells fail to establish all of the marks associated with silencing and are, therefore, more susceptible to X reactivation.

5.2 X Reactivation during Experimental Reprogramming

X reactivation has also been observed under specific experimental circumstances. It occurs during nuclear transfer of somatic nuclei to unfertilized oocytes and following fusion of somatic cells with totipotent cell types such as ES, embryonic germ (EG), or embryonal carcinoma (EC) cells (e.g., see Tada et al. 2000). Finally, X reactivation occurs when XX somatic cells are converted to induced pluripotent stem (iPS) cells (Maherali et al. 2007).

Nuclear transfer embryos provide a particularly interesting example. Experiments in mice (Eggan et al. 2000) showed rapid reactivation of a marker gene on Xi in cleavage stage nuclear transfer embryos. Despite this, the nucleus retained some memory of which X had been inactive because in cloned embryos the donor cell Xi was also the Xi in trophectoderm cells of the placenta. In contrast, cells of the embryo proper showed random X inactivation (see Fig. 12). Presumably, X reactivation and reprogramming that occurs in the developing ICM gives the embryo a second chance to reset epigenetic information from the donor nucleus (see Mekhoubad et al. 2012).

Recent studies have shown that ectopic *Xist* activation makes a significant contribution to the inefficiency of reproductive cloning in mice (Inoue et al. 2010). Using XY donor somatic nuclei, the single *Xist* allele is activated in cloned embryos. Similarly, using XX donor somatic nuclei, both *Xist* alleles are activated. Use of donor cells in which *Xist* is deleted dramatically improves cloning efficiency. Presumably, the host oocyte erases *Xist* gene repression present on the active X chromosome in XY and XX somatic cells. It follows that embryos cloned using donor nuclei with an intact *Xist* locus can only survive early preimplantation development if reprogramming of somatic *Xist* repression fails. This could explain why, in surviving embryos cloned from normal XX somatic cells, the donor cell Xi remains inactive in the extraembryonic lineages for which X inactivation patterns are normally determined at the two- to four-cell stage (Fig. 6).

Although X reactivation occurs during iPS reprogramming of mouse somatic cells, the situation in human iPS cells appears more complex. Early iPS cultures retain an Xi, but X reactivation can occur subsequently, dependent on

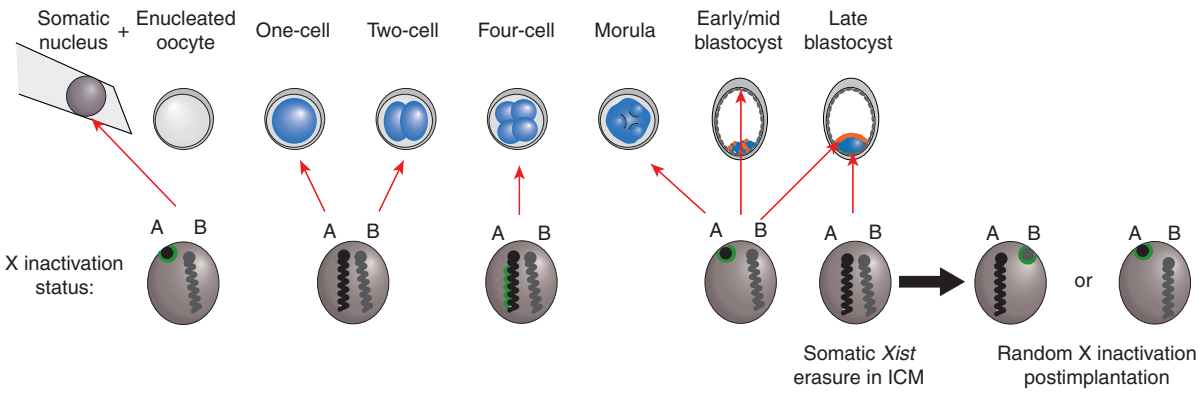

Figure 12. Regulation of X inactivation in cloned mouse embryos. The figure illustrates an XX donor cell with the inactive X chromosome (*A*) coated with *Xist* RNA (green line). In this model, transcription from the donor nucleus, including *Xist* RNA, is repressed by oocyte factors until the two-cell stage, resulting in X reactivation. Recommencement of *Xist* expression then occurs at the two-cell stage. *Xist* is then reexpressed again from the inactive X allele from the donor cell. This would be attributable to retention of a mark such as DNA methylation at the *Xist* promoter. This pattern is maintained in cells allocated to the TE and PE lineages, but not in pluripotent epiblast in which *Xist* expression is again extinguished, leading to a second reactivation event. In the ICM, erasure of the epigenetic marks governing donor *Xist* expression allows for subsequent random X inactivation in the embryo proper.

exact culture conditions. This is likely to relate to different levels of pluripotency as observed also in comparing mouse ES cells and epiblast stem cells (EpiSCs; Bao et al. 2009). Human ES cells (hESCs) are more similar to EpiSCs than to ES cells from mouse, and consistent with this, XX hESCs retain an inactive X chromosome (Tchieu et al. 2010).

5.3 Lessons from Inducible *Xist* Transgenes

A series of experiments using inducible *Xist* transgenes in ES cells has greatly increased our understanding of stability versus reversibility of X inactivation. First, it was shown that *Xist* RNA can establish X inactivation in undifferentiated ES cells and during very early stages of differentiation, but not subsequently (referred to as the "window of opportunity"; Wutz and Jaenisch 2000). Ectopic expression of the nuclear scaffold/matrix protein, SATB1, confers the ability to respond to *Xist* RNA in thymic lymphoma and fibroblast cells (Agrelo et al. 2009), suggesting that this is at least one component that is important for developmental competence (but see also Nechanitzky et al. 2012). The ability of cells to respond to *Xist* RNA broadly correlates with reversibility of X inactivation. Thus, silencing was reversed when the transgene was switched off in ES cells or during early differentiation stages, but not in later differentiation or somatic cells.

Returning to X reactivation and reprogramming, the inducible transgene data imply that in defined cellular environments, namely, undifferentiated ES cells, X reactivation will occur when expression of *Xist* RNA is extinguished. If we consider that those cells in which X reactivation has been documented to occur (i.e., PGCs, ICM cells, iPS, EG, and EC cells) are all similar to ES cells in terms of pluripotency and plasticity, then extinction of *Xist* expression may underlie X reactivation in all cases.

6 SUMMARY AND FUTURE DIRECTIONS

In recent years, there has been significant progress in our understanding of the molecular mechanism of X inactivation. To date, this progress has been fed by advances in related fields of epigenetic research and has, in turn, stimulated advances in other fields. An example of the latter is the growing evidence that some clusters of imprinted genes are regulated by *cis*-acting ncRNAs in much the same way that *Xist* regulates the X chromosome (see Barlow and Bartolomei 2014). Similarly, the independent evolution of ncRNA regulating X inactivation in marsupials further illustrates the potential generality of in *cis* silencing by ncRNA. Conversely, other studies point to distinct types of ncRNA that function in *trans* in gene silencing (e.g., HOTAIR [Rinn et al. 2007; Rinn 2014]). It will be interesting to see if mechanistic links with *Xist* and marsupial Rsx ncRNAs emerge in future studies.

There remain many unanswered questions. Although progress has been made in defining the *cis*-acting sequences and *trans*-acting factors that regulate counting and choice, their further elucidation provides an exciting challenge. Similarly, although we now know some of the chromatin-modifying complexes involved in maintaining X inactivation, such as the Polycomb-group complexes, the signal for establishing chromosome-wide silencing, triggered by *Xist* RNA, remains unknown. Similarly, the mechanistic links between chromosome-wide changes in Xi chromatin and the silencing of individual genes remain elusive. Other key questions are to understand how silencing spreads across the chromosome and what role, if any, way stations (perhaps LINE elements) play in this process and in the stabilization/maintenance of the silent state. This may relate to the intriguing question of how X inactivation is reversed in some cell types and stages of development, but is essentially irreversible in others. This latter question relates to the wider and crucially important issue of understanding genome plasticity and reprogramming through development. Finally, the recent validation of an old hypothesis, namely, the up-regulated expression of genes on the single active X in males and females, has given us a new view of the context in which X inactivation operates and a better understanding of the true complexity of dosage compensation in mammals.

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