Chromatin Remodeling in Dosage Compensation

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Abstract

In many multicellular organisms, males have one X chromosome and females have two. Dosage compensation refers to a regulatory mechanism that insures the equalization of X-linked gene products in males and females. The mechanism has been studied at the molecular level in model organisms belonging to three distantly related taxa; in these organisms, equalization is achieved by shutting down one of the two X chromosomes in the somatic cells of females, by decreasing the level of transcription of the two doses of X-linked genes in females relative to males, or by increasing the level of transcription of the single dose of X-linked genes in males. The study of dosage compensation in these different forms has revealed the existence of an amazing number of interacting chromatin remodeling mechanisms that affect the function of entire chromosomes.

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INTRODUCTION

Sex chromosome: a chromosome that is present in the somatic nuclei of only one sex or that is present singly in one sex and in two copies in the other

Heterogametic: an individual or a population of individuals that produce two types of gametes, for example, containing either an X or a Y chromosome

In diploid organisms, some of the genetic information responsible for sexual differentiation is present on one pair of homologous chromosomes, the sex chromosomes. In many cases, one of the sex chromosomes has become structurally modified while the other has remained unchanged. The structurally modified chromosome is limited to one sex; in some groups of organisms it is transmitted from males to sons (Y chromosome), in others, from females to daughters (W chromosome). The other sex chromosome, X in instances where the male is heterogametic (XY), or Z when the female is heterogametic (ZW), has remained structurally unchanged and is present in two doses in the homogametic sex. This system of sex determination results in an inequality in the dosage of the genes present on the X or Z chromosomes in males and females. Many of these genes are equally important to the development and maintenance of both sexes in a non-sex-specific manner, and an inequality in gene product levels would be inappropriate and may lead to differential selection between the sexes. It is not surprising, therefore, that mechanisms have evolved to prevent such inequalities and compensate for differences in the dosage of X-linked or W-linked genes between the sexes.

The phenomenon of dosage compensation was discovered in Drosophila by Herman Muller more than 70 years ago. It has been studied at the molecular level in model organisms belonging to three very distantly related taxa: round worms, dipterans, and mammals. In these organisms, the transcriptional regulation leading to equal products of X-linked genes in males and females has been achieved in different ways: by shutting down one of the

two X chromosomes in the somatic cells of females throughout most of its length (mammals), by decreasing the level of transcription of the two doses of X-linked genes in hermaphrodites relative to males (*Caenorhabditis elegans*), or by increasing the level of transcription of the single dose of most X-linked genes in males (Drosophila) (**Figure 1**). Some 25 years after Muller's discovery, Theodosius Dobzhansky noted that the polytenic X chromosome in salivary glands of male Drosophila larvae is wider and more diffuse than each X in females. The significance of this observation was provided by George Rudkin who determined that the DNA content of the male X is equal to that of each X chromosome in females, leading to the hypothesis that the difference in morphology of the X in the two sexes reflects a difference in levels of activity. This hypothesis was substantiated by A.S. Mukherjee in Wolfgang Beerman's laboratory using what at the time was a state-of-the art molecular technique: transcription autoradiography. Fifteen years later, John Belote identified four genes with loss-of-function mutations that were inconsequential in females but lethal in males. The X chromosomes of mutant males exhibited approximately half of the normal level of transcription and had lost the paler and somewhat distended appearance that had been interpreted as an indication of an enhanced level of activity in relation to each of the two X chromosomes in females. These results demonstrated that the equalization of X-linked gene products was achieved by doubling, on average, the transcriptional activity of the X chromosome in males rather than by halving the transcriptional activity of each X in females. Then, in 1991, the first of these genes (*mle*) was cloned by Mitzi Kuroda, issuing in the molecular biology study of dosage compensation in flies. More recently, two nontranslated RNA species (*roX* RNAs) were found to be associated with the X chromosome throughout most of its length in a manner that is dependent on the dosage compensation machinery [see references in (93, 94, 118)].

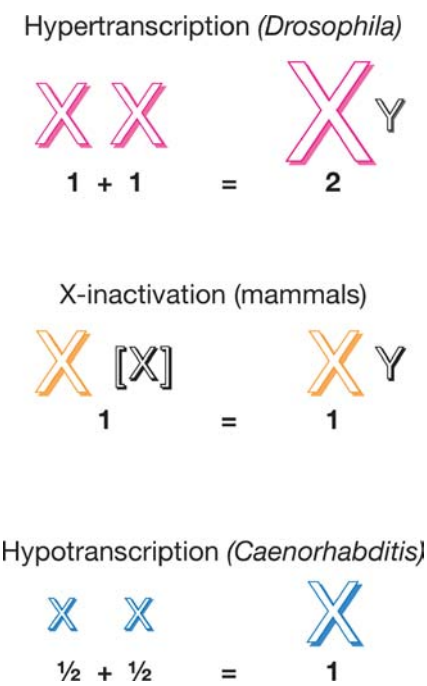

Figure 1

Dosage compensation mechanisms.

Following the discovery by Susumu Ohno that the "sex chromatin body" or Barr body visible in nuclei of somatic cells of females is an X chromosome, in 1961 Mary Lyon presented the mammalian X-chromosome inactivation hypothesis and laid the cornerstone of the study of dosage compensation in mammals. The major historical landmarks of this study are the demonstration of female mosaicism for X-linked enzyme variants by Ronald Davidson, Harold Nitowsky, and Barton Childs; the identification by Bruce Cattanach of a region of the X chromosome (the X chromosome controlling element, *Xce*) involved in modifying the randomness of inactivation; the general belief that inactivation must be initiated and controlled by a specific *cis*-acting site (the X-inactivation center or *XIC*); the mapping of this center and the discovery of a gene encoding a nontranslated RNA that is transcribed only by the inactive chromosome (X inactive-specific transcript or *XIST*) by Huntington Willard and his associates; the identification

Homogametic: an individual or a population of individuals that produce only one type of gametes with respect to chromosome content. Mammalian females are homogametic because all of their gametes contain an X chromosome and a set of autosomes

Autosomes: all chromosomes that are not the sex chromosomes and that are present in two doses in the somatic nuclei of both sexes

MSL: male-specific lethal

by Jennie Lee of a gene in the *XIC* region that is antisense to Xist, is transcribed by the active X, and protects it from inactivation by Xist [see references in (16, 64)].

The existence of a mechanism for dosage compensation in *C. elegans* was first noted by Barbara Meyer in 1986. Over the next few years, Meyer and co-workers isolated a number of genes responsible for dosage compensation components: These genes were identified by the hermaphrodite-specific lethal or dumpy phenotypes of their loss-of-function mutations, presumably caused by the overexpression of X-linked genes. In view of the fact that hermaphrodites have two X chromosomes and are somatically female, this overexpression indicated that dosage compensation in the worm is achieved by decreasing the activity of the two Xs. In addition, Meyer and co-workers established the existence of an important functional link between the dosage compensation mechanism and the genetic hierarchy responsible for sex differentiation [see references in (133)].

Over the past dozen years, the study of dosage compensation has provided a unique window into a variety of fundamental mechanisms of transcriptional regulation. In Drosophila, an RNA-containing regulatory complex that is responsible for doubling, on average, the rate of transcription of most X-linked genes in males may represent a unique example of a novel type of chromatin remodeling machines or complexes. In *C. elegans*, the limited downregulation of both X chromosomes in hermaphrodites involves a subset of proteins and factors that are normally engaged in chromosome condensation during cell division. Finally, in mammals, the facultative heterochromatization of one of the two X chromosomes in females involves the spread of noncoding RNA as a harbinger of covalent modifications of both DNA and histones. One purpose of this review is to highlight the progress that has been achieved by studying dosage compensation in understanding the role that chromatin architecture plays on the regulation of gene transcription. The other purpose is to highlight some of the areas where future investigations are sure to yield comparable or greater insights.

DOSAGE COMPENSATION IN DROSOPHILA

In Drosophila, sex is determined by the relative numbers of X chromosomes and sets of autosomes present in the fertilized egg. The molecular translation of this ratio is the responsibility of a small set of X-linked genes [the sisterless genes *sisA*, *sisB*, *sisC* and the runt gene (*run*)] that, in females, reach a sufficient level of expression to induce the transcription of the regulatory gene Sex lethal (Sxl). *Sxl* initiates differentiation in the female mode by regulating a cascade of sex-specific splicing events leading to the repression of male-specific realizer genes, thus achieving female sexual differentiation. In male embryos, female-specific realizer genes are repressed, resulting in male sexual differentiation [see references in (34, 157)]. The sex-lethal gene product is directly responsible for the absence of dosage compensation in females (see below). In males, it is the absence of SXL that allows the establishment of the mechanism of dosage compensation in very early embryos (61, 128, 170).

Assembling the MSL Complex

Five proteins whose absence is inconsequential in females but is lethal in males owing to their specific role in the mechanism of dosage compensation, have been shown to coimmunoprecipitate in various partial combinations (36) and to assemble into an RNA-containing complex—the MSL complex (189). These proteins are the products of the *msl1*, *msl2*, *msl3*, *mle*, and *mof* genes (collectively referred to as the *msl* genes to reflect the male-specific lethal mutant phenotype that characterizes them). In addition, the complex contains one of the roX RNAs (roX1 or roX2). Because of the frequent discovery of

proteins that are implicated in its function (see below), it is useful to view the complex consisting of the five MSL proteins and roX RNA as a "core complex" in order to leave open the possible existence of additional, albeit substochiometric subunits. MLE is an ATP-dependent RNA/DNA helicase (108); MOF is a histone acetyl transferase that specifically acetylates histone H4 at lysine 16 (2, 80, 189). In females, the complex does not form because SXL prevents the translation of the *msl2* gene transcript (8, 65, 121). The absence of MSL2 leads to a low level of MSL1 and although the MOF, MLE, and MSL3 proteins are present, the complex does not assemble. Expression of a cDNA lacking the SXL-binding sites will cause formation of the MSL complex in females with resulting hyperactivation of both X chromosomes and severe effects on viability (95).

Several efforts have been made to determine the order of assembly of the MSL proteins during complex formation (21, 67, 170) and to map their sites of interaction (36, 139, 183). Although there are slight variations in the reported order of assembly, perhaps due to the use of different tissues or cultured cells, it is clear that MSL1 and MSL2 provide the scaffold for the assembly of the other proteins and roX RNA. Furthermore, the interaction of MSL1 and MSL2 allows the complex to bind DNA. This is significant since neither protein nor any of the other members of the core complex contain any identifiable DNA-binding domain (36, 67, 121). The other components are added to the MSL1/MSL2 scaffold as follows. A predicted coiled-coil N-terminal domain of MSL1 associates with the RING finger of MSL2 and a PEHE domain associates with the zinc finger of MOF. In addition, MSL1 associates with MSL3 via a C-terminal domain (36, 139, 183; F. Li, D.A. Parry & M.J. Scott, manuscript in preparation). To be efficiently included in the forming complex, MSL3 must be acetylated at lysine 116 by MOF (21). Experiments that used baculoviruses expressing recombinant proteins in SF9 cells have shown that the interaction between MSL1, MSL3, and MOF greatly influences the latter's activity and specificity: While MOF acetylated MSL1 at very low levels when MSL3 was missing, the presence of all three proteins resulted in a dramatic enhancement of acetylation of histone H4 on reconstituted nucleosomal arrays (139). MLE does not seem to interact with the other MSL proteins; the presence of RNA-binding sites and the fact that it is released from chromosome-associated complexes by RNase treatment (175) suggest that MLE associates with the other MSL subunits by binding with the roX RNA. Two other subunits, MSL3 and MOF, can also bind RNA.

The roX1 and roX2 RNAs are very different in size (4.1 to 4.3 kb and 0.6 kb, respectively) and share no sequence similarity with the exception of a 30-bp region of high identity (60). The region appears to be dispensable to roX function and, therefore, the meaning of this homology is not understood. In spite of their differences, the roX RNAs are redundant: Deletion of either has no effect on the viability of males or females (129), whereas deletion of both has no effect in females but results in male lethality (60, 129). In a few rare cases, some roX-less males are able to form a sufficient amount of complex to allow their survival (129). It is not known whether these complexes use a different RNA for assembly and targeting. *roX1* or *roX2* transgenes relocated to the autosomes by germline transformation attract the MSL complex at their sites of insertion (96). Either type of transgenes can rescue males that lack both of the endogenous *roX* genes. In these instances, the complex associates with the X chromosome in reasonably normal fashion but also spreads significantly in *cis* from the locus of the transgene (155). This ectopic spreading, as well as cases of abnormal spreading along the X chromosome, can vary according to various parameters that include competition from active endogenous *roX* genes and the level of MSL proteins [see references in (92, 94)].

H4: histone 4

Transgene: a gene that is introduced into the genetic material of an organism or of cells in culture by molecular means

Ectopic: the presence of a gene or the manifestation of some aspect of gene function in a place in the genome other than the normal place

Targeting the Complex to the Sites of Action

In experimental circumstances, the MSL complex can spread along the autosomes. Why, then, is it normally restricted to the X? The roX RNAs are rapidly degraded unless they associate with the proteins of the complex, and since both roX genes are X-linked, assembly of the complex must occur at or near the sites of their transcription. From these sites, migration of the complex is restricted to the X chromosome because of its clear affinity for largely undefined chromosome sequences. The most pronounced level of affinity is for the so-called "high affinity" or "entry sites." These sites were originally defined by indirect immunofluorescence staining as 25 to 35 points along the X chromosome where partial (121) or inactive (68) complexes, or complexes formed in the presence of reduced amounts of MSL components (52), would be seen to bind. The sequences of three of these high-affinity sites have been characterized; two are associated with the *roX* genes and one is elsewhere on the X chromosome (91, 149, 155). Although a male-specific DNase I hypersensitive site is present at all three sites, no similarity in sequence exists among them or between them and the rest of the X chromosome. A number of observations suggest that in addition to these high-affinity sites, there are many sequences dispersed along the X chromosome for which the complex exhibits different levels of affinity. First, when immunofluorescence signals are progressively boosted, new sites continue to appear until the limits of resolution of this technique are reached. Second, there are regions of the X chromosome where no high-affinity sites have been mapped, which, nevertheless, attract the MSL complex when they are present as cosmids integrated into autosomes by transgenesis or in X-to-autosome transpositions (56, 149). Finally, an ectopic promoter located in regions of the X chromosome devoid of complex is able to attract the complex upon activation (179). In these instances, the complex association with the activated promoter does not appear to require its uninterrupted "flow" from adjacent regions where it is normally present. Rather, the dynamic association of complexes in these regions allows free complexes to access an isolated active gene located in relative proximity. It appears, then, that open transcriptional units on the X chromosome represent the final destination of the complex from its location on the X chromosome at sequences ranging from the high to the low end of the affinity scale.

The fact that the complex requires both active MLE and MOF subunits to spread beyond the high-affinity sites (68) indicates that spreading along the X chromosome is not a simple stochastic process; rather, it is a process that requires specific catalytic interactions. The acetylation of H4 by MOF may facilitate access to DNA sequences along the X chromosome and the affinity of MLE for RNA may help target transcribing genes.

The lack of binding to the autosomes could be due to the limited amount of complex present in the nucleus and to its sequestration into the X-chromosome compartment (even the smallest of the X-chromosome transpositions analyzed may pair back and enter this compartment). It could also be due to the absence on the autosomes of any affinity sequences found on the X, even those for which the complex has only limited attraction. This would explain the lack of spreading from the X into the autosomal element of translocations or transpositions (56). In contrast, if a sufficient level of complex is present and available at the site of a roX transgene (for example, by deleting one of the endogenous roX genes), a significant amount of spreading of the MSL complex can occur from the site of the transgene along the autosome (91). An explanation for this paradox could be that years of culturing in the laboratory have resulted in the selection of boundary elements or other regulatory sequences or factors that limit the spread of the MSL complex into the autosomal element of translocations or

insertions, thereby avoiding an imbalance in product levels even for a small number of autosomal genes.

Chromatin Modifications and a Model for the Mechanism of Compensation

The binding pattern of the MSL complex at hundreds of sites on the X chromosome in males, visible by immunofluorescence on larval salivary gland polytene chromosomes (**Figure 2**), is very similar to the distribution of histone H4 acetylated at lysine 16 (14, 199). This isoform of histone H4 is generally associated with active chromatin—witness its presence throughout the genome in budding yeast where it maintains the boundary between telomeric heterochromatin and euchromatin (98, 193), and in humans, where it is found ubiquitously on all chromosomes except for the inactive X (88). In Drosophila, H4K16ac, present only in males, is the result of the acetyl transferase activity of the MOF subunit of the MSL complex (2, 189). On the X chromosome of males, H4K16ac is not limited to the promoter region of genes that are compensated; rather, it is found throughout transcriptional units and beyond (188). This observation, the attraction of MSL complex for activated genes (179), and some circumstantial considerations have led to the hypothesis that the primary mechanistic result of the chromatin modifications responsible for dosage compensation is an enhancement in the rate of transcription elongation (188). The acetylation of histone H4 at lysine 16 may reduce the superhelical torsional stress to which DNA is subjected as it wraps around nucleosomes and facilitate the extension rate of the RNA polymerase. The fact that the transcription of X-linked genes is enhanced to the same twofold level by the compensation mechanism, irrespective of the promoter strength of the genes, and the fact that failure of compensation by the MSL complex decreases the output of X-linked genes but does not appear to alter their developmental program are concordant with the hypothesis. In order to achieve an increased steady-state level of X-linked gene transcripts, enhanced elongation must be coupled with enhanced re-initiation.

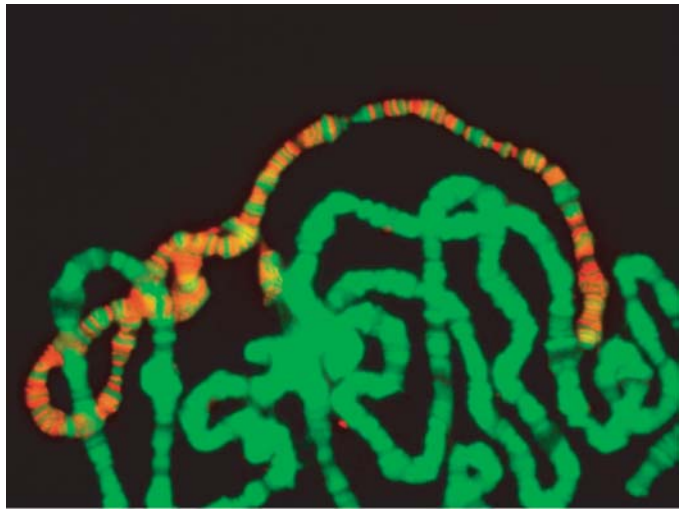

Figure 2

The MSL complex associates exclusively with the X chromosome in males. Indirect immunofluorescence staining of a chromosomal spread from a salivary gland of a third-instar male larva exposed to MSL1 antiserum.

James Birchler has proposed an alternative mechanism to compensate for the potential imbalance between X-linked gene products in relation to autosomal gene products in males and females. In this model, dosage compensation is achieved by decreasing the overall level of autosomal transcription in males: The MSL complex prevents an overexpression of X-linked genes in normal males, and the proper level of dosage compensation results from a twofold imbalance of transcription factors on the X relative to the autosomes, which causes a twofold negative dosage effect on target gene expression [for references see (152)]. Recent data do not support this model (P. Becker & M. Kuroda, personal communications).

Histone phosphorylation is another modification that has long been associated with active chromatin [see references in (123)]. The existence of a novel tandem kinase (JIL-1) was reported to be present on all chromosomes in both sexes of Drosophila but highly

Polytene chromosomes: very large chromosomes that result from multiple DNA replications without cell division. In some tissues newly formed chromosomes remain tightly associated, in register, providing easily scored landmarks that can be used for cytological mapping

enriched along the X chromosome in males (90). JIL-1, which was shown to phosphorylate histone H3 in vitro, localizes with the MSL complex on polytene chromosomes and can be coimmunoprecipitated with various MSL proteins (89). Loss-of-function mutations affect the viability of both sexes but have a more pronounced effect in males (205). Reduced levels of JIL-1 lead to the disintegration of chromatin structure in the nuclei of embryos and affect the morphology of polytene chromosomes in both sexes, with the X chromosome in males exhibiting a more severe effect (205). The concentration of histone H3 phosphorylated at serine 10 was also reported to be higher on the X chromosome than on the autosomes in males and to be significantly decreased in mutant individuals, although in the latter, the level of phosphorylation of mitotic chromosomes is normal (205). Recently, JIL-1 has been identified as the wild-type product of Su(var)3-1 (55). JIL-1 does not appear to be a component of the MSL complex [purified either by tandem immunoprecipitation (189) or by the TAP tag method (S. Medjan & A. Akhtar, personnal communication]; it is present on all chromosomes in both sexes and plays a general role in heterochromatin compaction and expansion (55). These considerations raise the possibility that this kinase is enriched on the X chromosome in males as a consequence of the chromatin modifications and the higher rate of transcription that underlie the process of dosage compensation. The same argument would explain the increased sensitivity of this chromosome to JIL-1 loss of function.

Several other nuclear factors have been reported recently to be enriched on the X chromosome in males (A. Akhtar, personal communications; H. Hirose, personal communications). Loss of function of these factors affects males more severely than females. At present it is unknown whether these factors are components of the MSL complex or if they directly participate in the mechanism of dosage compensation.

Heterochromatin: portion of the genome that is either constitutively (e.g., the region that surrounds the centromere) or facultatively (e.g., the inactive X in mammals or silenced genes in certain regions of a developing embryo) inactive

Euchromatin: portion of the genome that includes the vast majority of active genes. Regions of euchromatin can be reversibly inactivated in some tissues during certain periods in development and adult life

H3: histone H3

Su(var): suppressor of variegation

HP1: heterochromatin protein 1

Interactions with Other Chromatin Remodeling Complexes

The X chromosome in males responds dramatically to the loss of function of the general chromatin assembly complexes ACF and CHRAC and the nucleosome repositioning complex NURF. In vitro, ACF (ATP-dependent chromatin assembly and remodeling factor) and CHRAC (chromatin accessibility complex) establish regularly ordered arrays of nucleosomes (86, 202), whereas NURF disrupts nucleosome periodicity (72, 198). Loss-of-function mutations in ISWI (imitation switch protein), the ATPase common to all three complexes, transform the X chromosome in salivary gland preparations into a chromatin mass that has lost all morphological features (54). Loss-of-function mutations in a subunit unique to the NURF complex have the same effect on the X; this is unexpected given that the normal activity of this complex is to make chromatin more accessible (4). X-chromosome morphology can be rescued in males (or in females where formation of the complex has been induced and where mutations in ISWI have the same abnormal morphological effect on the X chromosomes as they do in males) by preventing the occurrence of H4K16ac. Since in vitro, the interaction of purified ISWI with nucleosomes is diminished if H4 is acetylated at lysine16, the possibility that MOF in the MSL complex enhances transcription by interfering with ISWI-mediated formation of regular nucleosomal arrays that are characteristic of inactive chromatin (37).

The most recent interaction of a chromatin factor with the male X chromosome involves the Su(var)3-7A protein, a structural component of heterochromatin that appears to colocalize with HP1 (heterochromatin protein 1) and with the histone methyltransferase Su(var)3-9. Overexpression of this heterochromatin protein results in morphological effects in the chromosomes of both males and females, but the male X is most affected as it

assumes a very small and highly compacted shape (49).

Do all of these interactions that appear so specific for the X chromosome in males reflect the existence of an extensive regulatory mechanism for dosage compensation in which the MSL complex is only one component? It is natural to speculate that a twofold enhancement of transcription represents a level of regulation so precise that it would require a balance between two or more opposing mechanisms. Yet, the general similarity of the effect of very different complexes or remodeling activities suggests that the common denominator may be the unique characteristic of the chromatin of the male X chromosome which renders it more sensitive to disturbances than autosomes or X chromosomes in females.

DOSAGE COMPENSATION IN *CAENORHABDITIS ELEGANS*

The soil nematode *C. elegans*, like many organisms, has a sex-determination system based on the number of X chromosomes inherited by the embryo. In *C. elegans*, XX embryos normally develop into self-fertile hermaphrodites and XO animals develop into males. This X-chromosome counting mechanism creates the potential for expression differences in X-linked genes between the sexes, and it is apparent that such differences are poorly tolerated. The compensation strategy chosen by *C. elegans* to rectify this dosage difference is to decrease the level of gene expression from each of the two hermaphrodite X chromosomes by one half. The particular mechanism adapted by this species bears little resemblance to dosage compensation mechanisms adapted by other organisms, illustrating different evolutionary paths to the creation of dosage compensation.

The X:A Ratio Determines Sex and Dosage Compensation

The ratio of X chromosome to autosome ploidy is the primary determinant of the sexual phenotype in *C. elegans* and is directly translated into extensive sex-specific phenotypes: approximately one third of the cells are structurally and functionally divergent in adult males and hermaphrodites (194). An animal with a single X chromosome per diploid autosomal set develops as an XO male, whereas an animal with two X chromosomes per diploid autosomal set develops as an XX hermaphrodite. Animals with an X:A ratio of 0.67 or less (e.g., 1X:2A or 2X:3A) always develop as males, whereas once an X:A threshold of 0.75 or greater is reached (e.g., in 3X:4A animals), hermaphrodite development results (34). XX hermaphrodites are somatically females that undergo a transient masculinization of the germ cells during larval development, giving rise to a limited number of stored spermatids that are used for self-reproduction; gametogenesis in the XX individual is strictly limited to the production of oocytes (reviewed in Reference 181).

Dosage compensation responds to the X:A ratio similarly, with full implementation of the mechanism occuring in animals in which the ratio reaches that of 2X:2A or 3X:4A. The effect of the mechanism is to reduce the abundance of X-linked transcripts in XX hermaphrodites to that of XO males by reducing by 50% the expression level of genes on each X chromosome. This effect is of the same magnitude as that achieved by dosage compensation in Drosophila, albeit in the opposite direction. The existence of completely different mechanisms that can achieve a global difference limited to a twofold effect reveals the powerful forces that act in evolution to equalize X-linked gene expression between the sexes.

The Dosage Compensation Complex

Given that the X:A ratio determines both sex and dosage compensation, it is not surprising that the regulation of both pathways is directed by overlapping sets of proteins. Genetic screens performed to look for mutations

that preferentially affected the rate of survival of one sex or the other identified a number of genes with defects in both sex determination and dosage compensation, named *sdc* genes (*s*ex determination and *d*osage *c*ompensation), as well as a number of genes that acted specifically in dosage compensation with more subtle effects on sex determination (for review, see Reference 134). Mutations in the latter genes result in increased XX-specific lethality, with survivors characteristically developing as poorly elongated, or dumpy (*dpy*) animals. The dumpy appearance of these animals, due to an overexpression of X-linked loci, is also observed in 3X:2A animals arising from X chromosome non-disjunction (82, 134). These mutations have no effect on the viability or appearance of XO individuals. They identified the components of a repressive complex, the DCC (dosage compensation complex) that specifically assembles onto the X chromosomes in an X:A ratio-dependent manner and is responsible for dosage compensation (34).

The DCC has been extensively characterized, both genetically and biochemically. The complex is composed of at least 10 polypeptides that are interdependent for colocalization and stability. The *sdc* and *dpy* gene products are included in potential "subcomplexes." One, the putative DPY subcomplex (**Figure 3*B***), is composed of DPY-26, DPY-27, DPY-28, and MIX-1, and shares components with a separate and highly conserved mitotic 13S condensin complex. The SDC subcomplex is composed of SDC-1, SDC-2, SDC-3, DPY-21, and DPY-30. The DCC has a single known target outside of the X chromosome, the autosomal sex-determination gene, *her-1*. Repression of *her-1* by the DCC is required for hermaphrodite development, thus providing the link between sex determination and dosage compensation. Curiously, whereas the DCC provides a twofold repression of X-linked loci, it mediates a 20-fold repression of *her-1* (31). The potential reason for this disparity probably reflects subtle differences in roles of different members of the complex, as well as complex components at the respective loci (210).

The DPY/Condensin-Like Components of the DCC

Components of the DCC are shared with another protein complex that is essential in all tissues of both sexes, the 13S condensin complex (**Figure 3*C***) (29). The condensin complexes functions during mitosis and meiosis for DNA compaction and sister chromatid resolution (29, 70).

MIX-1 is a component shared by the condensin and DC complexes (**Figure 3*a*,*b***). MIX-1 is an ortholog of SMC2 (*s*tructural *m*aintenance of *c*homosomes) that is a conserved component of condensin complexes in most eukaryotes, (70, 116) Mutations in the *mix-1* gene were not originally identified in dosage compensation genetic screens because of its essential role in mitotic and meiotic events in both sexes where it is required for centromere function and chromosome segregation. In this capacity, MIX-1 pairs with its conserved partner, SMC-4. In somatic cells of XX animals, MIX-1 is found on the X chromosomes in the DCC where it associates with DPY-27, which has sequence homology to SMC-4 (32, 69, 116), yet it is also observed in the condensin complex of mitotic cells in the soma and germ line (29). MIX-1's simultaneous presence in two separate complexes is likely guided by interactions with other components of the complexes; for example, SMC-4 and DPY-27 in the condensin and DCC complexes, respectively (70).

The other components of the condensin-like subcomplex are DPY-26 and DPY-28. Both proteins are expressed in germ cells, but their localization is not restricted to the X chromosome in this tissue (117). The stability and localization of each protein in germ cells and in somatic cells requires the presence of the other. Their activity in germ cells is consistent with a role in sister chromatid cohesion. *dpy-26* and *dpy-28* mutant animals exhibit a *him* phenotype (*h*igh *i*ncidence of *m*ales),

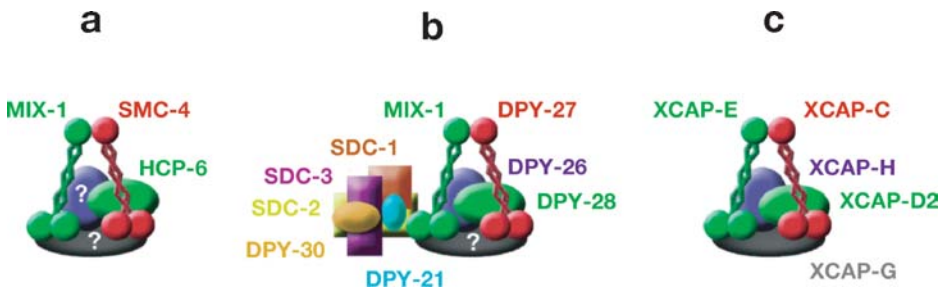

Figure 3

The *C. elegans* dosage compensation complex is an adapted form of the condensin complex. (*a*) The *C. elegans* condensin complex that functions during cell division. (*b*) The SDC subcomplex of the dosage compensation complex which is proposed to help adapt the condensin complex to target-specific repression. This subcomplex shares MIX-1 with the condensin complex, but uses the DPY proteins as XCAP subunits. (*c*) The condensin complex that is conserved in all eukaryotes; homologous components are shown as similar shapes with the same colors in the two worm complexes. Figure kindly provided by Barabara Meyer.

which correlates with an increased frequency of meiotic chromosome non-disjunction and points to roles for these proteins during meiosis (117, 165). DPY-26 and DPY-28 share sequence homology to the non-SMC condensin components XCAP-H and XCAP-D2, respectively (134). The DPY/condensin-like subcomplex thus appears to be a form of the 13S condensin complex that has been adapted to a specialized role in sex determination and dosage compensation. It is conceptually pleasing to consider that a form of a complex whose conserved role is to hypercondense DNA has evolved to mediate a twofold repression of the X chromosome. How the original mechanism has been "tuned" to accomplish a surprisingly precise 50% repression is a fascinating question.

The SDC Subcomplex

The SDC subcomplex consists of at least three components, SDC-1, SDC-2, SDC-3, DPY-21 and DPY-30, each of which can be coimmunoprecipitated with antibodies against the others (31). SDC-1 encodes a novel but conserved protein that has 7 N-terminal C2H2 zinc finger motifs. SDC-1 is expressed in both males and XX animals, so its cellular role is unlikely to be limited to the DCC, but the major apparent defect in *sdc-1* mutants is partial masculinization of XX animals (146). The linchpin of DCC assembly and localization to the X chromosomes is SDC-2. This protein is the only component that has no maternal contribution, is solely expressed in XX embryos, and associates with the X chromosome independently of the other DCC components (46, 147). *sdc-2* expression is repressed by XOL-1, the master sex-determination switch gene, and as such is the only DCC component that exhibits sex-specific regulation (135). This repression in XO males in turn gives *sdc-2* the property of a sex-specific switch gene for dosage compensation regulation.

Like SDC-2, SDC-3 is required to recruit the DPY/condensin-like complex to the X chromosome (45, 210). SDC-3 has a role in repression of the *her-1* locus that differs from its activity on the X. This is revealed in specific mutations called *sdc-3(Tra)*, which affect sex determination but not dosage compensation (51). SDC-3 has two protein domains that have separable functions in its roles on the X and at *her-1*: a C2H2 zinc finger domain that is required for DCC function, and a myosin-like ATP-binding domain that is required for *her-1* repression (100). The protein is maternally expressed in germ cells but does not preferentially accumulate on chromatin; its

accumulation and X-chromosome localization are normally seen only in XX embryos and are dependent on other members of the DCC (45).

DPY-21 is also a component of the DCC, but its absence has little obvious effects on stability or X- localization of other DCC components (32, 45, 46, 116, 117). DPY-21 recruitment to the X does depend on all other DCC proteins (except SDC-1) but its stability does not (210). This is different from many of the other DCC proteins, whose stability is decreased in the absence of other DCC components. DPY-21 also is unique among the DCC proteins in that it is absent from the complex that assembles at the *her-1* locus (210).

In addition to SDC-2 and SDC-3, recruitment of the DPY/condensin-like complex to the X chromosomes also requires DPY-30. DPY-30 is a ubiquitously expressed nuclear protein with homology to a component of the *S. cerevesiae* COMPASS complex, which contains Set1p, a histone H3 lysine 4-specific histone methyltransferase, and has recently been verified to be a DCC component (C. Hassig, T. Wu & B. Meyer, personal communication; 83, 84, 142). *dpy-30* mutants exhibit a maternal effect-mediated XX-specific lethality, but XO animals also have developmental defects, suggesting additional roles for DPY-30 outside of dosage compensation.

Sex-Specific Recruitment of the DCC to the *her-1* Locus

her-1 is a switch gene in sex determination. Loss-of-function mutations in *her-1* cause XO animals to develop into hermaphrodites; gain of function (or loss of *her-1* repression) masculinizes XX animals (81). Therefore *her-1* expression is required for male development and needs to be repressed for hermaphrodite development. The DCC localizes to both the *her-1* locus and on the X chromosomes, and almost all of the components found at one target are present at the other (31). A striking difference in the effects of the complex at the two targets is the level of repression that is achieved. Whereas repression at the X chromosomes is limited to twofold, the repression of *her-1* approaches 20-fold. SDC-2 provides the switch for hermaphrodite development by directly repressing *her-1* expression (45). It accomplishes this by, together with SDC-1 and SDC-3, recruiting the rest of the DCC. SDC-3 is thought to be a major player in the specificity of this recruitment, since it binds to the *her-1* locus in the absence of other components (210). The *sdc-3(Tra)* mutations that can cause defects in sex determination but not dosage compensation result in defective loading of the DCC to the *her-1* locus, but do not affect assembly on the X chromosomes (31). The DCC complex that binds to *her-1* is also different in composition than the one on the X, because DPY-21 is not present in the former but is observed in the latter (210). This difference in components between the complexes at the two loci may contribute to the difference in level of repression observed (134).

Sequence-Specific Recruitment of the DCC

Three DNA elements within the *her-1* locus are required for DCC recruitment and repression (31, 115). Two of these elements have a 15-nucleotide repeat in common that is essential for *her-1* repression, and these sequences can recruit SDC-3 binding (31), but none of the sequences are enriched on the X chromosome. This suggests that different sequence elements are required for recruitment of the DCC to its different targets. Since SDC-2 can localize independently to the X chromosomes, and SDC-3 can recognize and bind *her-1* elements, it has been suggested that these two proteins form the basis for the recruitment of the DCC to the different targets (210).

If SDC-2 directs the DCC to the X-chromosome, what sequence elements is it recognizing and what directs its global activity along the entire X chromosome? Four models have been proposed to account for these

properties (40). Model I, similar to the mechanism of X-inactivation initiation at the Xist locus in mammals, proposes a single site of DCC recruitment, followed by extensive spreading *in cis*. In Model II, which resembles a model proposed for Drosophila, a limited number of recruitment sites from which the DC complex spreads *in cis* would be present along the X chromosome. Model III is similar to Model II, except that instead of spreading in *cis*, repression occurs through long-range interactions. Finally, Model IV predicts a large number of recruitment sites densely arranged along the X, each of which recruits the complex for local repression.

These models were recently tested by using antibodies directed against components of the DCC and a combination of X chromosome deletions and detached duplications of the X chromosome (40). The concept was straightforward: if a single recruitment site was required (Model I) then only one detached duplication would recruit the DCC and conversely a single deletion could eliminate all DCC recruitment. If several, but not all, regions of the X could recruit, this would rule out Models I and IV, since IV requires a dense arrangement of recruitment sites with local repression. Model III would be ruled out if large regions were observed that failed to recruit the DCC as detached duplications, but did so when attached at their normal site, which would indicate spreading of the complex *in cis*. Model I was eliminated, since multiple regions were found to be independently capable of DCC recruitment. Although multiple recruitment sites were apparent, Model IV was ruled out since regions known to contain dosage-compensated loci were unable to recruit the DCC autonomously when detached, even though the DCC was found at these same regions on the intact X. This observation is more consistent with recruitment at a neighboring site (since the dosage compensated regions could not autonomously recruit the DCC), followed by spreading into these regions as in Model II , and is inconsistent with action over a distance as in Model III. Therefore, recruitment of the DCC to a limited number of loci along the X chromosome appears to be followed by spreading *in cis* of the complex along the chromosome (**Figure 4**). Curiously, the spreading *in cis* was apparently limited to X chromosome sequences, since no spreading into the autosome portion of X:autosome fusion chromosomes was detected. Whether this represents the presence of X-limited sequences that promote spreading, or autosomal sequences that prevent it is not known (40).

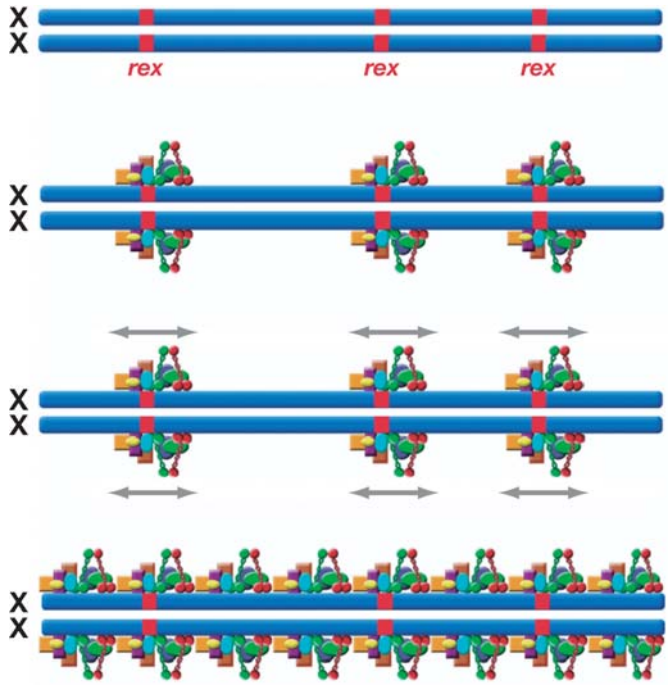

Figure 4

Recruitment and Spreading Model of *C. elegans* dosage compensation. The DCC is initially recruited to the X chromosome at a number of recruitment loci (recognition elements on X; *rex*) that are nonuniformly spread along the chromosome. The complex subsequently spreads in *cis* to neighboring regions for a near-complete coverage of the chromosome. (Figure kindly provided by Barabara Meyer)

X-Specific Recruitment Elements

Molecular mapping and identification of a DCC recruitment site was attempted using overlapping cosmids in repetitive transgenes, and further refined to a 4.5-kb fragment.

Multicopy transgenes carrying this element were capable not only of recruitment but also of titration of the DCC from the endogenous X chromosomes. The element contained a number of suggestive sequences, including an X-specific repetitive sequence and a region with syntenic conservation in *C. briggsae*, but none of these regions were sufficient for recruitment of the complex on their own (40). In conclusion, the mechanism for DCC recruitment seems to require X-specific sequence elements, but the nature of this attraction is not yet understood.

The X:A Ratio and XOL-1

The X:A ratio is read by its effect on the expression of the gene *xol-1* (*XO l*ethal), the master switch gene in dosage comp and sex determination. Loss of XOL-1 activity in XO animals results in inappropriate activation of dosage compensation, X-linked gene repression, and developmental arrest. Conversely, ectopic activation in XX animals inactivates dosage compensation, leading to overexpression of X-linked loci (135, 172). XOL-1 is structurally related to the GHMP family of small molecule kinases (e.g., homoserine kinase), but does not appear to have functional kinase activity (120). The role of *xol-1* as master regulator of both sex determination and dosage compensation is to repress *sdc-2*. Whether the repression is direct or indirect is not known. *sdc-2* repression prevents targeting of the DCC to the X and *her-1*, which serves to simultaneously prevent X-linked gene repression and activate male differentiation (23, 45, 46, 135, 146, 172). Controlling *xol-1* expression is therefore the functional read-out of the X:A ratio, so understanding how XOL-1 function is regulated is the key to understanding how this ratio is interpreted (**Figure 5**).

X-Signal Elements

The number of X chromosomes, or the numerator component of the X:A ratio, is obviously the essential quantifier of X-chromosome dosage. To find the genetic dosage elements linked to the X chromosome (signal elements components of the numerator signal), duplications and deletions spanning the X chromosome were assessed for effects on DC regulation. The logic of this approach was straightforward: Duplications containing X signal elements would be interpreted as an XX dosage when present in XO animals, and cause XO-specific lethality. Conversely, deletions of signal elements in XX animals would cause an underestimation of X dosage and prevent dosage compensation, causing XX-specific defects. A possible difficulty in isolating these potentially lethal mutations was overcome by screening for the genetic alterations in XO and XX animals carrying mutations in *sdc* and *xol-1* genes, respectively. Using this approach, Akerib & Meyer identified three nonoverlapping regions on the left end of the X chromosome that fulfilled the requirements for signal elements. Each of the regions showed the expected phenotypes, but in an additive fashion, illustrating that the numerator signal of the X:A ratio is polygenic. Of the three regions, region 3 was shown to have the strongest single effects on sex determination (1). Hodgkin and co-workers also identified region 3 using a novel duplication in a similar assay, and further refined it (using cosmid transgenes) to a 12–30-kb interval; they named the gene *fox-1* (feminizing locus on X) (82). The *fox-1* gene encodes a protein containing an RNP/RRM RNA-binding motif and is required for the posttranscriptional repression of *xol-1* (145a, 187a).

The genetic loci in regions 1 and 2 have not as yet been identified, but some information on their functions has been obtained by assessing their effects on *xol-1* expression. Both regions appear to repress *xol-1* activity at the transcriptional level (B. Meyer, personal communication). A potential partner in this activity encodes a fourth signal element, *sex-1* (*s*ignal *e*lement on *X*), which was identified in a mutant screen using ectopic expression

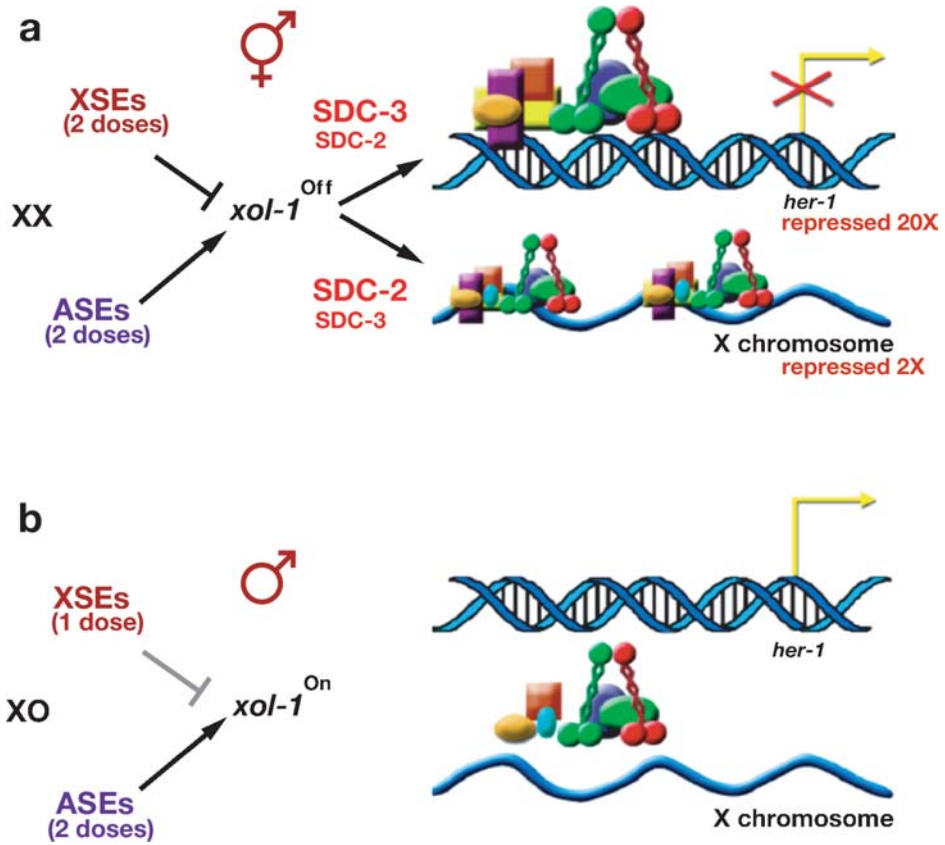

Figure 5

Sex-specific regulation of *C. elegans* dosage compensation. (*a*) In XX embryos, the dosage of X-linked signaling elements (XSEs) represses *xol-1* and allows SDC-2 and SDC-3 expression which target the DCC to the *her-1* promoter and X linked recruitment sites, leading to 20-fold and twofold repression, respectively, of the targets. Repression of *her-1* promotes the hermaphrodite mode of sex determination. (*b*) In XO embryos, the autosomal signal elements (ASEs) dosage overcomes the limited XSE dosage, which promotes *xol-1* expression and represses SDC-2 and SDC-3. (Figure kindly provided by Barabara Meyer)

of a *xol-1:lacZ* reporter in XX embryos (23). *sex-1* encodes a nuclear hormone receptor (NHR) and has been shown to repress the transcription *xol-1*. The dosage of *sex-1* manipulates the control of dosage compensation precisely as predicted for an essential numerator element. Indeed, increasing the dosage of *xol-1* promoter elements in multi-copy transgenes can dilute the XO lethality caused by extra copies of *sex-1*, suggesting a direct titration effect (23). The effect on *xol-1* transcription is likely to be direct, since SEX-1 binds to the *xol-1* promoter. The SEX-1 DNA-binding domain is homologous to retinoic acid and the Rev-Erb orphan NHRs, however no canonical NHR binding sequences have been identified in the *xol-1* promoter. An intact ligand-dependent activation domain exists in SEX-1, leaving open the possibility that a SEX-1 ligand is involved in its targeting and repressive activities at the *xol-1* locus (134).

The regulation of *xol-1* is thus a cumulative regulation, involving both transcriptional

and post-transcriptional repression. The level of the repression is determined by X chromosome dosage because the effectors of the repression are themselves X-linked. The cumulative regulation of *xol-1* also involves autosomal-linked activators, which counter X-linked repressors to signal the X:A ratio (166).

Xi: inactivated X chromosome

Xa: active X chromosome

Autosomal Signaling Elements

The denominator of the X:A ratio consists of autosomal signaling elements. Recent genetic screens have yielded several candidates that have been named *sea* genes (*s*ignal *e*lement on *a*utosomes) and that appear to function as *xol-1* activators. One of these, *sea-1*, has been identified and encodes a T-box transcription factors that opposes X signal elements by activating *xol-1* transcription (166). This provides a conceptually pleasing, if somewhat simplistic model in which the relative dosages of transcriptional activators and repressors, determined soley by the dose of the chromosomes on which they reside, control the level of a single switch protein. If the relative repressor/activator activity ratio is near that achieved by 2X:3A, repression of *xol-1* is insufficient and the switch is engaged to repress dosage compensation and activate male development. As the level of repressor/activator activity ratio increases (e.g., 3X:4A), repression is sufficient to prevent activation of the switch.

Dosage compensation in *C. elegans* thus provides an amazing example of a multiply adaptive response that was generated to compensate for the potentially disastrous consequences of sex chromosome evolution. In the case of *C. elegans*, an essential complex with ancient structural roles in chromatin compaction has been adapted for less efficient compaction and X-chromosome targeting. The amazing fine-tuning of this process to create a twofold reduction in transcription—only when it is located on the X chromosome—is still poorly understood. Its elucidation will make an important contribution to our understanding of genome regulation.

DOSAGE COMPENSATION IN MAMMALS

In the cells of female mammals, one X chromosome is transcriptionally silenced to achieve dosage compensation between XX females and XY males (122). The term X inactivation is used to describe the initial transition from a transcriptionally active to an inactive state and also the subsequent stable maintenance of the silent state. The inactive X chromosome (Xi) differs from the active X chromosome (Xa) and autosomes in differentiated cells, as it is characterized by a unique combination of epigenetic features including histone modifications and DNA methylation. These modifications are acquired sequentially during the onset of X inactivation and act redundantly to maintain X chromosome silencing. In this review we discuss the mechanisms by which the unique chromatin structure of the Xi is established and maintained, and the role of epigenetic modifications in regulating transcriptional silencing.

Histone Covalent Modifications Associated with Silencing

Different combinations of histone modifications are thought to establish transcriptionally active euchromatin and transcriptionally silent heterochromatin [see references in (87, 190, 200)]. According to this "histone code" hypothesis, epigenetic marks on the histone tails provide binding sites for proteins that regulate gene expression. Replacement of core histones with variant histones is another chromatin alteration that is employed to modulate gene expression. In addition to histone modifications, CpG methylation is a covalent DNA modification that is implicated in chromatin structure and transcription. Given that much transcriptional regulation is achieved via changes in chromatin

structure, it is not surprising that the Xi shows a distinct signature of chromatin marks when compared with the Xa and autosomes. Below we describe the features of chromatin that distinguish the Xi from the Xa and autosomes.

Enrichment for histone H3 methylated at lysine 9 is one of the hallmarks of heterochromatin (5, 87). There is enrichment of histone H3 dimethylated on lysine 9 (H3K9me2) on the Xi, specifically at the promoters of silenced genes, such that H3K9 methylation at the promoter correlates with transcriptional inactivity on the Xi (13, 79). Some human cell types also exhibit increased staining of histone H3 trimethylated on lysine 9 (H3K9me3) on the Xi in metaphase (28). The SET domain family of histone methyltransferases (HMTase) catalyzes the methylation of lysine residues (5). Five mammalian SET domain proteins, Suv39h1, Suv39h2, G9a, Eset/SETDB1, and EZH2, have HMTase activity on H3K9 in vitro and are candidates for catalyzing H3K9 methylation on the Xi (104). Eset/SETDB1 and Suv39h1 mediate formation of the trimethylated form of H3K9 (160, 203). As G9a catalyzes production of the H3K9me2 in vitro and in vivo (173), this enzyme is the most likely candidate, to serve as the HMTases that mediates H3K9 methylation on the Xi.

The significance of H3K9me2 enrichment on the Xi is likely to involve recruitment of H3K9me2 binding proteins that further regulate chromatin structure. HP1 binds methylated H3K9 in vitro, and this protein is required for formation of pericentric heterochromatin (6, 58, 103). All three forms of human HP1 appear to be enriched on the Xi as well as on pericentric regions (27), suggesting that H3K9me2 contributes to heterochromatin formation by recruiting HP1 to the Xi. None of the three forms of Hp1 are enriched on the Xi in mouse cells (161), suggesting that HP1 enrichment on the Xi may be specific to human cells. Alternatively, as adult human cells and embryonic mouse cells were examined, it is possible that HP1 enrichment on the Xi is specific to a particular developmental stage.

Regulated silencing of homeotic genes during *Drosophila* development requires methylation of H3 at lysine 27 (H3K27), mediated by the ESC-E(Z) complex (22, 141). Thus, this HMTase complex is involved in the formation of facultative heterochromatin. Loss of function of Eed, the murine ESC homolog, results in reactivation of X-linked genes, suggesting a role for H3K27 methylation in regulating X inactivation in female mammals (204). Further investigation revealed that Eed is present in a complex with Ezh2, the mouse homolog of E(Z) (53), and that both of these proteins are enriched on the Xi (124, 163, 186). Ezh2 is capable of methylating H3K27 in vitro (22, 43, 102, 141), and there is enrichment of the trimethylated form of H3K27 (H3K27me3) on the Xi in some cell types (66). These data argue that the Eed/Ezh2 complex mediates the accumulation of H3K27me3 on the Xi.

A model for the role of methylated H3K27 in transcriptional silencing is based on findings in *Drosophila*. In flies, H3K27me2 provides a binding site for the chromo-domain protein Polycomb (PC) (22, 43, 58, 102), a component of the Polycomb repressive complex (PRC1), which is essential for maintaining homeotic gene silencing (59, 187). Alterations in chromatin structure mediated by H3K27me3-bound mammalian PRC1 provide an attractive model for the role of this histone modification in mediating transcriptional repression on the Xi. PRC1 proteins are enriched on the Xi (47, 57, 164). Ring1/Ring1a, which is a histone H2A ubiquitin ligase, and the related gene product Rnf2/Ring1b, are PRC1 proteins that are required for increased amounts of monoubiquitinated histone H2A on the Xi (47, 57). In combination these results suggest that two PcG complexes contribute to stable X chromosome silencing. First, Eed/Ezh2 histone methyltransferase complex mediates H3K27 methylation, which in turn generates binding sites for PRC1, which results in histone

H3K9me2: H3 dimethylated at lysine 9

HMTase: histone methyltransferase

H3K27me3: H3 trimethylated at lysine 27

H3K4me2: H3 dimethylated at lysine 4

H3R17me2: H3 dimethylated at arginine 17

H3K36me2: H3 dimethylated at lysine 36

HDAC: histone deacetylase

H2A monoubiquitination. PRC1 can mediate silencing of target genes by interfering with SWI/SNF chromatin remodeling machinery, blocking transcriptional initiation, or recruiting additional silencing activities, though the contribution of histone ubiquitination to these silencing functions of PRC1 remains to be evaluated (50, 59, 99, 106, 184).

In contrast to H3K9 and H3K27 methylation, which correlate with transcriptional silencing, methylation of histone H3 at lysine 4 (H3K4) or arginine 17 (H3R17) shows a strong correlation with gene activity (192). Immunofluorescence and ChIP with antibodies directed against the dimethylated form of H3K4 (H3K4me2) shows that H3K4me2 is underrepresented on the Xi (13, 176). A similar result is obtained using antibodies raised against dimethylated H3R17 (H3R17me2) (30). The absence of these two methylation marks on the Xi is consistent with its silent state.

It has been suggested that histone H3 dimethylated on lysine 36 (H3K36me2) causes gene repression in yeast (105, 191). However, there is also evidence linking this modification to gene expression in yeast and *Tetrahymena* (101, 114, 180, 191, 208). H3K36me2 is underrepresented on the Xi (30), indicating that enrichment of this modification may be characteristic of active chromatin in mammals.

Hypoacetylation of histones H3 and H4 at lysine residues is commonly associated with heterochromatin and transcriptional inactivity (174). In agreement with this observation, the Xi appears devoid of histone acetyl modifications (88). When interphase cells are immunostained with an antibody raised against H3 acetylated at lysine 9, the Xi is understained, appearing as a hole (10, 12). The same result is observed using antibodies raised against H4 acetylated at lysines 5, 8, 12 and 16 (30, 79, 88, 97).

Histone acetylation is regulated by a combination of histone acetyltransferase and histone deacetylase (HDAC) activities that add and remove acetyl groups, respectively. Human EED and EZH2 interact with HDAC1 and HDAC2 in vitro and in vivo (201), suggesting that this HMTase complex may regulate acetylation on the Xi by recruiting deacetylases. However, Hdac1 and Hdac2 are not enriched on the Xi in mouse cells with Xi-enrichment of Eed and Ezh2 (124), indicating that the Eed/Ezh2 complex is insufficient to cause an enrichment of these Hdac's. HDAC1 and HDAC2 form corepressor complexes with SIN3A and SIN3B (209). SIN3A and SIN3B complexes are excluded from the Xi (27), further suggesting that these deacetylases do not regulate levels of acetylation on the Xi. There are at least 10 HDAC family members in mammals, providing a number of candidates for the HDACs that might mediate the decrease in histone H3 and H4 acetylation on the Xi. Alternatively, the under-acetylation of the Xi may be achieved by the exclusion of histone acetyltransferase activities from this chromosome.

Replacement of Canonical Histones by Histone Variants

In addition to exhibiting a unique combination of posttranslational modifications on core histones, the Xi contains a high proportion of nucleosomes in which canonical H2A is replaced by the variant histones macroH2A1.1, macroH2A1.2, or macroH2A2 (25, 38, 39). The N terminus of each variant is homologous to canonical H2A while the C termini or non-histone regions (NHRs) of macroH2A proteins show no H2A homology (25, 39, 156). Both the H2A-like domain and the NHR may be involved in proper localization of macroH2A to the Xi, as a truncated protein consisting of the H2A-like domain of macroH2A1 or macroH2A2 localizes to the Xi, and a fusion protein consisting of canonical H2A fused to the macroH2A1.2 NHR also localizes to the Xi (24).

The enrichment of macroH2A on the Xi suggests that this protein may contribute to

gene silencing. Indeed, ectopic macroH2A can downregulate gene expression in vivo. Using Gal4 to tether the macroH2A NHR to the promoter of the luciferase gene reduced luciferase activity more than twofold (159). MacroH2A NHR was not assembled into nucleosomes in this assay, indicating that the NHR may be sufficient for silencing outside the context of the nucleosome.

A recent study proposes two mechanisms for transcriptional repression by macroH2A: interference with transcription factor binding and resistance to nucleosome remodeling (3). The transcription factor NF-κB binds chromatin assembled with conventional histones, but not chromatin assembled with histone octamers containing macroH2A1.2. The NHR is necessary to prevent NF-κB binding. In addition, the DNA near the NF-κB binding site has different DNase accessibility in H2A- and macroH2A-containing nucleosomes. In combination, these results suggest that the NHR sterically blocks transcription factor access to its target DNA sequence. Nucleosome remodeling by SWI/SNF complexes usually promotes gene expression; however, nucleosomes containing macroH2A1.2 are resistant to SWI/SNF activity, suggesting that macroH2A may also regulate gene expression by inhibiting nucleosome remodeling (3). The H2A-like domain is responsible for resistance to SWI/SNF activity. Thus, both the H2A-like domain and the NHR may contribute to transcriptional regulation by macroH2A.

Two variant histones are less abundant on the Xi than on the Xa and autosomes, H2A-Barr body deficient (H2A-Bbd) and H2AZ (26, 27). Although little is known about the function of H2A-Bbd, H2AZ has been shown to antagonize silencing in *S. cerevisiae* (130). H2AZ may be underrepresented on the Xi because most genes on this chromosome are repressed. Localization of H2AZ to pericentric heterochromatin has been observed in mouse extraembryonic tissue (107, 167), indicating that this modification is not excluded from all heterochromatin. It therefore seems likely that variant histones are used in combination with other epigenetic marks to establish diverse forms of chromatin.

DNA Methylation

DNA methylation appears to play an important role in maintaining gene silencing on the Xi. Upstream sequences of genes on the X chromosome are hypermethylated on the Xi and hypomethylated on the Xa (7, 162, 206). Treatment with the DNA-demethylating agent 5-azadeoxycytidine results in reactivation of several X-linked genes (138).

ICF (Immunodeficiency, Centromeric instability, and Facial anomalies) syndrome results from a mutation in the DNA methyltransferase DNMT3b (78). Cells deficient for DNMT3b show hypomethylation of DNA and reactivation of genes on the Xi (75). In addition, one class of repetitive elements, LINE-1 elements, which are normally hypermethylated on both the Xi and Xa, are hypomethylated exclusively on the Xi in DNMT3b mutant cells (73). In combination, these data indicate that DNMT3b contributes to DNA methylation and gene silencing on the Xi. The two other DNA methyltransferases, DNMT3a and DNMT1 (11), may also play a role in DNA methylation on the Xi.

In *Neurospora* and *Arabidopsis*, trimethylation of H3K9 is required for DNA methylation (195). In *Neurospora*, HP1 is essential for DNA methylation, suggesting that HP1 binds methylated H3K9 and recruits a DNA methyltransferase (62). In mammalian cells, H3K9 trimethylation is required for Dnmt3b-dependent DNA methylation at pericentric heterochromatin (113). Therefore it is possible that H3K9 methylation directs DNA methylation on the Xi as well. A mouse H3K9 HMTase activity cofractionates with Dnmt3a (44). Hdac1 also cofractionates with Dnmt3a and the H3K9 HMTase activity, indicating that histone acetylation, histone methylation, and DNA methylation may be coordinately regulated in mammalian cells. The association

of DNA methyltransferase and histone methyltransferase activities may be important for the spread of heterochromatin as it is possible that methyltransferases recruited to one nucleosome can modify adjacent nucleosomes. Although a functional interaction of methylation and deacetylation complexes has yet to be identified on the Xi, it seems likely that this observation will be extended to X inactivation.

The Inactive X Exhibits Late Replication

Several types of heterochromatin, including silenced X chromatin, replicate late in S phase. Genes on the Xa replicate earlier than their counterparts on the Xi (76, 77, 182, 197). Analysis of two replicons on the X chromosome showed that the same origins fire both on the Xi and the Xa (35), suggesting that the same origins are differentially regulated on these chromosomes. This study raises an interesting question: what mechanisms are used to direct different behavior of the same origins on two homologous chromosomes within a single nucleus?

Studies in *Drosophila* may provide insight into the link between replication and silencing. Fly HP1 binds to components of the origin recognition complex (ORC) and flies mutant for an ORC protein show abnormalities in formation of heterochromatin (151). The three mammalian HP1 isoforms colocalize with heterochromatic regions, including the heterochromatin of the Xi (27). HP1 is thought to nucleate the spread of heterochromatin by binding methylated H3K9 and recruiting HMTases to methylate H3K9 on neighboring nucleosomes (6, 103). The interaction between HP1 and ORCs suggests two distinct models for the coregulation of replication and silencing. Late-replicating origins on the Xi may recruit HP1, which mediates silencing. Alternatively, HP1 on the Xi could mediate a change in chromatin structure that affects both gene expression and replication timing.

Late replication timing and DNA methylation on the Xi show an intriguing relationship. Treatment with the DNA-demethylating agent 5-azadeoxycytidine can trigger early replication of the Xi and reactivation of X-linked loci (74). In addition, cells deficient for DNMT3b show early replication of reactivated X-linked genes (75). In DNMT3b mutant cells, a number of X-linked genes are unmethylated. A subset of these unmethylated genes replicate early and are expressed, suggesting that DNA methylation can influence replication timing.

The Transcription of Xist Triggers and Orchestrates the Process of Inactivation

While most genes are silenced on the Xi and expressed from the Xa, the *XIST* gene shows the opposite expression pattern [reviewed in (16)]. *XIST* encodes a 17-Kb, spliced, polyadenylated, noncoding RNA that stably associates with the entire Xi, appearing to coat this chromosome (15, 17–19). *XIST* RNA's ability to coat the Xi depends on the tumor suppressor gene product BRCA1; in BRCA1 mutant cells, *XIST* RNA does not coat the Xi, although it is produced at normal levels (63). In cells that do not exhibit *XIST* RNA coating of the Xi, because of mutations in BRCA1 or deletion of the mouse *Xist* gene, macroH2A1.2 is not enriched on the Xi (9, 42, 63). These results suggest that *XIST* RNA acts as a scaffold that coordinates at least two related activities, chromosome coating and regulation of chromatin structure (**Figure 6**).

Thus far we have described a number of chromatin modifications specific to the Xi. Experiments were performed to address the importance of several of these modifications in stably maintaining the silent state of the Xi. Individually, deletion of *Xist*, DNA demethylation with 5-azadeoxycytidine, or hyperacetylation of histones by treatment with the HDAC inhibitor TSA result in some reactivation of Xi-linked genes (41). These three treatments in combination induce a

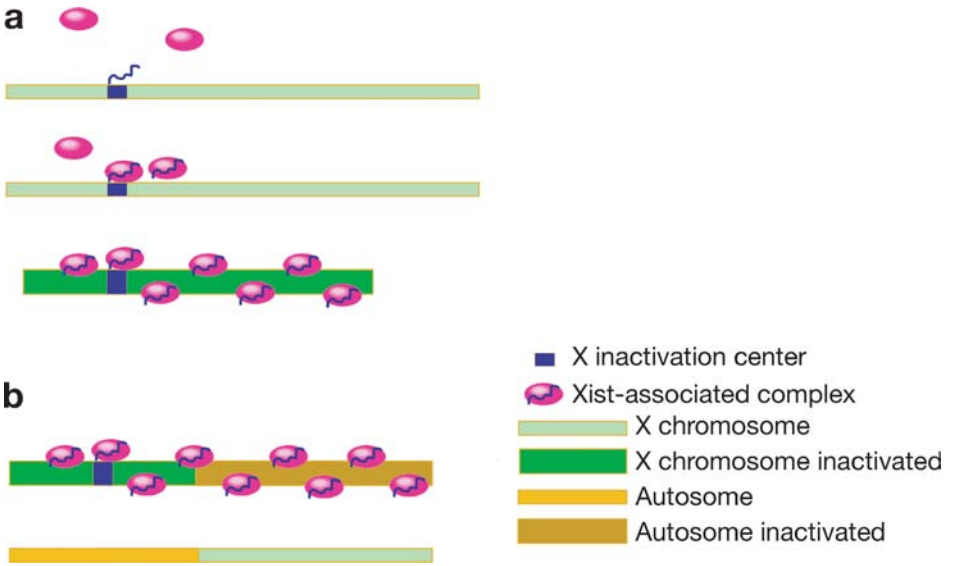

Figure 6

Spreading of the mammalian X-inactivation complex. (*a*) Following the transcription of Xist RNA in the XIC, the DCC complex spreads in *cis* to coat most of the X chromosome.

significantly higher frequency of reactivation than any one treatment alone. Thus, the chromatin modifications that characterize the Xi may work synergistically to maintain this chromosome in an inactive state, and redundant mechanisms may be employed for the extraordinarily stable maintenance of X chromosome silencing.

Cells in early female embryos have two active X chromosomes, one of which becomes silenced in a developmentally regulated fashion. Analysis of the appearance of the chromatin modifications that characterize the Xi indicates that these modifications first occur during the transition from a transcriptionally active to a silent state. This correlation suggests that Xi chromatin modifications contribute to this transition. The first noticeable event is the spread of *Xist* RNA from its site of transcription to coat the X chromosome. This initial *cis*-spread correlates closely with chromosome-wide silencing (153, 185, 207). *Xist* is necessary and sufficient for initiation of X chromosome silencing (127, 158, 207). Female embryonic stem (ES) cells provide a valuable model system to study alterations in chromatin structure that occur during X inactivation because this process is recapitulated when ES cells are induced to differentiate in vitro (169).

ES cells: embryonic stem cells

X inactivation occurs in at least three stages, as characterized by the requirement for *Xist*. The *cis*-spread of *Xist* RNA can be uncoupled from its developmental regulation in ES cells by expressing *Xist* from an inducible promoter. Normally, *Xist* RNA-mediated silencing occurs one to two days after female ES cells begin differentiation (153, 185). However, differentiation is not required for *Xist*-mediated transcriptional inactivation, as silencing occurs in undifferentiated male ES cells when *Xist* expression is driven from an inducible promoter (207). *Xist* RNA can coat the chromosome, but no longer causes silencing if expression is induced more than 36 h after the start of differentiation, suggesting that events that occur upon differentiation interfere with the ability of *Xist* RNA to mediate transcriptional silencing (33, 196, 207). Silencing is dependent on continued *Xist* expression for the first two and a half days after differentiation. In contrast, silencing is not dependent on continued expression of *Xist* RNA in differentiated cells (20, 42, 207). Taken in combination, these results indicate that silencing can be divided into three

stages: initiation, establishment, and maintenance. In the initiation phase, *Xist* RNA can cause silencing de novo, and this silencing is *Xist* dependent. During establishment, *Xist* expression can no longer trigger silencing, although silencing continues to be *Xist* RNA dependent. During maintenance, the transcriptional state of the chromosome is stable in that silencing can neither be induced by *Xist* expression nor reversed by loss of *Xist*. It seems likely that transition from one stage to the next is mediated by a precisely ordered series of chromatin modifications directed to the Xi by *Xist* RNA. In addition, these results suggest that the chromatin-modifying activities present in a cell at the time *Xist* expression is upregulated determine whether silencing will occur. Thus, differentiated cells either lack appropriate chromatin-modifying activities or are unable to recruit those activities to the Xi.

Ectopic *Xist* expression from an autosomal transgene in the differentiated HT-1080 human male fibrosarcoma cell line results in many of the same chromatin changes that are observed on the Xi in female cells (71). The autosome bearing an *XIST* transgene is coated by *XIST* RNA, hypoacetylated on histone H4, replicates late in S phase, and shows chromosome-wide silencing. HT-1080 is the sole differentiated cell line in which activation of *XIST* expression has been reported to induce chromosome-wide silencing, indicating that cell lines differ in their ability to enact the large chromatin structural changes associated with inactivating an entire chromosome. It will be interesting to see if the chromatin-modifying activities that normally direct initiation of X chromosome inactivation during differentiation are present in the already differentiated HT-1080 cell line.

Female ES cells undergo X chromosome silencing upon differentiation, facilitating temporal studies of alterations that occur during X inactivation. Changes in histone acetylation and methylation are the first chromatin modifications detected on the Xi in differentiating ES cells. H3K9me2 and H3K27me3 and deacetylation of H3K9 are first detected on the Xi concomitant with or shortly after the initial *cis*-spread of *Xist* RNA (30, 79, 97, 132, 163, 186). H4 hypoacetylation of the Xi occurs within the same time frame, but with slightly slower kinetics (30). The decrease in acetylation and increase in methylation of H3K9 on the Xi occur roughly simultaneously in differentiating ES cells (30, 79), suggesting these modifications are mutually exclusive. As acetylated H3K9 is a poor substrate for HMTases in vitro (171), it is possible that deacetylation must occur prior to methylation. It is unclear whether these deacetylation and methylation occur sequentially on the same histone or on different histones. H3K4, H3R17, and H3K36 methylation disappear from the Xi with the same kinetics as H3K9 deacetylation and H3K9 methylation (30) suggesting that H3 modifications are coordinately regulated during X inactivation.

Enrichment of H3K27me3 is detected transiently on the Xi during ES cell differentiation (163). The Eed/Ezh2 HMTase complex is also transiently enriched on the Xi when X inactivation is initiated in differentiating ES cells and in embryos (163, 186). H3K27me3 and Eed/Ezh2 Xi-enrichment immediately follows *Xist* RNA coating. Cells expressing a mutant form of *Xist* RNA that coats but does not silence the X chromosome display Xi-enrichment of Eed/Ezh2 complex and H3K27me3 (163), indicating that this modification is not sufficient for transcriptional silencing. As H3K27me3 Xi-enrichment can persist in some differentiated cell types (66), and since differentiated cells no longer show Eed-Ezh2 enrichment (124, 163, 186), it is possible that other HMTases may be required to maintain H3K27 methylation on the Xi after it is first established by Eed/Ezh2.

Compared to the chromosome-wide enrichment of H3K9me2, recruitment of macroH2A1.2 to the Xi is a relatively late event in ES cell differentiation, indicating that this histone variant contributes to

maintenance rather than initiation of X inactivation. In differentiating ES cells, full *Xist* RNA coating is visible in a fraction of cells at day 1 and is visible in most cells by day 3 (185); macroH2A1.2 recruitment to the Xi begins in some cells at day 6 or 7 and is present in a majority of cells around day 9, indicating at least a 3-day lag between *Xist* coating and macroH2A recruitment (131, 168). The central region of *Xist* RNA is required for macroH2A recruitment to the Xi during ES cell differentiation. Since the region of *Xist* RNA necessary for macroH2A recruitment is different from that necessary for transcriptional silencing, it seems likely that *Xist* RNA functions as a scaffold to direct multiple chromatin-modifying activities to the Xi in a developmentally regulated fashion.

Random Inactivation Versus Imprinting

In the female mouse embryo and in ES cells, X chromosome inactivation is random in that either the maternally or paternally inherited X chromosome (Xm and Xp, respectively) can be inactivated. However, in the extraembryonic, or placental, tissues of the mouse, X inactivation is imprinted such that the Xp is always inactivated.

Random and imprinted X inactivation differ in the timing of appearance of histone modifications relative to *Xist* RNA coating and silencing. During random X inactivation in ES cells, histone modifications occur concomitantly or very shortly after the initial *cis*-spread of *Xist* RNA that triggers silencing. In contrast, there is a noticeable delay between *Xist*-mediated silencing and the acquisition of histone modifications during imprinted X inactivation (85, 150) (**Figure 7**).

The Xi in extraembryonic cells shows the same chromatin modifications that are observed on the Xi in embryonic cells. However, the order in which these modifications appear on the Xi is different between random and imprinted X inactivation. In differentiating ES cells, hypoacetylation of H3K9,

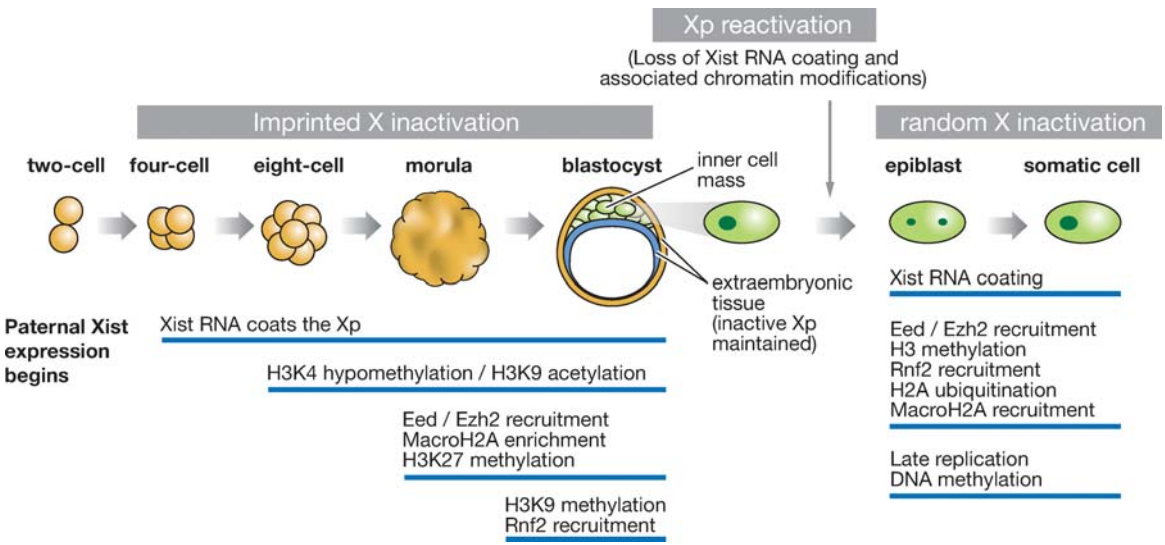

Figure 7

Patterns of X inactivation during the development of placental mammals. X chromosome inactivation is limited to the paternal X in the early embryo and is maintained in the extraembryonic membranes throughout development; it is erased in the inner cell mass and is replaced by random inactivation of either X chromosome. During inactivation, the DNA and nucleosomes undergo covalent modifications that result in the recruitment of a histone variant and heterochromatin-specific proteins.

hypomethylation of H3K4, and enrichment of H3K27me3 and H3K9me2 on the Xi occur in the same time frame, and enrichment of macroH2A is a much later event. In contrast, during imprinted X inactivation in early embryos, hypoacetylation of H4 and H3K9 and hypomethylation of H3K4 are observed first, followed by enrichment of H3K27me3 and macroH2A, and finally enrichment of H3K9me2 (125, 150). Thus, H3K9me2 accumulation on the Xi appears to be coincident with H3K27me3 accumulation during random X inactivation and is detected slightly later during imprinted X inactivation. This apparent difference in timing of H3K9me2 accumulation may be due to the finer temporal resolution of the acquisition of histone modifications in embryos than in differentiating ES cells, as all the cells in embryos are synchronized for initiation of X inactivation. The second difference between random and imprinted X inactivation, the early appearance of macroH2A during imprinted X inactivation, is too large to be explained by the difference in synchronization of initiation of X inactivation during imprinted and random X inactivation. The early appearance of macroH2A on the Xi during imprinted X inactivation suggests that this variant histone may be involved in initiation of X inactivation in extraembryonic cells.

In the preimplantation embryo, which gives rise first to the placenta and subsequently to the embryo proper, all cells undergo imprinted X inactivation. As a result, one would expect cells of both the embryonic and extraembryonic lineages to show imprinted X inactivation. As this is not the case, the Xp must undergo reactivation before random X inactivation can occur. Indeed, reactivation of the Xp has been observed. In the subset of cells that will give rise to the embryo, *Xist* expression is downregulated and a number of associated chromatin modifications are reversed (125, 150). As *Xist* levels drop, Eed/Ezh2 dissociates from the Xi and following this, H3K27me3 is lost from this chromosome. A second example of reactivation during development occurs in the cells that give rise to gametes, also known as primordial germ cells (143). It will be interesting to determine whether these cells also establish and reverse chromatin modifications characteristic of the Xi.

Antisense Regulation of Xist by Tsix

During X inactivation a counting mechanism measures X chromosome to autosome ratio to ensure only one X chromosome per diploid genome is silenced and a choice mechanism designates one X chromosome as the Xi and the other as the active X chromosome (Xa). During random and imprinted X inactivation, choice is determined by the differential regulation of *Xist*, and its antisense transcript, *Tsix*, on the two X chromosomes. In inner cell mass cells of the expanded blastocyst and in ES cells, both of which are poised to undergo random X inactivation, *Xist* RNA can be detected only at the site of transcription on the single Xa in male cells and from both active X chromosomes in female cells (154). The embryonic form of *Xist* RNA has a significantly shorter half-life than the somatic form (153, 185). *Tsix* transcription is always detected when the unstable embryonic form of *Xist* RNA is produced (110, 136). When X inactivation is initiated, *Tsix* transcription from what will become the Xi ceases. Concomitantly *Xist* transcripts show an increased half-life and spread *in cis* from the site of synthesis to coat the entire X chromosome (110). Several days after silencing of the Xi, embryonic transcription of unstable *Xist* RNA and *Tsix* from the Xa are extinguished (110).

Tsix affects the choice of which chromosome will become the Xa. Mutations that disrupt *Tsix* function cause skewing of X inactivation in female ES cells and embryos, such that the wild type chromosome is always selected as the Xa (112, 119, 178). *Xist* also plays a role in this choice process as chromosomes bearing *Xist* mutations are always selected to remain active (126), while chromosomes

carrying an allele in which *Xist* transcription is increased are rarely selected as the Xa (144, 145). The opposite effects of *Xist* and *Tsix* mutations on Xa choice suggest that the relative levels of these two transcripts is a major factor in determining which X chromosome will remain active and which will be silenced (137).

Xist and *Tsix* exhibit differential regulation on the maternal and paternal X chromosomes prior to imprinted X inactivation. *Tsix* and *Xist* pinpoints are expressed from the maternal X chromosome in early cleavage stage embryos, whereas the paternal X chromosome (Xp), which is at least partially coated by *Xist* RNA, exhibits no *Tsix* transcription (48, 109, 185). By the blastocyst stage, *Tsix* continues to be expressed exclusively from the maternal allele in future trophoblast cells and *Xist* RNA fully coats and silences the Xp (48, 109, 178). During imprinted X inactivation *Xist* deletions are lethal when inherited from the father and the lethality arises from a lack of X inactivation of the Xp in the extraembryonic tissues, indicating that *Xist* is required for silencing of the Xp (127). *Tsix* loss-of-function has opposite parent-of-origin-specific effects. Only maternally-inherited *Tsix* deletions are lethal, due to ectopic *Xist* spread and X inactivation of the Xp in many extraembryonic cells (112, 119, 178).

Xite is a *cis*-element that regulates *Tsix* (148). *Xite* harbors intergenic transcription start sites and DNaseI hypersensitive sites. Deletion of *Xite* downregulates *Tsix* in cis and skews X inactivation, suggesting that *Xite* promotes Tsix persistence on the Xa. Truncating *Xite* RNA does not affect randomness of X inactivation, indicating that *Xite* does not function via an RNA. These results suggest that allele-specific differences in *Xite* may cause differential regulation of *Tsix* on the two X chromosomes in female cells, leading to skewing of X inactivation.

In ES cell lines and embryos bearing a targeted disruption of *Tsix* in cis to a partial deletion of *Xist*, transcription of *Xist* is not silenced from the mutant chromosome (Xa), suggesting that *Tsix* negatively regulates *Xist* expression in cis (177). Disruption of *Tsix* impairs establishment of repressive epigenetic modifications and chromatin structure at the *Xist* locus (140, 177), suggesting that *Tsix* silences *Xist* through modification of the chromatin structure.

Xp: paternal X chromosome

The cis-elements that are important for X chromosome counting lie 3′ to *Xist* (111, 140). Like in worms and flies, X chromosome counting in mammals is thought to occur through X-linked numerator elements and autosomal denominator elements. Sequences within the *Tsix* and *Xite* show features of numerators, suggesting that counting is genetically separable from but molecularly coupled to choice. *Tsix* and *Xite* mutations affect XX and XY cells differently, demonstrating that counting requires a "competence" factor, a factor that is produced when cells have two X chromosomes and is necessary for silencing of the Xi (111).

The Xi differs from the Xa and autosomes in differentiated cells, as it is characterized by a unique combination of epigenetic features. The noncoding RNAs *Tsix* and *Xist* are crucial in establishing this difference. *Xist* is believed to act as a scaffold to coordinately recruit multiple chromatin-modifying activities to the Xi, including histone methyltransferases, histone deacetylases, and DNA methyltransferases. These activities are recruited during development in a temporally regulated manner that appears to be tissue specific. The marked presence or absence of specific chromatin modifications on the Xi suggests that these modifications are involved in establishing and/or maintaining a transcriptionally silent state on the Xi. Studies of these modifications show that they act in combination, underlining the importance of multiple redundant mechanisms to regulate the X-inactivation process. Identification of the enzymatic activities that mediate the changes in histone methylation and acetylation that occur during X chromosome silencing will be crucial to understanding how these activities are targeted by noncoding RNAs.

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SUMMARY POINTS

1. Dosage compensation is the equalization of sex-linked gene products between the sex that has one dose and the sex that has two doses of these genes.
2. Equalization is achieved by vastly different mechanisms.
3. The study of these mechanisms has offered a unique perspective on both traditional and unexpected chromatin remodeling mechanisms.
4. Some of the traditional mechanisms consist of histone and DNA covalent modifications that have been known to silence genes in other systems.
5. Unexpected features include nontranslated RNAs that are used for the regulation of entire chromosomes, the replacement of canonical histones with histone variants during chromosome silencing, the selective and attenuated use of factors that are responsible for chromosome condensation during cell division, and histone covalent modifications that affect the process of transcription in a novel way.
6. The extent of the regulation achieved by all of these regulatory mechanisms—a twofold reduction or enhancement in gene activity—is remarkable and relatively unique among gene-regulatory mechanisms.

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