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REVIEW ARTICLE

Transcriptional modulation of entire chromosomes: dosage compensation

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Abstract. Dosage compensation is a regulatory system designed to equalize the transcription output of the genes of the sex chromosomes that are present in different doses in the sexes (X or Z chromosome, depending on the animal species involved). Different mechanisms of dosage compensation have evolved in different animal groups. In Drosophila males, a complex (male-specific lethal) associates with the X chromosome and enhances the activity of most X-linked genes by increasing the rate of RNAPII elongation. In Caenorhabditis, a complex (dosage compensation complex) that contains a number of proteins involved in condensing chromosomes decreases the level of transcription of both X chromosomes in the XX hermaphrodite. In mammals, dosage compensation is achieved by the inactivation, early during development, of most X-linked genes on one of the two X chromosomes in females. The mechanism involves the synthesis of an RNA (Tsix) that protects one of the two Xs from inactivation, and of another RNA (Xist) that coats the other X chromosome and recruits histone and DNA modifying enzymes. This review will focus on the current progress in understanding the dosage compensation mechanisms in the three taxa where it has been best studied at the molecular level: flies, round worms and mammals.

Keywords. dosage compensation; sex chromosomes; sex determination; *Drosophila*; Caenorhabditis; mammals.

Introduction

In various groups of organisms, sex determination and differentiation are determined by particular sex chromosomes. In some organisms such as mammals and flies, females have two X chromosomes and males have one X and a chromosome that is different from the X in shape and genetic content, and is referred to as the Y chromosome. In some other insects and in roundworms, males have a single sex chromosome, the X. Still in other insects and birds, it is the males that have two identical sex chromosomes (Z chromosomes) while the females have one Z chromosome and another sex chromosome that is different from Z and is called the W chromosome. The commonly accepted theory is that sex chromosomes were derived from autosomes, and that, the morphological and functional differences that evolved between the X and Y chromosomes (or between the Z and W chromosomes) were initiated by the presence of a pair of sex-determining alleles, with one allele inducing the differentiation of one sex and, therefore, limited to that sex. Homozygosity for the other allele would lead to the differentiation of the other sex. A reduced level of genetic recombination in the individuals that received the sex-determining allele would favour the accumulation of random mutations and of transposable elements on the allele-bearing chromosome, leading to the eventual partial degeneration of that allele-bearing autosome into a Y or W chromosome or even to its loss from the genome (Lucchesi 1978).

In mammals, the Y chromosome is male-determining; while in birds, female differentiation is determined by the presence of the W chromosome. In fruit flies and round worms, it is the presence of two X chromosomes versus only one X that leads to sexual differentiation. In all organisms where sex chromosomes exist, males and females present with often striking phenotypic differences in physical, physiological and behavioural characteristics that must result from differential gene expression. The so-called 'sex biased' genes responsible for sexual dimorphism, as predicted (Rice 1984), have accumulated on the X and Z chromosomes. This has been evidenced in worms where the X chromosome is enriched for genes with female-biased expression (Albritton *et al.* 2014) and in fruit flies where the X chromosome is similarly enriched, and genes that are involved in male fertility are present on the Y chromosome (Kennison 1981; Parisi *et al.* 2003).

In humans, the X bears a disproportionate number of sex-related and reproduction-related genes (Saifi and Chandra 1999). In mice, X-linked sex-biased genes appeared to be predominantly male-biased genes (Wang *et al.* 2001). The difference in the female versus male sex-biased genes on the X chromosomes of flies and mammals in these earlier reports was later explained by taking into account that some tissues exhibit high degrees of sexual differentiation while others do not. For example, in liver and muscle, sex-biased gene expression is predominantly female-biased; in brain, both female-biased and male-biased genes are expressed, while in the male reproductive organs, male-biased genes are predominant (Meisel *et al.* 2012).

Yet, a common problem presented by all of these systems of sex determination is that many of the ancestral autosomal genes that have been retained or that were transposed over time on the X or Z chromosomes are not involved in establishing sexual characteristics and are equally important for the development and maintenance of both sexes; a difference in the level of product of these genes could lead to unequal selection in males and females. To avoid this problem, mechanisms have evolved to compensate for the difference in the dosage of X-linked (or Z-linked) genes between the sexes. These mechanisms, referred to as dosage compensation, extend to the entire chromosome or are limited to those genes for which the organism is particularly dose sensitive. They have been studied in distant organisms: round worms, fruit flies (where the first example of such a mechanism was discovered), mammals, birds (e.g., see Zimmer *et al.* 2016), and more recently in other groups such as mosquitos and reptiles (e.g., see Rose *et al.* 2016; Marin *et al.* 2017). Although they perform a similar function, dosage compensation mechanisms have evolved independently in these different groups. This review will recount some of the key initial studies and discuss the current progress in understanding the dosage compensation mechanisms in the three taxa where it has been best studied at the molecular level: flies, round worms and mammals.

Dosage compensation in *Drosophila melanogaster*

Herman Muller was the first to report that genes present on the X chromosome lead to the same phenotype in males and females. Muller noted that partial loss-of-function mutations present on the X chromosome that affected the pigmentation of the eye led to the same eye colour in males with a single X and therefore a single dose of the mutant alleles and in females with two X chromosomes and two doses of the alleles. Surprisingly, males with two doses had darker eyes than males with one dose and females with a single dose had a lighter eye colour than females with two doses. Yet, males with one dose of the mutant alleles have a phenotype similar to females with two doses. The equality in the phenotype between males and females indicated that a regulatory mechanism must have evolved to compensate for the normal difference in dosage of these alleles in the two sexes (Muller 1932). Using transcription autoradiography with larval salivary gland polytene chromosomes, a technique that was developed in W. Beermann's laboratory, A. S. Mukherjee demonstrated that the level of RNA synthesis on the single male X chromosome was equivalent to that of both X chromosomes in females and that dosage compensation operates at the level of transcription of the whole sex chromosome (Mukherjee and Beermann 1965). This observation was repeated in the mosaic salivary glands of females that had lost one of the two X chromosomes in some of the cells (Lakhotia and Mukherjee 1969). Again using transcription autoradiography, dosage compensation was shown to operate at the level of individual genes (Chatterjee and Mukherjee 1971). The more diffused appearance of the X chromosome in male salivary glands than either of the two X chromosomes of females, and the faster replication of the X in male glands than in females led to the hypothesis that dosage compensation is achieved by a hyperactivity of the X in males (Lakhotia and Mukherjee 1969, 1970). This hypothesis was eventually validated, and the equalization in the level of chromosomal RNA synthesis in males and females was shown to be achieved by enhancing the transcriptional rate of X-linked genes in males rather than decreasing it in females (Belote and Lucchesi 1980). This enhancement is the responsibility of the male-specific lethal (MSL) complex that associates with the X chromosome in male somatic cells. The MSL complex consists of five core subunits that include an ATPase/helicase that preferentially unwinds short RNA:DNA hybrid substrates *in vitro* (Lee *et al.* 1997), an E3 ubiquitin ligase (Wu *et al.* 2011), and a histone acetyltransferase that acetylates histone H4 at lysine 16 (H4K16ac) (Hilfiker *et al.* 1997). As a member of the MSL complex, this subunit globally acetylates the X chromosome genes in males (Smith *et al.* 2000). In addition to its protein subunits, the MSL complex contains one of two long noncoding RNAs that are transcribed from the two roX (RNA on the X) genes, *roX1* and *roX2,* located on the X chromosome (Amrein and Axel 1997; Meller *et al.* 1997). Although very different in size and having limited sequence similarity, either one of these RNAs can provide the initial scaffold for the formation of the complex (Ilik *et al.* 2013; Maenner *et al.* 2013). In females, the complex is prevented from forming by the presence of the sex lethal (SXL) female-determining protein that interferes with the translation of one of the protein subunits (male-specific lethal 2, MSL2), as well as by the absence of the roX RNAs that are expressed only in males.

In males, the MSL complex is found only on the X chromosome; it initiates its assembly at the site of roX RNA synthesis then binds a series of sites along the X chromosome—chromatin entry sites (CES) or high affinity sites (HAS)—for which it has a high degree of affinity and then spreads to numerous additional locations on the X (Alekseyenko *et al.* 2008; Straub *et al.* 2008). The first

of these sites to be bound by the MSL complex have a slightly different sequence signature and are referred to as PionX or pioneerig sites on the X (Villa *et al.* 2016). A domain in one of the MSL subunits. MSL2, binds roX RNA altering the conformation of MSL2 to allow it to target the GA-rich sequence common to the HASs (Li *et al.* 2008; Zheng *et al.* 2014; Villa *et al.* 2016). The ultimate targets of the MSL complex are the active genes along the X chromosome (Sass *et al.* 2003), with which it associates in a sequence-independent manner, via the affinity of one of its subunits (MSL3, male-specific lethal 3) for the H3K36me3 mark of active genes (Larschan *et al.* 2007).

As the DNA sequence present at the CESs is only slightly enriched on the X chromosome, a number of additional factors must participate in strictly limiting the presence of the MSL complex to this chromosome. One of these factors is a chromatin-linked adaptor (CLAMP) that binds to the DNA sequence common to CESs and is necessary for the recruitment of the MSL complex to these sites (Larschan *et al.* 2012; Soruco *et al.* 2013). It is interesting to note that absence of the CLAMP protein is lethal in both sexes and, therefore, it appears to be required in both sexes (Urban *et al.* 2017).

Another family of repetitive sequences has been implicated in the specific X chromosome targeting by the MSL complex. A particular repetitive DNA sequence that is unique to the X chromosome and is present as clusters in transcriptionally active regions (Waring and Pollack 1987; DiBartolomeis *et al.* 1992) is the source of an siRNA whose absence results in failure of the MSL complex to associate with the X chromosome (Menon *et al.* 2014). The repetitive DNA sequences ectopically relocated to autosomal sites can recruit the MSL complex; this recruitment and the spreading of the complex from the ectopic site are greatly increased if a roX-encoding gene is present at the autosomal site as well, indicating that the repeated sequences and roX RNA act synergistically (Joshi and Meller 2017).

The enhanced level of transcription of X-linked genes in males is most likely achieved by increasing the rate of elongation of the RNA polymerase II complexes (Larschan *et al.* 2011; Ferrari *et al.* 2013). The acetylation of H4K16 mediated by the MOF subunit of the complex occurs throughout the length of the genes but is particularly enhanced towards the 3′ end (Smith *et al.* 2001). This acetylation weakens nucleosome packing in reconstituted chromatin fibres, and results in a more disordered architecture (Dunlap *et al.* 2012) rendering it more accessible to factors or complexes (Bell *et al.* 2010), such as the elongation factor SPT5 (Prabhakaran and Kelley 2012). Further, the MSL complex targets the DNA-unwinding topoisomerase II to active X-linked genes resulting in reduced negative supercoiling, once again facilitating RNAPII elongation (Cugusi *et al.* 2013).

As is common with eukaryotic chromosomes, the X chromosome occupies its own intranuclear territory (Strukov *et al.* 2011) and exhibits a unique higher-order topology manifested by the physical clustering of the CESs. This clustering depends on the presence in the nucleus of those MSL complex subunits (MSL-1 and MSL-2) that bind to the CESs even in the absence of the other components (Grimaud and Becker 2009). Another feature of eukaryotic chromosomes is their organization in a series of domains whose boundaries are defined by the presence of insulator molecules. More recently, the fine-grained topological organization of the X chromosome was found to be identical in male and female cell lines (Ramírez *et al.* 2015; Ulianov *et al.* 2016). In males, though, dosage compensation results in an enhanced level of interaction among active X chromosome loci within individual chromosomal domains (Schauer *et al.* 2017).

Dosage compensation in *Caenorhabditis elegans*

In this organism, individuals are either males with a single X chromosome or hermaphrodites (somatically equivalent to females, although they do produce sperm during a brief period of their life cycle), with two X chromosomes. Therefore, given the observations made with *Drosophila* and in mammals (see below), it was reasonable to assume that some regulatory mechanism must exist to compensate for the difference in the dosage of X-linked genes between the sexes (Hodgkin 1983; Meneely and Wood 1984). This assumption was validated by the discovery that dosage compensation does occur in Caenorhabditis and that it is the responsibility of a multisubunit dosage compensation complex (DCC) that decreases the level of transcription of both X chromosomes in hermaphrodites (Meyer and Casson 1986). The DCC contains five proteins that are present only in this complex and an additional five proteins that are found in condensin I, a ubiquitous complex which functions to condense chromosomes in preparation for mitosis or meiosis.

The DCC forms only in hermaphrodites because one of the components that triggers its assembly and targets it to the X chromosome, sex and dosage compensation 2 (SDC-2), is synthesized only in hermaphrodites (Dawes *et al.* 1999). In this sex, the complex associates exclusively with the two X chromosomes, with a single exception: an autosomal sex-determination gene (*her-1*) that must be repressed to allow hermaphrodite development. As in *Drosophila*, there is a number of recruitment elements on the Caenorhabditis X chromosome (referred to as *rex*) that share a DNA sequence motif to which the DCC complex binds (Csankovszki *et al.* 2004; McDonel *et al.* 2006; Ercan *et al.* 2007). Although the rex consensus sequence is also found in the autosomes, recruitment to the X chromosome is initiated at a small number of high-affinity rex sites that contain an additional sequence identification mark (Albritton *et al.* 2017). From these rex elements the DCC spreads along the X chromosome to other rex sites in a sequence-independent manner, landing eventually near the promoter regions of active genes.

The DCC alters the compaction of X chromosome chromatin in interphase (Lau *et al.* 2014). This is most likely the result of an enrichment of H4K20me1. This modification, correlated with condensed chromatin, is achieved by one of the DCC proteins that specifically demethylates H4K20me2; inactivation of this DCC component reduces the levels of H4K20me1 and results in decreased X chromosome compaction and loss of dosage compensation function (Brejc *et al.* 2017).

The DCC also alters the topology of the X chromosomes. In Caenorhabditis, the genome is subdivided into functional units that are equivalent to the domains of flies and mammals. The presence of the DCC creates a unique redistribution of these domains that function to repress gene expression along the entire chromosome (Crane *et al.* 2015).

Dosage compensation in mammals

The seminal observation in this group was the presence of a heterochromatic body, seen for the first time in the interphase nuclei of female cat neurons (Barr and Bertram 1949). This body is formed by the condensation of one of the two X chromosomes (Ohno *et al.* 1959). These results led Mary Lyon to formulate the hypothesis that the condensed X chromosome is inactive (Lyon 1961). Inactivation of an X chromosome would allow only one copy of X-linked genes to be expressed in females and would, thereby represent the mechanism used by mammals to equalize the output of X-linked genes between the sexes (Davidson *et al.* 1963). In eutherian mammals, X inactivation is initiated by the transcription of a gene (*Xist*, X inactive specific transcript) located in a region of the X that is designated as the X chromosome inactivation centre (XIC). The transcription of *Xist* is induced by the RLIM protein (RING finger LIM-domain-interacting, formerly known as RNF12), an ubiquitin ligase that regulates the activity of different transcription factors and cofactors (Jonkers *et al.* 2009). The gene that encodes RLIM is present on the X chromosome leading to twice the amount of the protein in females in comparison to males. In females, the level of RLIM is sufficient to activate *Xist* in one of the two X chromosomes. The eutherian XIC contains additional genes that regulate the transcription of *Xist*. An example is just proximal to *Xis*t (*Jpx*, Tian *et al.* 2010). Only one of the two *Xist* alleles present in females is transcribed and the long noncoding RNA that it generates induces silencing of the X chromosome (Xi) from which it is expressed (Brown *et al.* 1991). On the other X chromosome, a gene that is also present in the XIC, *Tsix* produces a noncoding antisense RNA that inhibits the transcription of *Xist*, allowing this chromosome (Xa) to remain active (Lee and Lu 1999). Several elements (*Xite*, X-inactivation intergenic transcription elements), located in close proximity of the *Tsix* gene, function as enhancers of *Tsix* (Ogawa and Lee 2003). In very early embryos, *Xist* is repressed on both X chromosomes by a number of factors that include *Tsix* RNA and the major pluripotency factors, Oct4, Sox2 and Nanog. In marsupials, dosage compensation is also achieved via X inactivation, although different molecules and factors are involved (Gendrel and Heard 2014).

During development, X chromosome inactivation is concurrent with the onset of cellular differentiation; either of the two chromosomes can be inactivated, but once the decision to inactivate the maternal or paternal chromosome is made in a particular cell, the same chromosome will be inactivated in all of its descendants. Exceptions to the random nature of X inactivation are found in marsupials, where the paternal X chromosome is always inactivated (Sharman 1971), and in the extra-embryonic tissues of some eutherian mammals (Takagi and Sasaki 1975). These instances are clearly cases of parent of origin imprinting (Brown and Chandra 1973). Deviation from random X inactivation can occur in eutherian mammals, due to the presence of different alleles of modifiers at the Xic locus that either enhance the transcription of the *Xist* gene or lower the level of transcription of the *Tsix* gene (Lee and Lu 1999; Nesterova *et al.* 2003).

A significant number of genes on the X chromosome escape inactivation. Around 15% of X-linked genes are expressed from both X chromosomes in human females (Balaton *et al.* 2015). Some of these genes are expressed in some tissues but not in others. In addition, the genes that are present on the pseudoautosomal region remain active. This region has avoided degeneration and heterochromatization during the evolution of the Y chromosome; therefore, the genes that it includes are present in two active doses in males. Dosage compensation of these genes requires that both doses remain active in females.

To trigger X chromosome inactivation, a mechanism must exist to tell the cell that it has two X chromosomes. Such a surveillance mechanism insures that the single X chromosome in male embryos remains active. Evidence for the existence of a counting mechanism is provided by aneuploid embryos with supernumerary X chromosomes and the standard two sets of autosomes in their genome. In these individuals, a single X chromosome remains active and all of the others are inactivated. Various hypotheses attempting to account for the number of inactivated X chromosomes in aneuploid and polyploid embryos were advanced, with perhaps the most prescient suggesting the existence of an autosomal ‘informational entity’ that would interact with an X chromosome site (Brown and Chandra 1973). While the molecular basis of the counting process has remained undetermined for several decades, one of the components of the responsible regulatory mechanism has been identified recently in humans (Migeon *et al.* 2017). In a study of partial autosomal aneuploids (trisomics) foetuses that survive gestation, a region on chromosome 19 was discovered whose dosage regulates X inactivation. Importantly, the copy number of

the region determines that a single X chromosome remains active in diploid females and two X chromosomes are active in triploid females (XXX; AAA, where A represents one set of autosomes) or males (XXY; AAA). Presumably, the dosage of one or more genes in this region inhibits directly or indirectly the synthesis of Xist RNA.

As mentioned above, different noncoding regulatory RNAs are involved in X chromosome inactivation (Lee and Lu 1999). The Xist RNA spreads in *cis*, first to various entry points brought close to the XIC by the folding of the X chromosome in interphase nuclei; this is followed by a general association to gene-rich and then to gene-poor regions (Simon *et al.* 2013; Engreitz *et al.* 2013). Targeting of the Xist RNA does not seem to rely on the presence of particular sequences on the X chromosome, and the mechanism that achieves subsequent coating of the entire X chromosome is still not fully understood. Repetitive elements that are enriched on this chromosome (such as the long interspersed element Line1 or L1 that are enriched in the proximity of inactivated genes) were thought to facilitate Xist RNA spreading (Cotton *et al.* 2014; Bala Tannan *et al.* 2014). But the role of Line 1 elements as target sites has been challenged (Simon *et al.* 2013; Engreitz *et al.* 2013).

As expected, X chromosome inactivation involves a number of canonical epigenetic modifications. Histone H4 acetylation of lysines 5, 12, 8 and 16 is substantially abrogated (Jeppesen and Turner 1993), and the Xi exhibits other epigenetic marks associated with gene silencing: reduced H3K4me and H3K9ac with a corresponding increase of H3K9me3; a high level of DNA methylation in the CpG islands of silenced genes (Norris *et al.* 1991), and a high level of H4K20me1. The histone variant macroH2A is also incorporated into the Xi chromosome (Costanzi and Pehrson 1998). A number of proteins such as histone deacetylases and the polycomb repressive complexes associate with the Xist RNA (McHugh *et al.* 2015; Chu *et al.* 2015). In mouse embryonic stem cells for example, a noncanonical PRC1 complex associates with Xist RNA and ubiquitinates histone H2A (H2AK119ub); this modification mediates the binding of PRC2 that is responsible for the presence H3K27me3 along the inactive X chromosome (Almeida *et al.* 2017). Loading of PRC2 on the RNA appears to involve the ATRX (alpha thalassemia / mental retardation X-Linked), a protein that has an ATPase and a helicase domain (Sarma *et al.* 2014), and the cofactor JARID2 (Rocha *et al.* 2014). Xist RNA is edited and is marked by the presence of N6-methyladenosine (Patil *et al.* 2016). Among the many proteins that interact with Xist is the lamin B receptor (LBR) suggesting that it plays a role in the frequent presence of Xi at the nuclear periphery (Chen *et al.* 2016). A lncRNA, Firre, encoded by a gene on the X chromosome that escapes inactivation, is responsible for this association (Yang *et al.* 2015).

During the development of female embryos, the inactive X is reactivated in primordial germ cells. This is necessary to ensure the transmission of an active X chromosome to male progeny and to allow the random inactivation of the maternal or paternal X chromosomes in female progeny. The molecular signals that initiate and result in reactivation are not fully understood. Xist RNA transcription is abrogated but that may not be the key factor since the presence of an active *Xist* gene is not required for the maintenance of an inactive X chromosome in somatic cells (Brown and Willard 1994).

A doubling of gene activity is a necessary corollary to some dosage compensation mechanisms

In Drosophila females, both X chromosomes are active and the levels of the products of X-linked and autosomal genes are generally concordant. Drosophila males double, on average, the transcriptional level of their single X chromosome and reach the same concordance with autosomal genes. In worms and mammals, males with their single X chromosome should have a deficiency in the level of X-linked gene products in relation to autosomal gene products, and the same would be true in females and hermaphrodites, where dosage compensation reduces the level of X-linked gene products to that of the male. This realization led to the early hypothesis that the single X chromosome in mammalian males must have doubled its transcriptional level during the evolution of the sex chromosomes, bringing its output to the same level as that of autosomal genes. The presence of two such hyperactive X chromosomes in females, though, would lead to an unacceptable overexpression of X-linked genes and necessitated the concomitant development of a dosage compensation mechanism (Ohno 1967). This is, in fact, the case in both worms and mammals (Gupta *et al.* 2006; Nguyen and Disteche 2006). In mammalian cells, X chromosome upregulation involves an increased level of MOF-mediated H4K16 acetylation at X-linked gene promoters in comparison to a comparable set of autosomal genes; it also involves a greater frequency of paused RNAPII, and a greater stability of transcripts, although no evidence of differences at the 5′ or 3′ ends of these RNA molecules has been obtained (Deng *et al.* 2013).

Concluding remarks

Dosage compensation mechanisms have proven to be invaluable paradigms for the study of many fundamental aspects of genetic regulation: from the coordinate transcriptional modulation of large groups of functionally unrelated genes, to the pioneering identification and assignment of specific functions for long noncoding RNAs; from assigning specific functions to epigenetic modification such as histone acetylation, to the establishment and maintenance of chromatin domains. It is reasonable to

expect that the continued investigations of these mechanisms, in traditional model organisms as well as in many others, will generate novel and fundamental information on various parameters that regulate gene expression.

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