Dosage compensation: the beginning and end of generalization

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Abstract | The genomes of higher eukaryotes are carefully balanced systems of gene expression that compensate for the different numbers of sex chromosomes in the two sexes by adjusting gene expression levels. Different strategies for sex chromosome dosage compensation have evolved, which all involve modulating chromatin structure as a means to fine-tune transcription levels. As data accumulate, previous over-simplifications are being revised, and novel features of the compensation processes are gaining attention, many of which are of sufficient global validity to influence our view on gene expression beyond the realm of dosage compensation itself.

Heterochromatin
A condensed and transcriptionally silent form of chromatin that is found at telomeres and centromeres of all chromosomes. Heterochromatin components, such as heterochromatin protein 1 (HP1), can be recruited to contribute to gene repression in general.

Hemizygosity
When a diploid genotype has only one copy of a particular gene, as in X-chromosomal genes in human and *Drosophila melanogaster* males, or when the homologous chromosome is lost or carries a deletion.

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Sexual reproduction is frequently associated with dimorphic sex chromosomes that were derived from autosome pairs; such arrangements have evolved on several independent occasions[1–3]. For example, in humans and fruit-flies (*Drosophila melanogaster*), females are characterized by two X chromosomes, whereas male cells contain only a single X and a Y chromosome. The Y chromosomes of both species are gene-poor, are largely heterochromatic and have arisen from precursor chromosomes in complex, mainly degenerative processes that involve chromosomal rearrangements and the inactivation, as well as subsequent loss, of most genes[1–3]. In the worm (*Caenorhabditis elegans*) this process has apparently eliminated the Y chromosome altogether, such that males have an X0 genotype, whereas hermaphrodites are XX[4]. This degeneration of the Y chromosome has rendered large parts of the co-evolving X chromosome hemizygous, which has consequences for the functionality of the genome as a whole. Complex, diploid genomes are not just collections of genes, but fine-balanced networks of gene expression. Gene loss during the deterioration of the Y chromosome therefore requires regulatory compensation of the corresponding X-chromosomal alleles, a process called dosage compensation.

The coordinated fine-tuning of transcription of a large number of X-chromosomal genes has fascinated generations of researchers. The advent of new methods in genomics and proteomics has now added a wealth of information that changes prevailing concepts. We will compare the different dosage compensation strategies known to function in the three systems that have been studied so far: *D. melanogaster*, *C. elegans* and mammals, which are represented by mouse and human cells. We will describe new insights into the principles that restrict the action of the dosage compensation machinery to the X chromosomes and discuss hypotheses that are based on recent high-resolution mapping studies.

Male arithmetic: how X equals XX

It has been suspected for a long time that many genes on the single X chromosome in male *D. melanogaster* are subject to a roughly twofold increase in transcription[5], effectively re-establishing the global balance of diploid gene expression (FIG. 1). Most recently, circumstantial evidence has been obtained which might indicate that a similar, twofold upregulation of X-chromosomal genes also operates in human and *C. elegans* males[6,7] (FIG. 1). Global analyses of gene expression showed that, on average, genes on the single X chromosome give rise to as many transcripts as autosomal genes, which are present in two copies, pointing to an increased expression from the single alleles. Direct, mechanistic explanation for this phenomenon is, however, lacking because factors that could be involved in elevating gene activity on X chromosomes are not known in humans or worms. By contrast, clear-cut evidence for an active, transcriptional boost has recently been obtained in *D. melanogaster*[8,9]. Knockdown of *male-specific lethal 2* (*msl2*), a crucial component of the fly dosage compensation system (FIG. 2), resulted in an up to twofold decrease of a substantial fraction of X-chromosomal genes[8]. Absolute measurements of RNA levels by normalizing transcripts to gene copy number showed that indeed the activity of X-chromosomal genes dropped after the elimination of dosage compensation, whereas the expression of autosomal genes was largely unaffected[9].

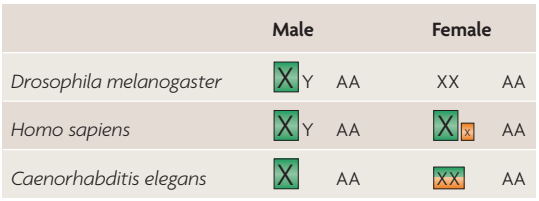

Figure 1 | **Principles of X-chromosome dosage compensation.** X-chromosome monosomy in fly, human and worm males is compensated by increased transcription from the single chromosome. *Drosophila melanogaster* males use a male-specific mechanism, leaving female expression unchanged. However, increased transcription of the two X chromosomes forces human females and hermaphrodite worms to respond by either inactivating one of the two chromosomes (human) or reducing the expression of both chromosomes by half (worm). Larger letters and green boxes symbolize activation. Orange boxes indicate repressed chromosomes.

Whereas this observation confirmed the prevalent concept, the details of the analysis challenged the generalization that dosage compensation involves uniform, twofold tuning of transcription. Of the genes that had sufficiently robust expression to allow the assessment of the relatively small changes following the removal of dosage compensation, only a fraction showed a clear twofold reduction in expression. For most of the genes the effect was subtler, reflecting a continuum of changes between a barely measurable and a twofold expression decrease[8]. Although measuring very small changes of gene expression on a global scale is demanding and could suffer from technical limitations, the data suggest that the extent of dosage compensation is a property of individual genes rather than a general property of the X chromosome. At the level of transcription no blunt 'twofold increase' rule seems to exist; instead genes seem to be compensated to a level that is sufficient not to compromise the fitness of the organism. Not all genes are haploinsufficient and it is intuitive that deviations from a twofold transcriptional compensation could be the rule, rather than the exception, depending on the function, stability and contribution from post-transcriptional regulation to the activity of a protein. In any case, recent data[8,10–12] indicate that X-linked genes in *D. melanogaster* have acquired dosage compensation individually and to different degrees during evolution, which is consistent with the stepwise decay of the Y chromosome[13,14].

Females strike back

Once a strategy has evolved that allows compensation for the loss of one X-chromosomal allele by increasing the transcription from the retained allele, it has to be applied only to the X chromosome in males. In *D. melanogaster*, this is achieved by the male-specific formation of the regulatory dosage compensation complex (DCC), which is due to the male-specific expression of the key protein, MSL2 (FIG. 2). In females the complex does not form and therefore the two X chromosomes are transcribed at their 'normal' rate (FIG. 1). The situation in worms and humans seems more complicated. Global gene expression profiling studies indicate that the principle(s) that increases the transcription of X-chromosomal genes is also at work in cells of the hermaphrodite *C. elegans* and female humans[6,7]. This indicates the existence of countermeasures that prevent inappropriately high expression of X-linked genes compared with autosomal genes. Curiously, worms and mammals have evolved different strategies. Female mammals inactivate large parts of one X chromosome entirely, effectively recreating the situation found in male cells[15] (FIG. 1). Hermaphrodite worms, however, counteract the twofold activation by reducing gene expression from both X chromosomes[16]. The complex interplay between twofold activation and a female-specific counteraction compensates for the different X-chromosome dosage in both sexes, but also adjusts the monosomic X to the diploid set of autosomes. *A priori* it is not obvious why genome function requires the global adjustment of gene expression between entire chromosomes (as opposed to gene-specific optimization), but the existence of intricate dosage compensation systems is evidence for the importance of gene expression homeostasis at the level of the genome.

Fine-tuning gene expression

Although dosage compensation has evolved independently and operates differently in the three model systems, it has an underlying common principle: transcriptional fine-tuning is achieved through modulating the permissiveness of chromatin to transcription, by adopting factors that also regulate chromatin structure and function in other contexts. At the phenomenological level this is best understood for the inactivation of one human female X chromosome[4,17]. A complete shutdown is achieved by selectively converting one female X chromosome into facultative heterochromatin using repression mechanisms that are known to be involved in gene silencing in other contexts, such as those that establish cell-type-specific or imprinted expression. These mechanisms are histone deacetylation, the placement of histone methylation and ubiquitylation marks that recruit repressor proteins such as heterochromatin protein 1 (HP1)[18] and Polycomb group complexes[19], the enrichment of the histone H2A variant macroH2A1 (mH2A1)[20] and, finally, DNA methylation[21]. Crucial for this targeting is the exclusive expression of the non-coding X-(inactive)-specific transcript (*XIST*) RNA from the future inactive X, which initiates in *cis* the cascade of chromatin modification events[4,17]. Although we still do not understand in mechanistic terms how any of these components contributes to the repression of transcription, we assume that a rigid and perhaps condensed structure is generated, which prevents the productive engagement of the RNA polymerase machinery.

Whereas one can imagine a chromatin organization that is sufficiently compact or rigid to prevent transcription completely, it is much less intuitive to think how chromatin structure can be fine-tuned to modulate

Haploinsufficiency
A gene dosage effect that occurs when a diploid organism requires both functional copies of a gene for a wild-type phenotype.

Monosomy
The lack of a counterpart for an entire chromosome.

Polycomb group
A set of developmental transcription repressors that interact to silence genes in an epigenetically stable manner involving histone methylation marks.

transcription in a twofold range. One of the parameters that might be adjusted is the folding of the nucleosomal array into the 30 nm fibre or the organization of this fibre into yet-to-be-described higher order structures. In *D. melanogaster* one of the hallmarks of the twofold increased transcription is the acetylation of histone H4 at lysine 16 (H4K16ac), which is restricted to the X chromosome owing to the targeting of DCC[4]. The N terminus of H4 is required for the folding of nucleosomal arrays into 30 nm fibres[22], and acetylation of H4K16 impedes fibre formation in the absence of other proteins *in vitro*[23]. This indicates that fine-tuning transcription for dosage compensation might be achieved by modulating chromatin folding. The relaxation of higher order chromosome structure becomes visible in the polytene chromosomes of *D. melanogaster*. In certain sensitized genetic backgrounds, the H4K16-acetylated male X chromosome (but not its female counterpart) loses its organization and appears 'bloated'[24,25] (FIG. 3). Experimental H4K16 hyperacetylation of autosomes in males or females leads to a similar disruption of chromatin organization[26]. Interestingly, decreased activity of the Janus kinase JIL1, an H3 kinase that is loosely associated with *D. melanogaster* DCC, also leads to a preferential loss of chromatin organization on the male X chromosome[27], which points to a subtle interplay between different histone modifications that is currently not understood in mechanistic terms.

If the twofold activation of transcription in *D. melanogaster* can be achieved by modulating the folding of nucleosomal fibres, the same principle might be used to bring about the twofold reduction of transcription in *C. elegans*. Exhaustive genetic analyses have identified nine proteins that constitute the regulatory DCC in the worm[28] (FIG. 4). Remarkably, none of them seems to be a histone deacetylase or phosphatase, as one might have expected. Rather, it seems that *C. elegans* uses an entirely different principle for the subtle modulation of chromatin compaction that is required for the twofold reduction of transcription. The worm DCC might do so by engaging condensins, as some DCC components are shared with the 13S condensin complex, which is responsible for chromatin compaction during mitosis and meiosis[4].

One conclusion that can be derived from the observations made so far is that modulating chromatin organization seems highly suited to fine-tuning transcription (alternative scenarios might have included amending X-chromosomal promoters with a sex-specific modifier of RNA polymerase efficacy). This might point to a property of chromatin organization that currently is not widely appreciated, namely the possibility of flexible tuning of a continuum of structures with corresponding functional read-out. Chromatin might also offer itself as a target for modulation owing to the existence of a large number of factors with relatively small contributions to the final structure. In evolutionary hindsight, using chromatin condensation for fine-tuning gene activity has the advantage of adding a layer of regulation to a large number of genes with different regulatory statuses.

30 nm fibre
A chromatin fibre with a diameter of roughly 30 nm, which originates from the folding of the nucleosomal array.

Polytene chromosomes
Specialized interphase chromosomes of the *Drosophila melanogaster* salivary gland, which consist of about 1,000 identical chromatids lined up together. DNA staining reveals a pattern of gene-poor, condensed 'bands' that are separated by decondensed, gene-rich interbands.

Complexes, complexities and complications

The worm DCC[28] (FIG. 4) comprises a hermaphrodite-specific core of SDC (sex determination and dosage compensation) proteins that are required for the stabilization and targeting of the complex to the X chromosome. The association of a condensin subcomplex (formed by the dumpy — shorter than wild-type — proteins DPY-26, DPY-27, DPY-28, and the mitosis and X-associated protein MIX-1) is thought to induce the chromatin changes necessary for compensation. Also essential

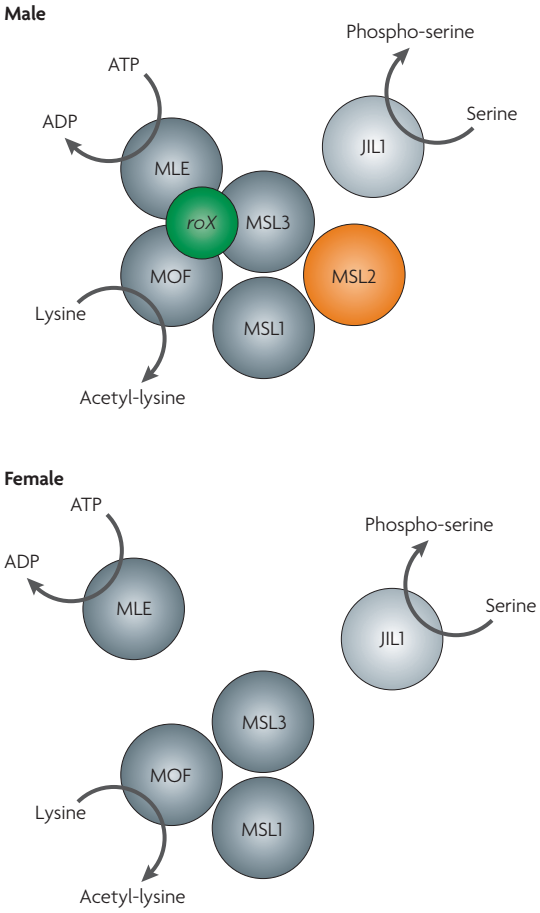

Figure 2 | **Composition of the *Drosophila melanogaster* DCC.** The dosage compensation complex (DCC) of *Drosophila melanogaster* only forms in male cells (upper panel) owing to the male-specific expression of the protein MSL2 (male-specific lethal 2). MSL2 stabilizes MSL1 by direct interaction. According to a widely used model MSL1 and MSL2 form a platform on chromatin that invites the association of the other subunits: MSL3, the histone acetyl transferase MOF (males absent on the first), the RNA helicase MLE (maleless) and *roX* (*RNA on the X*) RNA. MLE, MSL3 and MOF are able to bind RNA. The DCC interacts exclusively with the X chromosome, but the protein domains that are responsible are largely unknown. The Janus kinase JIL1 is only loosely associated with the complex. It is enriched on the X chromosome, but also present on autosomes. MSL2 is not expressed in female cells (lower panel). Consequently, *roX* RNA and MSL1 are unstable and the DCC does not form. Whereas roles for MLE in female cells have been proposed, functions for MOF outside the complex in female cells are not known.

for the modulation of X-linked gene expression is the association of DPY-21 and DPY-30. Apart from its X-chromosome-specific role, the worm DCC is involved in repressing the autosomal *her-1* (*hermaphrodization of X0 animals*) gene[29], which is responsible for suppressing hermaphrodite differentiation. Interestingly, this repression is far beyond the range of compensation — about 20-fold — and does not involve the binding of DPY-21. This could point at context-dependent differences in the extent of repression of DCC components. In an interesting analogy, the *D. melanogaster* DCC activates the male-specific expression of *roX* (*RNA on the X*) genes by much more than twofold[30–32].

In *D. melanogaster*, the male-specific lethality of loss-of-function phenotypes revealed a core of five proteins, collectively called 'male-specific lethal' proteins , which associate to form a DCC in male flies (FIG. 2). Progress has been made towards characterizing the molecular anatomy of this core complex[33–36], which has two catalytic activities — acetyl transferase and RNA–DNA helicase. The acetyl transferase MOF (males absent on the first) is likely to be involved in the activation of X-linked genes because its targeting to ectopic sites in order to acetylate H4K16 can lead to considerable derepression of transcription[37]. However, its function might not be restricted to histone acetylation because MOF can also acetylate MSL1 and MSL3 *in vitro*[35,38]. The acetylation of MSL3 has been proposed to affect the interaction of the protein with non-coding *roX* RNA *in vivo* (see below)[35,38].

A complex can be purified from human cells that resembles the fly DCC in that it contains orthologues of *D. melanogaster* MSL proteins and is responsible for most H4K16 acetylation in human cells[39,40]. This complex is not localized to any particular chromosome nor does it seem to be involved in dosage compensation. Rather, knockdown experiments of MYST histone acetyl transferase 1 (MYST1), an orthologue of the *D. melanogaster* MOF, and male-specific lethal 3-like 1 (MSL3L1) indicate an important role in nuclear organization and in signalling DNA double-strand breaks in the context of the ATM-dependent cell-cycle checkpoint[40,41]. These observations further illustrate the earlier suggestion that dosage compensation uses chromatin regulators that have other roles in other contexts.

The RNA–DNA helicase maleless (MLE) in the *D. melanogaster* DCC might help to integrate two non-coding RNAs, *roX1* and *roX2*, into the complex (FIG. 2). These RNAs are required for the proper targeting of DCC to the X chromosome (see below)[42,43], but the precise nature of their contribution is not known. Both *roX1* and *roX2* are transcribed from the X chromosome, in an interesting analogy to the *XIST* RNA that initiates X-chromosome inactivation in mammals. However, in contrast to *XIST,* the X-chromosomal location of *roX* genes is not an absolute requirement for dosage compensation[1,44].

In addition to the MSL proteins, more and more proteins are being identified that might contribute to raising transcription levels from the male X chromosome, but have other functions as well. A well-known example is JIL1, which is able to phosphorylate histone H3 at serine 10 (H3S10). JIL1 loosely associates with the MSL complex, is enriched on the male X chromosome wherever DCC is present and might also be required for increasing transcription[45]. JIL1 affects the balance between repressive heterochromatin and potentially active euchromatin on chromosomes, possibly because phosphorylation of H3S10 is incompatible with the binding of HP1 (REF. 46). The structure of polytene chromosomes in general, and of the male X chromosome in particular, is disrupted in *Jil1* mutants: the distinction between euchromatin and heterochromatin is lost[27]. A similar derangement of the dosage-compensated polytene X chromosome is observed in larvae deprived of suppressor of variegation 3-7 (SU(VAR)3-7), a protein involved in heterochromatin formation[47,48]. The functional interaction between dosage compensation in *D. melanogaster* and proteins known to define heterochromatin reinforces the concept that the increased transcriptional activity of the compensated X chromosome might be due to its increased ability to counteract the formation of repressive chromatin structures.

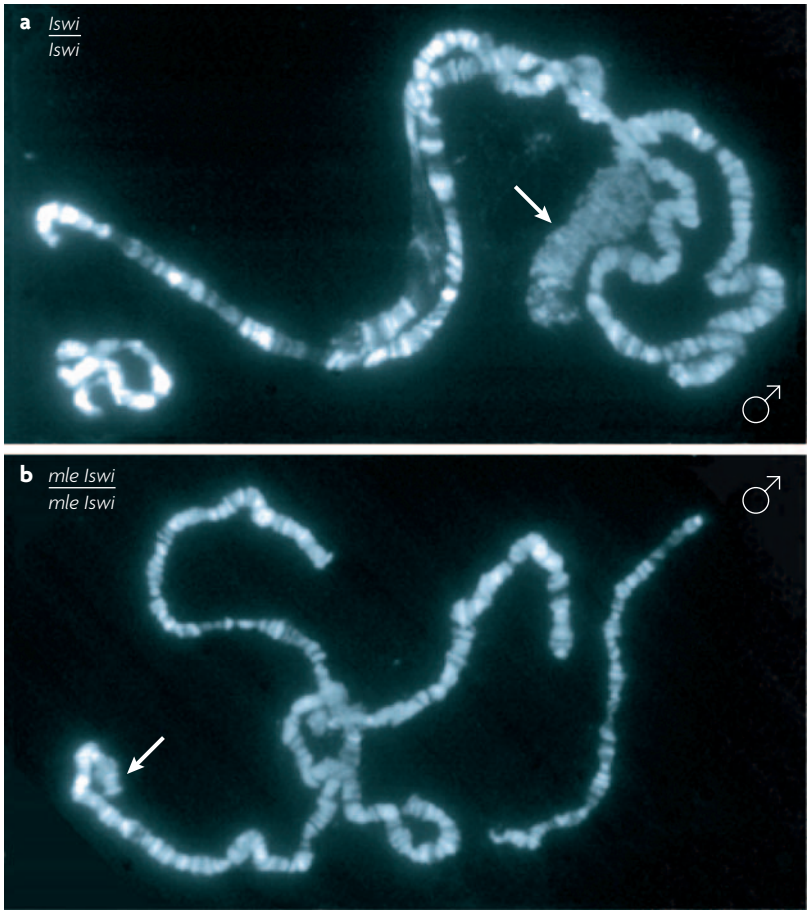

Figure 3 | **X-chromosome bloating in ISWI/ISWI males. a** | Knockdown of the gene for the chromatin remodelling enzyme ISWI (imitation SWI) in *Drosophila melanogaster* males leads to a specific distortion of X-chromosome structure. **b** | A *maleless/maleless* (*mle/mle*) background that disrupts dosage compensation and prevents histone H4 lysine 16 (H4K16) hyperacetylation of the X chromosome rescues the bloated X phenotype. The X chromosome is indicated by an arrow. Image reproduced with permission from *EMBO Reports* (REF. 26) © (2002) Macmillan Publishers Ltd.

In the search for further components of the dosage compensation system in *D. melanogaster*, Akhtar and colleagues recently purified MOF-associated proteins[49]. These studies yielded two new, important pieces of information. MOF is not only present in the DCC, but also in complex with a second set of proteins, for which no male-specific phenotype is known. Some of the subunits are also found in the mixed-lineage leukaemia (MLL)–MYST1 complex recently identified in human cells[50]. The study also revealed that the DCC interacts with nuclear-pore complex components. These interactions seem to be functional because knocking down the pore constituents *Megator* (*Mtor*) and *Nup153* disrupts the staining of X-associated MSL proteins and the activation of dosage compensation[49]. These observations are reminiscent of recent reports in yeast showing that optimal gene expression is achieved by tethering transcription units to nuclear pores[51,52]. Efficient transcription might depend on downstream events, such as the processing and transport of the newly synthesized RNA. Conversely, the silencing of genes might depend on physical compartmentalization into a repressive nuclear domain. Such a process has recently been postulated for X-chromosome inactivation in mammals[53].

Painting chromosomes

Dosage compensation necessitates a clear-cut distinction of a specific chromosome and a concomitant coating by the regulatory machinery[1,54,55] (FIG. 5). In theory this could be achieved if the compensation machinery was able to recognize DNA sequence elements, which would have to be enriched on the X chromosome and dispersed over its length. The existence of discrete DNA recognition elements has been inferred from studies in fruitflies and worms, but consensus sequences have been difficult to identify. Furthermore, some chromatin fragments seem to be unable to recruit DCC by themselves, but are nevertheless occupied if they are placed next to chromatin fragments that have been shown to contain strong targeting elements[55,56]. These findings have led to 'two-step' models, in which an initial chromosomal targeting of DCC (for example, by sequence elements) is followed by the distribution of the complex from the primary recruitment site to coat the neighbouring chromosomal domain. Secondary binding sites might not be defined so much by DNA sequence, but by other characteristics, such as active transcription or a transcription-associated epigenetic mark.

The most prominent example for such a two-step scenario is provided by mammalian X-chromosome inactivation, which is initiated from a single chromosomal region, the X-inactivation centre (*XIC*) from which heterochromatinization and gene silencing spread along the entire chromosome[57]. When the *XIC* is translocated onto autosomes, spreading of the characteristic silent chromatin structure into autosomal chromatin can occur, but is frequently attenuated and incomplete, indicating that other X-chromosomal factors are required for complete dissemination[58]. A recent study[59] correlated the attenuated spread of heterochromatinization from an autosomally translocated *XIC* with a low density of LINE-1 (long interspersed nuclear element)-repeats close to the insertion site. This strengthens an old hypothesis[60] that the LINE-1 elements, which are enriched on the X chromosome, might function as 'relay elements' to promote efficient distribution of the silencing marks.

It is important to point out that the inactivation of the human X chromosome is not complete: 15% of the X-linked genes escape the realm of repression (FIG. 5). Those 'escapers' tend to cluster in chromosomal regions where the density of LINE-1 repeats is around or below the genome average[61]. Interestingly, those regions are typically located in evolutionarily younger parts of the chromosome suggesting that X-chromosome inactivation evolves to include more parts of the chromosome by active enrichment of LINE-1 repeats, which facilitate the dissemination of the silent state.

In contrast to the single targeting site in mammals, X-to-autosome translocations and *P*-element mediated insertions of X-chromosomal DNA into autosomes in *D. melanogaster* indicated the existence of hundreds of X-chromosomal loci that are able to autonomously

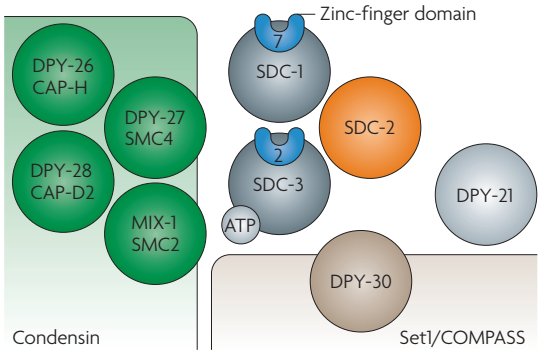

Figure 4 | **The *Caenorhabditis elegans* DCC.** Dosage compensation in worms requires the action of at least nine proteins. Mutation of any of these genes causes overexpression of X-linked genes and a *dumpy* (*dpy*; shorter than wild type) phenotype in hermaphrodites that manage to escape lethality. The dosage compensation complex (DCC) assembly and target recognition depend on the hermaphrodite-specific association of SDC (sex determination and dosage compensation) proteins. *sdc-2* is repressed in males and, therefore, serves as a sex-specific switch gene. The zinc-finger domains in SDC-1 and SDC-3 might be directly responsible for chromosome binding. The ATP binding domain of SDC-3 has been shown to be required for the repression of the autosomal *her-1* (*hermaphrodization of X0 animals*) gene. Four DCC proteins are similar to 13S-condensin complex members[79]: the mitosis and X-associated protein MIX-1, DPY-27, DPY-26 and DPY-28, which are homologues of SMC2 (structural maintenance of chromosomes 2), SMC4, CAP-H (non-SMC condensin I complex, subunit H) and CAP-D2, in corresponding order. DPY-30 is shared with another complex that is homologous to the yeast Set1/COMPASS histone H3 methylase complex[80,81] and might therefore have a more general role in regulating transcription in both XX and X0 animals. DPY-21 is only found on X-chromosomal targets and is not required for repression of the *her-1* gene. The numbers 2 and 7 represent the number of zinc-finger domains.

Epigenetic
Any heritable change in gene expression that is not caused by a change in DNA sequence.

LINE-1
Long interspersed nuclear element. This is a family of mammalian retrotransposons of high abundance (for example, the human genome contains about 900,000 LINEs, covering about 20% of the genome).

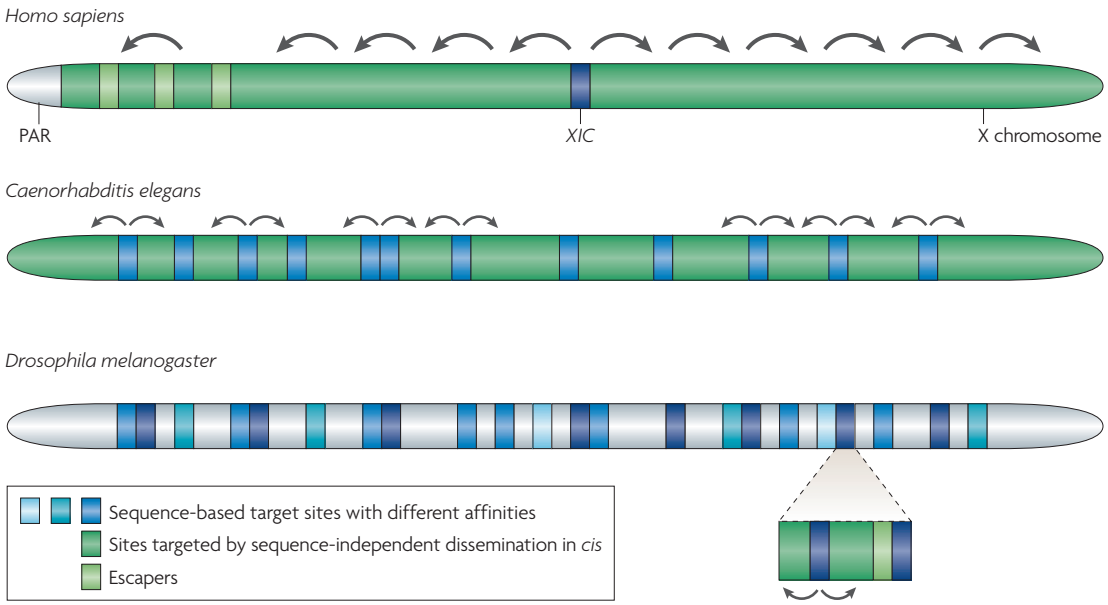

Figure 5 | **Chromosome-wide distribution of dosage compensation.** In *Homo sapiens* a single targeting element, the X-inactivation centre (*XIC*), serves as a nucleation site for the spreading of silent chromatin along the entire chromosome. Dispersed sequence-based 'relay stations' and epigenetic marks along the X chromosome are thought to support the spreading process. Genes within the pseudoautosomal region (PAR), which retains homology with the Y chromosome and several dispersed genes (escapers), remain active. Whether these genes also escape the recently described global activation of the X chromosome (FIG. 1) is not known. In *Caenorhabditis elegans*, several recruitment sites exist from which dissemination in *cis* is thought to help to cover the rest of the chromosome. In *Drosophila melanogaster*, many distinct targeting sites of different affinities establish a discontinuous pattern of bound and unbound genomic regions. Whereas spreading between those regions is negligible, the dissemination of the dosage compensation complex (DCC) within those regions (typically spanning thousands of kilobases and containing more than one primary targeting site) might well use a spreading process.

recruit MSL proteins[62–64]. However, visualizing DCC binding to polytene chromosomes by immunohistochemical staining reveals that the X chromosome is not uniformly bound, but that extended chromosomal segments, typically the gene-poor regions, remain unbound (FIG. 6). Polytene chromosome analyses do not resolve single DCC binding sites but the cumulative binding of DCC to entire chromosomal domains that contain many genes. However, progress has recently been made towards identifying DCC binding regions by inserting progressively shorter X-derived candidate fragments into autosomes and monitoring their ability to attract DCC[62–65]. The expression of increasing concentrations of MSL2 in female cells leads to the formation of corresponding levels of DCC, which associates with the two X chromosomes. Comparing the polytene binding patterns from female larvae that express different DCC levels revealed that the global concentration of DCC is an important determinant of target-sequence occupancy and that affinities of different binding regions for the DCC vary strongly. Accordingly, there are a few dozen high-affinity sites that will attract MSL proteins even when their concentration is very low and/or important complex components such as MSL3, MLE or MOF are missing. Sites of progressively lower affinity are filled as global DCC concentrations are experimentally increased[64]. This principle can be extended further. Low-affinity regions might profit from high local concentrations of DCCs that are due to nearby clustering of strong binding sites and so be bound in a context-dependent manner[64]. However, these observations do not reveal the relative contributions of DNA sequence, epigenetic histone modifications or as-yet-unknown factors to the affinity of a site for a DCC.

Similar principles might apply to the DCC binding determinants in *C. elegans*[55]. Large parts of the worm X chromosome seem to be secondary binding sites by the above definition (FIG. 5), but the nature of the 'spreading' from primary to secondary sites is so far unknown. Interestingly, extensive spreading does not occur when the primary targeting elements are translocated to autosomes[56]. It has been speculated that autosomal 'blocking' sequences might prevent the dissemination in *cis*. Alternatively, the spreading phenomenon could reflect the successive coverage of low-affinity binding regions located on the X chromosome that are not able to attract the DCC in an isolated context.

ChIP-on-chip analysis
A powerful method to map the distribution of chromatin proteins along genomes by chromatin immunoprecipitation (ChIP) and subsequent hybridization of the co-precipitated DNA to oligonucleotide microarrays (chip).

The resolution revolution

The banded pattern of *D. melanogaster* larval polytene chromosomes, stained to show MSL proteins, indicates a discontinuous dissemination of DCC with binding to gene-rich regions and skipping gene-poor ones (FIG. 6). Recent high-resolution ChIP-on-chip[66] profiling experiments have revealed the detailed distribution of the MSL complex on the X chromosome[10,11], disclosing surprising detail that is unmatched by any of

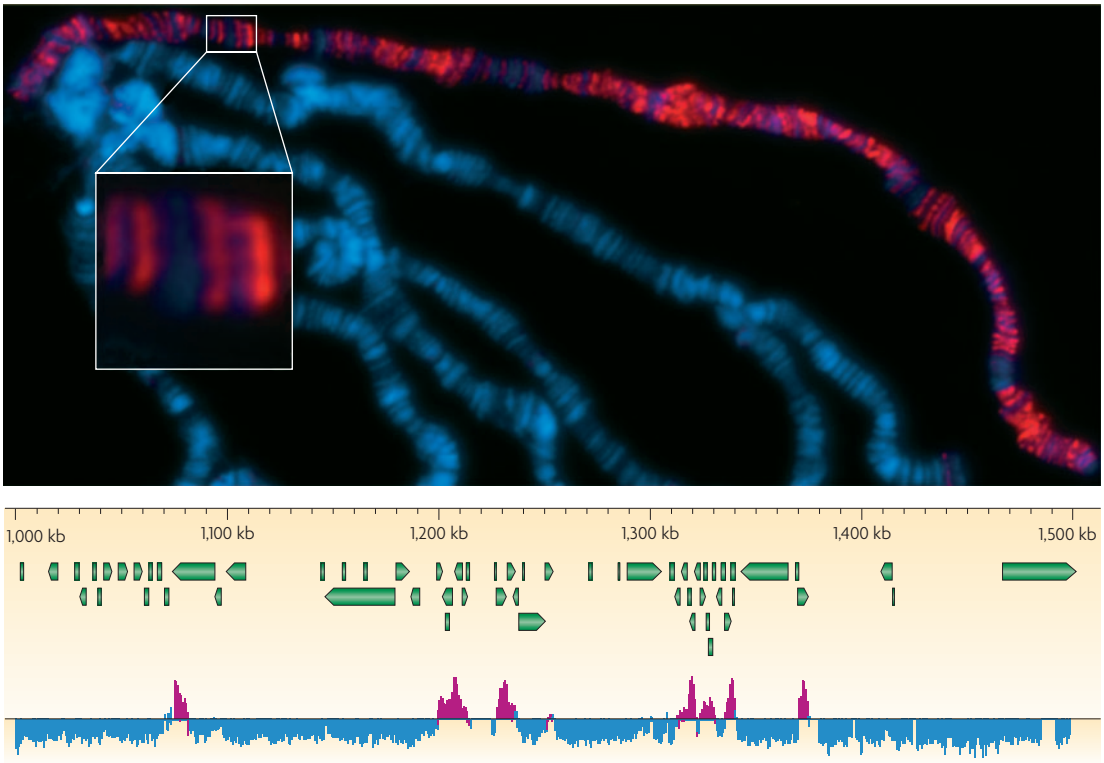

Figure 6 | **Gain of resolution by a ChIP-on-chip array.** In flies, chromosome binding of the the dosage compensation complex (DCC) can be illustrated by polytene chromosome staining (upper panel). The microscope image reveals that the DCC, shown here in red by male-specific lethal 1 (MSL1) antibodies, gathers almost exclusively on the male X chromosome (autosomes are illustrated by the blue DNA stain). The discontinuous distribution correlates with gene density, with DCC binding preferentially to gene-rich regions and skipping gene-poor ones (the inset shows a magnification of an X-chromosomal segment with four fluorescent bands). However, microscopy can not resolve the binding pattern within one fluorescent band that might span 100 kb and contain dozens of genes. Chromatin immunoprecipitation (ChIP) on tiling microarray chips (lower panel) overcame this limitation and allowed, for the first time, the resolution of the MSL binding pattern within the fluorescent bands seen on stained polytene chromosomes. A 500-kb segment of the X chromosome is shown with its corresponding genes (green arrows) and significant sites of MSL1 binding as revealed by ChIP-on-chip profiling (magenta).

the other model systems. The binding profiles clearly distinguish MSL proteins from classical transcription activators. Rather than homogeneously covering the gene-rich chromosomal domains, genes are targeted individually leaving about half of the X-linked genes unbound (FIG. 6). Regulatory regions such as promoters or enhancers are rarely, if at all, bound by DCC. Instead, the complex associates almost exclusively with transcribed gene sequences, where it covers broad regions, indicating that target-gene occupancy is not due to single binding sites. In addition, both MSL proteins analysed, MSL1 (REF. 11) and MSL3 (REF. 10), exhibited unexpected 3′ enrichment along the gene.

The discontinuous distribution even within gene clusters suggests that there are local determinants of complex recruitment (FIG. 7). Several observations argue for multiple targeting features working cooperatively. Although DNA sequences clearly contribute to defining the interaction of DCC with selected high-affinity binding sites (REFS 64,67; G.D. Gilfillan and P.B.B., unpublished observations) and even the global binding pattern of MSLs can be explained by DNA sequences to a limited extent[11], no simple recognition sequences shared by all DCC binding sites could be identified[10,11]. At present we assume that DCC binding is a function of variable combinations of several degenerate sequence motifs (FIG. 7A). A model involving complex, composite targeting sequences is consistent with the observed binding patterns, as a higher flexibility in target-sequence determinants increases the possibility of incorporating binding sites of reasonable affinity into the coding regions without compromising protein function. The increased codon bias of X-linked compared with autosomal genes[68] could point to subtle sequence changes on haploinsufficient genes that allow the generation of multiple degenerate recognition motifs without altering amino-acid composition. However, the observed codon bias might simply be a consequence of distinct selective forces on the X chromosome without having functional implications for complex targeting[68].

Because simple sequence motifs for DCC targeting could not be isolated by classical bioinformatic tools, and because MSLs are preferentially bound to active genes, Alekseyenko *et al.* proposed a sequestration

Codon bias
The preference for specific codons over others that code for the same amino acid.

model that largely depends on transcription[10] (FIG. 7B). This hypothesis reinforces a previous observation that MSL protein binding was ectopically induced following activation of a transgene on the X chromosome[69]. The skewed enrichment towards the 3′ end correlates with specific transcriptional elongation-associated alterations of chromatin structure, such as trimethylation of lysine 36 of histone H3 or the presence of other elongation factors[70,71]. Alternatively, the observed enrichment of DCC on the 3′ ends of coding regions might be the consequence of reduced binding of the complex at the front end of a gene owing to high transcription rates.

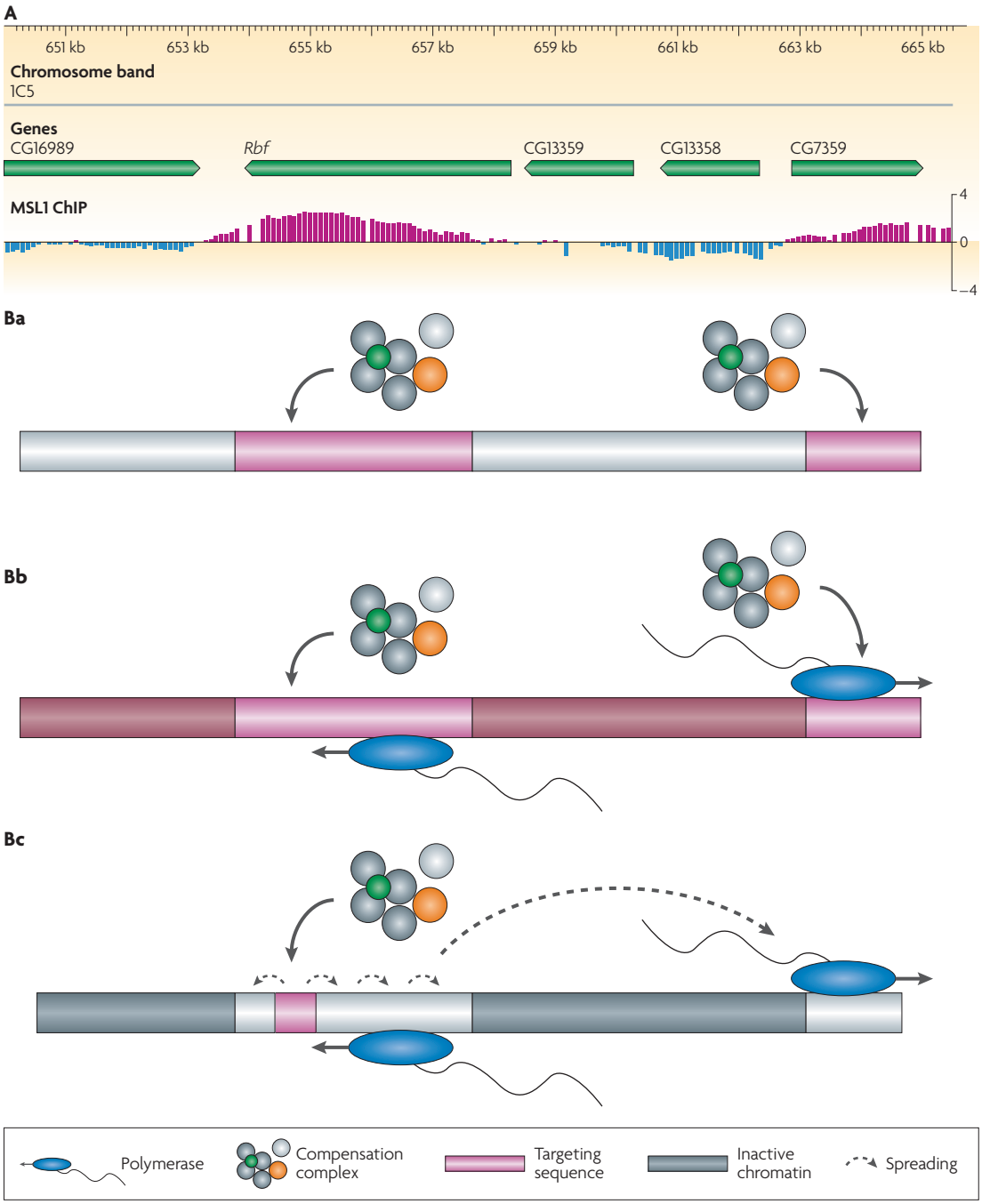

Figure 7 | **Targeting models for the *Drosophila melanogaster* DCC. A** | Specific binding of the male-specific lethal (MSL) proteins, as revealed by ChIP-on-chip analysis can be explained by various models. **Ba** | Binding depends on DNA sequence motifs only. Quantitative variations of binding could depend on the different affinities of the target sequences. **Bb** | Binding depends on transcriptional activity. RNA polymerase or other factors associated with active chromatin are required to expose X-chromosome-specific targeting elements. **Bc** | A two-step model of binding. The the dosage compensation complex (DCC) is recruited to primary targeting elements that do not depend on the chromatin state. Spreading in *cis* into adjacent regions occurs independently of sequence but requires transcriptional activity. *Rbf*, retinoblastoma family.

However, the situation does not allow overall generalizations: there are also inactive genes bound by MSLs and active genes that remain unbound[10–12]. Furthermore, examples have been described in which MSL recruitment does not coincide with transcription[72,73]. The observation of active, compensated genes not bound by MSL proteins might point to alternative mechanisms for dosage compensation in addition to the prevalent mode discussed here[12]. As far as MSL binding in the absence of obvious transcription is concerned it is appealing to speculate that the high-affinity sites identified by translocation experiments represent a subclass of targeting regions that recruit the DCC independently of transcription. Transcribed genes nearby might benefit from the high local concentration of MSLs and recruit compensation proteins through a transcription-dependent mechanism[64] (FIG. 7Bc).

Plasticity versus stability

In mammals, X-chromosome inactivation is maintained epigenetically. Once one of the two female X chromosomes has been selected for inactivation in early development, the silent state is perpetuated through all subsequent cell divisions even in the absence of factors, such as the *XIST* RNA, that are crucial for establishing silent chromatin[57]. Epigenetic inheritance of the silent state involves labelling the chromatin with DNA and histone methylation marks, which are recognized by proteins that render chromatin repressive for transcription, and which are propagated by the continuous recruitment of the corresponding methylases. It is currently not known whether and to what extent epigenetic marks contribute to defining fly DCC binding sites during the cell cycle. Given the DCC targeting models in flies discussed above, a specific inheritance mechanism is not implicitly required as the activated state could be constantly renewed or re-established at every cell division following the recognition of X-chromosomal targeting elements by the male-specific DCC. If targeting involved sensing transcription, dosage compensation could be adapted easily to the changing needs of developmental or cell-type-specific gene expression.

Surprisingly, most of MSL target regions do not vary between tissue types or stages of fly development[10,12,69,74]. A trivial explanation for the invariance of MSL binding could be provided if all of the stably bound regions mapped to housekeeping genes that are constitutively expressed. Systematic comparison of developmental and tissue-specific expression of dosage-compensated genes and DCC occupancy has not been carried out so far, but it promises to answer the open question of whether the interaction of DCC depends on active chromatin or is epigenetically inherited.

Given the lack of stable chromatin marks such as DNA or histone methylation, how is the DCC stably maintained in flies? Measurements of the dynamics of MSL2 in living *D. melanogaster* cells revealed that DCC remains associated with the X chromosome throughout the cell cycle and, furthermore, documented a surprising stability of protein binding to target sites in interphase[32]. The lack of mobility of MSL2 on the X chromosome is at odds with ordinary protein–DNA interactions, a fact that led to the speculation that the interaction of the MSL complex might convert into a topological linkage through a clamp-like configuration, conferring stability to the chromosomal association. The only chromatin modification that defines the activated male X chromosome, H4K16 acetylation, is thought to be labile. However, stable binding of the DCC to the X chromosome requires the histone acetyl transferase activity of MOF and it is possible that the acetylation contributes to stabilizing DCC on the chromosome as part of a reinforcing feedback loop[32,75]. Identifying H4K16ac interactors, should they exist, would be revealing.

Mechanistic considerations

The precise nature of the chromatin structure that leads to the inactivation of the mammalian female X chromosome is not known, but we assume that a conformation is generated that interferes with the productive initiation of transcription. It is even less clear how chromatin is tuned to modulate transcription in a twofold range. The particular distribution pattern of the *D. melanogaster* DCC on the transcribed portions of regulated genes indicates that the complex might act downstream of transcription initiation. So far, there is no evidence that compensated X-linked genes recruit more RNA polymerase than autosomal genes[11] but nucleosome turnover and histone H3.3 incorporation are increased[76], which indicates that transcription efficiency might be the tuning parameter. Transcription elongation through chromatin is highly regulated[70,71], and an increased rate of transcription at constant amounts of polymerase might therefore be explained by a more processive RNA polymerization, for example by preventing the rate-limiting premature termination[77]. If juxtaposition of transcription terminators and promoters of a transcription unit, which is supposed to facilitate the recycling of RNA polymerase for re-initiation[78], was a widespread principle, an improved elongation processivity would lead to more mRNA.

Although effects of DCC on processes downstream of transcription such as RNA processing or export cannot be excluded at present (see above), most of the data indicate that tuning transcription in the twofold range occurs by modulating the rate of transcription elongation through chromatin folding. Interestingly, the ChIP-on-chip analyses revealed a strong correlation between the amount of MSL1 at target genes and the precise levels of increased transcription[11]. The fact that incomplete compensation was observed at genes harbouring lower MSL1 levels indicates that compensation results from a cumulative effect of many DCC complexes. Accordingly, the precise level of compensation is determined by the affinity of the target sequences for DCC, which in turn depends on the number and type of degenerate sequence motifs that could be accommodated in the coding sequences.

Outlook: beginning and end of generalization

Plenty of recent data in all model systems highlight that dosage compensation is not homogenous with respect to both localization and functional read-out, allowing

for variations in the level of compensation and even complete escape. This strengthens the idea that the X chromosome as a whole does not elicit compensation, but individual genes or loci actively recruit dosage compensation machineries by a common but species-specific mechanism. Localization on the transcribed unit, as observed in *D. melanogaster*, indicates that DCC proteins are different from canonical transcription factors. Fine-tuning of compensated expression occurs mainly at the level of chromatin, conceivably affecting processes downstream of transcription initiation.

Owing to the large numbers of co-regulated genes, dosage compensation systems provide excellent opportunities for the analysis of gene regulation in general. The effects of specific chromatin modifications are reiterated in many independent, but mechanistically related observations such that global analyses have the statistical power to uncover common regulatory principles. However, information of high biological significance might lie in the variations and deviations from these common principles. Combining the profiling of gene expression patterns during development and of high-resolution chromosomal interactions between regulatory proteins using forward and reverse genetic manipulations promises to reveal more about these rules and exceptions, which will be necessary to fully understand dosage compensation.

Note added in proof

Barbara Meyer and colleagues have recently identified two disparate DNA motifs that are important for DCC recruitment in *C. elegans*[82]. Although the sequences on their own are not enriched on the X chromosome, the clustering of the two motifs seems to define specific binding sites. This is reminiscent of the situation in *D. melanogaster* in which combinations of various degenerate motifs have been postulated to constitute DCC binding regions[11,64].

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

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