Dosage compensation in *Drosophila melanogaster*: epigenetic fine-tuning of chromosome-wide transcription

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Abstract | Dosage compensation is an epigenetic mechanism that normalizes gene expression from unequal copy numbers of sex chromosomes. Different organisms have evolved alternative molecular solutions to this task. In *Drosophila melanogaster*, transcription of the single male X chromosome is upregulated by twofold in a process orchestrated by the dosage compensation complex. Despite this conceptual simplicity, dosage compensation involves multiple coordinated steps to recognize and activate the entire X chromosome. We are only beginning to understand the intriguing interplay between multiple levels of local and long-range chromatin regulation required for the fine-tuned transcriptional activation of a heterogeneous gene population. This Review highlights the known facts and open questions of dosage compensation in *D. melanogaster*.

Chromosomal aneuploidy
The presence of an abnormal number of chromosomes, either more or less than the diploid number. Associated with cell and organismal inviability, cancer and birth defects.

In many organisms, suppressed recombination between the two alleles of an autosomal sex-determining locus has led to the evolution of unequally distributed sex chromosomes. The most common forms of sex determination are the XX or XY and the ZZ or ZW systems, where male or female heterogamety are shown, respectively[1]. Compensating mechanisms have often emerged in parallel to overcome detrimental imbalances in gene expression that result from chromosomal aneuploidy (although recent data from an increasing number of organisms suggest that this compensation is less mandatory than was previously believed (reviewed in REF. 2)). In *Drosophila melanogaster*, a ribonucleoprotein dosage compensation complex (DCC; also known as the male-specific lethal (MSL) complex) is enriched on the single male X chromosome, where it mediates global acetylation of histone H4 at lysine 16 (H4K16ac)[3]. A large body of evidence from genome-wide as well as single-gene studies supports the view that the DCC causes a twofold upregulation of transcription from the X chromosome[4–7]. This basic result is not trivial, as alternative mechanisms for dosage compensation have been adopted by different organisms throughout evolution. For example, transcription on the two X chromosomes in *Caenorhabditis elegans* hermaphrodites is repressed by half to match the single X chromosome in males[8], whereas in mammals one of two female X chromosomes is randomly inactivated to equilibrate expression between the sexes[9]. Nevertheless, recent evidence indicates that transcription of the active X chromosomes is twofold enhanced in both sexes of *C. elegans* and mammals to equalize X-linked and autosomal gene expression[10–12].

All chromosome-wide dosage compensation systems provide exquisite models for the study of chromatin regulation at a local level as well as on a global scale. Two main questions have been the focal points of many years of research. First, how can a specific chromosome be recognized in its entirety and targeted by the epigenetic machinery in only one sex? And second, how can the concomitant alteration of its intrinsic properties coordinately adjust the transcriptional output from a diverse set of individually regulated genes by a fixed factor?

A model of *D. melanogaster* dosage compensation has become widely accepted during the past decade and, according to this model, DCCs spread from high-affinity binding sites on the X chromosome to low-affinity targeting cues in compensated genes, where they activate transcription[13]. In this Review, we discuss the tremendous advances in our understanding of the events that underlie *D. melanogaster* X-chromosome compensation, which have substantially refined this concept in recent years. It now appears that the dynamic interplay between MSL proteins, male-specific RNAs on the X (roXs) and a limited number of X-specific DNA sequence elements causes a dramatic structural and

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doi:10.1038/nrg3124

functional reprogramming of the entire male X chromosome. We will describe the principles that orchestrate each step of this process and present models for the resulting transcriptional activation, whose exact mechanism remains enigmatic to date.

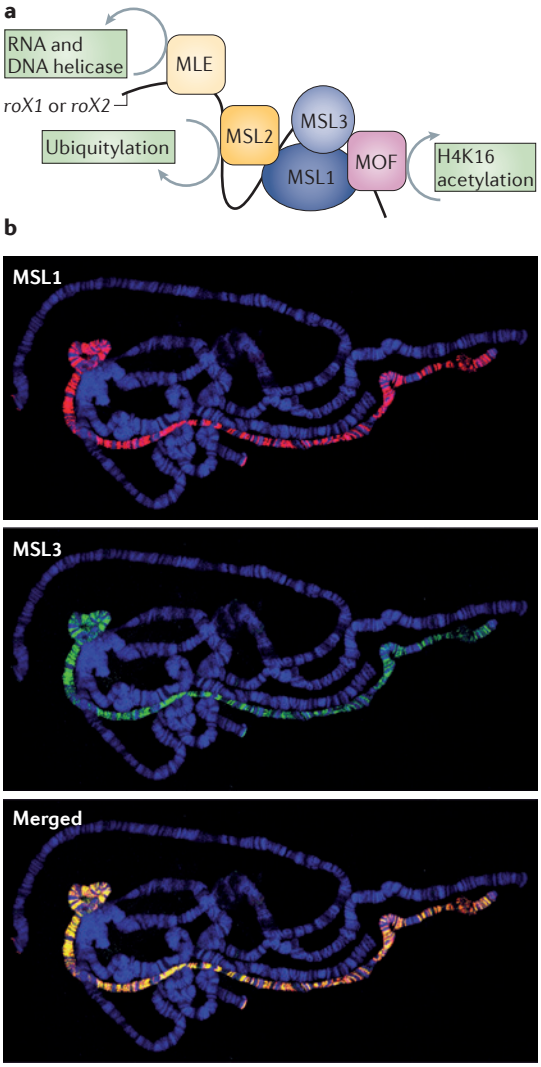

Figure 1 | **Composition and localization of the dosage compensation complex. a** | The dosage compensation complex (DCC), also known as the male-specific lethal (MSL) complex, is composed of at least five proteins (MSL1, MSL2, MSL3, maleless (MLE) and males absent on the first (MOF)), as well as two large non-coding RNAs on the X, *roX1* and *roX2*. Translation of the MSL2 protein is suppressed in females, preventing formation of the complex. Three components of the DCC have enzymatic functions. MLE is an RNA and DNA helicase, MOF acetylates histone H4 at lysine 16 (H4K16ac), and MSL2 shows ubiquitin E3 ligase activity. MSL1 is believed to act as a scaffold for complex assembly and triggers MOF enzymatic activity together with MSL3 (REFS 22,23). **b** | The DCC is specifically targeted to the male X chromosome. Polytene chromosomes isolated from third instar larvae were immunostained with MSL1 (red) and MSL3 (green) antibodies. DNA is stained with Hoechst 322 (blue). The 'merged' panel shows the overlap of the two signals (yellow).

DCC assembly and evolution

The coordinate alteration of chromatin structure appears to be the main principle of all chromosome-wide dosage compensation systems[14]. Each system has evolved dedicated machineries of tremendous complexity, despite the fact that the propagation of sex-restricted hemizygosity occurred stepwise through evolution[1] and dosage compensation systems are expected to evolve rapidly — and, in fact, they do[15]. Not surprisingly, a unifying theme in all studied model organisms is therefore the redirection of pre-existing chromatin regulators for the novel task of dosage compensation.

DCC assembly. In *D. melanogaster* males, dosage compensation is orchestrated by the MSL2 protein, which induces assembly of the DCC. Translation of *msl2* is specifically repressed in females by binding of the sex regulator sex lethal (SXL) together with Upstream of N-ras (UNR) to the 5′ and 3′ untranslated region (UTR) of the *msl2* mRNA[16–19]. By contrast, in males, an alternative splicing cascade prevents the expression of a functional SXL protein (reviewed in REF. 20), leading to translation of MSL2, and UNR contributes to DCC function under these conditions[21]. In addition to MSL2, the DCC consists of the H4K16ac-specific histone acetyltransferase males absent on the first (MOF), as well as MSL1, MSL3, maleless (MLE) and the *roX1* or *roX2* non-coding RNAs (ncRNAs) (FIG. 1a). Binding of MSL2 stabilizes MSL1, which acts as a scaffolding protein to mediate the integration of MSL3 and MOF into the complex[3,22,23]. In addition, and most likely in concert with MSL1 and MLE, MSL2 activates transcription of the *roX1* and *roX2* genes[24–28]. Incorporation of either roX RNA is then aided by the ATP-dependent DEXH box RNA and DNA helicase MLE[29,30], which remains peripherally associated with the complex by RNA interactions. Physical tethering of the DCC to the male X chromosome is beautifully illustrated by the banded staining pattern that is observed following immunolabelling of its complex members on polytene chromosomes from third instar larval salivary glands (FIG. 1b). The remarkable capacity of MSL2 to induce this binding is exemplified by its ability, when ectopically expressed in female flies, to lead to assembly of the DCC on the two X chromosomes and to cause female lethality[31]. However, MSL2 is not present in all male tissues or developmental stages, and alternative compensation mechanisms have been suggested for these special circumstances (BOX 1).

Novel tasks for old players. As mentioned above, orthologues of MSL proteins from yeast to humans have various other chromatin-related functions (BOX 2). The flexibility by which the epigenetic machinery can be differentially used even on individual chromosomes inside the same cell was further demonstrated by the recent finding that MOF also acts as a transcriptional regulator at gene promoters across the male and female genome, where it is part of the so-called non-specific lethal (NSL) complex[32,33]. However, although MOF binding to autosomal promoters correlates with

Box 1 | **MSL-independent dosage compensation**

Because the dosage compensation complex (DCC) and hyperacetylation of the X chromosome are absent from cells of the male germline, investigators asked if X-chromosome dosage remains uncompensated in diploid cells of germline-associated tissue. Although one study reported some reduction in X-derived transcript levels relative to autosomal transcript levels in the male testis[119], this observation might be explained by the general paucity of testis-specific genes on the X chromosome[120,121]. Most other evidence suggests that X-linked transcriptional output is indeed elevated in the germline by a DCC-independent mechanism of an as of yet unknown nature[11,121].

The DCC is similarly absent during a short window from the onset of zygotic transcription until after the blastoderm stage 60 to 90 minutes later, when accumulating male-specific lethal 2 (MSL2) triggers assembly of the DCC on the male X chromosome[122,123]. Nevertheless, recent high-throughput RNA sequencing (RNA-seq) data suggest that X-linked transcriptional output is to some degree compensated in the early embryo[124]. However, although key regulators of embryonic development were almost precisely equalized between the sexes, many X-linked genes showed little or no compensation, arguing against a chromosome-wide compensation mechanism being active at this stage. Instead, it was suggested that transcript levels might be buffered by gene-specific feedback loops or potentially by binding of sex lethal (SXL) to X-derived mRNAs in females. Although a direct effect of SXL on transcript levels has not been described yet, such a mechanism would explain why it is in fact the female X-linked expression that appears to be reduced relative to autosomal transcription in early embryos[124].

Interestingly, in addition to somatic X-chromosome-wide compensation, some degree of transcriptional buffering has also been observed in flies that are heterozygous for autosomal duplications or deficiencies. However, the magnitude of this effect remains somewhat unclear *in vivo*, as the values obtained by microarray analysis varied substantially depending on the normalization procedure and the included gene set[11,125]. More extensive buffering, especially of autosomal copy number variations, was detected in aneuploid S2 cells[126], but just as in fly tissues, it is not clear whether this effect is mediated by a global compensation mechanism of unknown nature or whether it is the result of gene-specific feedback regulation. Even if a general compensation mechanism for copy number aberrations exists in *Drosophila melanogaster*, as long as information on the molecular basis of this effect is lacking, it remains difficult to investigate its contribution to X-chromosome compensation.

H4K16ac and gene expression, transcriptional activation appears to be largely mediated by other members of the NSL complex, suggesting that MOF may serve to modulate this activity[33]. Likewise, although its function outside dosage compensation is poorly characterized, MLE associates with numerous transcriptionally active regions as well as heat-shock puffs on all chromosomes in both sexes, suggesting a general role in transcriptional regulation or in RNA processing[34]. In fact, with the exception of MSL2, all protein components of the *D. melanogaster* DCC are present in female flies, and additional functions of these proteins, which are possibly shared with their human orthologues, are likely to be found in the future. In addition to DCC components, several other epigenetic regulators with ubiquitous functions have been implicated in the specific regulation of the male X chromosome, including: the heterochromatin proteins suppressor of variegation 3-7 (SU(VAR)3-7) and heterochromatin protein 1 (HP1)[35–37]; the ISWI nucleosome remodelling complex[38,39]; DNA supercoiling factor (SCF)[40]; the JIL1 kinase[41–43]; and the nuclear pore components NUP153 and Megator[44,45]. However, the precise contributions of these factors to the dosage compensation mechanism remain to be elucidated.

Reprogramming the X chromosome

How can the sole presence of the MSL2 protein and subsequent DCC formation eventually alter the properties of an entire chromosome? During the 20 years since MLE was first visualized at hundreds of sites along the male X chromosome[46], much of the research on dosage compensation was focused on the mechanism of DCC targeting. It appears now that this process is much more dynamic than was initially believed and involves a dramatic reorganization of X-chromosome architecture. More specifically, it seems that the very structural changes that are brought about by the DCC[47] are themselves the basis for most DCC binding on X-linked target genes[29,48] and ultimately also cause X-linked transcription activation[49].

The role of roXs. How is X-chromosome recognition initiated? Prominent molecular labels of the male X chromosome are the *roX1* and *roX2* transcripts, which can be visualized along the entire male X chromosome as part of the DCC[50,51]. Both roXs originate directly from the X chromosome, where their genes are located. This is reminiscent of the situation in mammals, where heterochromatinization of the inactive X chromosome is triggered by expression of the X-linked X inactivation specific transcript (*XIST*) RNA[9]. Four of the five MSL proteins contain RNA-interacting modules[52–55] and, conversely, roX RNAs are unstable when they are not bound to the DCC[30], suggesting that incorporation at least into partial DCC subcomplexes is happening co-transcriptionally. This idea is further supported by immunofluorescence staining of polytene chromosomes from third instar larval salivary glands that show DCC binding proximal to autosomal insertions of roX transgenes[13,30]. DCC spreading into autosomal chromatin is dependent on the available concentrations of MSL proteins[56] and does not happen when the nascent roX RNA dissociates too rapidly owing to a high transcription rate[57]. An important conclusion from these studies is that the DCC can recognize autosomal chromatin under certain conditions and that its chromatin-binding capacity is substantially enhanced upon roX incorporation. Accordingly, the presence of at least one of the two roXs is required for efficient DCC targeting to X-linked chromatin *in vivo*[58], and more recent work has suggested that the specificity of DCC binding is altered upon incorporation of roX RNAs into the complex[26]. However, X-linked roX transcription cannot be the sole determinant of DCC targeting. In the presence of endogenous roX RNAs, local DCC recruitment also occurs around autosomal insertions of a non-transcribed *roX1* gene whose DNA sequence was by itself found to constitute a high-affinity target for DCC binding[59]. Furthermore, autosomal roX transgenes are able to restore the defective DCC targeting and reduced male viability associated with *roX1* and *roX2* double mutants in *trans*[58]. Both results are at odds with a linear progression of the DCC from the sites of roX synthesis. Instead, although chromatin binding is favoured at sites of high DCC concentration around the nascent roX RNAs, these data suggest that the DCC reaches its final target sites by free diffusion through the interchromatin space (FIG. 2a).

Box 2 | **MSL proteins in mammals**

All five protein components of the dosage compensation complex (DCC) are conserved in mammals. RNA helicase A (RHA), the orthologue of *Drosophila melanogaster* maleless (MLE), is involved in various aspects of RNA metabolism[127,128]; however, its relationship to other mammalian male-specific lethal (MSL) proteins remains unclear. More closely resembling the situation in *D. melanogaster*, a mammalian MSL complex consisting of human males absent on the first (MOF), MSL1, MSL2 and MSL3 shows histone H4 lysine 16 (H4K16)-specific acetylation activity *in vitro* and *in vivo*[44,129,130]. However, so far, no RNA component could be identified in this complex. Although accumulating evidence indicates that transcription on the active X chromosome is twofold enhanced in both mammalian sexes to match autosomal expression levels[10–12,131], it remains to be elucidated what role MSL proteins have in this process.

Clearly, MOF is essential for vertebrate development[132,133] and is involved in transcription regulation at mammalian gene promoters[110,111,134–136]. Interestingly, this also involves MOF-mediated acetylation of non-histone targets, depending on the subunit composition of MOF-containing complexes[136–138]. In addition to its functions in transcription regulation, the essential role that MOF has in the mammalian DNA damage pathway has become well-established in recent years[130,139–142]. It is believed that the enhanced chromatin accessibility resulting from MOF-mediated H4K16ac facilitates the recruitment of DNA repair proteins to sites of DNA damage[140]. The role of MOFs in DNA damage response appears to be highly conserved, as the yeast homologue of MOF, Esa1, is similarly required for the repair of DNA double-strand breaks[143], and a corresponding function has been reported for *D. melanogaster* MOF[144].

A completely novel aspect of MSL-mediated transcriptional control has recently been discovered with the finding that the RING finger domain of human MSL2 acts as a ubiquitin ligase with specificity towards H2BK34 (REF. 105) (and, most likely, p53 (REF. 145)). This activity appears to regulate transcription by crosstalk with H2B120 ubiquitylation, as well as H3K4 and K79 methylation. A ubiquitylation activity towards histone H2B was also observed for *D. melanogaster* MSL2 in that study, and because point mutations that disrupt this activity in flies lead to male lethality[146], a possible role of H2BK31ub in *D. melanogaster* dosage compensation will be an attractive topic of future research.

The role of high-affinity sites. If DCC spreading does not propagate linearly along the chromatin template, how can the DCC get homogeneously distributed across the entire X chromosome? It was an early observation that, in the absence of other MSL proteins, a core complex of MSL1 and MSL2 recognizes a restricted but defined set of ~35 binding sites across the X chromosome[60,61]. These regions also included the two roX genes and were termed as high-affinity sites (HASs; also known as chromatin entry sites), and their actual number was later determined to be ~140 through the use of more sensitive methodology[62,63] (FIG. 2a). It was then found that, like the roX genes, several of these sequence elements are able to attract the DCC and induce spreading when translocated to an autosome[62,64–66], which suggests that HASs might generally serve as nucleation sites for DCC binding.

Much research has since been devoted to a better understanding of the HAS–DCC interaction. HASs differ qualitatively from the bulk of X-linked DCC target sites. Although most DCC binding is found in the transcribed regions of ~75% of active X-linked genes[51,67–69], HASs are predominantly located in intronic or non-coding sequences[62,63]. The identification of DNA sequence elements that attract the DCC to these sites was initially complicated by the small number of well-defined HAS sequences and the low resolution of genome-wide DCC binding information. Nevertheless, a conserved GA-rich motif was found within the *roX1* and *roX2* HASs[70] and, accordingly, the clustering of low-complexity, short GA-rich sequences triggers strong DCC binding at autosomal insertion sites[66]. With the development of the high-throughput chromatin immunoprecipitation followed by microarray (ChIP–chip) and chromatin immunoprecipitation followed by sequencing (ChIP–seq) methods, large enough training data sets with DCC binding-site information could be yielded that allow the computational prediction of conserved HAS sequence motifs. Two such studies have found that a GA-rich motif that is similar to the GAGA factor (GAF) binding site is significantly enriched in HASs[62,63]. However, little overlap has been observed between the target sites of GAF and the DCC[62,71], and interfering with the function of GAF in flies only subtly affects DCC binding to HASs[72], making it unlikely that GAF has a direct role in HAS recognition. It should also be noted that, although the described DNA motif is found in 91% of all HASs, most genomic occurrences of this motif are outside HASs, and by itself this motif is not sufficient to predict the presence of a HAS[62]. This suggests that additional sequence elements, local features such as reduced nucleosome occupancy[62,63] or the broader chromatin context have an impact on HAS recognition.

Despite the lack of classical DNA-binding domains in MSL proteins, recent work has shed more light on the molecular basis of the recruitment of MSL1 and MSL2 to HASs. MSL1 has been shown to bind nucleosomes in cell-free *in vitro* assays[23], and the amino-terminal region of MSL1 is required for its self-association, for binding to MSL2 and for targeting to HASs *in vivo*[73]. Nevertheless, it appears that most direct DNA binding capacity resides within MSL2 (REF. 53). Although other regions in MSL2 clearly contribute to chromatin binding *in vivo*[26], the CXC domain turned out to be of particular importance for direct DNA interaction, and mutations in this domain prevented the recruitment of MSL2 to a HAS in a reporter assay[53]. Curiously, although *in vivo* targeting of MSL2 was dependent on the HAS sequence, this specificity was not observed in cell-free *in vitro* assays, where MSL2 or the MSL1–MSL2 heteromer indiscriminately interacted with DNA. Additional factors, such as roXs, might therefore convey targeting specificity *in vivo*. Indeed, it has been found that a carboxy-terminal, basic, proline-rich region in MSL2 — that contributes to direct RNA binding[53] and is required for incorporation of a roX into the DCC — is also necessary for targeting to most HASs on the X chromosome[26].

Mechanisms of DCC spreading. The observation of autosomal DCC spreading surrounding translocated HAS sequences suggested that elevated DCC concentrations around HASs might induce DCC binding to some universal feature of active chromatin. Alternatively, the covalent modification of histones and concomitant alteration in chromatin structure that is mediated by the DCC might at the same time allow DCC spreading from its nucleation sites at HASs (FIG. 2b).

Training data sets
The known examples of an object (for example, an exon) that are used to train prediction algorithms, so that they learn the rules for predicting an object. They can be positive training sets (consisting of true objects, such as exons) or negative training sets (consisting of false objects, such as pseudoexons).

CXC domain
A frequent protein structure module, characterized by the occurrence of one to three CXC motifs amino-terminal to a $CX_4CXCX_6CX_{4-5}CX_2C$ sequence. A general role of CXC domains for DNA binding has been proposed.

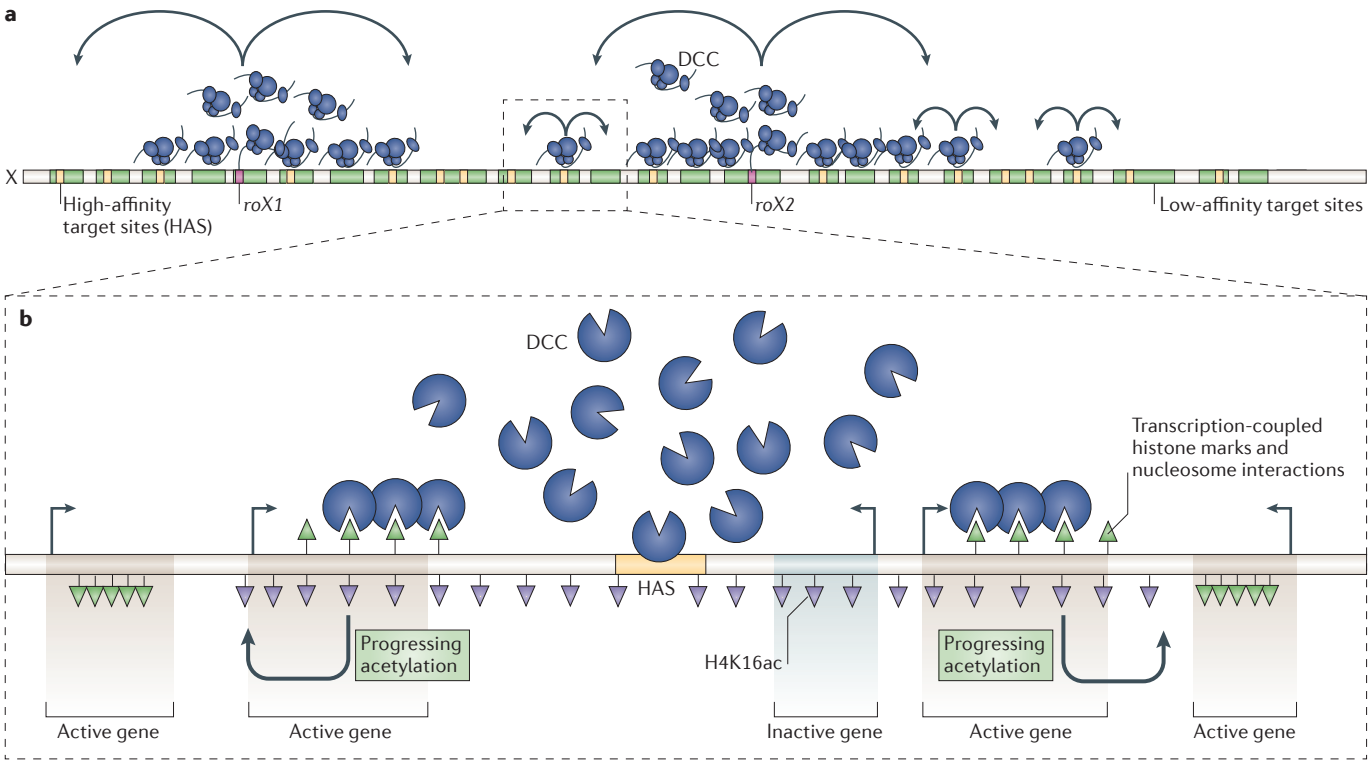

Figure 2 | **DCC nucleation and spreading.** Identification of the male X chromosome by the dosage compensation complex (DCC) is the first step in the dosage compensation process. **a** | The DCC becomes fully competent for chromatin targeting following incorporation of RNAs on the X (roXs) at the sites of their synthesis on the X chromosome. The DCC then reaches its final target sites by diffusion through the interchromatin space. DNA sequence elements at high-affinity sites (HASs) attract the DCC to multiple loci across the X chromosome from where it spreads to its low-affinity binding sites. **b** | It has been proposed that physical attraction of the DCC to a HAS would lead to locally elevated DCC concentrations. This local shift in biochemical equilibrium would then trigger DCC binding to lower-affinity targeting cues, which include transcription-coupled histone modifications in the transcribed region of active genes and involves nucleosome interactions by multiple male-specific lethal (MSL) proteins. DCC-mediated acetylation of histone H4 at lysine 16 (H4K16ac) is also required for this last targeting step, most likely owing to the enhanced chromatin accessibility associated with this histone mark. As the DCC is able to modify histones during transient interactions in the three-dimensional space of the nucleus, H4K16ac can spread several kilobases around DCC binding sites. The resulting exposure of additional binding sites might thus constitute an alternative or complementary DCC spreading mechanism.

Support for the first model came from the observation that the number of DCC-bound loci on the X chromosome is progressively reduced to a reproducible subset of target regions when concentrations of MSL proteins are limited[17,74]. These results suggested the existence of a hierarchy of DCC binding sites that are targeted by the DCC in a concentration-dependent manner. Accordingly, HASs would act as hubs that physically attract the DCC and thus elevate the local concentrations of DCCs in their proximity. The resulting local shift in biochemical equilibrium would then induce DCC binding to low-affinity target sites (FIG. 3). To enrich freely diffusing DCC components locally, DCC binding to HASs should be in a constant dynamic turnover. Indeed, it seems to be a widespread property of transcription factors to undergo rapid cycles of chromatin binding and dissociation, and dwell times last in the order of only a few seconds[75]. However, fluorescence recovery after photobleaching (FRAP) experiments have revealed a remarkably stable association of GFP-tagged MSL2 with the male X chromosome[76]. Nevertheless, it remains possible that cooperative interactions between multiple DCCs, potentially bridged by roXs, stabilize chromatin binding after a more dynamic recruitment phase. This interpretation would also reconcile between the seemingly contradictory observations that DCCs remain stably associated with the mitotic X chromosome[77], but they are responsive to changes in the transcriptional status of their target genes[78]. A structural role of roXs for the stabilization of DCC interactions may furthermore explain the specific requirement of the RNA helicase activity of MLE for DCC spreading[79].

The second model for DCC spreading from HASs does not rely on elevated DCC concentrations. It has been shown that spreading is absolutely dependent on H4K16ac, the transcriptionally activating histone acetylation that is mediated by the DCC itself. This is exemplified by flies that express a catalytically inactive version of the histone acetyltransferase MOF, as DCC binding is restricted to HASs in these animals[29,48]. H4K16ac has a direct impact on chromatin structure by preventing its compaction into 30-nanometre fibres (30 nm

Fluorescence recovery after photobleaching
(FRAP). In this technique, a laser pulse is used to bleach fluorescently labelled molecules (such as an ectopically expressed GFP fusion protein) within a restricted volume of the cell. The increase of fluorescence signal within the bleached area is then measured over time to determine diffusion rates of the labelled molecules.

30-nanometre fibres
(30 nm fibres). A helical arrangement of adjacent nucleosomes, which is believed to be the first level of chromatin compaction and appears as fibres of ~30 nm diameter in electron micrographs.

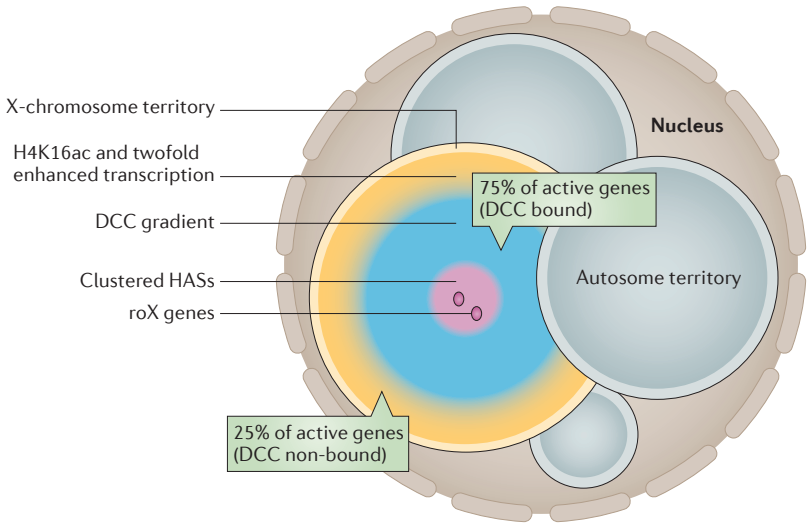

Figure 3 | **The X-chromosome territory represents a uniquely active compartment.** It has been proposed that reciprocal interactions between male-specific lethal (MSL) proteins, RNAs on the X (roXs) and high-affinity targeting elements may eventually create a male-specific higher-order architecture of the X chromosome. Recent work provided evidence for long-range interactions between high-affinity sites (HASs), which were found to be clustered in close proximity within the dosage compensation complex (DCC)-stained region of the nucleus in an MSL1- and MSL2-dependent manner[95]. By contrast, regions that appear to be devoid of DCC binding in chromatin immunoprecipitation (ChIP) experiments were localized towards the periphery of the X-chromosome territory. A model was suggested in which the higher-order structural arrangements resulting from HAS clustering support the formation of a DCC concentration gradient that originates from the sites of DCC assembly at the roX genes[96]. Progressively lower DCC concentrations towards the periphery of the X-chromosome territory would prevent spreading of the DCC and of histone H4 acetylated at lysine 16 (H4K16ac) into neighbouring chromatin on autosomes. Note that the DCC can mediate H4K16 acetylation over several kilobases distal to its actual binding sites on chromatin[49]; this is most likely to occur during transient encounters in three-dimensional space, and H4K16ac thus covers most active genes on the X chromosome. A large body of evidence suggests that dosage compensation of X-linked genes is a direct consequence of the distinct conditions within the X-chromosome territory. This is demonstrated by the observation that autosomal genes show DCC binding and transcriptional activation when they become translocated in this permissive environment[104].

fibres) *in vitro*[80,81] and increases chromatin accessibility *in vivo*[47], which seems to be a prerequisite for DCC binding to its low-affinity target sites. At first glance, the dependence of DCC targeting on the modification that is established by the complex itself appears to be a 'chicken and egg' problem. However, the strong DCC-binding sequence determinants at HASs, together with other chromatin features, such as low nucleosome occupancy, allow initial DCC recruitment even in the absence of H4K16ac[29,48]. Spreading of DCCs outside HASs would then require these nucleation sites to propagate acetylation (and, consequently, DCC binding) through the surrounding chromatin. Indeed, genome-wide profiling has revealed that MOF-mediated H4K16ac extends in continuous domains on the X chromosome that include intergenic regions and bridge the gaps between sites of DCC binding[49]. These data suggested that, when in context with other DCC components, the activated MOF enzyme is able to modify neighbouring H4 tails during transient interactions in the three-dimensional space of the nucleus. Consequently, the modification of nearby nucleosomes, which can in fact be located several kilobases away from sites of DCC binding, would expose additional DCC target sites and thus constitute a bona fide spreading mechanism (FIG. 2b). To ensure the specificity of this process, the full acetylation capacity of MOF should only be unleashed after the DCC has been fully assembled on chromatin. Indeed, the enzymatic activity of MOF depends on its interaction with MSL1 and MSL3 *in vitro*[23]. Furthermore, in the absence of *roX1*, deletion of a conserved stem–loop in *roX2* results in defective H4K16 acetylation, although X-chromosome targeting of DCCs (most likely to HASs) is still observed[82], indicating that a checkpoint for proper complex assembly is functional *in vivo*.

Binding site choice during DCC spreading. Although the model described above predicts that DCC concentrations are homogeneously elevated in the nuclear space around HASs, and although X-linked chromatin is globally hyperacetylated and thus accessible[47], the spreading process specifically distributes the DCC to the transcribed regions of active genes (FIG. 2b). How is this specificity achieved? One candidate for a transcription-coupled signal that might serve as a targeting cue for DCC binding is trimethylated H3K36 (H3K36me3), which is enriched in the transcribed regions of active genes. MSL3 may bind to H3K36me3 because the chromobarrel domain of Eaf3 — the yeast orthologue of MSL3 — physically recognizes this modification[83,84]. In fact, DCC occupancy outside HASs closely overlaps the H3K36me3 distribution, which is an even better predictor for DCC binding than transcription itself[85]. Furthermore, depletion of the enzyme that is responsible for H3K36me3 led to compromised DCC targeting[85,86]. Conversely, deletion of the MSL3 chromobarrel domain impaired male viability and caused a loss of DCC binding from the transcribed regions of X-linked genes[87,88]. Surprisingly, however, recent structural data obtained in two independent laboratories have questioned a direct relationship between H3K36me3 and MSL3, because no such interaction could be observed *in vitro*. Instead, the human MSL3 chromobarrel bound to H4K20me1 and H4K20me2 peptides[89] and co-crystallized in a complex with DNA and an H4K20me1 peptide[90], whereas the resolved protein structure appeared to be unfavourable for binding to trimethylated lysine residues. Like H3K36me3, the H4K20me1 mark is enriched in the transcribed region of genes in humans and *D. melanogaster*[91,92], but its roles in dosage compensation remain to be elucidated, as does a potential crosstalk between the two marks.

It is clear, however, that recognition of either H3K36me3 or H4K20me1 is not sufficient for DCC recruitment. As mentioned above, the presence of at least H4K16ac is also required. Besides catalysing this acetylation, MOF is also likely to contribute structurally to chromatin binding by DCCs, as it directly interacts with nucleosomes *in vitro*[93]. MOF shares this feature with MSL1 and MSL3, and all three bind to nucleosomes in a cooperative manner[23]. H4K16ac disrupts

internucleosome interactions[80], and it is therefore likely that it is the concomitant exposure of additional nucleosomal surfaces that, together with the recognition of transcription coupled histone marks, is required for DCC binding. It seems that multiple MSL proteins contribute to these interactions, and several chromatin features are read simultaneously to ensure robustness and specificity of the targeting process. Accordingly, only fully assembled DCCs can bind to X-linked genes, and DCCs remain restricted to HASs in the absence of MSL3, MOF or roXs or if MLE function is compromised[48,54,58,61]. It is clear that only comprehensive structural information will enable us to fully understand all targeting principles of the DCC. An increasing number of partial structures have already emerged that are invaluable resources and guidelines for the study of DCC targeting *in vivo*[22,89,90,94]. Certainly, exciting findings in this field of research can be expected in the future.

Higher-order alterations in X-chromosome architecture. Both models for DCC spreading — either by enhanced local DCC concentrations or by H4K16ac-mediated chromatin accessibility — are by no means mutually exclusive, and the concerted action of both mechanisms would in fact constitute an even more robust targeting system. However, neither model can fully account for the absolute X-chromosome specificity of DCC targeting *in vivo*. Locally elevated DCC concentrations as well as promiscuous acetylation of neighbouring nucleosomes over distances of several kilobases from the sites of DCC binding are both expected to act in *trans* and should affect any chromatin region (on the X chromosome or on autosomes) that comes in proximity to a HAS in the three-dimensional space of the nucleus. Under these conditions, how would H4K16ac and high DCC concentrations be prevented from leaking into proximal chromatin on autosomes? First hints came from a recent study using fluorescence *in situ* hybridization (FISH) that demonstrated male-specific long-range interactions between HASs, which were dependent on the presence of MSL1 and MSL2 (REF. 95). Accordingly, it was proposed that the cell may solve the problem of spurious DCC diffusion to autosomes by organizing the three-dimensional spatial arrangement of HASs as centrally as possible inside the X-chromosome territory, effectively creating a DCC concentration gradient that is nucleated from the sites of complex assembly at the roX genes[96] (FIG. 3). One prediction from this model would be that many of the ~25% of active X-linked genes that escape stable DCC binding are located towards the periphery of the X-chromosomal territory at which DCC concentrations diminish, and this has indeed been observed for at least two such loci[95]. A growing body of evidence suggests that the organization of chromosomes into distinct conformations is not determined by structural scaffolds but is instead the result of stochastic and self-organizing processes, which are a combined function of the concentrations, affinities and reciprocal interactions of all chromatin-binding factors[96,97]. Accordingly, it was proposed that sequences with a high affinity for the DCC might themselves become attracted by centres of locally elevated DCC concentrations, thus creating a positive-feedback loop, wherein local HAS clustering leads to further DCC enrichment[96]. Such a dynamic self-organizing model appears to be very attractive, although the possibility remains that DCCs might also establish direct physical contacts between distal HASs after they have become juxtaposed in three-dimensional space, which would in some ways provide a better explanation for the stable association of MSL2 with the X chromosome[76]. Stable DCC association throughout mitosis would ensure the fast reorganization of this special configuration after each cell division[77].

How is transcription upregulated?

The ultimate question in the field of *D. melanogaster* dosage compensation is how the transcription of a heterogeneous population of genes can get coordinately upregulated by precisely twofold. In contrast to the wealth of information that has been gathered on the DCC-targeting process, surprisingly little is known about the actual mechanism of transcriptional activation, and for a long time all speculations were based on entirely circumstantial evidence (FIG. 4).

Transcriptional elongation. Traditionally, owing to the observation that H4K16ac and DCCs were found in the transcribed regions of active genes and owing to the relative absence of the DCC at gene promoters, it was believed that the DCC might function by directly enhancing the elongation efficiency of RNA polymerase II (Pol II)[67–69,98,99]. However, only recently have the first attempts been made to directly identify the step in the Pol II transcription cycle that is targeted by the dosage compensation mechanism. Global run-on sequencing (GRO-seq) in the male-derived S2 cell line suggested that autosomal genes are generally suffering from inefficient transcription elongation, as the density of actively transcribing Pol II diminished towards the 3′ end of the transcribed units[100]. By contrast, on the X chromosome, transcribing Pol II reaches the 3′ end of genes with slightly but significantly higher efficiency, and this was dependent on the presence of MSL2 and thus also H4K16ac. Accordingly, the authors argue that the relaxed X-linked chromatin structure associated with H4K16ac would impose fewer obstacles to Pol II progression and would thereby mediate dosage compensation (FIG. 4c). These results are in agreement with an elongation-based mechanism, but they raise several interesting conceptual questions. First, if the steric hindrance imposed by the chromatin substrate causes a probability of premature transcription termination during Pol II progression, this effect should disproportionally affect long genes compared to short ones. Second, if the speed of Pol II progression is affected, this should become rate-limiting, particularly for highly expressed genes, which would therefore disproportionally benefit from accelerated elongation. Accordingly, it will be interesting to investigate in more detail how an elongation-based mechanism could enhance the expression of individual male X-linked genes by a common factor, despite these genes showing a huge variation in length and spanning a wide range of expression levels.

Fluorescence *in situ* hybridization
(FISH). A technique that uses fluorescently labelled hybridization probes to determine the abundance of RNA species or the spatial organization of genomic loci in fixed cells.

Chromosome territory
A domain of the nucleus occupied by a pair of homologous chromosomes.

Stochastic
Probabilistic; governed by chance.

Self-organizing
A process in which pattern at the global level of a system emerges solely from numerous interactions among the lower-level components of the system. The rules specifying interactions among the system's components are executed using only local information, without reference to the global pattern.

Global run-on sequencing
(GRO-seq). A method for the genome-wide mapping of the position, amount and orientation of transcriptionally engaged RNA polymerases.

Hit-and-run
Targeting mechanism in which a DNA-binding protein undergoes repeated random and short-lived interactions with DNA until it encounters its cognate binding sequence.

Transcriptional initiation. Interestingly, it has recently been observed that the nature of the basal promoter and associated transcription factor binding sites can influence the ability of a reporter gene to undergo dosage compensation[101]. Accordingly, transcriptional activation by the DCC correlated with a corresponding increase in Pol II recruitment to a reporter gene promoter in male flies[4], suggesting that an earlier step in the transcription cycle might also be affected by the dosage compensation mechanism. Despite targeting of the DCC to the transcribed regions of genes, ~25% of all transcribed X-linked genes are in fact devoid of substantial DCC binding[32,49] but are dosage-compensated[11]. The common feature of compensated genes is their location within extended domains of elevated H4K16ac, which include promoters as well as intergenic regions. Two complementary mechanisms are conceivable for how the globally enhanced chromatin accessibility associated with H4K16ac[47] may raise the frequency of transcription initiation. First, elevation of H4K16ac throughout the X chromosome might be a simple evolutionary strategy to similarly increase the accessibility of promoter elements and distal gene regulatory sequences for transcription factor binding (FIG. 4a). This way, initiation rates might be coordinately enhanced, whereas the stoichiometry of transcription factors at enhancers and promoters remains preserved across the diverse set of X-linked genes. At the same time, recruitment of DCCs to the transcribed regions of genes would not interfere with transcription factor binding at gene promoters. Second, DNA-binding proteins are thought to find cognate sequences by a hit-and-run mechanism[102], and more frequent encounters with unspecific binding sites are expected to increase their residence time in a certain environment. The exposed structural properties of the male X chromosome might therefore cause a global enrichment of general transcription factors throughout the X-chromosome territory, thus

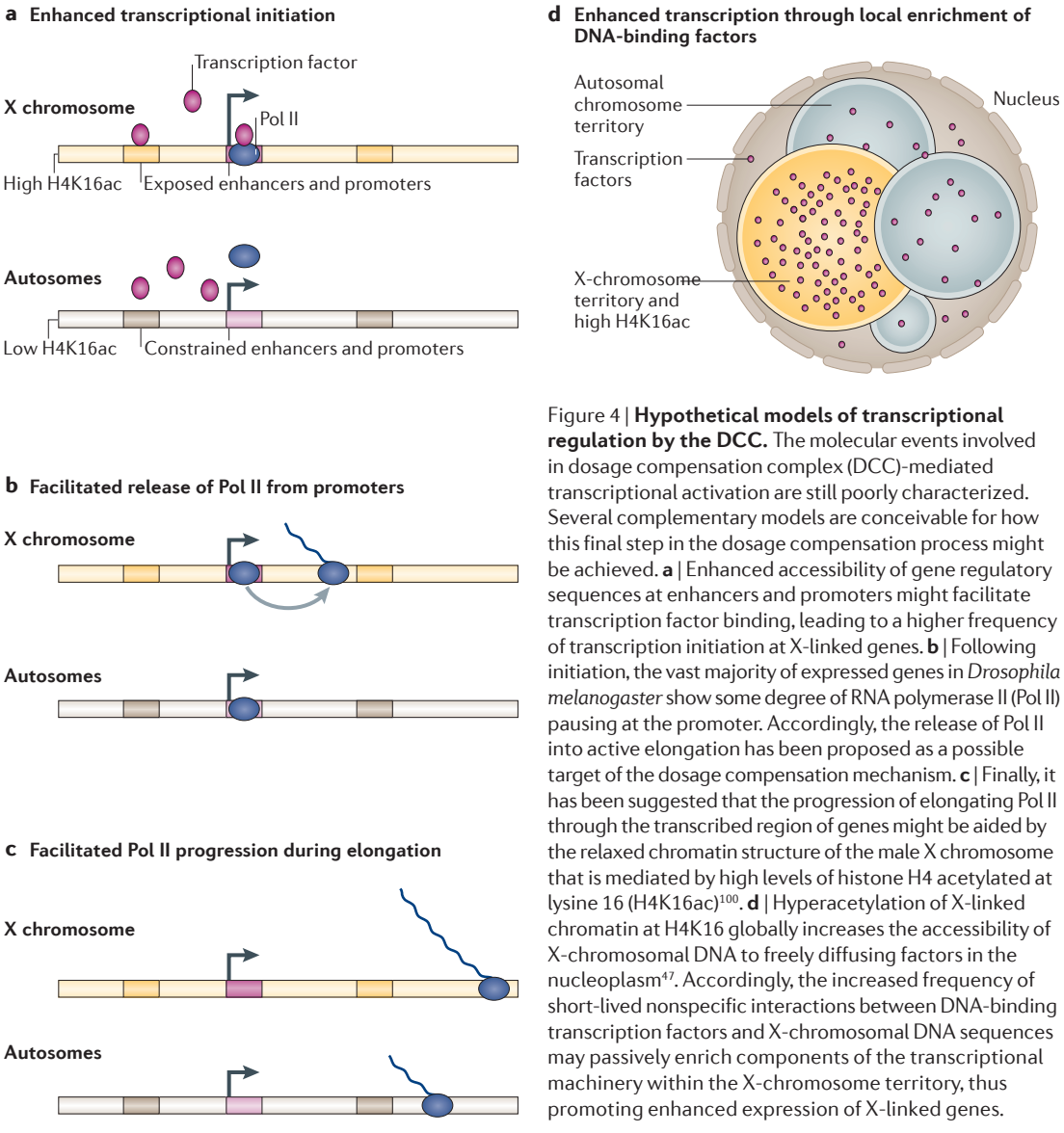

Figure 4 | **Hypothetical models of transcriptional regulation by the DCC.** The molecular events involved in dosage compensation complex (DCC)-mediated transcriptional activation are still poorly characterized. Several complementary models are conceivable for how this final step in the dosage compensation process might be achieved. **a** | Enhanced accessibility of gene regulatory sequences at enhancers and promoters might facilitate transcription factor binding, leading to a higher frequency of transcription initiation at X-linked genes. **b** | Following initiation, the vast majority of expressed genes in *Drosophila melanogaster* show some degree of RNA polymerase II (Pol II) pausing at the promoter. Accordingly, the release of Pol II into active elongation has been proposed as a possible target of the dosage compensation mechanism. **c** | Finally, it has been suggested that the progression of elongating Pol II through the transcribed region of genes might be aided by the relaxed chromatin structure of the male X chromosome that is mediated by high levels of histone H4 acetylated at lysine 16 (H4K16ac)[100]. **d** | Hyperacetylation of X-linked chromatin at H4K16 globally increases the accessibility of X-chromosomal DNA to freely diffusing factors in the nucleoplasm[47]. Accordingly, the increased frequency of short-lived nonspecific interactions between DNA-binding transcription factors and X-chromosomal DNA sequences may passively enrich components of the transcriptional machinery within the X-chromosome territory, thus promoting enhanced expression of X-linked genes.

Deterministic
Not governed by stochastic processes.

Chromosome conformation capture
(3C). A technique that is used to study the long-distance interactions between genomic regions, which in turn can be used to study the three-dimensional architecture of chromosomes within a cell nucleus.

shifting the biochemical equilibrium towards enhanced pre-initiation complex formation (FIG. 4d).

In both of the described mechanisms, based on enhanced initiation or elongation, transcriptional activation is a consequence of the structural and biochemical properties within the X-chromosomal environment. An outstanding feature of these non-deterministic systems is the remarkable evolutionary flexibility that they convey, as newly derived X-linked genes would be compensated without the need for the evolution of DCC-targeting sequences. A recent study provided compelling evidence for this plasticity. Although DCC recruitment to the transcribed region of genes is dependent on their transcriptional activity[78,103], it has been shown that active transcription and localization on the male X chromosome are also sufficient to induce DCC binding and transcriptional upregulation of autosome-derived genes, even if they reside in large translocated segments of up to 65 kb[104].

Nevertheless, further studies are clearly required to dissect whether the dosage compensation mechanism enhances transcription initiation, elongation or both. A recent study suggests that enhancing both might indeed be the case, at least for the subset of genes bound by the DCC. It was shown that human and *D. melanogaster* MSL2 act as E3 ubiquitin ligases for H2B and that this modification regulates H3K4 and H3K79 methylation through *trans*-tail crosstalk in human cells[105] — two histone marks that are known to facilitate transcription initiation and elongation, respectively[106–108]. Finally, the transition between initiation and active elongation, another regulated step in the Pol II transcription cycle, has been suggested as a possible target of the DCC[109] (FIG. 4b). Interestingly, it has been shown that MOF-mediated H4K16ac can enhance the release of paused Pol II from mammalian promoters by recruitment of BRD4 and positive transcription elongation factor b (PTEFb)[110,111]. It remains to be explored whether a similar mechanism may contribute to enhanced transcription during *D. melanogaster* dosage compensation.

A repressive role for DCCs? Whichever step of Pol II transcription is regulated, it is clear that elevated transcription rates are not mediated by a direct activating effect of DCCs in the transcribed region of genes. This is not only exemplified by the fact that H4K16ac rather than DCC binding is the common feature of compensated genes[32,49]. Interestingly, and resembling some aspects of the long-debated 'inverse dosage model' (BOX 3), two recent studies have also found that DCC binding in fact reduces the strong H4K16ac-mediated transcriptional activation down to twofold[4,112]. Although it is still unclear how this effect is mediated, it is possible that the repressive functions of ISWI-mediated chromatin remodelling are involved, as an increase in X-linked transcription has been suggested following ISWI disruption[113]. However, a separate study showed that upregulated genes are randomly distributed between the X chromosome and autosomes in ISWI mutants[114]. Also, HP1, which appears to be enriched on the male X chromosome[37], might somehow act in concert with the DCC to dampen exceeding expression levels, but no direct interactions between MSL proteins and HP1 or ISWI have been observed. Alternatively, it is tempting to speculate that DCC binding directly counteracts chromatin relaxation and the free exposure of DNA, thereby balancing the above mentioned activating effects of H4K16ac. What makes such a model attractive is the inherent possibility to fine-tune the resulting transcriptional output, which could be achieved by modulating the affinities and concentrations of DCC components until precisely twofold activation is achieved. The 25% of compensated X-linked genes that escape DCC binding *in vivo*[32,49] may be testimony to this adjustment process.

Concluding remarks

Despite the advances that we have presented in this Review, crucial questions remain. Although the advent of genome-wide profiling technologies such as ChIP–chip and ChIP–seq has allowed a better definition of the DCC targeting cues on chromatin, we have only begun to fully appreciate the dramatic alterations in local and global X-chromosome architecture that are the consequence and cause of this interaction. The recent combination of deep sequencing with chromosome conformation capture

Box 3 | **The inverse dosage model**

Interestingly, a repressive function of the dosage compensation complex (DCC) was postulated many years ago by proponents of the inverse dosage model (reviewed in REF. 147). The inverse dosage effect describes a genome-wide increase in transcriptional activity that is sometimes observed in response to genomic deletions. In the context of *Drosophila melanogaster* dosage compensation, it was proposed that the reduced levels of X-chromosome-encoded transcriptional repressors in males would cause a genome-wide upregulation of transcription that is directly proportional to the twofold reduction in X-chromosome copy number. Accordingly, in this model, transcriptional upregulation of the X chromosome would occur completely independently of the DCC and of histone H4 acetylated at lysine 16 (H4K16ac). The purpose of the DCC would instead be to sequester the transcriptional activator males absent on the first (MOF) and thus H4K16ac away from autosomes specifically to nullify the autosomal activation that is caused by the inverse dosage effect, thus leading to balanced expression between the X chromosome and autosomes. The activating effects of the resulting excess H4K16ac on the male X chromosome would simultaneously be counteracted by a repressive function residing within the DCC. At the same time, to allow dosage compensation, this repressive function would not interfere with the twofold activation of the X chromosome that is mediated by the inverse dosage effect[147,148]. However, it has been argued that this concept suffers from too many assumptions, and although inverse dosage effects have been observed in plants and insects[149], studies in support of this model for X-chromosome compensation were based on a small number of genes[113,148].

The central question that arises is whether the activation of the male X chromosome primarily occurs because of the direct action of DCCs or instead whether it results from the reduced dose of X-encoded transcriptional repressors, as proposed by the inverse dosage model. Indeed, most evidence points towards a more direct role of the DCC and concomitant H4K16ac for X-linked transcriptional activation. Besides the general activation potential of MOF[150], several studies demonstrated enhanced expression following tethering of DCC components to plasmid-based or genomic reporter constructs *in vivo*, often leading to precisely twofold elevated transcription[4,151–153]. Furthermore, genes in proximity to an autosomal roX insertion appeared to be collectively upregulated when targeted by local DCC spreading[7]. Taken together, owing to the large body of evidence from global as well as single-gene studies[5–7], as well as the striking structural reorganization of the X chromosome that can be observed as a consequence of DCC function, it is the prevailing view today that enhanced X-linked transcription is directly mediated by the DCC.

(3C)-like approaches has provided novel tools for the comprehensive genome-wide mapping of physical interloci interactions in a cell population[115,116]. These techniques will help to unravel the specific architecture of the male X chromosome in unprecedented detail, which will be pivotal in fully understanding the interdependence of global chromosome structure and local DCC recruitment. Even more work is required to identify the mechanism of transcriptional activation unequivocally. Additional studies using endogenous tissues, cell-based assays or *in vitro* transcription systems will elucidate the newly discovered repressive function of the DCC and clarify the extent to which transcription initiation and elongation are enhanced on the compensated X chromosome.

It has to be emphasized that the principles that orchestrate the structural and functional reprogramming of the X chromosome in male flies are most likely not restricted to *D. melanogaster* dosage compensation. Twofold enhanced transcription has also been observed for the active mammalian X chromosome[10–12], and future experiments will reveal whether a complex similar to the *D. melanogaster* DCC is responsible for this upregulation. Furthermore, global regulatory mechanisms are not limited to sex chromosomes. Beyond the organization of eukaryotic genomes into chromosomal territories and nuclear subcompartments, such as heterochromatic regions or the nucleoli[117], the folding of chromatin into distinct three-dimensional architectures reflects dynamic yet clearly nonrandom and functionally important spatial arrangements[118]. These discrete conformations may thus facilitate the coordinate regulation of genes during development or in response to environmental cues, and X-chromosome compensation is just one paramount example for this type of regulation. Certainly, as for the past decades, its rich phenomenology and tremendous impact on a wealth of different regulators, and chromatin-related processes will therefore continue to make *D. melanogaster* dosage compensation one of the most exciting model systems for epigenetic regulation.

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Acknowledgements
We thank members of the laboratory for critical reading. This work was supported by the EU-funded network of excellence 'EpiGeneSys' awarded to A.A.

FURTHER INFORMATION
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