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Dosage compensation and nuclear organization: cluster to control chromosome-wide gene expression

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In many species, male and female animals differ in the number of X chromosomes they possess. As a consequence, large scale differences in gene dosage exist between sexes; a phenomenon that is rarely tolerated by the organism for changes in autosome dosage. Several strategies have evolved independently to balance X-linked gene dosage between sexes, named dosage compensation (DC). The molecular basis of DC differs among the three best-studied examples: mammals, fruit fly and nematodes. In this short review, we summarize recent microscopic and chromosome conformation capture data that reveal key features of the compensated X chromosome and highlight the events leading to the establishment of a functional, specialized nuclear compartment, the X domain.

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Introduction

Eukaryotic genomes are accommodated inside the small nuclear space while staying functional. A century of microscopic studies have demonstrated that this functionality is correlated to some degree to spatial organization of chromosomes. However these experiments had limited spatial resolution due to the use of light microscopy and the small number of loci whose location could be determined simultaneously. In the last 15 years, the advent of chromosome conformation capture techniques (C-techniques, Box 1) enabled the generation of genome-wide probabilistic physical contact maps, allowing investigation of the spatial organization of the entire genome [1]. One of the main discoveries using C-techniques is the folding of chromatin into large-scale (100 kb–1 Mb) topologically associated domains (TADs, also named chromosomal interacting domains) in all studied genomes ([2,3] and [4] for review). Sequences inside TADs show higher contact frequencies among themselves than with sequences in neighboring TADs (also called insulation). TADs cluster together inside the nuclear space and preferential clustering is correlated with the chromatin state and the transcriptional status of genes located inside the TADs [2,5]. This is highly reminiscent of previously described nuclear 'bodies' or 'foci' of functional domains such as Polycomb or transcriptional factories [6,7].

Organization of genomes into TADs may contribute to long range transcriptional regulation. An example of such long range control is dosage compensation (DC), where simultaneous regulation of multiple genes on a single chromosome occurs. The X chromosome provides therefore a fascinating model to investigate the formation and function of a specialized nuclear body and how chromosome organization is linked to chromosome-wide gene regulation. In the three best studied model systems (mammals, fruit fly and nematodes), the common underlying feature of DC is the direct interaction of a dosage compensation complex (DCC) with the X chromosome. The DCC binds the X at specific sites from which it spreads to control transcription. Recent microscopic and molecular data demonstrate that DCC binding modifies X chromosome conformation and nuclear positioning of the X chromosome, which in turn could modulate X-linked gene expression. Here we review the latest developments toward understanding the link between nuclear organization and dosage compensation in these model organisms.

Mammals: a long non-coding RNA-dependent silent compartment

In mammals, females randomly inactivate one of two X chromosomes during early development (X chromosome inactivation, XCI) and stably maintain this silenced state. Microscopically, the inactive X chromosome forms a compact heterochromatic structure, the Barr body while the active X is indistinguishable from an autosome [8]. XCI is caused by the monoallelic expression of the 17 kb long non-coding *Xist* RNA from one of the two X chromosomes. The Barr body is coated with *Xist*, carries high levels of histone 3 lysine 27 methylation (deposited by the Polycomb complex 2), high levels of DNA methylation and is often localized at the nuclear periphery or close to the nucleolus — two places where autosomal heterochromatin is clustered (Figure 1A(c)) [9,10]. Super resolution microscopy revealed that the Barr body is not

Box 1 Chromosome conformation capture techniques

Chromosome conformation capture (3C) techniques are based on the principle that in cross-linked chromatin, restriction fragments far apart on a chromosome or even on different chromosomes but close in the nuclear space can be ligated together. Different variations of 3C techniques exist (see below), but the initial steps are the same. Chromatin is cross-linked with formaldehyde and cut with a restriction enzyme. Fragments are ligated together, leading to ligation products consisting of distant fragments of the linear genome.

One to one and one to many techniques

3C is based on (semi-)quantitative PCR with a pair of primers hybridizing near the ends of restriction fragments of interest [59]. When repeated for many pairs, this gives a matrix of relative ligation efficiency for all studied fragments.

The 4C methodology (circularized 3C) is based on the creation of small DNA circles by another round of restriction digest and ligation [60]. The target locus (often called viewpoint) is circularized, capturing a ligated distant fragment. These circles are amplified using PCR with primers in the viewpoint pointing outwards. Amplicons are then either hybridized to microarrays or sequenced. This approach gives a genomic view of all possible contact partners from one site with the rest of the genome at high resolution.

Many to many technique

The 5C technology (carbon copy 3C) gives an overview of contacts between multiple sequences [61]. Instead of using a single oligonucleotide, numerous oligonucleotides corresponding to the different restriction sites in the genomic region of interest are hybridized. The 5′ end of all these nucleotides carry the same T7/T3 sequence, which is used for PCR amplification. PCR products can be either hybridized to microarray or sequenced. The result is a matrix of contact frequencies for many sites.

All to all technique

For Hi-C, restriction ends are labelled using biotin-tagged nucleotides [62–64]. After ligation, purification and shearing, ligated fragments are pulled-down using biotin and sequenced. A matrix of contact frequencies between all restriction fragments in the genome can be constructed. Importantly, one restriction fragment can only ligate once in a given haploid cell. Therefore, contact maps constructed using C techniques are probabilistic and represent the likelihood of two given fragments contacting together.

The resolution of these techniques depends on the size in base pairs of the recognition sequence of the restriction enzyme used to create the fragments and the sequencing depth of libraries. Whereas initial studies achieved only megabase resolution, the latest study with 15 billion contact reads reached kilobase resolution [15].

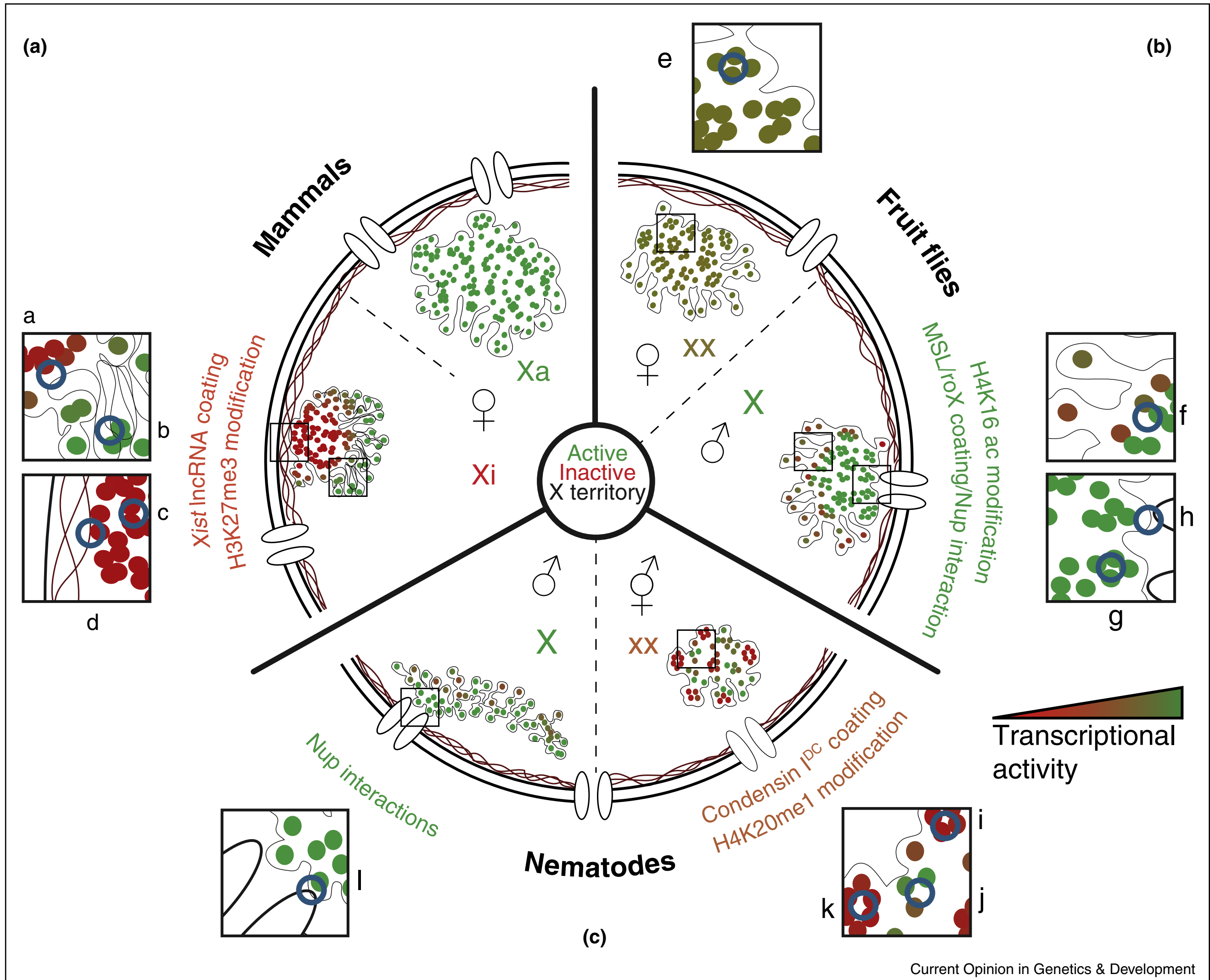
uniformly compact, but contains several interchromatin spaces harboring active chromatin [9,11], suggesting a spatial separation of active segments (escapee genes) and a core repressive compartment within the inactive X territory (Figure 1A(a)) [12,13].

In mice, allele specific Hi-C experiments distinguished the active X from the inactive X, providing molecular evidence supporting microscopic differences between the active and the inactive X. The active X is structurally similar to autosomes and consists of several small TADs separated by boundary regions enriched for cohesin and CTCF. In contrast, the inactive X shows a bi-partite structure with two large megadomains devoid of cohesin and CTCF. These large domains include several long range contacts between active regions that escape silencing (Figure 1A(b)) [14•,15•,16••]. This bi-partite structure is a consequence of *Xist* coating, as ablation of *Xist* from the inactive X resulted in both transcriptional reactivation and acquisition of several TADs separated by cohesion enriched boundaries, resembling the ones on the active X. Cohesin repulsion was therefore suggested as a way to establish the core repressive subcompartment of the inactive X [16••]. The boundary region between the two megadomains is the DXZ4 macrosatellite repeat locus [14•,15•,16••]. Unlike most of the genes on the inactive X, this locus remains transcriptionally active and harbors long range contacts with other macrosatellite regions on the inactive X [15•,17,18]. Moreover, the DXZ4 appears to anchor the inactive X close to the nucleolus, suggesting interplay between megadomain formation, nuclear localization and X chromosome silencing. Further experiments including Hi-C mapping in cells lacking DXZ4 locus will provide insights into the function of the megadomain boundary.

The locus encoding *Xist*, named X inactivation center (Xic), encodes many XCI regulators. In mouse, the most important one is *Tsix*, an antisense non-coding RNA suppressing *Xist* transcription before XCI. If a *Tsix* homolog is present in humans, its function in human *XIST* regulation is still debated (for review [19]). The murine conformational landscape of Xic consists of two TADs spatially separating *Xist* and *Tsix* regulators [2,3]. This separation is essential for correct regulation of the locus, as deletion of the boundary between these TADs leads to transcriptional misregulation of Xic ([3,20], reviewed in [21]). Once *Xist* transcription initiates, the RNA spreads along the X chromosome, targeting gene-rich distant regions of the chromosome before spreading to gene-poor regions [22]. Correlation between Hi-C and genome-wide chromatin mapping of *Xist*-interacting sites showed that the primary regions bound by *Xist* are in close spatial proximity to the *Xist* locus [22–24]. This is likely because these regions are transcriptionally active, as is the *Xist* locus, and transcriptionally active regions tend to cluster inside the nucleus [2,5,22,23]. A notable exception is highly expressed genes that seem to resist *Xist* interaction during the early stages of spreading (such as the Ocrl gene [23]). They however integrate later into the *Xist* compartments once the ncRNA has coated most of the X chromosome. This selective binding of *Xist* is only observed during the establishment of inactivation. In the maintenance phase, once silencing is robust *Xist* binds homogenously along the inactive X [22]. At this stage however, *Xist* appears dispensable for the maintenance of silencing [25].

Together, these observations suggests a two-step model for the establishment and maintenance of the inactive X compartment: *Xist* first targets moderately active regions

Figure 1



X chromosome functional organization and nuclear positioning in mammals (humans, mice), *Drosophila melanogaster* and *Caenorhabditis elegans*. **(A)** In mammals, females inactivate one of the two X chromosomes. The inactive X (Xi) is coated with the long non-coding RNA *Xist*, compacted into a dense structure comprising two large contact domains (megadomains) often located at the nuclear periphery or close to the nucleolus. In contrast, the active X is euchromatic. *Inset*: (a) Inactive and active regions in the Xi territory are spatially separated, with silent regions clustered inside the Xi chromosome territory. (b) The active regions on the Xi engage in long-range contacts. (c) *Xist* coated regions tend to cluster together forming superdomains and interact with Polycomb to mediate the creation of the silent subcompartment within the Xi. (d) *Xist* interacts with lamin A/C and lamin B receptor (LBR), which could position the Xi at the nuclear periphery. **(B)** In *Drosophila*, the single X chromosome in males is coated with the MSL complex which mediates twofold upregulation of X-linked genes. *Inset*: (e and g) MSL binding sites (High Affinity Sites (HAS)/ Chromatin Entry Sites (CES)) interact and cluster in both sexes, and the overall TAD organization of the X remains largely similar. (f) In males, transcriptionally unaffected or silent regions of the X chromosome are spatially excluded from the MSL territory. (g) Upon recruitment at HAS/CES, MSL spreading is restricted to spatially proximal regions. (h) MSL bound regions on the X chromosome are enriched for nucleoporins interactions and the MSL complex co-purifies with two nuclear pore subunits. **(C)** In *C. elegans*, the two X chromosomes in hermaphrodites are bound by the condensin-like dosage compensation complex (DCC), which leads to overall chromatin compaction. Both X are randomly positioned and undergo a twofold downregulation. The single X in males is less compact and transcriptionally upregulated. *Inset*: (i and k) In hermaphrodites, DCC spreads from its loading sites (recruitment element on X, *rex*). These *rex* sites cluster in a DCC dependent manner. (j) No spatial segregation of active/ repressive regions has been reported in *C. elegans*, while the DCC binds preferentially active genes. (l) The male X chromosome is upregulated, located at the nuclear periphery and interacts with a nucleoporin. Color codes for all systems: Black — the X territory; color gradient represents transcriptional activity ranging from red (inactive/silent) to green (upregulated). Blue circles represent region of interest.

located in close proximity to the *Xist* locus, recruiting downstream effectors mediating chromatin and DNA modifications, thus creating a silent core. *Xist* then progressively coats the entire chromosome, integrating highly expressed genes into the silent compartment while escapees stay at the periphery of the inactive X nuclear domain. Once the initial coating and silencing is achieved, chromatin and DNA modifications downstream of *Xist* can maintain silencing autonomously.

Three independent purification studies of *Xist*-associated proteins uncovered a number of interacting factors, some of which help explain structural and functional aspects of the inactive X domain (10-200 interactors, depending on the individual study) [16••,26•,27••]. *Xist* recruits Spen/SHARP, a transcriptional repressor that interacts with the histone deacetylase HDAC3. Deacetylated histones were proposed to recruit Polycomb complexes directly or *via* Polycomb/*Xist* interaction to mediate H3K27 methylation. This would ultimately lead to transcriptional silencing of X-linked genes [26•,27••]. Other factors repeatedly found associated with *Xist* are the nuclear matrix proteins hnRNPU/SP120/SAF-A and SAF-B. hnRNPU is essential for *Xist* spreading on the X and setup of the silent chromatin domain, requiring both the hnRNPU nuclear matrix interaction domain and *Xist* RNA binding domain [28]. The exact function of the nuclear matrix interaction remains, however, elusive. Finally, another common *Xist* interactor was the lamin B receptor (LBR), a nuclear envelope component, which could potentially mediate the perinuclear targeting of the inactive X (Figure 1A(d)) [16••,26•,29]. Knockdown of LBR led to desilencing of genes on the inactive X, even though the Polycomb complex is present [26•]. Interestingly, such mechanisms in which sets of proteins mediate silencing and dictate nuclear localization are a recurrent theme linking nuclear domain formation and gene expression regulation [30].

Drosophila: a ribonucleoprotein-based active compartment

In the fruit fly, males (XY) show twofold transcriptional upregulation of X-linked genes to balance expression with females (XX). The male X chromosome is often located close to the nuclear periphery in S2 cultured cells [31] and harbors high level of histone 4 lysine 16 acetylation [32]. The *Drosophila* dosage compensation complex (DCC) consists of 5 subunits: MSL1, 2 and 3, MOF, an H4K16 histone acetyl-transferase, and MLE, an RNA/DNA helicase with ATPase activity. *msl2* is expressed exclusively in males and assembles the DCC. MLE associates with the rest of the MSL-MOF complex *via* two essential but redundant long non-coding X-encoded RNAs *roX1* and *roX2* (reviewed in [33]). The fly DCC is recruited to the X chromosome in males at High Affinity Sites (HAS) or Chromatin Entry Sites (CES) characterized by a 21 bp motif (MSL recognition element or MRE) including the *roX1* and *roX2* loci. 150–300 of these sites are present on the X chromosome [34,35]. MSL2 interacts directly with the MREs, however the number of MRE sites that effectively recruit the MSL complex *via* direct MSL-DNA interaction is not clear [36]. Most MRE sites seem to recruit DCC *via* CLAMP (chromatin-linked adaptor for MSL proteins), a protein expressed in both sexes and bound to the MREs [37]. Unlike *Xist*, when one of the *roX* RNAs is expressed from an autosome, it spreads in *trans* and this leads to MSL complex specifically coating the X chromosome [38,39••]. An autosomal *roX* transgene containing HAS sites is also coated with MSL, yet the complex cannot spread and is restricted to active loci located nearby in three dimensions [39••]. Together, this suggests a model in which MSL spreading results from a combination of targeting by HAS sites and local spreading from these sites (Figure 1B(g)) [40].

Unlike the mammalian inactive X, the overall TAD structure of the X chromosome in flies does not differ between male and female cell lines [39••]. Interestingly, HAS are preferentially located at TAD boundaries and different HAS seem to interact independent of MSL binding. At least for one particular HAS, the chromosomal contact network is very similar in the presence or absence of the DCC (Figure 1B(e,g)) [39••]. A similar tridimensional network of interacting HAS was independently observed by comparing *roX2*-bound loci and Hi-C data of mixed-sex embryos [41••]. However, DCC loading led to increased long-range contacts (between different TADs along the chromosome), reminiscent of early FISH studies analyzing distances between HAS sites [42]. These data suggest that DCC takes advantage of spatially clustered HAS to target the male X chromosome and upon binding to HAS, further creates a specific 3D conformation of the entire X chromosome by increasing long range contacts. DCC-bound sites were scored as located closer to each other in males than in females, while DCC targets and unbound sequences (mostly inactive genes) were equally distant or even located further away in males than females. Together, these experiments suggest that similar to *Xist*, the MSL complex creates a nuclear domain with active genes clustered internally and spatially separated from inactive loci located at the edge or outside of the MSL territory (Figure 1B(f)). In this model, DCC bound regions form an active subcompartment within the X territory for systematic transcriptional upregulation.

The nuclear organization of the X chromosome also appears affected by loading of the DCC. Two subunits of the nuclear pore basket, NUP153 and Megator/TPR, co-purify with the MSL complex [31,43]. These subunits bind autosomal regions, marking transcriptionally active regions of the genome named Nucleoporin Associated Domains (NARs) [43]. Knockdown of TPR or Nup153 led to downregulation of X-linked genes and impaired perinuclear localization of the X chromosome [31,43], but

did not seem to change X chromosome conformation as measured by FISH [42]. Although the exact function of these proteins remains unclear and somewhat controversial, interaction with nuclear pores may provide a scaffold to build a stable active subcompartment. Alternatively, as NUP153 and Megator are shuttling subunits between the pore and the nucleoplasm, they might simply facilitate pore-proximal positioning of the X chromosome, therefore indirectly activating transcription (Figure 1B(h)).

Nematodes: sex-specific nuclear compartments to fine-tune transcription

Another strategy for DC was selected in nematodes: in hermaphrodites (XX), expression from both X chromosomes is downregulated two-fold by DCC, thereby matching the expression of X0 males [44]. DCC in worms comprises of a subset of proteins involved in sex-determination and dosage compensation (SDC-1, SDC-2 and SDC-3) and another subset exclusively involved in dosage compensation (MIX-1, DPY-21, -26, -27, -28 and DPY-30). Together, these subsets assemble to form a multiprotein complex that resembles the mitotic/meiotic condensin complexes and is therefore also referred to as the condensin I^{DC}. SDC-2 is essential for the loading of the complex onto X chromosomes and expressed exclusively in hermaphrodites [45]. The DCC is loaded at *rex* sites (*r*ecruitment *e*lement on *X*) characterized by a 12 bp MEX motif (Motif Enriched on X) [46]. About 38 of these sites have been formally characterized as able to recruit the DCC, although sequence analysis and chromatin immunoprecipitation experiments predict 200–300 *rex* sites on the X chromosome. Once loaded on *rex* sites, the DCC spreads in *cis*, leading to high levels of monomethylation of histone H4 lysine 20 and partial eviction of RNA polymerase II [47–50].

The precise molecular mechanism linking these DCC loading, chromatin modifications and transcriptional regulation remains unclear. Early on, the condensin-like structure of the DCC led to the suggestion that it could modify higher order chromatin structures to control gene expression [51]. Recent results show that indeed the DCC alters both the structural organization and the nuclear localization of the X chromosome.

Whole chromosome paints and distance measurements linked DCC binding to a slight increase in overall compaction of the X chromosome (Figure 1C) [52••,53]. Hi-C maps revealed that the TADs on the X chromosome in hermaphrodites showed higher internal contact frequencies than on autosomes. X chromosome TADs spanned ~1 Mb, separated by boundary regions that are enriched for *rex* sites. In addition, a subset of *rex* sites contacted each other more frequently compared to non-*rex* sites (Figure 1C(i,k)) [52••,54••]. Upon DCC knock-down, many but not all X TAD boundaries became weaker, in particular in the middle part of the chromosome [54••]. Correlated with this partial disappearance of TAD boundaries, most *rex-rex* contacts disappeared, suggesting *rex* sites cluster in a DCC dependent manner. Although X chromosome large scale structure is clearly modified by DCC loading, the functional importance of these changes in terms of gene regulation remains, however, elusive.

DCC binding to the X chromosome changes its subnuclear positioning. In hermaphrodite embryos, the outer third of all autosomes show high levels of interaction with the nuclear lamina, while the X chromosome only loosely interacts with the lamina at subtelomeric regions [55,56]. In contrast, in male embryos probes distributed along the X chromosome showed preferential perinuclear localization (Figure 1C(l)) [52••]. Perinuclear enrichment of the X chromosome in males appeared to be at least partially mediated by *rex* sites. Indeed, a single *rex* site is able to autonomously target an autosomal locus toward the nuclear periphery in males specifically. However, there might be additional factors like non-coding RNA genes, highly enriched on the X chromosomes, some of which were shown previously to interact with nuclear pores [57]. The male X chromosome interacts broadly with a component of the nuclear pore (Figure 1C(l)), similar to *Drosophila* [31,43,52••]. The loading of the DCC onto X chromosomes in hermaphrodites impaired both perinuclear chromosome localization as well as nucleoporin interaction [52••].

Together, molecular and microscopy data suggest the presence of two distinct sex-specific compartments in *Caenorhabditis elegans*. In males, the X chromosome interacts with nucleoporins, creating an activating compartment able to increase X-linked genes expression. In hermaphrodites, DCC loading onto X chromatin on the one hand increases overall chromosome compaction and TAD structure while on the other hand it impairs activation at pores by masking the interaction sites and altering X chromosome position [52••,58]. How these changes ultimately impact RNA polymerase levels on the X remains to be elucidated.

Nuclear organization and dosage compensation: where is the link?

Dosage compensation equates expression between X-linked and autosomal genes by either up-regulating or down-regulating X-linked transcription. In the three systems where DC is mechanistically best understood, DC undoubtedly leads to changes in the X chromosome large scale organization, nuclear localization and chromatin composition. Interestingly, DC always makes use of chromosomal topology for DCC initial targeting and/or spreading along the X chromosome before modifying this organization to stabilize gene expression. Setting up a specialized compartment for the X chromosome provides an additional layer of gene regulation, segregating activating factors (male flies and nematodes) or silencing

complexes (mammals and nematodes) from the rest of the genome in a self-reinforcing compartment. Understanding which factors setup the different X domains and stabilize these over the course of development will shed light on the importance of nuclear organization for X chromosome transcriptional regulation, providing useful paradigms for the plethora of other nuclear compartments.

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Using a new technique iDRiP (identification of direct RNA interacting proteins), the authors identified ~200 unique interactors of Xist lncRNA. Apart from known interactors like hnRNPU and ATRX, many structural proteins like lamin B receptor, cohesin, condensin were found as novel interactors of Xist and knockdown of these influenced X-linked gene regulation. Allele-specific Hi-C experiments demonstrated a prominent loss of TAD structure on inactive X compared to active X, proposed to be a consequence of cohesion repulsion.

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Using RNA antisense purification Mass spectrometry (RAP-MS) in mouse ES cells, authors identified 10 proteins that directly and specifically interact with the Xist lncRNA. The list includes previously known candidates such as hnRNPU/SAF-A and new candidates like SHARP/SPEN, the lamin B receptor, RBM15 and others. Using single molecule FISH (smFISH) to measure X-linked transcripts, they showed that most candidates lead to desilencing of X-linked genes upon knockdown. Moreover, knockdown of SHARP and HDAC3 revealed enhanced RNA polymerase II staining and exclusion of EZH2 (Polycomb complex) from the Xist territory, suggesting that histone deacetylation by HDAC3 might be important to recruit Polycomb and create a silent compartment. Interestingly, LBR knockdown led to desilencing in the absence of RNA PolII and presence of EZH2, but the actual mechanism remains unexplained.

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The authors generated Hi-C profiles in a *Drosophila* male cell lines (clone 8 and S2) and compared these with previously published Hi-C profiles established in female Kc or embryonic mixed sex cells. They reported the striking similarity between TAD structures in both sexes. They also discovered that TAD boundaries are enriched for High affinity DCC binding sites (HAS). These HAS sites contact each other in a sex independent manner. Moreover, they showed that the roX RNA, part of the male DCC can coat the X chromosome with MSL even when expressed from an autosomal locus. In contrast, the ectopic HAS site created by an autosomal insertion of the roX RNA locus is able to recruit MSL but the complex spreads only locally to spatially proximal regions.

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