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3D genome organisation in *Drosophila*

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Abstract

Ever since Thomas Hunt Morgan's discovery of the chromosomal basis of inheritance by using *Drosophila melanogaster* as a model organism, the fruit fly has remained an essential model system in studies of genome biology, including chromatin organisation. Very much as in vertebrates, in *Drosophila* the genome is organised in territories, compartments, and topologically-associated domains (TADs). However, these domains might be formed through a slightly different mechanism than in vertebrates due to the presence of a large and potentially redundant set of insulator proteins and the minor role of dCTCF in TAD boundary formation. Here, we review the different levels of chromatin organisation in *Drosophila* and discuss mechanisms and factors that might be involved in TAD formation. The dynamics of TADs and enhancer-promoter interactions in the context of transcription are covered in the light of currently conflicting results. Finally, we illustrate the value of polymer modelling approaches to infer the principles governing the 3-dimensional organisation of the *Drosophila* genome.

Authors biography

Charlotte Moretti is a postdoctoral fellow in the Ghavi-Helm team. She received her Ph.D. in biology from the René Descartes University in Paris.

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Yad Ghavi-Helm is a group leader of the Centre National de la Recherche Scientifique (CNRS) at the Institute of Functional Genomics of Lyon (IGFL). Her team focuses on transcription regulation, chromatin organisation, and enhancer-promoter interaction functionality during early *Drosophila* embryogenesis.

Key points

- *Drosophila* is a key model system to study the 3-dimensional organisation of the genome
- The *Drosophila* genome is organised in territories, A/B compartments, and topologically-associated domains (TADs)
- The relative contribution of transcription, architectural proteins, and epigenetics domains in TAD formation remains unclear
- Some TAD borders and chromatin loops appear dynamic across conditions, while others are stable
- Polymer physics can provide valuable models of chromatin organisation

49 **Keywords**

50 Chromatin organisation; *Drosophila*; topologically-associated domains; transcription

Introduction

The tight regulation of gene expression during development, cell differentiation, and cell identity maintenance involves the 3-dimensional (3D) organisation of the genome. This organisation is apparent at different levels, from large chromosome territories and compartments to more restricted structures such as TADs and local loops (**Figure 1**). As general principles of chromatin organisation are largely conserved across metazoans, *Drosophila melanogaster* rapidly emerged as a model of choice for the field. Over the years, fly researchers have developed an extremely flexible genetic toolbox including balancer chromosomes, the GAL4/UAS expression system, efficient transgenesis, and genome editing by CRISPR-Cas9 [1]. In addition, the *Drosophila* genome is relatively small (180 Mb with only three pairs of autosomes) and thoroughly annotated (it was one of the first genomes to be sequenced, in 2000 [2]). Overall, this makes *Drosophila* an excellent model organism to study chromatin organisation. In this review, we first introduce the different levels of chromatin organisation in *Drosophila* nuclei, before describing in detail TAD borders and their dynamics with respect to transcription. We conclude by illustrating the benefits of polymer modelling in deciphering the essential mechanisms underlying TAD formation in *Drosophila*.

Different levels of chromatin organisation

During interphase, eukaryotic chromosomes are confined within the nucleus, where a globally compact and efficient folding of chromatin is enforced, while appropriate regions remain accessible for normal gene expression. This compaction clearly does not occur at random. Since the 19th century, thanks to the rapid development of microscopy, we know that chromosomes occupy different territories within the nucleus (**Figure 1a**, reviewed in [3]). They are locally constrained [4] and segregated in different nuclear regions, with partial and stochastic overlaps in the periphery of the territories [5]. These territories are not identical from cell to cell, nonetheless large and gene-poor chromosomes tend to be located close to the periphery of the nucleus, while short and gene-rich chromosomes are more frequently found at the centre of the nucleus. Similarly, the level of folding and compaction of chromosomes depends on their localisation within the nucleus. At the periphery of the nucleus, chromatin is associated to the nuclear lamina and appears very compact and generally transcriptionally inactive, while closer to the centre of the nucleus chromatin is less compact and enriched in transcriptionally active regions [6–8]. Clusters of genes associated with the nuclear lamina are dynamically co-regulated during development via a transient reduction of Lamin binding and relocation towards the nuclear interior [6]. In addition, *Drosophila* chromosomes adopt a polarised orientation, known as the Rabl configuration, with

centromeres and telomeres clustering separately at two opposite nuclear poles (**Figure 1a**) [4,5,9–11]. Finally, in *Drosophila* genomes, homologous chromosomes are paired throughout the cell cycle, interacting with each other in tight and loose *trans*-interactions [12,13]. As a consequence, gene expression can be affected by a phenomenon called transvection [14], where *cis*-regulatory elements regulate the expression of genes located on the homologous chromosome [15].

The recent development of the chromosome conformation capture (3C) technique and its genome-wide derivatives (such as Hi-C) allows the identification, in a high-throughput manner, of genomic regions that are in close proximity *in vivo*. Seminal Hi-C experiments performed in *Drosophila* embryos revealed the presence of large (100 kb on average) and discrete domains along the linear chromosomes [16]. Such “topologically-associated domains” (TADs), are defined as chromosomal regions displaying a high self-interaction frequency delimited by a dramatic decrease of interaction frequencies with the regions on either side of the domains that defines their boundaries (**Figure 1b,c**). Hi-C experiments also revealed a segregation of the genome into two types of compartments, called A and B compartments, which represent the first principal component of the Hi-C contact matrix and can be visualised as a plaid-like pattern on Hi-C contact maps (**Figure 1b**) [17]. The A compartment contains gene-rich and transcriptionally active open chromatin, while the B compartment is gene-poor and enriched for closed chromatin regions [17]. Compartments tend to interact more frequently within their own class, probably owing to the preferential affinity of the protein set carried on each compartment for their siblings.

TADs and compartments can be classified into four main classes according to their global chromatin signature: one active class (corresponding to the A compartment) and three inactive classes (corresponding to the B compartment). Indeed, TADs strongly correlate with epigenetic domains, and their boundaries are enriched with active histone post-translational modifications and insulator protein binding sites [16,18] (developed below). While chromatin state is quite consistent within a same TAD, the segregation of active and repressive marks is not always perfect and TADs can display heterogeneous chromatin states [19]. Active TADs are enriched in transcriptionally active genes and therefore display active chromatin marks (H3K4me3 and H3K36me3) [20]. Inactive TADs are more frequently found associated with the nuclear lamina compared to active TADs [6]. The first class of inactive TADs corresponds to Polycomb-repressed loci that are enriched in histone H3 trimethylated at lysine 27 (H3K27me3) [16,18,21]. These Polycomb-associated TADs represent approximately 10% of the fly genome and contain a significant number of developmental genes and transcription factors, notably involved in tissue patterning. The second class of inactive TADs is composed of heterochromatin enriched in H3K9me2, HP1, and Su(var)3-9 [16]. Finally, the third class, covering up to 50% of the

euchromatin, is devoid of specifically-enriched chromatin marks [16,20,22]. However, recent studies showed that the H3K27me2 chromatin mark is pervasive in transcriptionally repressed regions and thus is likely to be present in this “void” inactive TAD class [23,24].

TADs and compartments are evolutionarily conserved structures [25] present in a large variety of species, including mammals [26,27], zebrafish [28], *Caenorhabditis elegans* [29], and yeast [30,31], suggesting that they represent robust functional units of the genome. *Drosophila* TADs are generally smaller than mammalian TADs (1 Mb on average in mouse and human). However, the detection of TADs is highly dependent on the resolution of the Hi-C assays and the detection threshold used in the TAD discovery algorithms, generating a large discrepancy between studies. Indeed, first reports indicated that the *Drosophila* genome may contain about 1300 TADs [16,18], while more recent studies using higher resolution Hi-C assays distinguished 2000 to 4000 or more TADs [32–34]. Because TADs are weakly defined at many loci, it has been proposed that the *Drosophila* genome is partitioned in TADs that are separated either by short boundaries or by longer unstructured “inter-TADs” regions [19]. More recently, it was reported that these inter-TAD regions actually contain small contiguous active TADs with an average size of tens of kilobases [34], which might establish gene loops between transcriptional start and termination sites, and have also been referred to as “mini-domains” [17]. Larger TADs would instead be enriched in inactive regions [32]. In addition, TADs and inter-TADs have been shown to correspond to the cytological bands and interbands of the polytene chromosomes [19,35–37].

In addition to the uncertainty in the number and location of TAD boundaries, their actual function in transcription regulation is also heavily debated. TADs are thought to define isolated/insulated structural domains within which chromatin looping brings genes in close proximity with their regulators. Indeed, the expression of genes located within the same TAD is more highly correlated than that of genes separated by TAD boundaries [32], probably because *cis*-regulatory elements are predominantly located in the same TADs as their target promoters. However, engineered chromosomal inversions affecting the genomic organisation of three testis-specific gene clusters or deficiencies affecting the left arm of chromosome 2 only mildly affect gene expression [38,39]. Similarly, the analysis of nested chromosomal inversions present within the second and third chromosomes of a highly rearranged “balancer” line revealed that changes in chromatin organisation and changes in gene expression are largely uncoupled [40]. At many loci, breaking, shuffling, or fusing TADs can have surprisingly little effect on gene expression, suggesting that TAD boundaries are not always essential for correct gene expression [40]. The role of chromatin organisation in the regulation of gene expression appears to be more complex than previously thought.

Within TADs, chromatin loops between regulatory elements are involved in the fine regulation of gene expression. Genes are positively regulated through enhancer-promoter interactions, where enhancers are brought in close proximity to their target promoter(s) and activate transcription via the release of paused RNA polymerase II (RNA Pol II) [41] (**Figure 1d**). In some cases, distal enhancer-promoter interactions are facilitated by the pairing of insulator proteins that dimerise to form a loop [42,43]. In other cases, enhancer-promoter interactions have been associated with the presence of paused RNA Pol II [41]. While most chromatin interactions are limited within a single TAD, examples of long-range contacts across TAD boundaries have also been reported [16]. Some of these long-range contacts involve Polycomb domains, initially visualised by microscopy as distinct foci called “Polycomb bodies”. Long-range interactions driven by Polycomb-mediated looping are responsible for the negative regulation of developmental gene expression. These interactions involve Polycomb response elements (PREs) and the recruitment of the Polycomb repressive complex PRC1 [33,44–48] (extensively reviewed in [49]). Long-range gene-gene interactions can also involve non-Polycomb repressed genes [36], some of which can be co-expressed, such as the *hbs* (*hibris*) and *sns* (*sticks and stones*) genes [16,40].

Drivers of chromatin organisation

The genetic and epigenetic rules that may drive TAD formation and maintenance have been the subject of intense research in recent years. In particular, great efforts have been directed towards the precise identification and description of TAD boundaries and the different factors bound in those regions, to disclose the molecular mechanisms at stake.

Active chromatin and transcription

While TADs can be classified based on their respective epigenetic states, their borders exhibit constant properties: they tend to be located in gene-rich and highly-transcribed open chromatin regions characterised by the presence of particular chromatin marks. More specifically, TAD boundaries are enriched with active chromatin marks such as H3K27ac, H3K4me1, H3K4me3, H3K36me3, and H4K16ac [16,18,19,32,50], and depleted in the repressive H3K27me3 chromatin mark [24,36]. Boundaries are also particularly sensitive to DNase I treatment, a hallmark of open chromatin, and thus significantly depleted in nucleosomes [36]. In addition, the RNA Pol II itself [19,51] as well as the general transcription factor TFIIC [36] are enriched at TAD boundaries. snoRNAs, known to mediate the stable opening of chromatin [52], have also been identified as positive drivers for boundary demarcation [53]. A vast majority (77%) of boundaries overlap with the promoter of ubiquitous, stable, and highly-expressed housekeeping genes and their enhancers [16,18,19,32,51,54]. Tissue-specific enhancers are also occasionally associated

with TAD borders [19,54]. Conversely, tightly-regulated developmental genes are predominantly embedded in H3K27me3-rich domains, which mostly correspond to inactive Polycomb-repressed domains [24]. This enrichment of actively transcribed genes at TAD borders suggests that transcription *per se* might be involved in their formation. In line with this hypothesis, simulated Hi-C maps based on GRO-seq data can accurately recapitulate contact heat maps [17]. Besides, the genome-wide inhibition of transcription observed in cells exposed to a temperature stress (heat shock) is accompanied by a drastic reorganisation of TAD borders and a general decrease in border strength [55]. However, transcription might not be the sole driver of TAD boundary formation, as suggested by the relative absence of coordinated changes in TAD boundary formation upon chemical perturbation of gene expression. Indeed, a more direct transcriptional inhibition with flavopiridol or triptolide causes a milder decrease in border strength [17,55], and inhibiting transcription by triptolide does not prevent the establishment of chromatin organisation during genome activation in early embryos [51]. Furthermore, the consistency of TAD border coordinates across different sections of stage 5 embryos, suggests an uncoupling between boundary formation and transcriptional programs [36], and calls for other mechanisms than transcription to be involved in TAD boundary formation. Actually, the deletion of two different 3 and 4 kb-long regions, each encompassing a different TAD border bound by multiple insulator proteins (including dCTCF and CP190) as well as the Rad21 cohesin subunit, was sufficient to cause TAD fusion [56]. It has thus been proposed that architectural proteins such as insulators might be better predictors of TAD borders than transcription itself [34,55].

Insulators and other architectural proteins

Insulators were initially identified through their ability to prevent long-range interactions between promoters and enhancers when positioned between them [57–60], and to block the spreading of repressive epigenetic marks [61–65]. More recently, Hi-C experiments performed on *Drosophila* embryos [16] and Kc167 cells [18], revealed that some insulator proteins are significantly enriched at TAD borders, suggesting a more global role in chromatin organisation.

Multiple studies have confirmed that insulators and other architectural proteins, including the B box binding subunit of TFIIC, are enriched at TAD borders [19,32,34,36,53–55,66]. Whereas vertebrate genomes are characterised by the preponderant role of the zinc finger CTCF insulator protein [67], in *Drosophila*, a large number of insulator proteins have been identified, including dCTCF, BEAF-32, CP190, Chromator, ZW5, mod(mdg4), GAF, M1BP, Su(Hw), Ibf1, Ibf2, Pita, ZIPIC *etc.* [68]. These insulator proteins can be divided in different classes, each binding different regions of the genome [69]. A large number of proteins are referred to as “insulators” in *Drosophila*, although their actual insulation function

has often not been clearly established. Many insulators might in fact be involved in transcription regulation rather than in chromatin organisation and TAD boundary formation [70]. Proteins involved in chromatin organisation are thus globally referred to as “architectural proteins”.

dCTCF, the main driver of TAD formation in vertebrates, is enriched at gene promoters, 5' UTRs, and intergenic regions, suggesting that it might be involved in the regulation of gene expression [69]. Yet, it is only found at 28% of TAD borders, with no evidence for a specific motif orientation contrary to what is observed in vertebrates [17]. Moreover, it is not required during embryonic development, and its deletion is not lethal until late pupal stages. Instead, it is involved in the correct expression of *Hox* genes, during both embryogenesis and larvae stages [71]. The relatively minor role of dCTCF in *Drosophila* is surprising given the high enrichment of CTCF at TAD borders in vertebrates and its proposed role, in combination with the cohesin complex, in TAD formation through the loop extrusion mechanism [67]. In *Drosophila*, the cohesin complex is enriched at TAD boundaries, as expected from its location at the promoter of active genes [66,72], and could therefore also participate in TAD formation.. However, a concerted action between dCTCF and cohesin in *Drosophila* remains uncertain. Indeed, initial experiments suggested that only 25% of dCTCF binding sites overlap with the binding of the Stromalin cohesin subunit [72], while a latter study found that nearly 50% of dCTCF binding sites overlap with the binding of the Rad21 cohesin subunit [66]. In addition to its potential role in TAD formation, cohesin might be involved in long-range enhancer-promoter interactions. Indeed, the cohesin complex, and its loading factor Nipped-B, are bound to the promoter of many active [73] and paused [74] genes, as well as to enhancers [66,75]. Moreover, Nipped-B was identified in a genetic screen designed to detect architectural proteins involved in the activation of the *cut* gene by its remote wing enhancer [76]. Cohesin has also been proposed to mediate interactions between Polycomb response elements (PREs) by controlling the binding of the Polycomb Repressive Complex 1 (PCR1) [77]. Altogether, while cohesin probably has an important role in regulating gene expression, for example by promoting the formation of mini-domains [78] or by mediating long-range enhancer-promoter interactions, its role in TAD formation through loop extrusion has not been demonstrated to date. In addition, condensin II, which largely co-localizes with cohesin [66], might act as an antagonist to cohesin [78].

In flies, other architectural proteins such as BEAF-32, CP190, and Chromator are probably more important than dCTCF in TAD boundary formation. In particular, CP190 and BEAF-32, enriched at gene promoters, 5' UTRs, and intergenic regions [69], are likely involved in TAD boundary formation. In synchronised cells, approximately 75% of the protein pairs BEAF-32/Chromator and BEAF-32/CP190 are found at TAD borders [34]. Conversely, 77% of TAD borders harbour these two protein pairs [34].

243 Additionally, expressing the dominant-negative BEAF-32 protein in the salivary glands resulted in less
244 condensed and 'puffy' polytene chromosomes [79]. However, siRNA depletion of BEAF-32 in S2 cells has
245 no significant effect on TAD boundaries and chromatin interactions [32], a discrepancy that could be
246 explained by the redundancy between BEAF-32 and the transcription factor DREF, which are sharing the
247 exact same binding sites and might play redundant roles in chromatin organisation at TAD borders [32].
248 A similar 'puffy' polytene chromosome phenotype was also observed upon depletion of the JIL-1 histone
249 H3S10 kinase [80]. Interestingly, Chromator, which recruits JIL-1 on polytene chromosomes [81], is found
250 significantly enriched at TAD borders [34,50]. Altogether, these results suggest that many factors could
251 act as architectural proteins in TAD border formation. This idea is supported by *in silico* enhancer-
252 blocking assays, that estimate the blocking effect of combinations of different proteins and DNA-binding
253 motifs [82]. It is likely that TAD border strength, defined as the ratio of intra- versus inter-TAD interaction
254 frequencies, correlates directly with the number and combination of architectural proteins bound at TAD
255 borders [32,34,53,55,66,82]. In this view, architectural proteins would act synergistically, creating
256 regulatory hubs of highly- or lowly- occupied borders that correspond to more stable or more dynamic
257 TADs.

258
259 **Dynamics of chromatin organisation**

260 After fertilisation, early *Drosophila* embryos develop as a syncytium through 13 rapid and synchronised
261 nuclear divisions (in about 120 minutes), before cellularisation occurs at nuclear cycle (nc) 14 [83]. While
262 zygotic transcription already starts at nc8, the major wave of zygotic transcription occurs at nc14. The
263 *Drosophila* genome becomes gradually compartmentalised from nc12 (about 100 minutes after egg lay)
264 into TADs, a process that continues throughout zygotic genome activation (ZGA) [44,51,84], despite
265 being interspersed by TAD disruptions at each cell cycle (**Figure 2**). Although not well separated until late
266 embryogenesis, active and Polycomb-repressed compartments start to appear soon after the major
267 wave of ZGA, reflecting the dynamic establishment of chromatin organisation during embryogenesis
268 [44]. Importantly, RNA Pol II is enriched at the boundary of newly-formed TADs and the dynamics of its
269 recruitment strongly correlates with TAD formation. However, chemical inhibition of transcription with
270 alpha-amanitin or triptolide before the onset of zygotic transcription (before nc8) showed that TADs can
271 form independently of transcription, albeit with changes in intra- and inter-TAD organisation [51]. At
272 nc12, in addition to RNA Pol II, TAD borders are also enriched for the binding of Zelda, a pioneering
273 transcription factor essential to open chromatin and activate early gene expression during ZGA [85,86].
274 However, the depletion of Zelda only results in a partial loss of TAD boundaries at Zelda-specific TAD

boundaries, demonstrating that its possible role in TAD boundary formation is limited to only a subset of loci. Zelda is also required to induce long-range active gene contacts during the midblastula transition (nc9 to nc13). Soon afterwards it associates with GAF to determine the open chromatin structures of active gene promoters and insulators [44,86]. Starting from nc14, GAF also plays a role in forming repressive short-distance loops within Polycomb domains, potentially via the recruitment of PRC1 proteins [44].

During cell type specification, it has been postulated that TADs might be partially rearranged in order to promote the expression of cell-type specific genes and repress alternative cell fate choices. Comparing Hi-C data from Kc167 cells (a 8-12h embryo-derived cell line) to BG3 differentiated neuronal cells, revealed that only some TADs are conserved in both cell lines, while others are cell-type specific [87]. Besides, enhancers controlled by neuronal-specific transcription factors are enriched in neuronal-specific TADs, suggesting that this change in topology is driven by cell differentiation [87]. However, comparing Hi-C data from Kc167 cells to either fly embryos or polytene chromosomes (from salivary glands of third instar larvae) revealed that TADs are invariant across these different conditions [18,37]. These apparently conflicting results might be explained by discrepancies in the identification of TADs between Hi-C methods and datasets sequenced at different depths, as explained above.

Microscopy-based assays offer an interesting alternative to compare chromatin organisation across conditions and assess the timing of TAD formation with respect to transcription in single cells. Such an approach (Hi-M) in nc14 *Drosophila* embryos revealed important intra-TAD rearrangements at the *snail* locus when comparing *snail*-expressing and non-expressing cells [84]. Intra-TAD interactions, such as enhancer-promoter interactions, could therefore be much more dynamic and correlated to transcription than TADs boundaries. However, comparing enhancer-promoter interactions at two early embryogenesis stages (3-4h and 6-8h after egg lay) uncovered much less dynamics than expected from transcriptional changes [41]. Unexpectedly, enhancers are already in close proximity to the promoter of their target gene prior to gene expression and these stable interactions are associated with the presence of paused RNA Pol II at the promoter. This suggests that enhancer-promoter interactions can be pre-formed before gene expression, and that these paused transcriptional hubs can be rapidly activated upon the recruitment of additional co-factors to release the paused polymerase [41]. Stable interactions might however be specific to early stages of embryogenesis, characterised by rapid cell cycles and extensive RNA Pol II pausing at gene promoters. In addition, comparing sorted mesodermal nuclei to whole embryos revealed that enhancer-promoter interactions may form in tissues where the gene will never be expressed [41]. The artificial tethering of *eve* enhancers to a reporter gene using *homie* insulator

elements confirmed that the close proximity between an enhancer and its target promoter is not sufficient to activate transcription [43]. However, physical proximity is necessary for sustained transcription, which in turn stabilizes and reinforces the physical association of paired regulatory elements [43]. Besides, this physical association might not necessarily imply direct contact between a distal enhancer and its promoter target(s). Indeed, a recent study based on live-cell imaging reported that co-expressed genes sharing the same enhancer are separated by large distances (at least 100-200 nm) during transcription [88]. The recruitment of numerous transcription factors and associated co-factors that regulate gene expression could prevent any direct contact [88].

Using polymer physics to model chromatin organisation

The association of recent high-resolution techniques, namely Hi-C [16,18,34] and super-resolution imaging [89], unambiguously demonstrated a strong link between the 1D chromatin epigenetic landscape and its 3D conformation inside the nucleus. However, it is still unclear whether chromatin organisation is a by-product of epigenetic marks and transcription or is genuinely involved in regulating gene expression. Computational modelling proposes an attractive approach to investigate this question and infer which factors are essential to chromatin organisation.

Modelling treats chromatin as a semi-flexible, self-avoiding, and self-interacting copolymer obeying basic constraints of thermal motion and steric repulsion. This copolymer is composed of blocks of consecutive monomers sharing the same epigenetic state. Each monomer (*i.e.* genomic region) preferentially interacts with other segments in the same epigenetic state. Applying polymer modelling to *Drosophila* chromosomes remarkably recapitulates the organisation of the fly genome into TADs, as well as A and B compartmental organisation [31,80–82]. In this model, transcriptionally inactive ‘null’ domains, devoid of specific epigenetic marks, behave as polymers in a fractal globule [89]. There, nucleosome-nucleosome interactions are thought to be a dominant, potentially sufficient, mechanism for the high compaction of inactive domains [92]. Conversely, the relatively decondensed active domains might correspond to low nucleosome interactions [19,89,92,93]. Experimentally, the acetylation of histones is known to promote chromatin unfolding by decreasing nucleosome-nucleosome interactions, as the neutralisation of positive charges on histone tails on a nucleosome hinders interaction with the acidic patch of another nucleosome [94]. Besides nucleosome-nucleosome interactions, computational analyses give strong interaction values for Polycomb-repressed domains, suggesting a prominent role for the molecular bridging action of Polycomb-group proteins and linker histone H1 [89,92].

Although epigenetic modifications appear to be primary drivers of spatial organisation, recent studies suggest that precise spatial organisation strongly influences the stability and spreading of epigenetic states [91,95–97]. Chromatin folding would create hubs enriched for specific regulatory proteins, increasing the local concentration of these molecular actors and their binding affinities. These “bio-chemical nano-reactors” would participate in the *trans* spreading of an epigenetic state to other chromatin segments [96]. Importantly, current models assuming that chromatin organisation is solely driven by the epigenetic state of interacting domains are certainly over-simplified. Indeed, the presence of TAD borders between segments in the same epigenetic state demonstrates that TAD formation simulations should include additional mechanisms besides homotypic chromatin folding [56]. Nevertheless, polymer modelling is a valuable tool to challenge the current biological hypotheses regarding TADs formation during embryonic development, differentiation, and cell cycle. In addition, the theoretical framework developed in computational modelling studies will enable investigations under perturbed conditions and undoubtedly provide clues regarding the identity and function of essential factors driving chromatin organisation.

Concluding remarks

Over the last few years, sustained efforts have been carried out to precisely describe and understand the mechanisms driving TAD formation and dynamics. Complementary approaches, such as Hi-C experiments, super-resolution imaging, and *in silico* modelling have allowed an integrative and powerful analysis of spatial chromatin organisation in various model organisms, from yeast to human. In *Drosophila*, TAD formation has been proposed to arise from the combinatorial action of (i) specific interactions between chromatin segments sharing the same epigenetic state, (ii) binding of insulators, chromatin remodelers, and other architectural proteins at TAD orders, and (iii) active transcription. This combination of mechanisms could explain the robustness of TAD organisation and the mild phenotypes observed upon challenges as diverse as protein depletion, genomic rearrangements, or chemical inhibition. The comparative analysis of TAD organisation in different model organisms also raises a number of puzzling questions. For example, how do insulators and cohesin function in TAD border formation and long-range interactions in *Drosophila* compared to vertebrates? Is the function of TADs evolutionary conserved? While topological domains are conserved among most metazoan, the mechanisms that organise and maintain them have evolved. Chromatin organisation thus appears to be an important functional feature of the genome rather than a by-product of gene expression and regulation. With a large and expanding toolbox including the maternal knock-out of genes during

embryogenesis, CRISPR-Cas9 induced mutations, super-resolution imaging, and single-cell -omic technologies, *Drosophila* will certainly remain a model of choice to address outstanding questions regarding the 3D genome organisation.

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Figure legends

Figure 1: Hierarchical organisation of the *Drosophila* genome.

a. Chromosomes are compacted within the nuclei in a Rabl-like configuration, i.e. the centromeres of the chromosomes cluster together to one pole of the nucleus in close proximity to the nucleolus, while the telomeres are found in the opposite pole. **b.** Chromosomes are divided in small and compact domains called topologically-associated domains (or TADs) that cluster together according to their chromatin state and form the A (blue) and the B (pink) compartments. **c.** TADs bring into close proximity genes and their *cis*-regulatory regions and are thought to form isolated transcriptional units within which the expression of genes is correlated. TADs are delimited by the presence of architectural protein binding sites at their borders. *Drosophila* TADs form relatively few loop domains compared to mammals, thus contacts between borders might be less frequent. **d.** Within TADs, enhancers are brought into close proximity to the promoter of the genes they regulate through loops that can be mediated by insulator pairing or RNA Pol II pausing. Active enhancers and promoters are bound by RNA Pol II, transcription factors (TFs) and co-factors.

Figure 2: Schematic representation of Hi-C contact maps of inactive and active *Drosophila* genome states.

Before zygotic genome activation (ZGA) and during cell cycle, when the genome is transcriptionally inactive, the *Drosophila* genome is unstructured, as shown by uniform contact frequencies in Hi-C contact maps. At the onset of ZGA, weak TADs, loops, and compartments start to appear and gradually become stronger at later stages of development. TAD formation is concomitant with the transcription of the genes present at their borders.

a **b** **c** **d**

Prior to ZGA and during mitosis

During ZGA

After ZGA and in differentiated cells