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Review

Principles of long-range gene regulation

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Transcription from gene promoters occurs in specific spatiotemporal patterns in multicellular organisms, controlled by genomic regulatory elements. The communication between a regulatory element and a promoter requires a certain degree of physical proximity between them; hence, most gene regulation occurs locally in the genome. However, recent discoveries have revealed long-range gene regulation strategies that enhance interactions between regulatory elements and promoters by overcoming the distances between them in the linear genome. These new findings challenge the traditional view of how gene expression patterns are controlled. This review examines long-range gene regulation strategies recently reported in Drosophila and mammals, offering insights into their mechanisms and evolution.

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Introduction

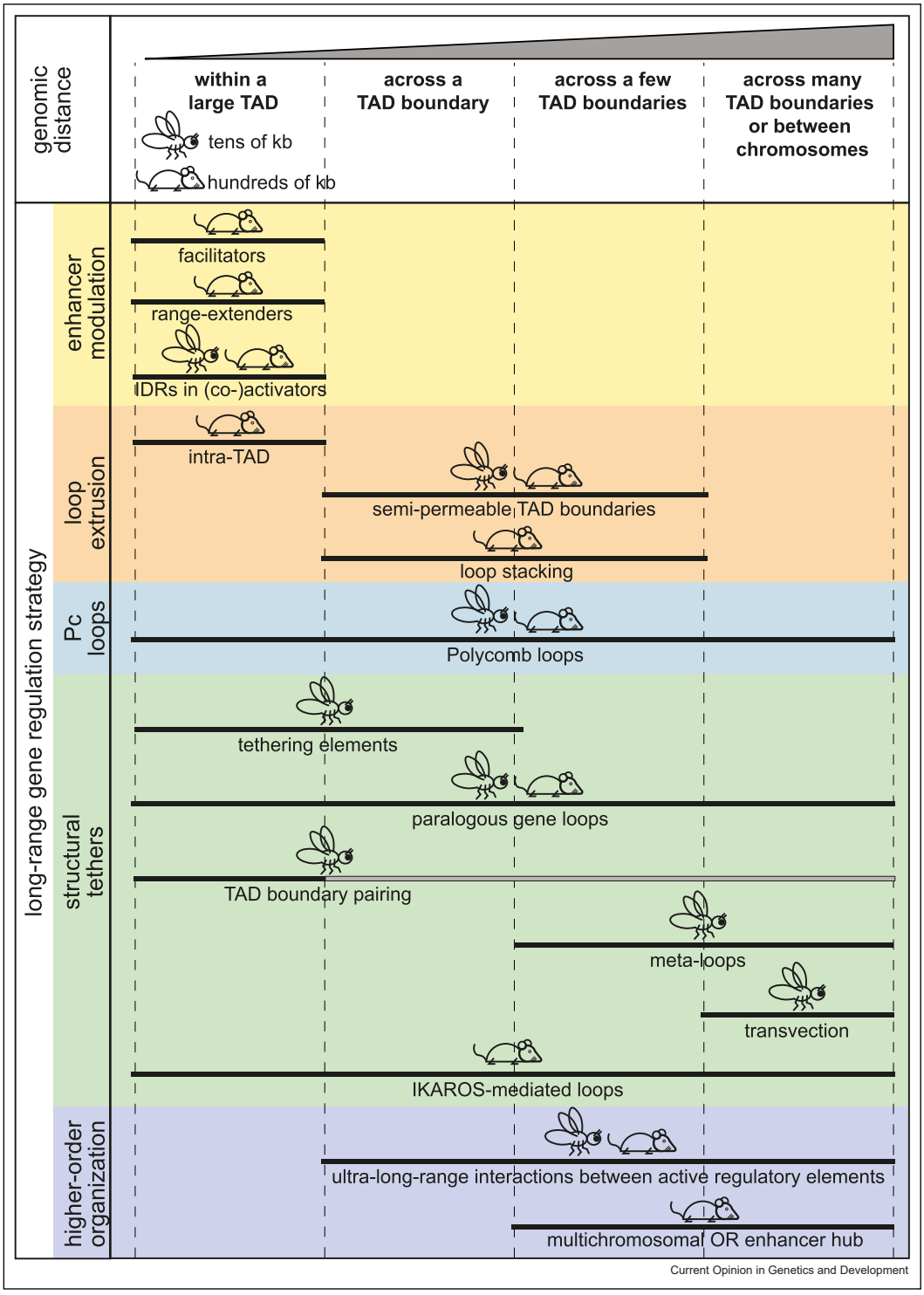
Gene transcription in multicellular organisms occurs in specific spatiotemporal patterns controlled by regulatory elements (REs), such as enhancers and silencers, that recruit transcription factors to activate or silence genes. Functional communication between REs and target promoters depends on physical proximity, though proximity does not always correlate with regulation. We refer to three-dimensional (3D) proximity events as 'interactions', which generally occur locally within dynamic structures called Topologically Associating Domains (TADs) typically spanning tens of kilobases (kb) in flies and hundreds of kb in mammals, where REs and promoters interact more frequently with each other than with those in other TADs. However, recent studies in flies and mammals, reviewed here (Figure 1), have expanded our understanding of how genes can be regulated at long-range, revealing interactions between distant elements, even across separate TADs. We refer to long-range gene regulation strategies as those thought to enhance promoter–RE interactions by overcoming prohibitive distances separating these elements in the linear genome.

These studies mainly used chromosome conformation capture techniques to create genome-wide maps of formaldehyde cross-linking frequencies between pairs of genomic loci at restriction fragment (in Hi-C) or nucleosome (in Micro-C)-resolution, or multiplexed DNA *in situ* hybridization (DNA-FISH) to image multiple loci in the same cell. The discovered distant genomic interactions, some of which are regulatory, form through diverse mechanisms. We tentatively classify them into five categories (Figure 2): (i) enhancer modulation, (ii) chromosomal loop extrusion, (iii) Polycomb loops, (iv) structural tethers, and (v) higher-order genome organization. These findings revise our understanding of how regulatory interactions are organized in the nucleus.

Enhancer modulation

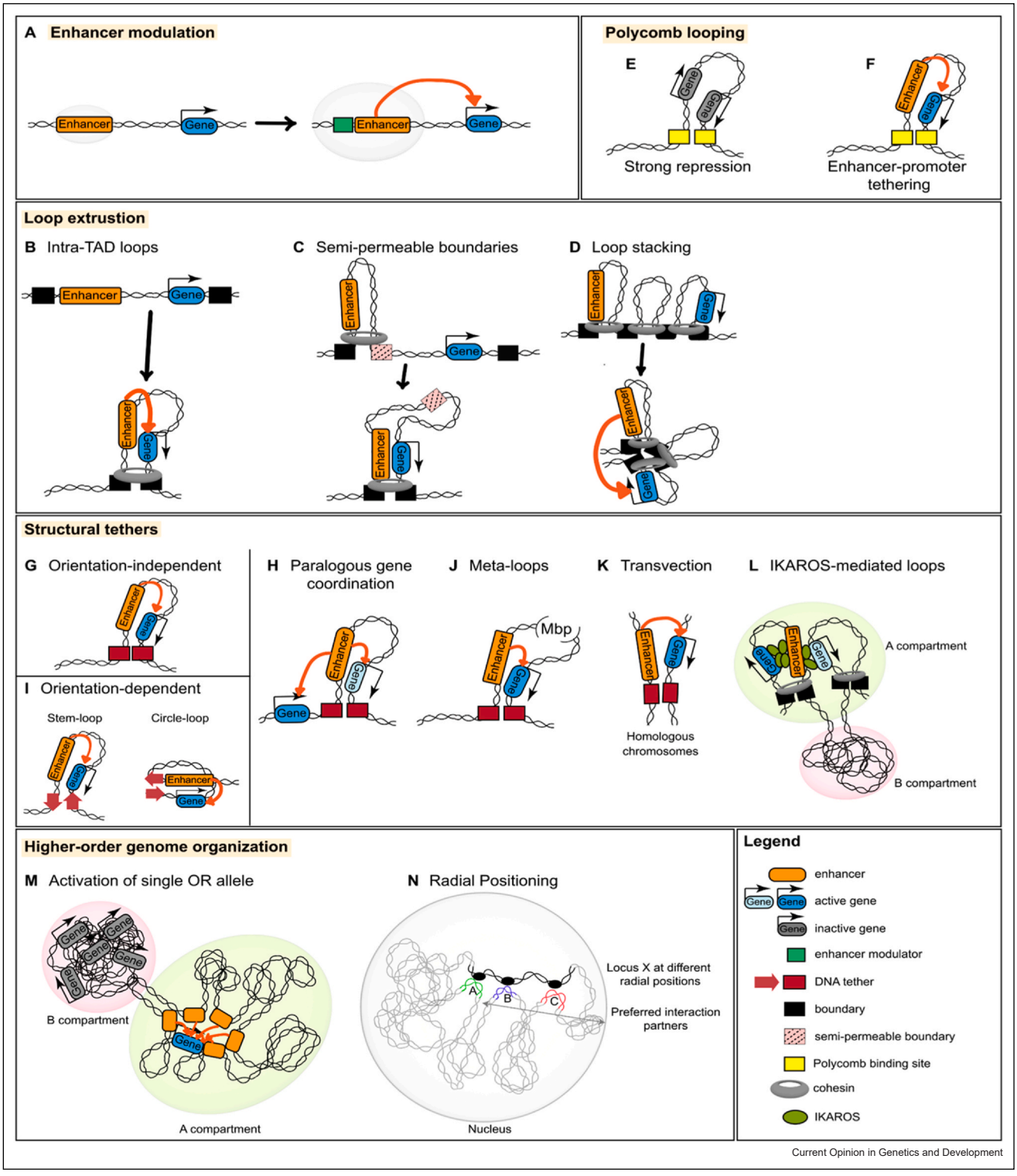
Enhancers can be modulated to extend their influence on promoters within the same TAD (Figure 2a). For example, sequences present in the mouse alpha-globin super-enhancer, called 'facilitators', boost the activity of nearby enhancers and increase their interactions with target promoters without acting as enhancers themselves, possibly by aiding transcription factor recruitment [1]. Another sequence, called 'range extender' (REX), confers long-range activity to mouse embryonic limb bud enhancers without acting as an enhancer itself [2]. Most mammalian enhancers regulate promoters over tens of kb, but long-range enhancers, for example, relying on REX, can act over hundreds of kb [2]. REX and other range-extending sequences found in long-range mouse embryonic limb bud enhancers contain conserved homeodomain motifs bound by LHX2, LHX9, and LEF1 transcription factors that may boost enhancer activity or tether enhancers to distant promoters. Additionally, enhancers recruit transcriptional (co-)activators with intrinsically disordered regions, which may promote clustering with distant promoters, aiding in long-range gene activation as recently demonstrated in fly embryos [3].

Figure 1



Overview of recently described long-range gene regulation strategies in flies and mice. The grey line for 'TAD boundary pairing' indicates its function across nonconsecutive TAD boundaries in non-native, transgenic settings. In most cases, the molecular basis of these strategies remains to be confirmed.

Figure 2



Potential mechanisms underlying long-range gene regulation strategies. In most cases, the molecular basis of these strategies requires further investigation.

Loop extrusion

In vertebrates, TADs form when cohesin extrudes a chromosomal loop and stalls at TAD boundaries bound by the DNA-binding protein CTCF (Figure 2b). In flies, CTCF and other insulator proteins form TAD boundaries [4–7], but whether they similarly stall cohesin has not yet been demonstrated.

TAD boundaries generally limit enhancer–promoter interactions between adjacent TADs [4,8,9], although they can be semi-permeable. Semi-permeability of TAD boundaries may arise from their dynamic nature and the fact that individual CTCF binding sites incompletely block loop extrusion [10] (Figure 2c). Alternatively, some enhancer–promoter interactions may be resistant to TAD boundaries. Up to 20% of enhancer loops in mice and fly embryos cross TAD boundaries [11–14], and some boundary-crossing interactions remain functional. For example, a developmental enhancer moved to an adjacent TAD still activated its target gene in fly embryos [13], and an endogenous developmental enhancer could cross an inserted insulator in pupae [15]. In mouse embryos, certain tissue-specific *Sox2* enhancers activated expression across an artificially introduced TAD boundary, indicating that the ability to overcome TAD boundaries can be tissue specific [16]. In mouse embryonic stem cells (mESCs), the insulating functions of specific TAD boundaries are insufficient to prevent regulatory cross-talk across the boundary and require additional mechanisms, such as promoter competition, to reinforce regulatory insulation [17].

Unexpectedly, most enhancer–promoter interactions are unaffected by acute CTCF or cohesin depletion in mammalian cells [11]. However, cohesin is essential for long-range activation of certain genes (Figure 2b) [18], such as of *Shh* by its enhancer present 400 kb away within its large TAD in mESCs [19]. Another study in human cells found that enhancers recruit cohesin to increase contact frequencies within a CTCF-delimited domain, which is crucial for activating a promoter across hundreds of kb [20]. Loop extrusion can thus facilitate long-range enhancer–promoter communication by compacting chromatin in mammals, whereas this function has so far been less documented in Drosophila.

When cohesin complexes collide during loop extrusion, they can stack successive loops. In mESCs, stacked loops visualized by DNA-FISH appeared as clustered TAD boundaries in the centre, from which intervening TADs radiated as separated loops [21] (Figure 2d). In stacked loops, enhancers and promoters located near boundaries are physically close and bridged to each other across intervening boundaries. This explains how the *Pitx1* gene is activated by its enhancer two TAD boundaries away [22]. Although the *Pitx1* enhancer is active in both mouse forelimbs and hindlimbs, it only activates *Pitx1* in hindlimbs, where stronger TAD boundaries facilitate stacking [22]. More generally, in mice and humans, enhancers that interact across TAD boundaries are often located near TAD boundaries [12,22]. Loop stacking can also bridge promoters and enhancers beyond the reach of a single cohesin molecule by stacking multiple cohesin loops rather than forming a single loop [22,23]. For example, the human *SOX9* gene is bridged to its distant enhancers over one megabase (Mb) away by loop stacking [23].

Polycomb loops

The conserved Polycomb group (PcG) transcriptional repressors form long-range loops in flies and mammals, visible in Hi-C and Micro-C maps (Figure 2e,f). PcG proteins are recruited to DNA elements called Polycomb response elements (PREs) in flies and to nonmethylated CpG islands (CGIs) in mammals. PcG-bound loci loop within TADs but also at longer ranges in flies [15,24–30] as in mESCs, in which they loop over tens of kb to tens of Mb [31,32], in a cohesin-independent manner [32–34], probably relying specifically on the activity of the canonical Polycomb Repressive Complex 1 (cPRC1, composed of a specific subset of PcG proteins) [28,30].

In both flies and mESCs, PcG loops are relatively infrequent and rarely multiway in single cells [30,32,34,35]. In flies, many PRE loops persist independently of gene transcription [15,24], paralleling the constitutive presence of PcG proteins at PREs [36]. Other PRE loops are dynamic over development, such as those connecting *HOX* loci, which are stronger in cells in which both loci are repressed [30]. In mESCs, PcG loops are dynamic over cell differentiation [32], being strong in mESCs but progressively weakened during neuronal differentiation, though they are retained or enhanced at specific loci in a manner correlating with PRC1 recruitment and gene silencing [37].

PcG loop perturbations further suggest that these loops reinforce gene silencing, despite their transient nature (Figure 2e). In flies, disrupting a PRE loop tethering the *dachshund* promoter to a distant PRE led to ectopic activation of *dachshund* and nearby genes by a local enhancer [15]. In mESCs, depletion of cohesin, which normally disrupts PcG loops, strengthened both looping and silencing of the associated PcG-bound genes [34]. Also, deletion of a PcG loop anchor resulted in derepression of the formerly paired gene [38].

Somewhat counterintuitively, PcG loops may also facilitate long-range enhancer–promoter communication (Figure 2f). In flies, some enhancers and promoters gain PRC1 binding and loop when they become activated during cell differentiation [26]. Mutations in PRC1 led to downregulation of looping genes, suggesting that

PRC1 is required for gene activation [26]. In differentiating mESCs, certain enhancers and promoters also form PRC1-dependent loops [26]. Promoter-distal PRC1 binding sites loop to distant genes (~100 kb away) in the same TAD and help bridge poised enhancers and promoters that are near each loop anchor [39].

Structural tethers

Diverse examples of DNA loci that can recruit specific transcription factors (or combinations thereof) to establish physical interactions with other distantly bound loci have been described in mESCs and Drosophila. We broadly refer to such loci that lack inherent regulatory activity but help bridge promoters and distant REs as 'tethering elements' [39–41].

In early fly embryos, tethering elements form hundreds of loops identified by Micro-C, mostly confined within TADs and averaging ~40 kb in size [40,41]. Most of these loops connect promoters or promoter-distal elements and are present in many cell types, independently of the transcriptional state of genes at promoter anchors [40,41]. Promoter-distal tethering elements bridge nearby REs to target genes to which they loop, facilitating rapid activation of certain developmental genes (Figure 2g). For example, deleting a tethering element or nearby enhancer in a *HOX* gene locus delayed transcriptional activation of the formerly paired *HOX* gene during zygotic genome activation [41]. One-third of early embryonic segmentation genes are in tethering element loops, suggesting that this is a widespread gene regulation strategy in early fly embryos [40].

Many tethering element loops in Drosophila connect promoters of paralogous genes, which evolved through gene duplication [40,42,43] (Figure 2h). Many paralogous gene loops are constant across development and cell types [40,42], while others form in specific differentiated cells [42,44]. Conversely, many paralogous genes form chromosomal loops in flies and mammals, usually in the same TAD but sometimes in distant TADs [40,42,44,45]. Paralogous gene loops likely allow co-regulation by shared REs, leading to both positive and negative cross-regulation [42]. Disrupting these loops can uncouple paralogous gene expression, and loop disruption can in some cases be rescued by re-introducing a formerly shared enhancer near the gene that lost access to it [40].

In Drosophila, some TAD boundaries act as tethering elements, forming long-range interactions that bridge promoters and REs near each boundary. The *even-skipped* gene boundaries, known as the Nhomie and Homie insulators, are well-known examples. A transgenic copy of Homie inserted several TADs away can loop to endogenous Nhomie and Homie in an orientation-dependent manner [46,47], forming cohesin-independent stem or circle loops depending on their orientation [48,49] (Figure 2i). It remains unclear how many Drosophila TAD boundaries share this tethering ability.

In differentiated fly neurons, tethering elements form 'meta-loops' identified by Micro-C, spanning 0.5–22 Mb and tens of TADs [44]. Meta-loops connect promoters of genes involved in axon guidance or signalling and promoter-distal elements [44]. Deleting specific intergenic meta-loop anchors or mutating transcription factors that form these loops reduced the expression of the formerly paired gene, showing that meta-loops regulate transcription over megabases [44]. Meta-loop anchors lack enhancer activity and may bridge neuronal gene promoters to distant REs [40–42] (Figure 2j). Meta-loops may have evolved to maintain proximity between formerly syntenic loci after they are separated by chromosomal rearrangements [44].

Finally, other tethering elements in Drosophila mediate long-range regulation between physically paired homologous chromosomes, a phenomenon called transvection [46,50,51] (Figure 2k).

How do tethering element loops form in Drosophila? Tethering elements in fly embryos are enriched for GAGA factor (GAF) and PcG protein binding [25,42,44]. GAF is required to form 7% of these loops [52], while the formation of the remaining 93% is unknown. GAF is required to form 20% of paralogous gene loops [44,52], with some genes being downregulated in *GAF* mutants, suggesting that GAF bridges paralogous promoters to shared enhancers [52]. In contrast, meta-loop anchors are not generally bound by GAF or PcG proteins [42,44], and instead, each relies on different DNA-binding proteins to form independent meta-loops [44]. The specific and long-range pairing of meta-loop anchors may require higher selectivity and binding affinity compared to intra-TAD loops.

Architectural elements that promote enhancer–promoter proximity independently of cohesin also exist in the mammalian genome. In mouse lymphoid cells, the transcription factor IKAROS binds to enhancers and facilitates their megabase-range interactions, bypassing TAD boundaries and heterochromatin [53] (Figure 2l). Loss of IKAROS in mouse pre-B cells disrupts these loops, downregulates B cell differentiation genes, and shifts loci from active (A) to inactive (B) compartments [53]. The mechanism of IKAROS-mediated looping may involve IKAROS oligomerization, though it remains to be clarified whether IKAROS functions as a structural tether or rather through a different higher-order genome organizing ability. Additionally, mouse post-meiotic spermatids show megabase range and trans-chromosomal interactions that maintain loci that were formerly syntenic in the mouse ancestral genome close in 3D [54].

Similar to Drosophila meta-loops, these interactions involve genes implicated in sensory perception and cell surface receptor signalling (among other processes), though their relevance remains unknown.

Finally, it is interesting to note parallels between PcG loops (described in the previous section) and tethering element loops. In fly embryos, many tethering elements overlap PRC1-binding sites [25,42,44], raising the question of whether PRE loops could be a subclass of tethering element loops. While long-range contacts between *HOX* loci are polycomb dependent [28,30], tethering elements overlapping PcG-binding sites within fly embryo TADs have not (yet) been shown to be PcG dependent. It therefore remains unclear whether tethering elements overlapping PREs function as PcG loops or through different mechanisms. In mESCs, PcG-recruiting CGIs function as tethering elements, bridging promoters to distal poised enhancers for activation [39]. Future research may clarify the relationship between PcG loops and non-PcG tethers.

Higher-order genome organization

Long-range gene regulation can also involve higher-order genome organization, such as compartmentalization of transcriptionally active or inactive chromatin and radial positioning of loci in the nucleus.

Hi-C and Micro-C maps in flies and vertebrates show 'ultra-long-range interactions' between active elements, including gene promoters, spanning up to tens of megabases [31]. These interactions correlate with transcription but form independently of RNA polymerase II, individual transcriptional cofactors, cohesin, or Polycomb [31]. These interactions are rare in single cells between individual pairs of active elements [31], even between superenhancers [55], and their function remains unclear.

A striking example of long-range gene regulation is olfactory receptor (OR) gene activation in mammals. Olfactory sensory neurons activate one allele from 1000 OR genes scattered across different chromosomes using OR enhancers from multiple chromosomes [56], while silent OR genes and OR enhancers group into a heterochromatic hub [57] (Figure 2m). Weak activation properties of OR gene promoters may require multi-enhancer hubs for their activation and may be a prerequisite for stochastic OR selection [58].

Finally, the radial position of loci relative to the nuclear periphery influences their ability to engage in long-range interactions (Figure 2n). The nuclear periphery, where constitutive heterochromatin domains localize, is broadly repressive [59–61]. DNA-FISH studies in human cells showed that loci have preferred interaction partners at different radial positions [62]. This suggests that moving a locus to another nuclear region introduces it to a new set of partners, rather than maintaining its existing interactions [62].

Concluding remarks

Diverse mechanisms for long-range gene regulation are being discovered in flies and mammals. Long-range gene regulation may be more common in differentiated cell types like neurons, which exhibit extensive 3D contacts [37,42,44,56–58,63]. Despite its compact genome, Drosophila shows megabase-range gene regulation as in mammals [44]. In flies, tethering elements bridge distant promoters and REs, often independently of cohesin [40–42,44,48,49,52]. In mammals, cohesin-driven loop extrusion is important for long-range regulation within or across TADs [19–23], but cohesin-independent interactions also occur [33,39,53,56–58,64]. Pairwise long-range interactions in Drosophila often contrast with complex, multiway interactions in mammals and have been instrumental in demonstrating the relevance of specific long-range gene regulation paradigms.

These findings raise questions about the diversity of factors mediating long-range interactions and whether they follow shared or independent principles across species. For example, homeodomain transcription factors such as LHX2 are implicated in long-range contacts in various contexts in mice, such as interchromosomal OR gene hubs and long-range limb bud enhancers [2,57]. Also, there is evidence that long-range interactions in flies and mammals are driven by chromosomal rearrangements in evolution [40,42,44,54]. Future research will help clarify the mechanisms driving long-range regulation and their evolution.

Author contributions

SZ and MCG wrote the manuscript.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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