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Chromosomes and Gene Expression

Chromosome Structure I: Loop extrusion or boundary:boundary pairing?

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Abstract

Two different models have been proposed to explain how the endpoints of chromatin looped domains ("TADs") in eukaryotic chromosomes are determined. In the first, a cohesin complex extrudes a loop until it encounters a boundary element roadblock, generating a stem-loop. In this model, boundaries are functionally autonomous: they have an intrinsic ability to halt the movement of incoming cohesin complexes that is independent of the properties of neighboring boundaries. In the second, loops are generated by boundary:boundary pairing. In this model, boundaries are functionally non-autonomous, and their ability to form a loop depends upon how well they match with their neighbors. Moreover, unlike the loop-extrusion model, pairing interactions can generate both stem-loops and circle-loops. We have used a combination of MicroC to analyze how TADs are organized, and experimental manipulations of the *even skipped* TAD boundary, *homie*, to test the predictions of the "loop-extrusion" and the "boundary-pairing" models. Our findings are incompatible with the loop-extrusion model and instead suggest that the endpoints of TADs in flies are determined by a mechanism in which boundary elements physically pair with their partners, either head-to-head or head-to-tail, with varying degrees of specificity. Although our experiments do not address how partners find each other, the mechanism is unlikely to require loop extrusion.

eLife assessment

This **valuable** work presents elegant experimental data from the Drosophila embryo supporting the notion that interactions among specific loci, called boundary elements, contribute to topologically associated domain (TAD) formation and gene regulation. The evidence supporting boundary:boundary pairing as a determinant of 3D structures is **compelling**; however, an inability to deplete loop extruders formally leaves open a possible contribution of loop extrusion. This study will be of interest to the nuclear structure community, particularly those using Drosophila as a model.

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Introduction

The chromosomes of multicellular animals have a regular and inheritable physical organization. This was first recognized in studies on the lampbrush chromosomes in amphibian oocytes and the polytene chromosomes of insects (Callan 1963; Nora et al. 2020; Zhimulev and Belyaeva 1991; Zhimulev et al. 1983). Subsequent research has shown that the architectural features inferred from analysis of lampbrush and polytene chromosomes are present in chromosomes throughout much of the animal kingdom. The key organizational principle is the subdivision of the chromatin fiber into a series of independent looped domains, commonly called "TADs" (Cavalheiro et al. 2021; Chetverina et al. 2017; Jerković et al. 2020; Matthews and White 2019; Rowley and Corces 2018). With important exceptions, the arrangement of TADs along a given chromosome tend to be invariant and are largely independent of the cell type or developmental stage. This regular and inheritable organization is a reflection of the underlying mechanism of TAD formation. TADs are separated from each other by special elements called boundaries or insulators. While these elements have been found in many different species, they have been most fully characterized in *Drosophila* (Cavalheiro et al. 2021; Chetverina et al. 2017). Fly boundaries span DNA sequences of 150 bp to 1.5 kb in length and contain one or more nucleosome-free nuclease-hypersensitive regions. These nuclease-hypersensitive regions are targets for a large collection of DNA binding proteins that have been implicated in boundary function. Some of the fly boundary factors are widely conserved (CTCF, BEN family proteins, GAF), while others appear to be restricted to insect linages (Su(Hw), Pita, Zw5, Zipic, Mod(mdg4), BEAF) (Heger et al. 2013; Heger and Wiehe 2014; Schoborg and Labrador 2010).

Boundary elements in flies are not only responsible for organizing the chromatin fiber, they also have genetic activities. When interposed between enhancers or silencers and target promoters, boundary elements block regulatory interactions (Bell et al. 2001; Chetverina et al. 2014; Chetverina et al. 2017). This insulating activity provides a mechanism for delimiting units of independent gene activity: genes located between a pair of compatible boundaries are subject to regulatory interactions with enhancers/silencers present in the same chromosomal interval, while they are insulated from the effects of enhancers/silencers located beyond either boundary in adjacent neighborhoods. Genetic studies suggest that the insulating activity of boundary elements is a consequence of subdividing the chromosome into a series of topologically independent domains (Cai and Shen 2001; Gohl et al. 2011; Muravyova et al. 2001). Organizing the chromatin fiber into looped domains enhances contacts between sequences within the loop, while it suppresses contacts with sequences outside of the loop. While it is not known whether a similar insulation mechanism is at play in mammals, the fact that mammalian chromosomes are also subdivided into TADs by boundary-like elements suggests that it might be.

A critical question in chromosome biology is the mechanism(s) responsible for determining the endpoints of the loop domains, the TADs. In mammals, a novel loop-extrusion model has been proposed to not only generate chromosomal loops but also determine the endpoints of those loops (Alipour and Marko 2012; Fudenberg et al. 2016; Guo et al. 2012; Guo et al. 2015; Nuebler et al. 2018; Sanborn et al. 2015). In this model, a cohesin complex initiates loop formation at a loading site within the "loop-to-be" and then extrudes a chromatin loop until it bumps into CTCF-dependent roadblocks, one on each side of the extruding loop. The location of these roadblocks combined with the processive action of the cohesin complex determines the endpoints of each TAD (Davidson and Peters 2021; Ghosh and Meyer 2021; Mirny and Dekker 2022; Perea-Resa et al. 2021). A key assumption of the loop-extrusion model is that mammalian boundaries are fully autonomous: they are roadblocks, and their physical presence in and of itself is sufficient to define the loop endpoint, independent of the functional properties of neighboring boundaries. In a more specific variant of this model, the relative orientation of the paired CTCF roadblocks is also important—in order to halt cohesin-mediated extrusion, the CTCF sites on each side of the loop

must have a convergent orientation. In this model the CTCF roadblocks are able to block an oncoming cohesin complex in only one direction; however, their intrinsic blocking activity is independent of the orientation of other CTCF sites in their neighborhood.

While this loop extrusion mechanism is widely thought to be operative in mammals, the evidence regarding TAD formation and function in flies is seemingly inconsistent with this mechanism. Genetics studies have shown that fly boundaries are functionally non-autonomous and that their activities in both loop formation and gene regulation depend upon their ability to engage in direct physical interactions with other boundaries (Chetverina et al. 2014; Chetverina et al. 2017). That fly boundaries might function by physical pairing was first suggested by studies which showed that the *gypsy* transposon *su(Hw)* boundary and the bithorax complex (BX-C) *Mcp* boundary can mediate regulatory interactions (enhancer/silencer: reporter) between transgenes inserted at sites separated by 15-32 MB or more (Muller et al. 1999; Sigrist and Pirrotta 1997; Vazquez et al. 1993). Consistent with direct physical interactions, these distant transgenes co-localize *in vivo*, and in living tissue remain in contact for extended periods of time (Chen et al. 2018; Li et al. 2011; Vazquez et al. 2006).

Some of the parameters that govern pairing interactions have been defined in two different transgene assays, insulator bypass and boundary competition. In one version of the bypass assay, a set of enhancers are placed upstream of two different reporters (Cai and Shen 2001; Kyrchanova et al. 2008a; Muravyova et al. 2001). When a single boundary is introduced between the enhancers and the closest reporter, the enhancers are unable to activate either reporter. However, if the same boundary is placed downstream of the closest reporter, bypass is observed. In this case the closest reporter, which is bracketed by the two boundaries, is still insulated from the enhancers; however, the downstream reporter is activated (Muravyova et al. 2001). Kyrchanova et al. (Kyrchanova et al. 2008a) showed that bypass activity is, in most cases, orientation-dependent. When the same boundary is placed in the upstream and downstream position, they typically need to be introduced in the opposite orientation. The reason for this is that self-pairing is head-to-head, and this configuration generates a stem-loop topology, bringing the upstream enhancers into close proximity to the downstream reporter. When the two boundaries are introduced in the same orientation, the enhancers do not activate the downstream reporter because a circle-loop rather than a stem-loop is formed (Chetverina et al. 2017; Kyrchanova et al. 2008a). Head-to-head pairing seems to be a common feature of self-pairing interactions between fly boundaries and was observed for *scs*, *scs'*, *iA2* and *wari* (Kyrchanova et al. 2008a). A preference for head-to-head interactions is also observed for heterologous pairing interactions between different boundaries in the *Abdominal-B* (*Abd-B*) region of the BX-C (Kyrchanova et al. 2011; Kyrchanova et al. 2008b). There are some exceptions. The BX-C *Fab-7* boundary pairs with itself and with its neighbor *Fab-8* in both orientations (Kyrchanova et al. 2011; Kyrchanova et al. 2008b). Bypass activity also seems to depend upon finding the proper match. For example, when multimerized dCTCF or Zw5 binding sites are placed both upstream and downstream of the closest reporter, bypass is observed. However, there is no bypass when multimerized dCTCF sites are tested with multimerized Zw5 sites (Kyrchanova et al. 2008a). Similar results have been reported for the Zipic and Pita factors (Zolotarev et al. 2016). Boundary:boundary pairing preferences are also observed in competition assays in which different boundaries are introduced into the same transgene (Gohl et al. 2011). In these assays, the insulating activity of a boundary in the blocking position (between an enhancer and a reporter that is flanked by a 3' boundary) is challenged by placing a heterologous boundary upstream of the enhancer. If the boundary upstream of the enhancer is a better match for the boundary located downstream of the reporter, then the insulating activity of the boundary in the blocking position can be compromised or lost altogether. These and other experiments argue that fly boundaries are functionally non-autonomous—that is, their activities depend upon their ability to physically interact with other boundaries.

However, the differences between the two models are not limited to the mechanisms for determining the endpoints of TADs. The models also differ in the possible topologies of the chromatin loops that form TADs. Only two loop topologies can be generated by the cohesin-extrusion mechanism. One is a stem-loop while the other is an unanchored loop. Depending upon the location of the road-blocks that halt cohesin extrusion and the cohesin loading sites, the chromatin fiber would be organized into a series of stem-loops. These stem-loops will be separated from each other by unanchored loops (see **Fig. 1B**). This is the configuration that is often illustrated in articles discussing the loop-extrusion model. If the relevant road-blocks are very closely juxtaposed, then the unanchored connecting loops will disappear and be replaced by a series of stem-loops which point in opposite orientations, as illustrated in **Fig. 1C**. Stem-loops are also possible in the boundary-pairing model, and they will be formed when two heterologous boundaries pair with each other head-to-tail in *cis* (see **Fig. 1D**). Like loop-extrusion, the stem-loops could be separated from each other by unanchored loops (**Fig. 1E**). If the boundaries in the neighborhood pair with each other head-to-tail, a series of connected stem-loops pointing in opposite orientations will be generated. In this case, the main axis of the chromosome would be a series of paired boundaries (**Fig. 1F**).

Stem-loops are not, however, the only loop topology that can be generated in the boundary-pairing model. If the neighboring boundaries pair with each other head-to-head instead of head-to-tail, a circle-loop structure will be generated (**Fig. 1G**). Like stem-loops, the circle-loops could be connected by unanchored loops (**Fig. 1H**). Alternatively, if boundaries pair with both neighbors head-to-head this will generate a series of linked circle-loops oriented in (more or less) the same direction (see **Fig. 1I**).

In the studies reported here, we have critically evaluated these two models. We have used a combination of MicroC to analyze how TADs are organized and experimental manipulations to test the predictions of the "loop-extrusion" and the "boundary-pairing" models. For these experimental manipulations, we have used the well-characterized *homie* boundary from the *even skipped* (*eve*) locus. *homie* together with *nhomie* flank the 16 kb *eve* locus, and these two elements share many of the properties of other fly boundaries (Fujioka et al. 2013; Fujioka et al. 2009). Like the *gypsy* and *Mcp* boundaries, *nhomie* and *homie* can mediate long-distance regulatory interactions. For example, a *lacZ* reporter transgene containing *homie* (or *nhomie*) inserted on one homolog can be activated by an *eve* enhancer in a *homie*- (or *nhomie*-) containing transgene inserted on the other homolog over a distance of 2 Mb (Fujioka et al. 2016). Long-distance activation is also observed in heterologous combinations of *nhomie* and *homie*, but not in combinations with phage *lambda* DNA. Similarly, regulatory interactions are observed when a *homie*- or *nhomie*- containing reporter is inserted at an attP landing site in the *hebe* gene, 142 kb upstream of the *eve* locus (Chen et al. 2018; Fujioka et al. 2016; Fujioka et al. 2009). In this case, the *eve* enhancers drive reporter expression during development in a pattern that mimics the stage- and tissue-specific expression of the endogenous *eve* gene. Activation also depends upon the orientation of the boundary (5'→3') relative to the reporter in the transgene. For *homie*, the reporter must be located "upstream" of the boundary (5'→3'), just like the *eve* enhancers (and *eve* gene) are "upstream" of endogenous *homie* (see **Fig. 2B**). Little or no activation is observed when the relative orientation is reversed so that the reporter is located "downstream" of *homie*. For *nhomie*, the reporter must be located "downstream", in the same relative position as that in which the *eve* enhancers and *eve* gene are located with respect to endogenous *nhomie* (5'→3': **Fig. 2B**).

Results

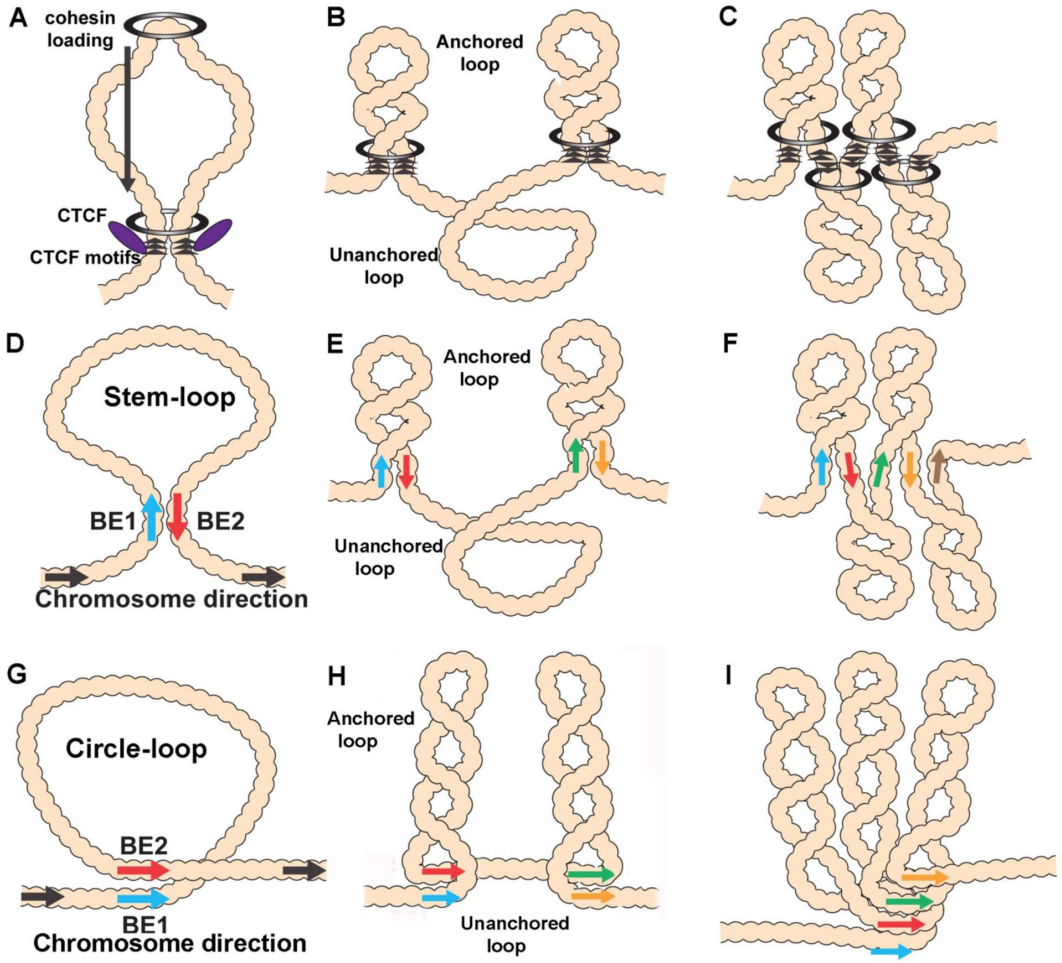

Figure 1.

Diagrams of possible loop topologies generated by loop extrusion and boundary:boundary pairing.

A) Cohesin embraces a loop at a loading site somewhere within the TAD-to-be and then extrudes a loop at an equal rate on both strands. Extrusion continues until cohesin encounters boundary roadblocks on both strands. B) In one model, the orientation of the roadblock is important. As a consequence, the chromatin fiber will be organized into a series of stem-loops separated from each other by unanchored loops. C) If the presence of a boundary roadblock, but not its orientation, is sufficient to halt extrusion, the chromatin fiber will be organized into a series of linked stem-loops. The main axis of the chromosome will be defined by the cohesin:CTCF roadblocks. D) The pairing of two boundaries head-to-tail generates a stem-loop. E) If boundary pairing interactions are strictly pairwise, head-to-tail pairing will generate a series of stem-loops separated from each other by unanchored loops. F) If boundaries can engage in multiple head-to-tail pairing interactions, the chromosome will be organized into a series of linked stem-loops. The main axis of the chromosome will be defined by a series of paired boundaries. G) The pairing of two boundaries head-to-head generates a circle-loop. H) If boundary pairing interactions are strictly pairwise there will be a series of circle-loops separated from each other by unanchored loops. I) If boundaries can engage in multiple head-to-head pairing interactions, the chromatin fiber will be organized into a series of circle-loops connected to each other at their base. Pairing interactions between boundaries #1 and #2 need not be in register with pairing of boundaries #2 and #3. In this case, the main axis of the chromosome may bend and twist, and this could impact the relative orientations of the circle-loops.

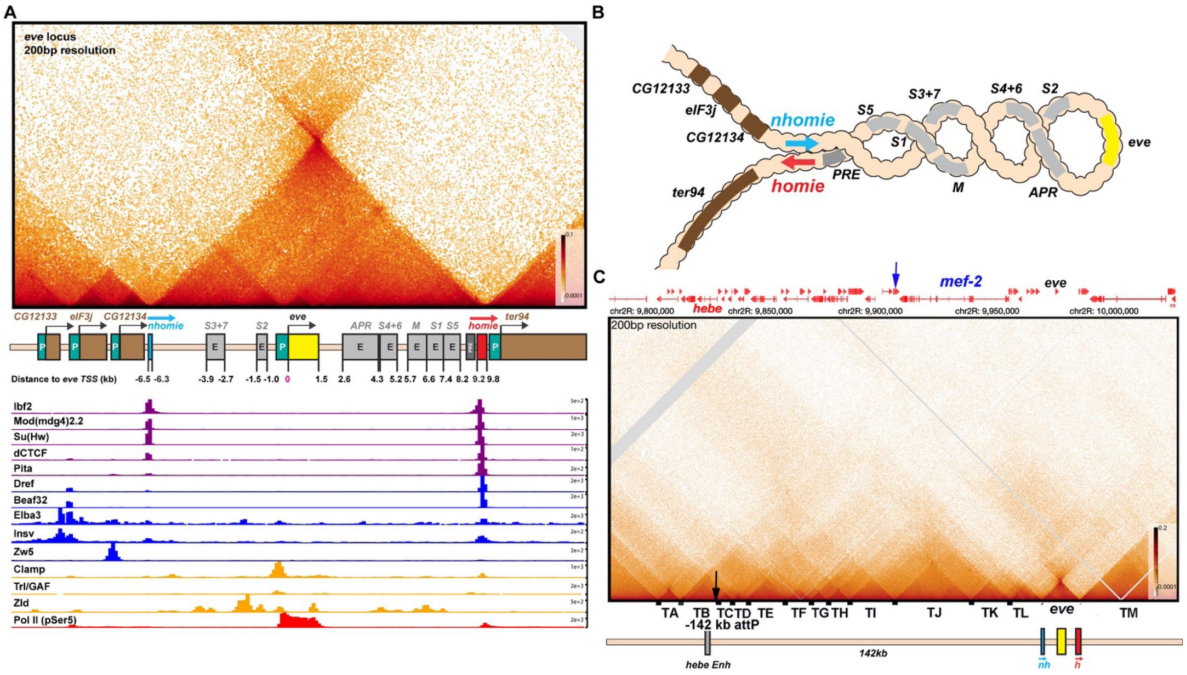

Figure 2.

TAD organization of the *even-skipped* locus and neighboring sequences.

A) The *eve* TAD is a volcano with a plume that is anchored by *nhomie* and *homie*. ChIP-seq data below the MicroC map indicate that many of the known fly chromosomal architectural proteins are associated with the two *eve* boundary elements *in vivo* (Cuartero et al. 2014; Duan et al. 2021; Gaskill et al. 2021; Li et al. 2015; Sun et al. 2015; Ueberschär et al. 2019; Zolotarev et al. 2016). B) The *eve* locus forms a stem-loop structure. In this illustration, *nhomie* pairs with *homie* head-to-tail, and this forms a stem-loop which brings sequences upstream of *nhomie* and downstream of *homie* into contact, as is observed in insulator bypass assays (Kyrchanova et al. 2008a). The *eve* locus is shown assembled into a coiled "30 nM" chromatin fiber. C) MicroC contact pattern for the chromosomal region spanning the attP site at –142 kb and the *eve* locus. Like the *eve* volcano, this contact pattern was generated using aggregated previously published NC14 data (Batut et al. 2022; Levo et al. 2022). A black arrow indicates the –142kb locus where transgenes are integrated into the genome. A blue arrow indicates the position of the *Etf-QO* gene. Note the numerous TADs between the –142kb *hebe* locus from the *eve* "volcano TAD". Individual TADs are labeled TA—TM. Black boxes indicate positions of sequences that, based on ChIP experiments, are bound by one or more known insulator proteins *in vivo*.

The eve TAD

We used MicroC to probe the TAD organization of the *eve* locus and the genomic region that extends from *eve* to just beyond the attP site at –142 kb. For this purpose, we chose nuclear cycle 14 embryos. DNA replication commences soon after nuclei exit the previous mitosis and continues for nearly an hour until the nuclei arrest in G2, and the process of cellularization is completed. As sister chromosomes in flies pair with each other in register, there will be two juxtaposed copies of this region of the chromosome in most nuclei. The sister chromosomes should be linked to each other by cohesin in preparation for the asynchronous cellular divisions at the end of NC14 (Collier and Nasmyth 2022; Gligoris et al. 2014). While homolog pairing is limited in pre-cellular blastoderm embryos, there are regions of the chromosome that already show evidence of pairing (Erceg et al. 2019; Fung et al. 1998). In fact, transvection mediated by transgenes containing the *gypsy* insulator and the *homie* and *Fab-8* boundaries has been observed in live imaging studies of nuclear cycle 14 embryos (Lim et al. 2018). Since the NC14 nuclei are just emerging from an earlier mitosis and are undergoing DNA synthesis, one would imagine that other key features of chromosome organization might also be in the process of being established. Thus, it should be possible to capture evidence of intermediates like those expected to be generated by extruding cohesin complexes as they move through the *eve* TAD and other nearby loci at this stage of development.

Shown in **Fig. 2A** is a blow-up of the TAD organization of the *eve* locus. As expected, the left and right boundaries of the *eve* TAD correspond to *nhomie* and *homie*, respectively, and sequences containing these two boundaries converge at the apex of the *eve* interaction triangle. All of the sequences within the TAD appear to come into contact with each other and define a high density of internal contact domain (HDIC). These contacts are expected to be generated, at least in part, by the sliding of the chromatin fiber within the TAD against itself, much like sequences in circular/supercoiled DNA can interact with each other (**Fig. 1E**). There are also sequences within the *eve* TAD that show enhanced interactions. For example, there is a darker interaction triangle connecting *nhomie* and the promoter region, while another triangle connects *homie* and the PRE adjacent to *homie* to the promoter region. These enhanced internal contacts would be expected to help insulate the *eve* gene from external regulatory elements and at the same time facilitate interactions between *eve* and its upstream and downstream enhancers.

As predicted by both the loop-extrusion and boundary-pairing models, the *eve* TAD is a stem-loop, not a circle-loop (**Fig. 2B**). This is evident from the prominent “volcano plume” at the apex of the *eve* TAD (volcano triangle). A volcano plume is observed because sequences flanking *eve* are brought into close proximity when *homie* and *nhomie* are linked to each other by either cohesin or by head-to-tail pairing (see **Fig.1**: see also Ke et al., 2024). **Fig. Supplemental 1** shows the hierarchy of interactions between TADs to either side of *eve* (L-M, K-M and J-M in **Fig. Supplemental 1**). This conclusion is supported by previous transgene studies. As first described in the boundary bypass experiments of Cai and Shen (Cai and Shen 2001) and Muravyova et al. (Muravyova et al. 2001), the formation of a stem-loop structure when two boundaries are linked together brings sequences immediately beyond the linked boundaries into close proximity with each other. In this assay, a stem-loop structure is required, as it enables enhancers flanking the “upstream” boundary to interact with reporters flanking the downstream boundary (Kyrchanova et al. 2008a). In contrast, a circle-loop configuration does not bring the neighbors to either side of *eve* into close proximity.

While a stem-loop is the topology expected for the *eve* TAD in both models, one feature of the TAD is inconsistent with the loop-extrusion model. According to this model, loops (TADs) are generated by a cohesin complex that forms a small bubble at a loading site somewhere in the TAD-to-be and then extrudes both strands of the growing loop at an equal rate until it bumps into the boundary road-blocks (Davidson and Peters 2021; Ghosh and Meyer 2021; Mirny and Dekker 2022; Perea-Resa et al. 2021). If the loading site were located in the middle of the TAD-to-be, and the

extrusion intermediates in a population of nuclei were captured by crosslinking, one should observe a crosslink-generated stripe that begins at the loading site and extends perpendicular to the chromosomal DNA until it reaches the apex that links the two TAD boundaries with imaginary lines of 45° (**Fig. Supplemental 2B**). This is the pattern that would be generated by a "broken zipper" when it transiently links the zipper teeth equidistant from the initial "loading site". If the loading site is off center to the left, the crosslinking of the passing cohesin complex in a population of nuclei will generate a perpendicular stripe until cohesin come to a halt when it encounters the roadblock on the left (**Fig. Supplemental 2C**). Assuming that the complex continues to extrude the right strand, it will then generate a stripe at 45° degrees that extends to the apex of the TAD. If the loading site corresponds to one of the CTCF roadblocks or is located close to the roadblock, the cohesin complex will generate a 45° stripe that comes to a halt at the apex of the triangle when it encounters the neighboring CTCF roadblock (**Fig. Supplemental 2D** and E). If there are multiple loading sites within the TAD, there should be a series of perpendicular stripes that generate and sequentially reinforce the stripe(s) at 45° (**Fig. Supplemental 2F**). However, in spite of the fact that NC14 fly embryos should provide by far the best prospect of actually capturing loop extrusion intermediates, there are no vertical stripes in the *eve* TAD, nor are there stripes at 45° that extend to the apex of the *eve* TAD. Nor do we observe a population of perpendicular stripes that convert into a series of reinforced 45° stripes. There is also no evidence of stripes that are initially tilted to the left or right of a "loading site", as might be observed if the rate of extrusion were unequal on the two strands, and then subsequently convert to a 45° stripe as cohesin comes to a halt at the first boundary encountered.

TADs in the eve environs

The attP insertion site at –142 kb is near the end of the first exon of the *hebe* transcription unit (**Fig. 2C**). It is located just within a ~14 kb TAD, TB, that has a high density of internal contacts, and extends from the *hebe* promoter to the 3' end of the neighboring *dila* gene. In between *hebe* and the *eve* locus there are at least ten distinct TADs, TC-TL (**Fig. 2C**). The endpoints of most of these TADs correspond to sequences that are associated with one or more known chromosomal architectural factors (dots along the horizontal axis in **Fig. 2C**).

These TADs correspond to the fundamental building blocks for organizing the 3D architecture of this chromosomal segment. Superimposed upon these TADs are regions that exhibit lower density of contacts (LDC). For example, the *mef2* gene spans two TADs, TJ and TK. TK contains the *mef2* distal promoter region and extends to the internal *mef2* promoter, while TJ extends to near the promoter of the divergently transcribed *Etf-QO* gene (blue arrow). Both of these TADs are linked together by a rectangular LDC domain, J-K (see **Fig. Supplemental 1**). Likewise, TJ is linked to its immediate neighbor TI by the LDC domain I-J. In the next level, TADs separated by a single TAD are linked together. In the region immediately above TJ, the LDC domain I-K links TI to TK (**Fig. Supplemental 1**). Similarly, TJ is linked to TL by J-L while TJ is also linked to the large TAD TM (which contains *TER94* and the neighboring gene, *pka-R2*) on the other side of *eve* by J-M. The same pattern of a hierarchical series of LDC domains linking TAD neighbors, TAD next-door neighbors and next-next-door neighbors is observed in the DNA segment that includes the *hebe* gene.

--*cohesin-mediated loop extrusion:* Like *eve*, the TADs in the region between *eve* and the attP site in the *hebe* locus are defined by right-angle triangles (volcanos) with a high density of internal contacts. However, unlike *eve*, these volcano triangles do not have a plume, and instead are surrounded by a series of LDC rectangular "clouds". This is not the contact pattern that one might expect for either a series of stem-loops connected by unanchored loops or a series of stem-loops connected to each other. Moreover, there are no perpendicular or angled crosslinking stripes (broken zippers) emanating from the base of these triangles, nor are their stripes along their 45° legs. Thus, in spite of the fact that the NC14 nuclei used in this analysis are just emerging from an

earlier mitosis and a round of DNA synthesis and should be in the process of assembling TADs, the crosslinking signatures that should be generated by the embrace of passing cohesin complexes are completely absent.

In the loop-extrusion model, the LDC domains would arise in a subset of nuclei because the extruding cohesin complex breaks through one or more boundary roadblocks before its progression is halted (**Fig. Supplemental 3A** versus B, C and D) (Hsieh et al. 2020; Krietenstein et al. 2020). Breakthroughs could occur at only one roadblock on one side of the extruding loop (**Fig. Supplemental 3B** and C), or at multiple roadblocks on one or both sides (**Fig. Supplemental 3D**), giving rise to a set of overlying LDC domains like that evident in the region between *hebe* and *eve*. However, while there would have to be multiple breakthrough events to account for the hierarchical array of LDC domains observed in these NC14 embryos, there is no evidence of the 45° crosslinking stripes that would be expected to mark the legs of the LDC domains following the breakthrough. For example, the I-J LDC domain (**Fig. Supplemental 1**) could be generated by cohesin complexes that initiated extrusion in either TI or TJ and then failed to halt at the TI:TJ boundary (green arrowhead). In either case, there should be a 45° stripe of crosslinking that marks (at least part of) the left (TI) and/or the right (TJ) leg of the I-J LDC domain and extends to the apex of the TI-TJ LDC triangle; however, there is no evidence of such stripes. Nor is there evidence of crosslinking stripes marking one or both legs of the J-K or I-K LDC domains (**Fig. Supplemental 1**).

While the LDC domains associated with the TADs located between *eve* and the *hebe* locus could potentially be explained by cohesin breaking through one or more boundaries (even though we do not observe the expected stripe intermediates), it is important to note that the *eve* boundaries, *nhomie* and *homie,* do not appear to be subject to as frequent break-through events. This is because, unlike the other TADs in the neighborhood, the LDC domains that link *eve* to TA and TL are much more lightly populated compared to the LDC domain that links TA to TL. -- *boundary:boundary pairing:* At least two different mechanisms are expected to account for the TADs and LDC domains in the region between *eve* and the attP insertion site at -142 kb. First, since none of the TADs in this DNA segment (with the possible exception of TA) are topped by volcano plumes, they could correspond to circle-loops. As circle-loops are generated by head-to-head pairing, the boundaries in the region between *nhomie* and *hebe* would be expected to pair with each other head-to-head. (The TAD to TAD contact maps generated by circle-loops and stem-loops are considered further in the accompanying paper (Ke et al. 2024).)

As illustrated in **Fig. Supplemental 3E-G**, the coiled circle-loop TADs, though topologically independent, are expected to be in relatively close proximity to each other. This means that in addition to crosslinking events within the TADs, there will be crosslinking events between neighboring TADs. These crosslinking events could generate the LDCs seen in **Fig. 2C** and **Fig. Supplemental 1**. Since TADs next to each other (**Fig. Supplemental 3E** and F; **Fig. Supplemental 1**, I-J or J-K) would typically be expected to interact more frequently than TADs separated by one, two or more TADs (**Fig. Supplemental 3G**; **Fig. Supplemental 1**, I-K or J-L), there should be a progressive reduction in contact frequency at each step. This is generally what is seen (see **Fig. Supplemental 1**). While connected stem-loops generated by cohesin extrusion should also bump into one another, contacts between next-door neighbors might be expected to be less frequent than contacts between next-next-door neighbors (**Fig. 1B, C** and **Fig. Supplemental 3A**). (TAD-to-TAD crosslinking and the impact of loop topology is further examined in Ke et al. (Ke et al. 2024).

The second mechanism for generating LDC domains would be switching and/or combining pairing partners (**Fig. Supplemental 3H-J**). Though imaging studies suggest that pairing interactions can be of relatively long duration (>30 min: Chen et al. 2018; Vazquez et al. 2006), they are not permanent, and other nearby boundaries can compete for pairing interactions (Gohl et al. 2011). If switching/combining occurs in NC14 embryos (see **Fig. Supplemental 3H-J**), then the

precise pattern of TADs and LDC domains could also be impacted by the relative avidity of potential pairing partners in the neighborhood and also the distances separating potential partners. For example, the rectangle linking TH and TI (H-I in **Fig. Supplemental 1**) could represent partner switching, so that the boundary between TI and TJ (green arrowhead in **Fig. Supplemental 1**) sometimes pairs with the boundary separating TH and TI (blue arrowhead in **Fig. Supplemental 1**) and sometimes with the boundary separating TG and TH (red arrowhead in **Fig. Supplemental 1**). Distant boundary elements could also occasionally interact with each other, generating a supra-TAD that contains multiple TAD domains. For example, at the apex of the LDC domain B-I there is an interaction dot (small black arrow, **Fig. Supplemental 1**) that links the TA-TB boundary to the TI-TJ boundary (green arrowhead).

Activation of a distant reporter by the eve enhancers

In previous studies we found that a minimal 367 bp *homie* fragment can orchestrate regulatory interactions between the *eve* enhancers and reporters inserted at an attP site in the first intron of the *hebe* transcription unit, 142 kb upstream of the *eve* gene (Fujioka et al. 2016). This minimal fragment lacks the one potential *homie* CTCF site, but has sites for several other generic boundary proteins including BEAF, Zipic, Su(Hw), Pita and Ibf2. In blastoderm stage embryos, *lacZ* reporter expression was observed in a 7-stripe pattern that coincided with the stripes of the endogenous gene. However, at this stage only a subset of *eve*-expressing nuclei also expressed the reporter. Later in development, during mid-embryogenesis, reporter expression was observed in cells in the dorsal mesoderm, the ventral CNS, and in the anal plate region, in a pattern that recapitulated *eve* gene expression (Fujioka et al. 2016). At these later stages, most of the *eve*-expressing cells also appear to express the reporter. The *eve*-dependent activation of the reporter in a stripe pattern has also been visualized in live imaging of pre-cellular blastoderm-stage embryos (Chen et al. 2018). Within the 7 *eve* stripes, the majority of the nuclei (~80-85%) did not express the *lacZ* reporter. In these nuclei, the transgene was found to be at a considerable average physical distance from the *eve* locus (~700 nM). In the remaining nuclei, the transgene was located in closer proximity to the *eve* locus (~330 nM, on average). Within this subset, the transgene was expressed in most of the nuclei. Moreover, in most cases in which the transgene was active, it remained in close proximity and continued to express *lacZ* for the duration of the experiment (~30 min).

In the two models for TAD formation, quite different mechanisms must be invoked to account for activation of the reporter at –142 kb by the *eve* enhancers. In the boundary-pairing model, the transgene *homie* boundary at –142 kb loops over the intervening TADs and pairs with the *nhomie:homie* complex flanking the *eve* TAD (c.f., **Fig. 3B and C**). In the loop-extrusion model, a cohesin complex initiating loop extrusion in the *eve* TAD must break through the *nhomie* roadblock at the upstream end of the *eve* TAD. It must then make its way past the boundaries that separate *eve* from the *attP* site in the *hebe* gene, and come to a halt at the *homie* boundary associated with the *lacZ* reporter. This would generate a novel TAD, *eveMammoth* (*eveMa*), that extends from the *eve homie* all the way to the *homie* fragment at –142 kb, and encompasses both the reporter and the *eve* gene, including its enhancers (c.f., **Fig. 3D**). Of course, the *eveMa* TAD could also be generated by a cohesin complex that initiated in, for example, TF. However, in this case, the runaway cohesin complex would have to break through the intervening boundary roadblocks in both directions. In both the boundary-pairing and loop-extrusion models, the configuration of the chromatin fiber would lead to the activation of *lacZ* expression by the *eve* enhancers, while the reporter would be protected from the *hebe* enhancers by the *homie* boundary.

To test these two models, we used a transgene that has two divergently transcribed reporters, *lacZ* and *gfp*, that are each under the control of an *eve* promoter (**Fig. 3A**). Two different versions of the transgene were generated. In the first, the *eve-lacZ* reporter is located “upstream” of *homie* (with respect to the 5’→3’orientation of *homie* in the *eve* locus), while *eve-gfp* is located downstream. In the second, the orientation of *homie* within the transgene is reversed, so that *eve-*

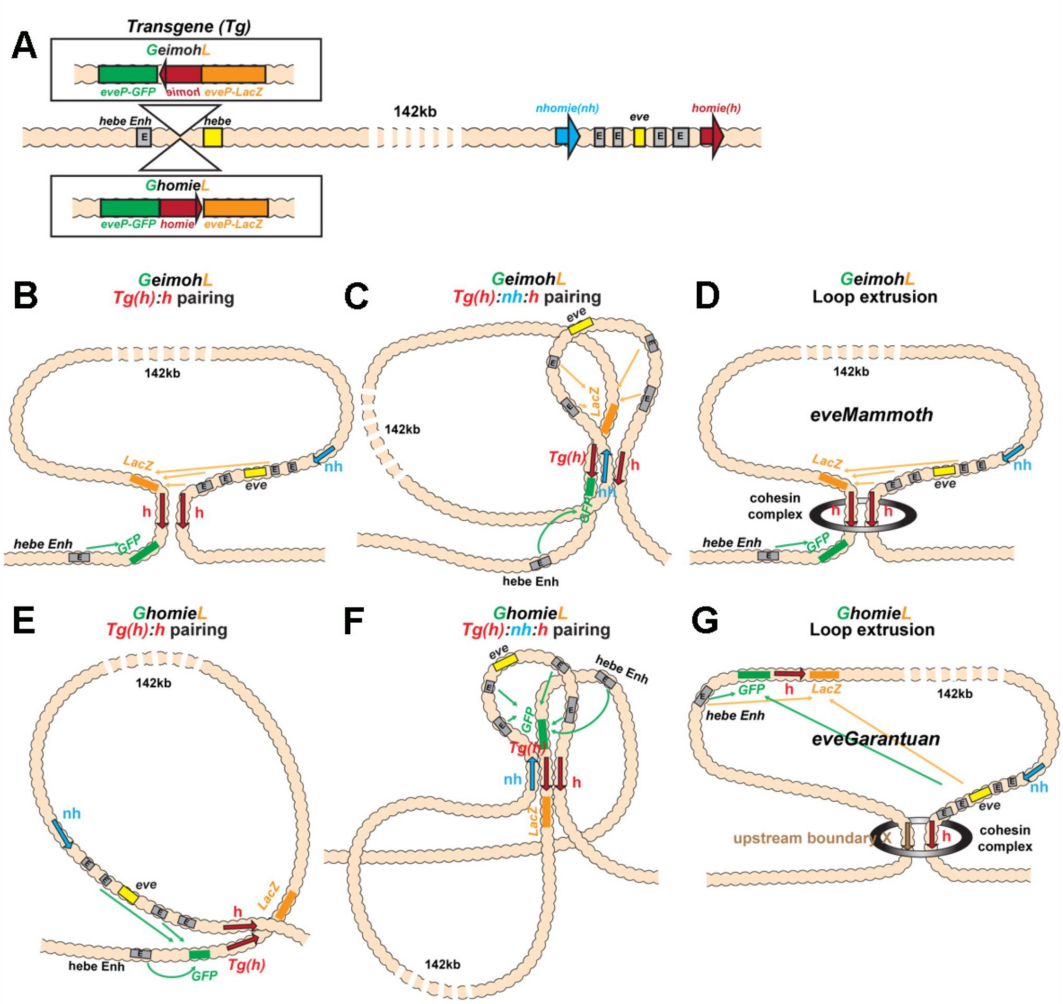

Figure 3.

Schematics of boundary pairing and loop extrusion.

A) *GeimohL* and *GhomieL* transgenes and the *eve* locus on a linear map. The same color codes are used throughout. In the *eve* locus, *nhomie* (blue arrow) and *homie* (red arrow) are oriented, by convention, so that they are pointing toward the right. This convention is maintained in the two transgenes. In *GeimohL*, *homie* (top) is in the opposite orientation from *homie* in the *eve* TAD and so is pointing away from the *eve* TAD. In *GhomieL* (bottom) *homie* is in the same orientation as *homie* in the *eve* locus and so is pointing towards the *eve* TAD. B) Predicted boundary pairing interactions between *GeimohL* and the *eve* TAD. *homie* in the transgene pairs with *homie* in the *eve* TAD head-to-head. Since *homie* in the transgene is pointing in the opposite orientation from *homie* in the *eve* TAD, a stem-loop will be generated. C) If *homie* in the transgene also pairs with *nhomie* in the *eve* TAD head-to-tail, a loop structure like that shown in C will be generated. In this topology, *eve-lacZ* is in close proximity to *eve* enhancers, and *eve-gfp* is in contact with the *hebe* enhancer. D) Loop-extrusion model for *GeimohL*. Transgene *homie* and endogenous *homie* determine the endpoints of the extruded *eveMammoth* (*eveMa*) loop. Like B, the topology of *eveMa* is a stem-loop. This topology brings *eve-lacZ* into close proximity to the *eve* enhancers, while *eve-gfp* is in another loop that contains the *hebe* enhancers. E) Predicted boundary pairing between *GhomieL* and the *eve* locus. The *GhomieL* transgene is inserted in the same chromosomal orientation as *GeimohL*; however, the *homie* boundary is inverted so that it is in the same orientation as the *eve homie*, and so it is pointing towards the *eve* TAD. *homie* in the transgene will pair with *homie* in the *eve* locus head-to-head, and this generates a circle-loop. F) If *homie* in the transgene also pairs with *nhomie* in the *eve* TAD, a loop structure like that shown in F will be generated. In this topology *eve-gfp* will be activated by both the *eve* and *hebe* enhancers. G) Loop-extrusion model for *GhomieL*. The cohesin complex bypasses transgenic *homie* and is stopped at an upstream boundary X, to generate a novel *eveGarantuan* (*eveGa*) loop. Both *eve-gfp* and *eve-lacZ* are located within the same *eveGa* TAD, and thus should interact with *eve*. Since the *hebe* enhancers are within this TAD as well, they would also be able to activate both reporters.

gfp is upstream of *homie*, while *eve-lacZ* is downstream. These two transgenes were then individually inserted into the *attP* site at –142 kb. For the pair shown in **Fig. 3A**, *GeimohL* and *GhomieL*, the transgenes were inserted so that *eve-lacZ* is on the *eve* side of the *homie* boundary. The *eve-gfp* reporter is on the opposite side of the *homie* boundary and is farther away from the *eve* locus. This places the *eve-gfp* reporter next to a series of *hebe* enhancers located farther upstream (**Fig. 2C**), in the intron of the *hebe* gene, and thus it should be subject to regulation by these enhancers. In the other pair, *LhomieG* and *LeimohG*, the entire transgene is inserted in the opposite orientation in the chromosome so that *eve-gfp* is on the *eve* side of *homie*, while *eve-lacZ* is next to the *hebe* enhancers (**Fig. 6A**). As a control, we also generated a transgene in which *lambda* DNA instead of *homie* was inserted between the *eve-gfp* and *eve-lacZ* reporters. This transgene was oriented so that *eve-lacZ* was on the *eve* side of the *lambda* DNA, and *eve-gfp* was close to the *hebe* enhancers.

The eve enhancers drive lacZ expression in the GeimohL insert

The results for transgenes *GeimohL* and the control *GlambdaL* are most straightforward and will be considered first. In both the loop-extrusion and boundary-pairing models, the *GlambdaL* control is not expected to display any regulatory interactions with the *eve* locus. **Fig. 4C** shows that this is the case: the *eve-lacZ* and *eve-gfp* reporters in *GlambdaL* embryos are silent at the blastoderm stage, and transcripts are only very rarely detected. Later in development, both reporters are expressed in a repeating pattern along the dorsal midline (**Fig. 4E**). This expression is driven by the *hebe* gene enhancers located just beyond the *gfp*-reporter (Fujioka et al. 2009).

In the case of the *GeimohL* insert, regulatory interactions between the transgene and the *eve* locus will be observed if a stem-loop is established that links the transgene to *eve*. In the loop-extrusion model, the breakthrough cohesin complexes would generate a stem-loop, *eveMammoth* (*eveMa*), by coming to a halt at the *homie* boundary in the transgene and the *homie* boundary in *eve*, as illustrated in **Fig. 3D**. This will place the *lacZ* reporter in close proximity to the *eve* enhancers. It will also disrupt the *eve* TAD, as *nhomie* is no longer linked to the *homie* boundary by cohesin. In the boundary-pairing model, the *lacZ* reporter in the transgene is “upstream” of *homie* just like the *eve* gene and its enhancers are “upstream” of *homie* in the *eve* locus. Since *homie* pairs with itself head-to-head, pairing between *homie* in the transgene and *homie* in the *eve* locus will generate the same stem-loop as that generated by loop-extrusion (**Fig. 3B**). However, because *homie* pairs with *nhomie* head-to-tail, a more complex multi-loop structure like that in **Fig. 3C** would be expected if the pairing interactions with the transgene do not disrupt the *eve* TAD. Consistent with a tripartite structure of the sort shown here, previous studies have shown that three *Mcp* transgenes on three different chromosomes can interact with each other genetically (Muller et al. 1999). Subsequent direct visualization of *Mcp*-mediated pairing interactions in imaginal discs by Vazquez et al. (Vazquez et al. 2006) showed that four *Mcp*-containing transgenes separated by Mbs (at Bridges cytogenetic intervals ~65 on the left arm of chromosome 3, and ~83 and ~95 on the right arm of chromosome 3) and/or located on different homologs, clustered in the same nuclear foci in 94% of the nuclei.

As predicted from the formation of a stem-loop linking the transgene to the *eve* locus, *lacZ* is expressed in blastoderm-stage embryos in 7 stripes that coincide with the 7 stripes of the endogenous *eve* gene. However, unlike *eve*, not all nuclei in the seven stripes express *lacZ* (compare with the *eve* control in **Fig. 4A**). Later, during mid-embryogenesis, *lacZ* is expressed in the mesoderm, the CNS, and the anal plate in a pattern that mimics the endogenous *eve* gene. At this stage almost all *eve*-positive cells are also positive for *lacZ*. Unlike the *GlambdaL* transgene, the *hebe* enhancers do not drive *eve-lacZ* expression, as they are beyond the *homie* boundary (**Fig. 4E**). The *eve-gfp* reporter in the *GeimohL* transgene responds differently. As was observed for the *GlambdaL* control, the *eve* enhancers drive little if any *gfp* expression, either at the blastoderm stage or later in development (**Fig. 4C, E**). Instead, *gfp* is expressed in the midline during mid-

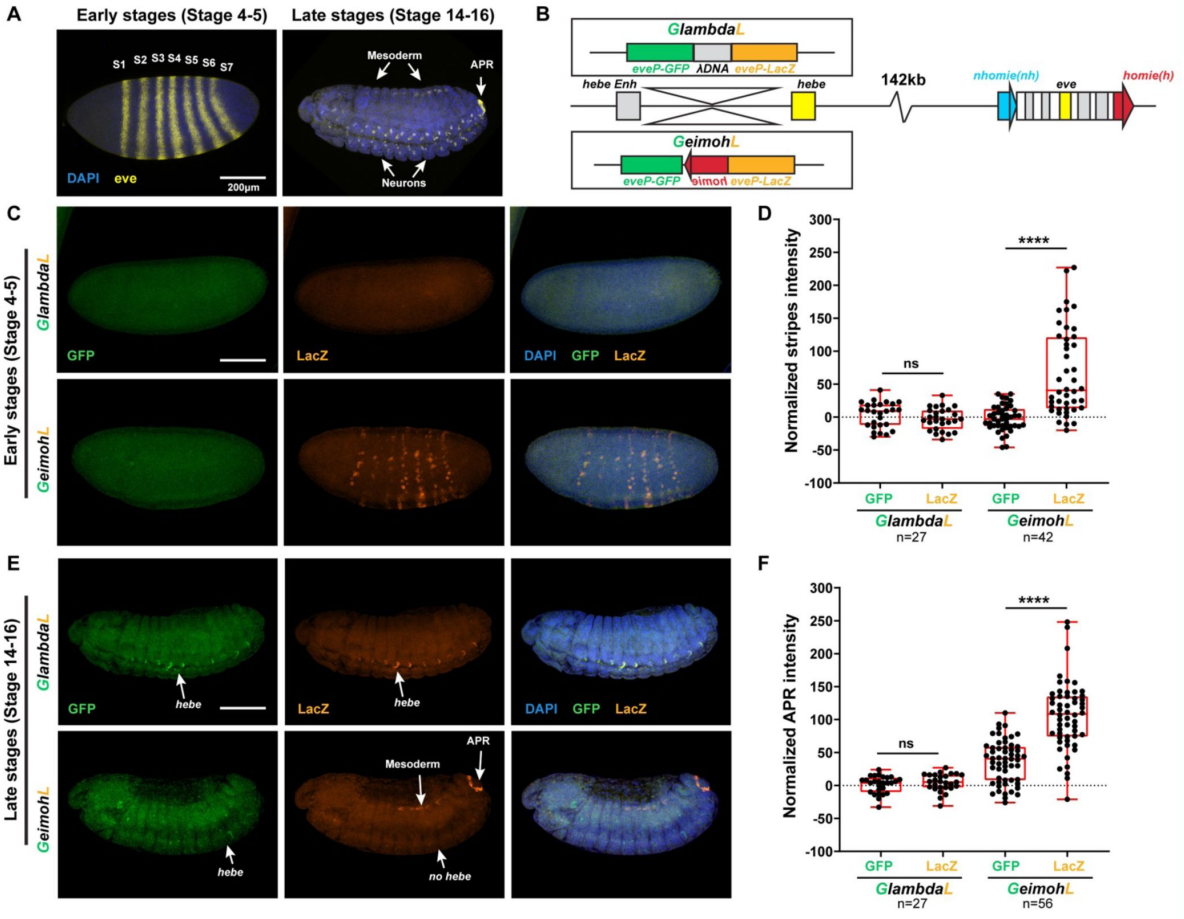

Figure 4.

The *lacZ* reporter is activated by *eve* enhancers in the *GeimohL* insert.

A) Expression patterns of endogenous *eve* in early (stage 4-5) and late (stage 14-16) embryos by smFISH. *eve* (Atto 633) probes were used to hybridize with *eve* mRNA. *eve* is shown in yellow and DAPI in blue. N>3. B) Schematics of transgenes. C) Expression patterns of transgenic reporters in early-stage embryos. Top: *GlambdaL*; bottom: *GeimohL*. *GFP* is in green, *lacZ* is in orange, and DAPI is in blue, here and in E. D) Quantification of normalized stripe signals for transgenes shown in C. The reporter smFISH intensity (signal to background), here and in F, was measured, normalized, and plotted as described in Methods. N>3. n=27 for *GlambdaL* and n=42 for *GeimohL*. E) Expression patterns of transgenic reporters in late-stage embryos. Top: *GlambdaL*; bottom: *GeimohL*. F) Quantification of normalized stripe signals for transgenes shown in E. N>3. n=27 for *GlambdaL*, n=56 for *GeimohL*. Scale bars = 200µm. N = # of independent biological replicates. n = # of embryos. The paired two-tailed t-test was used for statistical analysis. ****p < 0.0001, ns: not significant. Raw measurements are available in the Source Data files.

embryogenesis under the control of the *hebe* enhancers (**Fig. 4E**), which should be in the same TAD as *eve-gfp*. These results would be consistent with both the loop-extrusion (*eveMa*) and boundary-pairing models (**Fig. 3B**/C and D).

The eve enhancers drive gfp expression in the GhomieL insert

The situation is more complicated for the *GhomieL* transgene. This transgene is inserted in the same chromosomal orientation as *GeimohL*: *eve-lacZ* is on the *eve* side of the *homie* boundary and *eve-gfp* is on the *hebe* enhancer side of the boundary. However, the orientation of the boundary within the transgene is switched so that *eve-gfp* rather than *eve-lacZ* is "upstream" of *homie*. In the boundary-pairing model, flipping the orientation of *homie* in the transgene but keeping the same orientation of the transgene in the chromosome will have two consequences. First, as illustrated in **Fig. 3E**, the topology of the chromatin loop connecting the *homie* boundary in the transgene and the *homie* boundary in the *eve* locus will change from a stem-loop to a circle-loop. The reason for the change in loop topology is that *homie* pairs with *homie* head-to-head (Fujioka et al. 2016). As before, the transgene *homie* will pair with *eve homie* head-to-head to generate a simple circle-loop (**Fig. 3E**); however, if, as expected, it also pairs with *nhomie* so that the *eve* TAD remains intact, a more complicated loop structure would be generated (**Fig. 3F**). Second, the reporter that is preferentially activated by the *eve* enhancers will switch from *eve-lacZ* to *eve-gfp*. In order to be activated by the *eve* enhancer, the reporter must be "upstream" of the transgene *homie*. This physical relationship means that the *eve-gfp* reporter will be activated by the *eve* enhancers independent of the orientation of the transgene in the chromosome. On the other hand, the *eve-lacZ* reporter will not be activated by the *eve* enhancers. In addition, it will still be insulated from the *hebe* enhancers by the *homie* boundary.

It is not possible to form a circle-loop in the loop-extrusion model. Instead, the breakthrough cohesin complex will come to a halt as before when it encounters the inverted *homie* boundary at –142 kb (**Fig. 3D**). This means that the *eve-lacZ* reporter will be in the same TAD (*eveMa*) as the *eve* enhancers and will be activated by the *eve* enhancers. In contrast, the *eve-gfp* reporter will be in the neighboring TAD and will not be activated by the *eve* enhancers. It will, however, be regulated by the nearby *hebe* enhancers.

Fig. 5 shows that the four predictions of the boundary-pairing model are correct: a) the *eve* enhancers drive *eve-gfp* expression, b) the *hebe* enhancers drive *eve-gfp* expression, c) *eve-lacZ* is not subject to regulation by the *eve* enhancers, and d) *eve-lacZ* is insulated from the *hebe* enhancers. This would also mean that pairing of the transgene *homie* in the *GhomieL* insert with *homie* in the *eve* locus generates a circle-loop (or a more complicated multi-loop structure if it also pairs with *nhomie*), not a stem-loop, as was the case for *GeimohL*. In contrast, since loop-extrusion cannot generate circle-loops, the key predictions of this model, namely that *eve-gfp* will be silent while *eve-lacZ* will be regulated by the *eve* enhancers, are not satisfied. Instead, *eve-gfp* is activated by the *eve* enhancers, while *eve-lacZ* is not.

While the results described above for the *GhomieL* transgene are inconsistent with a "simple" loop-extrusion model, there is one potential caveat: the 5'→3' orientation of the *homie* boundary is inverted so that it is "pointing" towards the *eve* locus rather than away from it like the endogenous *homie* boundary. In this orientation, it is possible that *homie* no longer functions as an effective roadblock, and instead the cohesin complex emanating from the *eve* locus (or from a loading site somewhere in between) slips past this *homie* fragment and continues until it is blocked by a properly oriented boundary further upstream. In this case the newly formed stem-loop, *eveGargantuan* (*eveGa*) (**Fig. 3G**), would include the *eve-gfp* reporter, and it would then be activated by the *eve* enhancers. However, there are two problems with postulating this novel *eveGa* TAD. First, *eve-lacZ* would be in the same *eveGa* TAD as *eve-gfp*, and it should also be

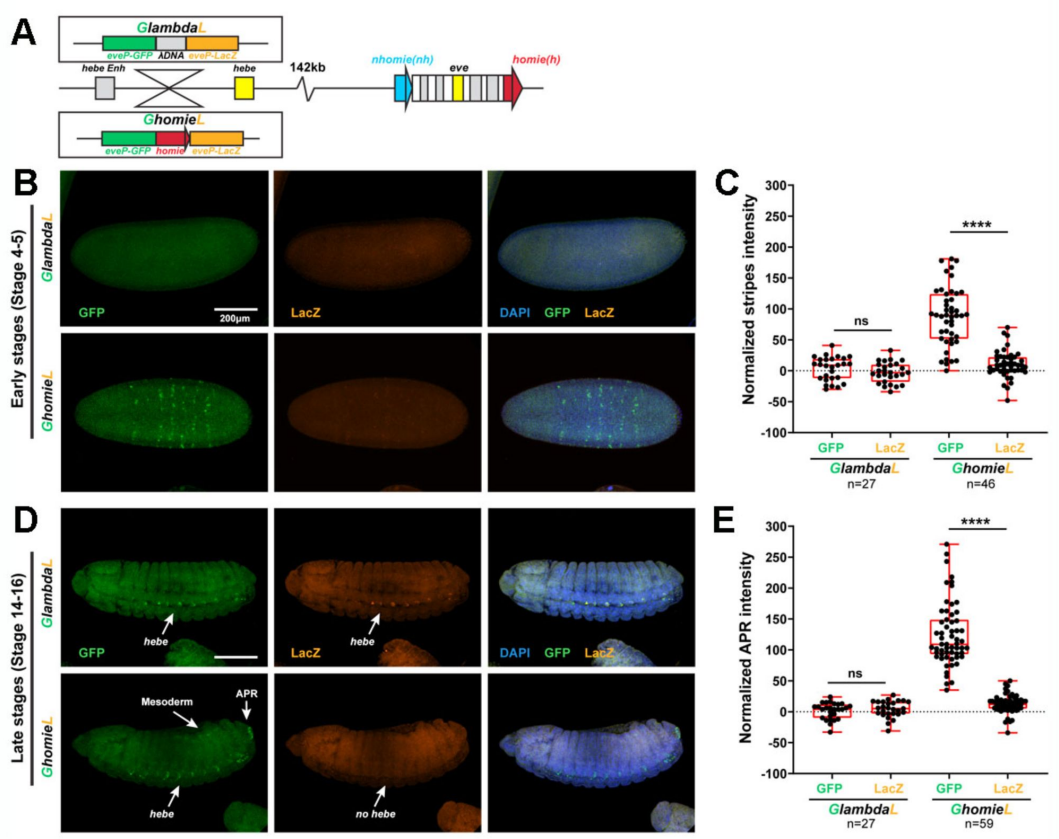

Figure 5.

The *GFP* reporter is activated by both *eve* and *hebe* enhancers in the *GhomieL* insert.

A) Schematics of transgenes. B) Expression patterns of transgenic reporters in early-stage embryos. Top: *GlambdaL*; bottom: *GhomieL*. *GFP* is in green, *lacZ* is in orange, and DAPI is in blue, here and in D. C) Quantification of normalized stripe signals as represented in B. The reporter smFISH intensity (signal to background) was measured, normalized, and plotted as described in Methods, here and in E. N>3. n=27 for *GlambdaL,* n=46 for *GeimohL*. D) Expression patterns of transgenic reporters in late-stage embryos. Top: *GlambdaL*; bottom: *GhomieL*. E) Quantification of normalized stripe signals for transgenes shown in D. N>3. n=27 for *GlambdaL*, n=59 for *GeimohL*. Scale bars = 200μm. N = # of independent biological replicates. n = # of embryos. The paired two-tailed t-test were used for statistical analysis. ****p < 0.0001, ns: not significant. Raw measurements are available in the Source Data files.

activated by the *eve* enhancers. Second, since the *homie* boundary was bypassed by the cohesin complex to form the larger *eveGa* TAD, it should not be able to block the *hebe* enhancers from activating *eve-lacZ*. Neither of these predictions is correct.

LeimohG and LhomieG: Orientation of homie in the transgenes determines which reporter is activated

To confirm these conclusions, we examined the expression patterns of the reporters when the transgene is inserted in the opposite orientation in the –142 kb attP site. In this transgene orientation, *eve-gfp* is on the *eve* side of the *homie* boundary while *eve-lacZ* is close to the *hebe* enhancers. In the boundary-pairing model, the orientation of *homie* in the transgene determines which reporter will be preferentially activated, while the orientation of the transgene *homie* in the chromosome determines topology of the resulting loop(s). The transgene *homie* will pair with the endogenous *homie* head-to-head. Since the transgene *homie* in *LeimohG* is in the opposite orientation from the endogenous *homie*, this pairing interaction will generate a stem-loop, if pairing interactions are exclusively pairwise. A more complicated multi-loop structure will be formed if the transgene *homie* interacts simultaneously with both endogenous boundaries (**Fig. 6B**). In both cases, *eve-gfp* will be activated by the *eve* enhancers. On the other hand, since this orientation places *eve-lacZ* on the same side of the transgene *homie* as the *hebe* enhancers, they will activate it rather than the *eve-gfp* reporter (**Fig. 6B**). In *LhomieG*, the topology of the loop (or multi-loop) will switch, and *eve-lacZ* will be activated by both the *eve* and *hebe* enhancers (**Fig. 6C**).

In the first version of the loop-extrusion model, the *eve-gfp* reporter in both *LeimohG* (**Fig. 6D**) and *LhomieG* (not shown) should be activated by the *eve* enhancers, since it is included in the *eveMa* TAD when the transgenes are inserted so that *eve-gfp* is on the *eve* side of the *homie* boundary, and *eve-lacZ* will be regulated by the *hebe* enhancers (**Fig. 6D**). In the second version of the loop-extrusion model, the functioning of the *homie* roadblock depends upon its orientation relative to endogenous *homie*. In this model, *LeimohG* is expected to form the *eveMa* TAD (**Fig. 6D**), while *LhomieG* will form the larger *eveGa* TAD (**Fig. 6E**).

As would be predicted by all three models, the *eve* enhancers activate the *eve-gfp* reporter in the *LeimohG* insert (**Fig. 6F and G**). Likewise, *homie* blocks the *hebe* enhancers from activating the *eve-gfp* reporter, while they turn on *eve-lacZ* expression instead. As predicted by the boundary-pairing model, *eve*-dependent expression of the reporters switches from *gfp* to *lacZ* in the *LhomieG* insert (**Fig. 6F and G**). This would not be expected in the first version of the loop-extrusion model, but it is predicted in the second version. However, as indicated in the diagram in **Fig. 6E**, the two reporters, along with the *hebe* enhancers, are included in the larger *eveGa* TAD, and for this reason both reporters should be activated equally by both the *eve* and *hebe* enhancers. This is not the case. The *hebe* enhancers are blocked by the intervening *homie* boundary, while there is little or no *eve*-dependent *gfp* expression.

TAD formation by transgenes inserted at –142 kb

We used MicroC to examine the TAD organization of the genomic region extending from upstream of the attP site through the *eve* locus in 12-16 hr embryos carrying different transgene insertions. At this stage, *eve* is expressed in only a small number of cells in the CNS, mesoderm and anal plate, and *hebe* is also expressed in only a small subset of cells. This means that most of the interactions detected by MicroC are in cells in which both the *eve* gene and the two reporters are inactive. In addition, while the transgene co-localizes with *eve* in less than about a fifth of blastoderm stage nuclei (based on the fraction of cells within *eve* stripes that activate a transgene reporter) the frequency of physical contact is expected to be much higher in 12-16 hr embryos (c.f., Vazquez et al.: Vazquez et al. 2006). This is because at this stage of development, cell cycles are much longer, providing more time for transgene – endogenous *eve* pairing to occur. The key findings are summarized below.

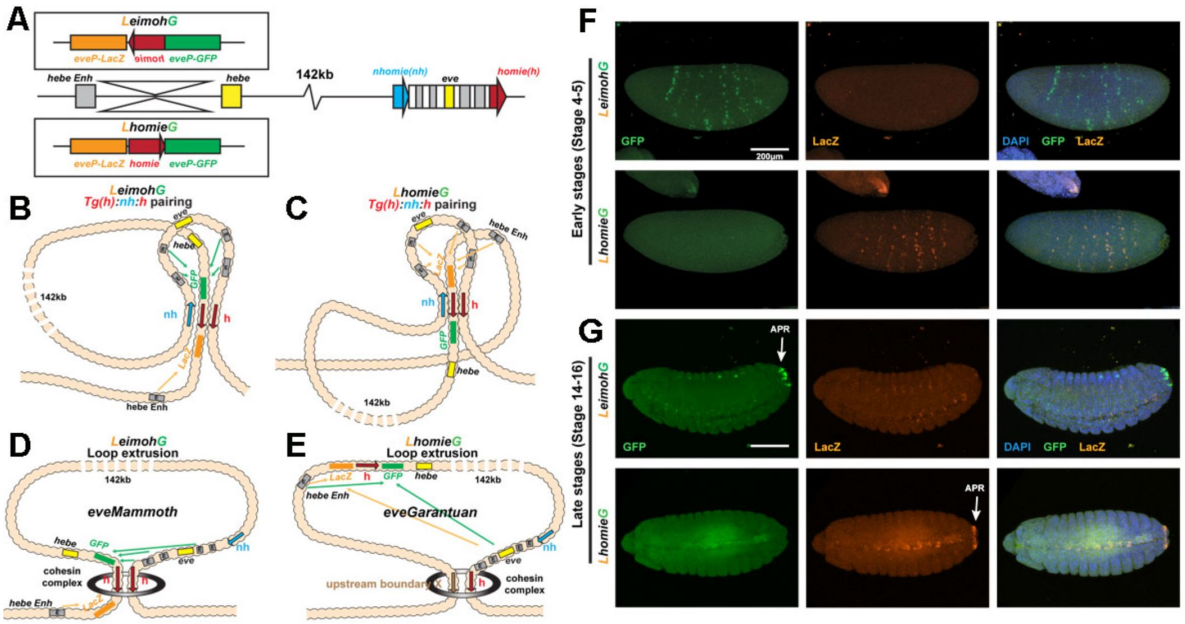

Figure 6.

Expression of reporters in the *LeimohG* and *LhomieG* inserts.

A) Schematics of GE transgenes. B) Boundary pairing for *LeimohG* transgene long-range interactions. In this topology, *GFP* is in close proximity to *eve* enhancers, and *lacZ* is in contact with the *hebe* enhancer region. C) Boundary pairing for the *LhomieG* transgene. In this topology, *lacZ* is in close proximity to both the *eve* enhancers and the *hebe* enhancers. D) Loop-extrusion model for *LeimohG*. Transgenic *homie* and endogenous *homie* flank the *eveMammoth* loop. In this topology, *GFP* is in close proximity to *eve* enhancers, and *lacZ* is close to the *hebe* enhancers. E) Loop-extrusion model for the *LhomieG* transgene. The cohesin complex bypasses transgenic *homie* and is stopped at an upstream boundary X, to create the *eveGarantuan* loop. Both *GFP* and *lacZ* are at a similar physical distance from *eve* enhancers and *hebe* enhancers in this topology. F) Expression patterns of transgenic reporters in early-stage embryos. Top: *LeimohG*; bottom: *LhomieG*. *GFP* is in green, *lacZ* is in orange, and DAPI is in blue. N=3, n=24 for both *LeimohG* and *LhomieG*. G) Expression patterns of transgenic reporters in late-stage embryos. Top: *LeimohG*; bottom: *LhomieG*. N=3, n=24 for both *LeimohG* and *LhomieG*. Scale bars = 200μm, N = # of independent biological replicates, n = # of embryos. Statistical analysis is in Table Supplemental #1.

GlambdaL: While there is no evidence of contact between sequences in *eve* and sequences around the attP site at –142 kb in wild-type embryos, this not true for embryos carrying the dual reporter transgene. Perhaps the most unexpected finding is a weak, but clearly discernable pattern of interaction linking the *GlambdaL* transgene to the *eve* locus (**Fig. 7A**). However, since the *GlambdaL* transgene does not respond to the *eve* enhancers, this low level of physical interaction is not sufficient to drive detectable expression of the reporters. Though our experiments do not allow us to unequivocally identify which sequences in the transgene are responsible for this long-distance interaction, the most likely candidates are the two *eve* promoters in the *GlambdaL* transgene.

GeimohL: Unlike *GlambdaL*, the *eve-lacZ* reporter in the *GeimohL* insert is activated by the *eve* enhancers, and this regulatory interaction is perfectly paralleled by the strong pattern of crosslinking between the transgene and the *eve* locus (**Fig. 7B**). On the *eve* side, the interactions between the transgene and the *eve* locus generate a heavily populated band of crosslinked sequences that span the 16 kb *eve* TAD. On the transgene side, there is an unequal distribution of crosslinked sequences to either side of the transgene *homie* boundary. As shown in the inset, the heaviest density of crosslinked sequences is on the *eve-lacZ* side of the transgene, consistent with the activation of this reporter by *eve* enhancers. To confirm that the interaction bias mimics the differences in activity of the two reporters in cells in which the reporters are expressed, we analyzed the interaction profiles from viewpoints within either the *eve-lacZ* or *eve-gfp* genes. **Fig. 7D** shows that *eve-lacZ* interacts with sequences extending across the *eve* TAD, while *eve-gfp* interactions are largely restricted to the *eve homie* element. While these results would seemingly be consistent with both the boundary-pairing and loop-extrusion models, there is no evidence of cohesin breaking through multiple intervening boundaries into order to establish the novel *eve(Mam)* TAD. Instead, the organization of the TADs and LDC domains in the interval in between the transgene and *eve* (**Fig. 7B**) resemble that seen in wild-type NC14 embryos (see **Fig. 2**) (with the caveat that there are significantly fewer reads in the transgene experiment). Equally important, the same is true for the *eve* TAD. In order to generate the *eve*(*Mam*) TAD, the cohesin complex must break through the *nhomie* boundary and, in so doing, disrupt the *eve* TAD. However, there are no obvious alterations in either the *eve* volcano or plume with respect to their structure or relative contact density compared to the *GlambdaL* and wild type NC14 embryos. Likewise, there is no evidence of novel vertical and 45° crosslinking stripes that would reflect the boundary breakthrough events that are expected in the loop-extrusion model to accompany the formation of a physical connection between the transgene and the *eve* locus.

We next used the transgene *homie* as the viewpoint to test the predictions of the boundary-pairing and loop-extrusion models. Since *homie* can pair with itself (head-to-head) and with *nhomie* (head-to-tail) in transvection assays (Fujioka et al. 2016), a prediction of the boundary-pairing model is that there will be physical interactions between the transgene *homie* and ***both*** *homie* and *nhomie* in the *eve* locus (c.f., Vazquez et al. 2006). Also based on what is known about how fly boundaries pair with each other (Chetverina et al. 2014; Chetverina et al. 2017; Kyrchanova et al. 2008a), self-interactions are expected to be stronger than heterologous interactions. In contrast, in the loop-extrusion model, the cohesin complex forms the *eveMa* TAD by linking *homie* in the transgene to *homie* in the *eve* locus, and this requires the cohesin complex to break through *nhomie* and disrupt the connections between *nhomie* and *homie* in the *eve* locus. In this case, *transgene:homie*←→*eve:homie* interactions should be observed, while *transgene:homie*←→*eve:nhomie* should not. **Fig. 8A** shows that, as predicted by the boundary-pairing model, the transgene *homie* interacts with both *homie* and *nhomie* in the *eve* locus.

Since the *eve* TAD does not appear to be disturbed by the presence of the *GeimohL* transgene, these findings would suggest that the transgene *homie* likely pairs with both *nhomie* and *homie* in a pattern somewhat like that illustrated in **Fig. 3C**. While the physical interactions between the transgene and *eve* boundaries are inconsistent with the loop-extrusion model, one could make the *ad hoc* assumption that runaway cohesin complexes form not one but two different novel TADs.

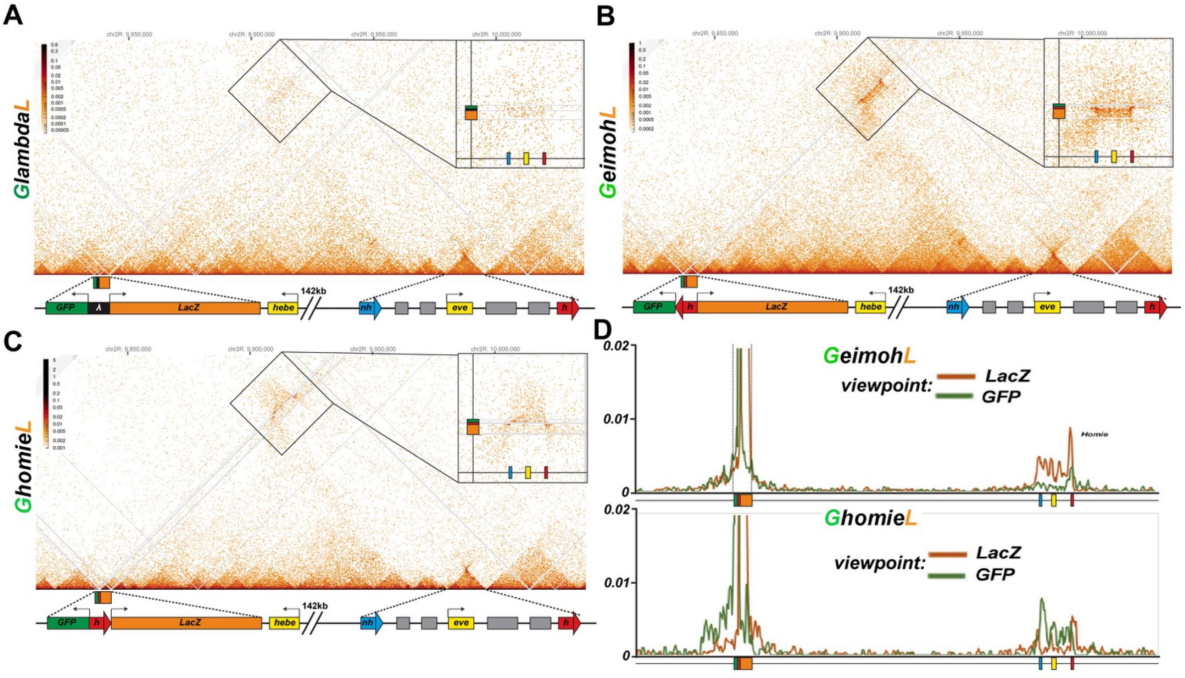

Figure 7.

homie in transgenes *LhomieG* and *LhomieG* mediates long-range physical interactions with the *eve* TAD.

Diagrams for panels A, B, and C. yellow box: 5' end of the *hebe* transcription unit. Transgene: green box: *eve-gfp* reporter, orange box: *eve-lacZ* reporter, black box: *lambda* DNA, red block arrow: *homie* DNA. *eve* TAD: blue block arrow: endogenous *nhomie*, red block arrow: endogenous *homie*; the directions of block arrows follows the established convention for *nhomie* and *homie*; gray boxes: *eve* enhancers, yellow box: *eve* transcription unit. A) MicroC contact profile of the control *GlambdaL*. Inset shows a blow-up of the contacts between the *GlambdaL* transgene and the *eve* TAD. Note a slight increase in interaction frequency (compare to **Figure 1A**). B, C**)** MicroC map of *GeimohL* and *GhomieL*, respectively. The key difference between the two inserts is the contacts between transgenes and sequences in the *eve* TAD. Note the changed pattern of interaction with the endogenous *eve* locus due to the orientation switch. D) "Virtual 4C" maps obtained from MicroC maps of "B" (top panel) and "C" (bottom panel). Viewpoints are shown in both panels from either the *lacZ* gene (orange) or the *GFP* gene (green).

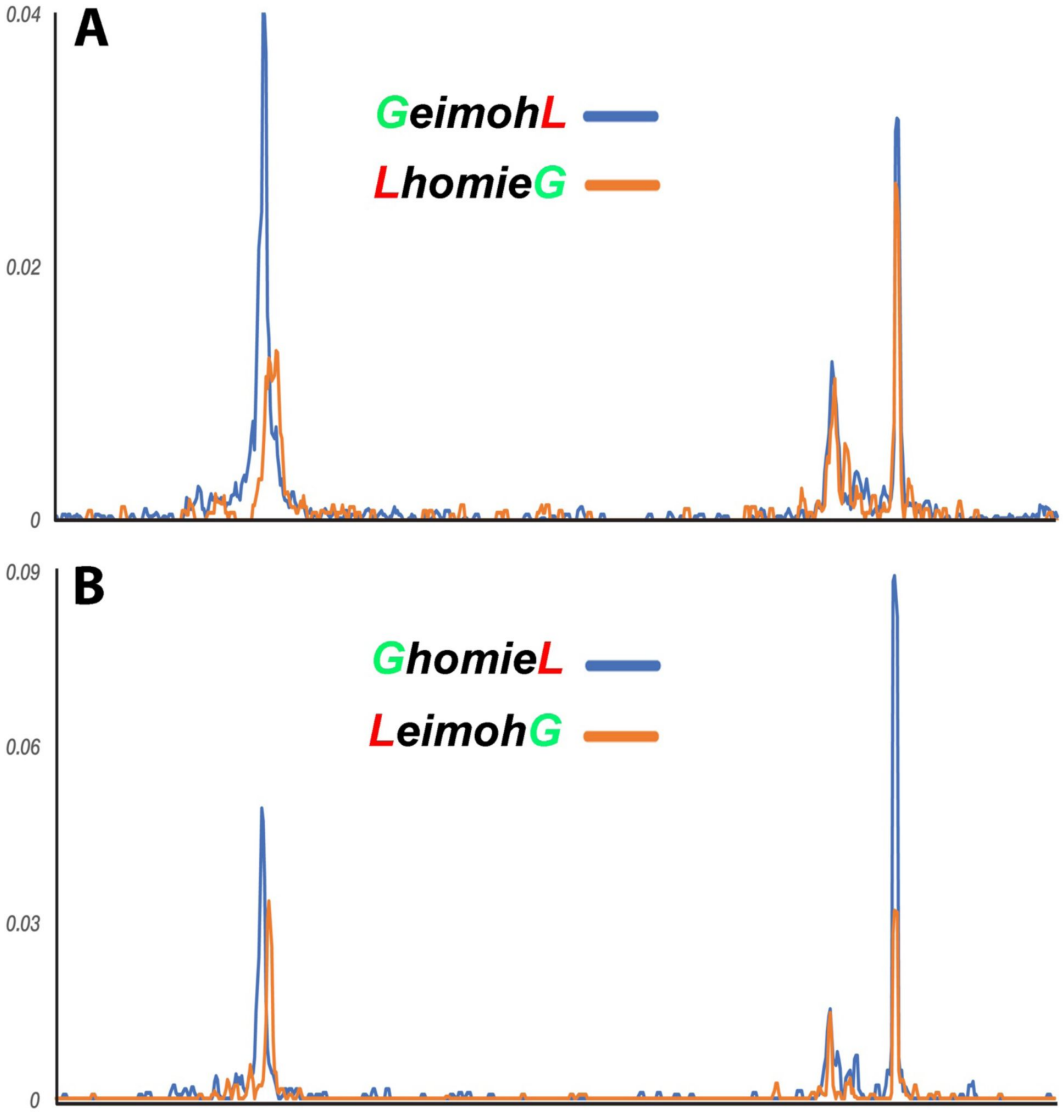

Figure 8.

homie* in the transgene interacts with *eve homie* and *nhomie.

"Virtual 4C" maps of the *GeimohL* and *LhomieG* (A) or *GhomieL* and *LeimohG* (B) transgenes oriented so that *lacZ* (blue) or *gfp* (orange) is located on the *eve* side of the *homie* boundary. Viewpoints are taken from the transgenic *homie* sequence. Note that transgenic *homie* preferentially interacts with endogenous *homie* in both orientations.

One would be *eveMa*. In the other, *eveElephant* (*eveEl*), a cohesin complex that initiates in, for example, the TE TAD would come to a halt on one side at the *homie* boundary in the transgene, and on the other side at the *nhomie* boundary in the *eve* locus (**Fig. Supplemental 4**). However, this configuration would not explain how the transgene reporter is activated by *eve* enhancers. Since the *eve* enhancers in *eveEl* are located in a different TAD than the TADs containing *eve-lacZ* and *eve-gfp*, they would not be able to activate either reporter.

GhomieL: When *homie* is reversed in the *GhomieL* transgene, the *eve-gfp* reporter responds to the *eve* enhancers instead of *eve-lacZ*. In the boundary-pairing model, *eve-gfp* is activated because self- and heterologous-pairing interactions between *homie* and *nhomie* are orientation-dependent. As a result, the topology of the loop formed between the transgene and *eve* is transformed in a way that brings the *eve-gfp* reporter instead of the *eve-lacZ* reporter into close proximity to the *eve* enhancers (see **Fig. 3E, F**). Since only stem-loops can be generated in the loop-extrusion model, activation of the distal *eve-gfp* reporter would require the formation of a larger *eveGa* TAD in order to encompass both the transgene and the *eve* enhancers in the same looped domain (**Fig. 3G**). However, as shown in **Fig. 7C**, we do not observe the cross-linking pattern expected for a new *eveGa* TAD that includes the entire transgene at –142 kb and then extends to an even more distal boundary. Nor are there any novel vertical/45° crosslinking stripes or obvious perturbations in the basic TAD organization in the region beyond –142 kb, or between –142 kb and the *eve* locus, as might be expected for cohesin complexes breaking through multiple intervening roadblocks. Instead, as was the case for *GeimohL*, the *GhomieL* transgene is physically linked to the *eve* locus, and there is a prominent band of crosslinked transgene sequences that spans the *eve* TAD (**Fig. 7C**). Consistent with the expression patterns of the two reporters, the heaviest density of crosslinked sequences is on the *eve-gfp* side of the transgene (see inset). This is confirmed by the interaction profiles generated using either the *eve-gfp* or *eve-lacZ* gene body as the viewpoint: the most frequent contacts are between sequences within *eve* and the *eve-gfp* reporter (**Fig. 7D**). In addition, as was the case for *GeimohL*, when the transgene *homie* is used as the viewpoint, interactions are observed between both *homie* and *nhomie* in the *eve* locus, with the most frequent corresponding to self-pairing interactions (**Fig. 8B**). Other than postulating the *ad hoc eveEl* TAD (**Fig. Supplemental 4**), this would not be expected for either version of the loop-extrusion model.

LhomieG and *LeimohG*: To confirm that the orientation of the *homie* boundary in the transgene is the key determinant of the physical interactions between the transgene reporters and the *eve* TAD, we used MicroC to analyze the TAD organization of *LhomieG* and *LeimohG* (Figs. Supplemental 5 and 6). Like their counterparts *GhomieL* and *GeimohL*, both show a strong band of interaction between the transgene and sequences spanning the *eve* locus. In the case of *LeimohG*, the pairing of the transgene *homie* with the endogenous *homie* is predicted to generate a stem-loop, while a more complicated multi-loop structure will be generated if it also pairs with *nhomie* (see **Fig. 6B**). As shown in **Fig. Supplemental 5A**, these pairing interactions bring *eve-gfp* into contact with the *eve* enhancers and, as expected, the *eve-gfp* sequences interact more frequently with the *eve locus* than does *eve-lacZ* (inset **Fig. Supplemental 5A**). When *homie* is inserted in the transgene in opposite orientation, *homie* self-pairing is expected to generate a circle-loop, while the more complicated structure shown in **Fig. 6C** will be formed if it also pairs with *nhomie*. While the sample preparation for MicroC was of poorer quality than the others, the inset in **Fig. Supplemental 6A** shows that, as expected, *eve-lacZ* interacts more frequently with sequences in the *eve* locus than does *eve-gfp*. On the other hand, there is no evidence of a larger *eveGa* TAD, even though *eve-lacZ* is activated by the *eve* enhancers. These interactions are confirmed when either *eve-lacZ* or *eve-GFP* are used as viewpoints to plot the physical contacts between the *eve* TAD and the two reporters in *LhomieG* and *LeimohG* (Figs. Supplemental 5B and 6B). In addition, as was the case for the *GhomieL* and *GeimohL* transgenes, when the transgene *homie* is used as the viewpoint, interactions are observed between *homie* in the transgene and both *homie* and *nhomie* in the *eve* locus (**Fig. 8A and B**). Again, the most frequent interactions are self-pairing interactions between *homie* in the transgene and the *eve homie*.

Loop topology and interactions with neighbors: The results in the previous sections demonstrate that the orientation of *homie* in the transgene determines which of the two reporters physically interacts with sequences in the *eve* TAD, and this is independent of the orientation of the transgene in the chromosome. On the other hand, the orientation of the transgene *homie* in the chromosome impacts how sequences flanking the transgene *homie* and the *eve* TAD interact with each other. With the important caveat that the depth of our MicroC sequencing is limited, the interaction profiles reflect the topology of the transgene-induced loop. For *homie* pointing away from *eve* (*GeimohL* and *LeimohG*), sequences to the *TER94* side of *eve* and to the *hebe* enhancer side of the transgene are more frequently crosslinked with each other (**Fig. Supplemental 7**). These interactions would be expected for either a stem-loop structure in **Fig. 3B** or the more complicated multi-loop structure shown in **Fig. 3C**. For *homie* pointing towards *eve*, sequences on the *TER94* side of *eve* interact with sequences on the *eve* side of the transgene. This would be expected for either a circle-loop structure in **Fig. 3E** or the more complicated multi-loop structure shown in **Fig. 3F** (neither of which can be generated by a simple loop-extrusion mechanism).

Discussion

Two different mechanisms have been proposed to explain how TADs are formed and their endpoints determined. In the first, a cohesin complex first induces a small loop at a loading site within the TAD-to-be and then progressively extrudes one or both strands until the complex encounters boundary roadblocks. If the loading site is located asymmetrically within the TAD-to-be, extrusion will continue on the unblocked strand until a roadblock is encountered. The endpoints of the loops were initially thought to be determined by the location of the first two roadblocks encountered by the cohesin complex (Alipour and Marko 2012). In a refinement of this model, the orientation of a given roadblock relative to the direction of movement of the cohesin complex determines whether it will come to a halt (Davidson and Peters 2021; Fudenberg et al. 2016; Guo et al. 2012; Guo et al. 2015; Sanborn et al. 2015). It has also been suggested that cohesin complexes can break through a roadblock and come to a halt at more distant roadblocks, so that in a population of cells analyzed by Hi-C or MicroC, the basic units of organization, the TADs, are overlaid by a hierarchy of LDC domains (Hsieh et al. 2020; Krietenstein et al. 2020). However, the only loop topologies that are possible in the loop-extrusion model are a stem-loop and an unanchored loop.

In the other mechanism, TAD formation is governed by boundary:boundary pairing interactions. In this case, the critical factors are the pairing and orientation preferences of potential partners, together with proximity. Since boundary pairing interactions often exhibit an orientation preference, the topology of the loop formed by paired boundaries can be either a stem-loop or a circle-loop, depending on their relative orientation in the chromosome. In this respect it differs from the loop-extrusion model in that switching the orientation of the boundary does not preclude the formation of a TAD, whereas it can in some versions of the loop-extrusion model.

In the studies reported here, we have used MicroC to analyze the TAD organization of a ~150 kb chromosomal segment flanking the *eve* locus, and have experimentally tested the predictions of these two models for the mechanisms of TAD formation. Our studies do not support a loop-extrusion mechanism, and are inconsistent with this model on multiple levels. *Manipulating "TAD" formation:* In our experimental paradigm, a transgene containing two divergently transcribed reporters and the *eve* boundary *homie* is inserted 142 kb upstream of the *eve* TAD. Depending upon the orientation of the *homie* boundary within the transgene, either *eve-gfp* or *eve-lacZ* is activated by developmentally regulated enhancers in the *eve* TAD. Since there are a series of well-defined TADs and their associated boundary elements separating the transgene from *eve*, a version of the loop-extrusion model in which the cohesin complex invariably arrests upon encountering *nhomie* and *homie* at the ends of the *eve* TAD cannot account for this long-distance regulation.

Instead, the extruding cohesin complex must be able to break through the boundaries located between *homie* in the transgene and *homie* in the *eve* TAD. This would generate a novel TAD, *eveMa*, with endpoints corresponding to the two *homie* boundaries (**Fig. 3D**). In this case, the reporter on the *eve* side of the transgene *homie* would be in the same domain as the *eve* enhancers, and thus potentially subject to regulation. While *eveMa* could account for reporter expression in inserts in which the transgene *homie* is pointing away from *eve* (and also away from the reporter on the *eve* side of the transgene insert), it does not explain reporter expression when the transgene *homie* is pointing towards *eve* (and in this case towards the reporter on the *eve* side of the transgene insert). In the latter case, the reporter activated by the *eve* enhancers lies outside of the *eveMa* (cf., **Fig. 3D**), and thus is not included in the same TAD as the *eve* enhancers. In order to account for activation of the distal reporter, one must imagine that the cohesin complex breaks through the *homie* boundary in the transgene and continues on until extrusion is halted by an even more distal boundary. In this revision of the loop-extrusion model, both reporters would be included in the same TAD as the *eve* enhancers (c.f., **Fig. 3G**, *eveGa*), and both reporters should be activated. However, this is not observed. *TAD organization: loop extrusion versus boundary pairing:* Analysis of the TAD organization in the region spanning the transgene insertion site and *eve* also does not fit with the expectations of the loop-extrusion model. To account for reporter activation, the cohesin complex must be able to break through the *nhomie* boundary. This breakthrough could be engineered by a cohesin complex that initiated loop extrusion either from within the *eve* TAD or from one of the TADs located between the *nhomie* boundary and the transgene at –142 kb. However, in the absence of the transgene, there is no indication that *nhomie* is particularly prone to break-through events. In fact, the *eve* TAD differs from most of the TADs in between *eve* and the transgene in that it is incorporated into larger LDC domains much less frequently. There is also no reason to imagine that the presence of a transgene carrying *homie* (or *nhomie*: see Fujioka et al. 2016) at –142 kb would somehow induce *nhomie* break-through events.

As expected from the regulatory interactions, *homie*-containing transgenes at –142 kb generate a strong band of crosslinking events that physically link the transgene to the *eve* locus. In the loop-extrusion model, this strong band of interaction should generate a novel interaction triangle (LDC or HDIC) with endpoints corresponding to the *homie* elements in the transgene and *eve*. Contrary to this expectation, a triangle of increased interaction frequencies spanning the entire region between –142 kb and *homie* is not observed for any of the transgene inserts. In viewpoints from the *homie* element in the *GeimohL* and *LeimohG* transgenes, there are two peaks in the *eve* TAD. The more prominent peak maps to *homie*, while the less prominent peak maps to *nhomie*. To account for these interaction peaks, the loop-extrusion model would have to assume that the presence of the *GeimohL* and *LeimohG* transgenes induces the formation of two novel TADs, the *eveMa* (*homie:homie*) TAD in **Fig. 3D and another** TAD, *eveElephant*, that links the transgene *homie* to the *nhomie* element in the *eve* locus (**Fig. Supplemental 4B**). However, while the *eveMa* TAD could potentially explain the activation of reporters on the proximal side of the transgene *homie* by the *eve* enhancers, these TADs do not explain how the *eve* enhancers are able to activate reporters on the distal (*hebe* enhancer) side of the *GhomieL* and *LhomieG* transgene. This would require the formation of yet another TAD, *eveGa*, that encompasses *eve*, the transgene, and one or more TADs beyond the *hebe* gene (**Fig. 3G**). However, there is no indication of physical interactions between the *homie* boundary in *eve* and a boundary located beyond the *hebe* gene. Moreover, like *eveMa*, *eveGa* would also have to be accompanied by the formation of *eveEl*, since the *homie* element in *GhomieL* and *LhomieG* interacts with both *nhomie* and *homie*.

While these results are inconsistent with the expectations of the loop-extrusion model, they dovetail nicely with many of the predictions of the boundary-pairing model. First, in all four transgenes, *homie* interacts with both *homie* and *nhomie* in the *eve* locus, but does not interact with the multiple boundary elements located in between. This is consistent with other studies which show that boundaries often have strong partner preferences (Gohl et al. 2011; Kyrchanova et al. 2011; Kyrchanova et al. 2008b). Second, interaction frequencies are greater for self-pairing than for heterologous pairing. This fits with what is known about how partner

preferences are determined (Blanton et al. 2003; Chetverina et al. 2017; Erokhin et al. 2021; Kyrchanova et al. 2008a; Zolotarev et al. 2016). Third, the pattern of reporter activation in the four transgenes is precisely as expected from previous studies on the orientation dependence of *homie* pairing with itself (head-to-head) and with *nhomie* (head-to-tail) (Chen et al. 2018; Fujioka et al. 2016). This orientation dependence means that the reporter located "upstream" of *homie* is the one that is activated, independent of the orientation of the transgene in the chromosome. Fourth, the pattern of physical interactions between the *eve* locus and the transgenes recapitulates the pattern of reporter activation. That is, when *eve-lacZ* sequences are preferentially crosslinked to sequences in the *eve* locus, then the *eve-lacZ* reporter is activated by the *eve* enhancers. Likewise, *eve-gfp* is preferentially crosslinked to sequences in *eve* when it is activated by the *eve* enhancers. Again, this is dependent on the orientation of *homie* in the transgene, but not on the orientation of the transgene in the chromosome. Fifth, unlike the loop-extrusion model where only stem-loops or unanchored loops can be generated, stem-loops, circle-loops and even more complicated structures can be, and, as we have shown here, actually are generated by boundary:boundary pairing.

Are the pairing interactions of homie and nhomie unusual? There are two reasons to think that the properties of most fly boundaries are similar to those of *homie* and *nhomie*. First, all of the fly boundaries that have been tested in assays that are expected to require pairing do have this activity (Blanton et al. 2003; Cai and Shen 2001; Erokhin et al. 2011; Gohl et al. 2011; Kyrchanova et al. 2008a; Kyrchanova et al. 2011; Kyrchanova et al. 2007; Kyrchanova et al. 2008b; Muravyova et al. 2001).

Second, multimerized binding sites for generic polydactyl zinc finger proteins like Su(Hw), Pita, dCTCF, Zw5, and Zipic that are found associated with many fly boundaries are not only able to block enhancer/silencer action but can also mediate pairing interactions in insulator bypass and transvection assays (Erokhin et al. 2021; Kyrchanova et al. 2008a; Zolotarev et al. 2016). Moreover, as is the case for *homie* and *nhomie*, pairing interactions require the appropriate partners: multimerized sites for Zw5 do not pair with multimerized sites for dCTCF (Erokhin et al. 2021; Kyrchanova et al. 2008a; Zolotarev et al. 2016). This makes good sense, since these proteins form dimers/multimers, as do other fly boundary factors like BEAF, Mod(mdg4), the BEN-domain protein Insensitive and the Elba complex (Avva and Hart 2016; Bonchuk et al. 2021; Bonchuk et al. 2011; Bonchuk et al. 2020; Fedotova et al. 2018; Fedotova et al. 2019; Fedotova et al. 2017; Hart et al. 1997). The ability to multimerize means that boundaries that share binding sites for the same protein, i.e., for Pita, Zipic or CTCF, can be physically linked to each other by a single protein complex.

Third, *homie* and *nhomie* are not the only examples of fly boundary elements that engage in specific orientation-dependent physical pairing interactions. In addition to *gypsy*, *Mcp* and *Fab-7*, Mohana, et al. (Mohana et al. 2023) identified nearly 60 meta-loops in which distant TADs are linked together by what appears in most instances to be orientation-dependent boundary pairing interactions. **Fig. 9** shows the MicroC contact profiles generated for two such meta-loops in 12-16 hr embryos. As observed in our *homie* transgene experiments, the meta-loop in panel A is formed by specific and orientation-dependent boundary:boundary (blue:purple) pairing interactions that bring together distant TADs. The blue boundary splits the *Leukocyte-antigen-related like* (*Lar*) gene into a small upstream TAD that contains the most distal promoter (black arrowhead), and a larger downstream TAD whose endpoint is close to an internal *lar* promoter (green arrowhead). The purple boundary is located ~600 kb away on the left arm of the 2nd chromosome between two TADs, Pa and Pb, in a gene poor region. The pairing of the blue and purple boundaries generates two rectangles of enhanced contacts. The one on the upper left corresponds to interactions of the sequences in the small TAD just upstream of the blue boundary with sequences in the Pa TAD located upstream of the purple boundary. The other box on the lower right is generated by interactions between sequences in the TAD downstream of the blue

boundary and sequences downstream of the purple boundary. Based on the locations of interacting TADs, the blue and purple boundaries pair with each other head-to-head, generating the circle-loop in the diagram.

The meta-loop in panel B links a boundary (blue arrow) to a boundary (purple arrow) ~5 Mb away on the right arm of the 3rd chromosome. The blue boundary is located between a small TAD that contains three genes encoding UDP-glycosyltransferases and a larger TAD that includes *CG10164* and the most distal *beat IV* (*beaten path IV*) promoter. The purple boundary separates a large TAD containing five genes (*Sid*, *CG33346*, *CG31050*, *CG14062* and *CG9988*) from a small TAD that contains the most distal promoter of the *snu* (*snustorr*) ABC transporter. The blue and purple boundaries pair with each other head-to-tail, and this generates a stem-loop meta-loop (see diagram). In this pairing configuration, sequences upstream of the blue boundary come into contact with sequences downstream of the purple boundary. This generates a box of interactions between the small TAD that contains the UDP-glycosyltransferase gene cluster and the small TAD that contains the most distal *snu* promoter. (Another weaker rectangular block of interactions links sequences in the TAD immediately upstream of the UDP-glycosyltransferase gene cluster, which contains *CG10175*, and the small TAD containing the distal *snu* promoter). A second, larger rectangular box is generated by contacts between sequences in the TADs downstream of the blue boundary and upstream of the purple boundary. Note that the positioning of the rectangular interaction boxes differs for circle-loops and stem-loops.

The pairing interactions in the *lar* and *beat IV* meta-loops are much simpler than those seen for *homie* in the dual reporter. In both cases, there is only a single contact point (blue:purple boundaries). In contrast, as shown in **Fig. 7 and in** Figs. Supplemental 5 and 6, the interaction between the dual reporter *homie* and the *eve* TAD generates a “stripe” that spans the *eve* TAD together with crosslinking events between TADs upstream and downstream of the dual reporters and TADs located to either side of the *eve* TAD. This more complicated interaction pattern is consistent with the viewpoint analysis (**Fig. 8**), which shows that the transgene *homie* physically interacts with both *homie* and *nhomie* in the *eve* TAD.

Boundary pairing orientation and loop topology: Our analysis of the TAD organization in the *eve* neighborhood suggests that the predominant loop topology may be a circle-loop, not a stem-loop. Only the *eve* TAD (and perhaps the very small TAD TA) is clearly a stem-loop. It is topped by a distinctive volcano plume and is not overlaid by a hierarchy of LDC domains, indicating that it must be “insulated” from crosslinkable interactions with other nearby TADs. By contrast, none of the TADs in the region spanning *eve* and the *hebe* gene have volcano plumes at their apex. Instead, all of these TADs are overlaid by a hierarchal organization of LDC domains (clouds) that diminish in crosslinking intensity as the number of in-between TADs increases. As discussed above, the contact pattern of the TADs to the left of *eve* are likely to arise from partner switching and/or head-to-head boundary pairing interactions that generate circle-loops. Pairing orientation versus loop topology is considered further in the accompanying paper (Ke et al. 2024).

Orientation and long-distance regulatory interactions: Our experiments highlight an important constraint on long-distance regulatory interactions that are mediated not only by boundary elements like *homie*, *nhomie*, and the meta-loop boundaries in **Fig. 9**, but also by the GAF-dependent (LBC) tethering and bypass elements (Batut et al. 2022; Kyrchanova et al. 2023; Kyrchanova et al. 2018; Kyrchanova et al. 2019a; Kyrchanova et al. 2019b; Levo et al. 2022; Li et al. 2023; Mohana et al. 2023), namely the orientation of the physical interactions that bring distant enhancers (or silencers) into close proximity with their gene targets. As we have shown here, the orientation of the physical interactions that bring distant sequences close together determines whether the enhancer will be able to activate its target gene. In principle the effects of orientation could be mitigated by increasing the distance between the boundary/tethering elements and either the enhancers (silencers) or the promoter. However, distance can be a critical, rate-limiting step in transcriptional activation. For example, live imaging experiments have shown

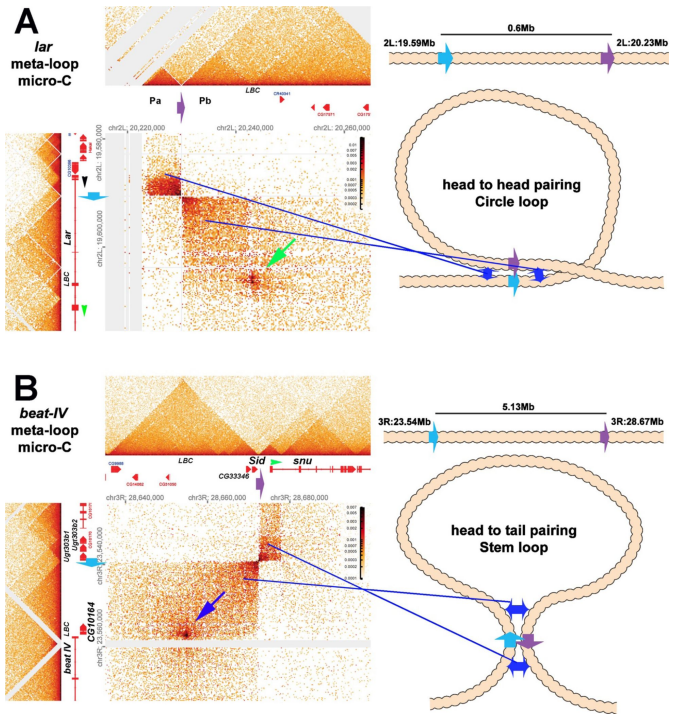

Figure 9.

Circle-loop and stem-loop meta-loops.

Distant boundary elements can find and pair with each other, forming large meta-loops. **Panel A** shows a circle-loop meta-loop formed by the head-to-head pairing of the blue (blue arrow) and purple (purple arrow) boundaries. As described in the text, the blue boundary separates a small TAD that contains the most distal *Lar* promoter (indicated by the black arrowhead) and a larger TAD that contains part of the *Lar* transcription unit (and is upstream of an internal *Lar* promoter: green arrowhead). The purple boundary is located between two TADS, Pa and Pb, in a gene-poor region of the 2nd chromosome approximately 600 kb away downstream of the *Lar* gene. When the blue and purple boundaries pair, sequences in the small TAD upstream of the blue boundary come into contact with sequences in the Pa TAD upstream of the purple boundary. This interaction generates the dark rectangle on the upper left side of the interaction plot. Sequences downstream of the blue boundary in the TAD containing part of the *Lar* transcription unit are also brought into contact with sequences in the Pb TAD downstream of the purple boundary. This interaction generates the larger rectangular box on the bottom right side of the interaction plot. Shown in the diagram on the right is a schematic illustrating that the head-to-head pairing of the blue and purple boundaries generates a ~600 kb circle-loop. In the circle loop configuration, sequences in the TADs upstream of the blue and purple boundaries come into contact with each other, as do sequences downstream of the blue and purple boundaries (blue double arrows). **Panel B** shows a stem-loop meta-loop formed by the head-to-tail pairing of a boundary (blue arrow) located upstream of the *beat IV* gene with another boundary (purple arrow) located ~6 Mb away, between the *Sid* and *snu* genes. The blue boundary separates a small TAD containing three UDP-glycosyltransferase genes from a larger TAD containing the *CG10164* gene and the distal-most promoter of the *beat IV* gene. The purple boundary separates a ~20 kb TAD containing *Sid* and four other genes from a small TAD that contains the most distal *snu* promoter (green arrowhead). When the blue and purple boundaries pair with each other head-to-tail (see diagram on right), this interaction brings sequences in the large TAD downstream of the blue boundary into contact with sequences upstream of the purple boundary in the 20 kb *Sid* TAD, and this generates the large rectangular box to the lower left of interaction map. The stem-loop configuration also brings sequences upstream of blue boundary in the small TAD containing the three UDP-glycosyltransferase genes into contact with the TAD containing the distal-most *snu* promoter. This interaction gives a small rectangular box with a high contact density. Sequences in the TAD upstream of the UDP-glycosyltransferase TAD are also brought into contact with the small TAD containing the *snu* promoter. These two interacting "zones" are indicated by blue double arrows in the diagram on right. In addition to boundary:boundary pairing, both meta-loops have a prominent dot in the larger regular boxes indicated by the green (panel A) and blue (panel B) arrows. Based on the ChIP signature of the sequences giving rise to these dots, they likely correspond to elements that are bound by the GAF containing LBC complex, as indicated in the figure ("*LBC*" in both panels). LBC elements in other contexts function to bring enhancers into contact with promoters (Batut et al. 2022; Kyrchanova et al. 2023; Kyrchanova et al. 2019a; Kyrchanova et al. 2019b; Levo et al. 2022).

that the frequency and amplitude of transcriptional bursts depend on the distance between the enhancer and target promoter. Nearly continuous bursting and high amplitude transcription was observed when the enhancer was located close the promoter (Fukaya et al. 2016). However, at distances as little as 6 kb, transcription became discontinuous with discrete bursts, while at 9 kb, the bursts were much less frequent, and the amplitude/output was substantially reduced (Yokoshi et al. 2020). If similar distance-dependent reductions in transcriptional efficiency are common, then it would not be possible to overcome the topological constraints on regulatory interactions by simply increasing the distance between the boundary/tethering elements and the enhancers/promoters. While our studies do not directly address how chromosome architecture impacts regulatory interactions in vertebrates, it is clear that long-distance regulatory interactions in vertebrates (c.f., Tan et al. 2023) will be subject to the same types of topological constraints as those that apply in flies.

Acknowledgements

We would like to thank Gordon Grey for running the fly food facility at Princeton, members of the Lewis Sigler Genomics Core facility for their invaluable assistance with DNA sequencing, and Sergey Ryabichko for his help with smFISH probes. We would also like to thank members of MOL431 for creative input. Special thanks to Qing Liu for invaluable technical assistance, and to Olga Kyrchanova, Daria Chetverina, Maksim Erokhin, Pavel Georigev, Tsutomu Aoki, Girish Deshpande, Airat Ibragimov, Sergey Ryabichko, Yuri Pritykin and Alex Ostrin for stimulating discussions and sharing unpublished data.

Materials and methods

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
n-Heptane	Fisher Chemical	O3008-4
Paraformaldehyde 20% solution, EM Grade	Electron Microscopy Sciences	15713-S
Formaldehyde, 16%, methanol free, Ultra Pure	Polysciences Inc	18814-10
PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher	AM9624
Tween 20	Sigma	P1379
Triton X-100	Bio-Rad	161-0407
Tris base	Sigma	11814273001
Methanol	Fisher Chemical	203403
SSC, 20X	Thermo Fisher	15557044
Formamide	Thermo Fisher	17899
Dextran sulfate	Sigma	D8906
Salmon Sperm DNA	Thermo Fisher	AM9680
Ribonucleoside Vanadyl Complex	NEB	S1402S
Nuclease-free BSA	Sigma	126609
Triethylammonium Acetate	Sigma	625718
dGTP (100 MM)	VWR	76510-208
dTTP (100 MM)	VWR	76510-224
Lonza NuSieve 3:1 Agarose	Thermo Fisher	BMA50090
T4 DNA ligase	NEB	M0202L
Biotin-11-dCTP	Jena Bioscience	NU-809-BIOX

Biotin-14-dATP	Jena Bioscience	NU-835-BIO14
Qubit dsDNA HS Assay Kit	Life Technologies Corp	Q32851
Atto 633 NHS ester	Sigma	01464
Phase Lock Gel™, QuantaBio - 2302830, Phase Lock Gel Heavy	VMR	10847-802
NEBNext Ultra II DNA Library Prep Kit for Illumina	NEB	E7645S
Ampure Xp 5ml Kit	Thermo Fisher	NC9959336
Hifi Hotstart Ready Mix	Thermo Fisher	501965217
Dynabeads MyOne Streptavidin C1	Life Technologies Corp	65001
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Sigma	11873580001
N,N-Dimethylformamide	Sigma	227056
Potassium acetate solution	Sigma	95843
DSG (disuccinimidyl glutarate)	Thermo Fisher	PI20593
T4 Polynucleotide Kinase - 500 units	NEB	M0201S
DNA Polymerase I, Large (Klenow) Fragment - 1000 units	NEB	M0210L
End-it DNA End Repair Kit	Thermo Fisher	NC0105678
Proteinase K recomb. 100 mg	Sigma	3115879001
Nuclease Micrococcal (s7)	Thermo Fisher	NC9391488
EGS (ethylene glycol bis(succinimidyl succinate))	Thermo Fisher	PI21565
Atto 565 NHS ester	Sigma	72464
Oligonucleotides		
WK_eve_probeset Sequence see “Supplemental Data S1”	Biosearch Technologies	
WK_LacZ_probeset Sequence see “Supplemental Data S1”	Biosearch Technologies	

WK_GFP_probeset Sequence see “Supplemental Data S1”	Biosearch Technologies	
NEBNext Multiplex Oligos for Illumina	NEB	E7335S
Software and algorithms		
Fiji (ImageJ)	Schindelin et al. (Schindelin et al. 2012)	fiji.sc
NIS element	Nikon	microscope.healthcare.nikon.com/products/software/nis-elements
Graphpad Prism 8	GraphPad Software	graphpad.com
HiGlass	Kerpedjiev et al. (Kerpedjiev et al. 2018)	higlass.io
bwa	Li H. and Durbin R. (Li and Durbin 2009)	bio-bwa.sourceforge.net/
samtools	Github/open source	samtools.github.io
pairsamtools	Github/open source	github.com/mirnylab/pairsamtools
pairix	Github/open source	github.com/4dn-dcic/pairix
cooler	Abdennur, N., and Mirny, L. (Abdennur and Mirny 2020)	github.com/mirnylab/cooler
Miniconda	Anaconda	docs.conda.io/en/latest/miniconda.html
Snakemake	Github/open source	snakemake.github.io

Plasmid construction and transgenic lines

The dual reporters construct was described previously (Fujioka et al. 2016). In short, both reporters contain the *eve* basal promoter (-275 to +106 bp relative to *eve* start site), the *lacZ* (*eve-lacZ*) or *EGFP* (*eve-gfp*) coding region, and the *eve* 3’ UTR (+1300 to +1525 bp). These two reporters are divergently transcribed. The 367 bp *homie* fragment, or a 500 bp fragment from *lambda* phage DNA, was placed between the two reporters. For the *homie* fragment, we inserted it in the transgene in both orientations, giving *eve-gfp:homie:eve-lacZ* and *eve-gfp:eimoh:eve-lacZ*.

The -142 kb attP landing site was described previously (Fujioka et al. 2009). The -142 kb site contains two attP target sites for phiC31 recombinase-mediated cassette exchange (RMCE) (Bateman et al. 2006) and *mini-white* as a marker. RMCE can result in the insertion of the

transgene in either orientation, and all four possible insertions of the two transgenes were recovered. For two of these, *GeimohL* and *GhomieL*, the *eve-lacZ* reporter is on the *eve* side of the transgene *homie*, while *eve-gfp* is on the *hebe* enhancers side of the transgene *homie*. For the other two, *LeimohG* and *LhomieG*, the *eve-gfp* reporter is on the *eve* side of transgene *homie*, while *eve-lacZ* is on the *hebe* enhancers side of the transgene *homie*. RMCE events were identified by loss of *mini-white*, and the orientation of each insert was determined by PCR.

smFISH probe preparation

The sequences of target genes were obtained from Flybase (*flybase.org*, Gramates et al. 2022). To design probes, the target gene sequences were submitted to the Biosearch Technologies Stellaris RNA FISH probe designer tool (*biosearchtech.com*), with parameters set to 20nt probes, 48 probes per gene, and mask level 5 for *Drosophila melanogaster*. The sequences of the probes used are listed in Supplemental Data S1. The probes with 3' MdC(TEG-Amino) modifications were then ordered from Biosearch Technologies in 96-well plate format, cartridge purification and salt free conditions, and in 5 nmol scale. To prepare the fluorophore for probe labeling, the Sigma Atto NHS ester fluorophore (Atto 565 NHS or Atto 633 NHS) was dissolved in dimethylformamide at 5mg/mL as the stock solution. For probe labeling, 1 nmol of each probe was combined and mixed with Atto NHS ester fluorophore at 1mg/mL in 500µL of 0.1M sodium bicarbonate (pH 8.0-8.5) for 2 hours, and the probes were then precipitated with 0.5M potassium acetate (pH 5.2) in 75% EtOH. Then, the probes were dried and resuspended in 50µL of DePC-treated H_2O. The probes were then injected into HPLC columns in which the percentage 0.1M triethylammonium acetate varied from 7% to 30% with a flow rate of 1mL/min. To monitor coupled probes during the HPLC purification, the detector was set to 260nm for DNA and to the absorption wavelength of the coupled fluorophore. The coupled probes were collected when both 260nm and the absorption wavelength channel were detected. The coupled probes were then dried and diluted to the appropriate concentration for smFISH experiments in DePC-treated H_2O.

smFISH

smFISH methods were adapted and modified from previous studies (Little and Gregor 2018; Trcek et al. 2017). 100-200 flies were placed in a cage with an apple juice plate at the bottom of the cage. Early-stage embryos were collected for 7 hours, while for later-stage embryos, collection was overnight. Embryos from each plate were washed into collection mesh and dechorionated in bleach for 2min, then fixed in 5mL of 4% paraformaldehyde in 1X PBS and 5mL of heptane for 15min with horizontal shaking. The paraformaldehyde was then removed and replaced with 5mL methanol. The embryos were then devitellinized by vortexing for 30s, and washed in 1mL of methanol twice. Methanol was then removed and replaced by PTw (1X PBS with 0.1% Tween-20) through serial dilution at 7:3, 1:1, and 3:7 methanol:PTw. The embryos were washed twice in 1mL PTw and then twice in 1mL smFISH wash buffer (4X SSC, 35% formamide and 0.1% Tween-20). Embryos were incubated with ~5nM coupled smFISH probes in hybridization buffer (0.1g/mL dextran sulfate, 0.1mg/mL salmon sperm ssDNA, 2mM ribonucleoside vanadyl complex, 20µg/mL RNAse-free BSA, 4X SSC, 1% Tween-20 and 35% formamide) for 12-16h. Embryos were then washed twice for 2 hours in 1mL smFISH wash buffer, followed by 4X washing in 1mL PTw. For DAPI/Hoechst staining, the embryos were stained with 1ug/mL DAPI or Hoechst in PTw, and washed 3X with 1mL PTw. F inally, the embryos were mounted on microscope slides with Aqua PolyMount and a #1.5 coverslip for imaging.

Imaging, image analysis and statistics

Embryos from smFISH were imaged using a Nikon A1 confocal microscope system with a Plan Apo 20X/0.75 DIC objective. Z-stack images were taken at intervals of 2µm, 4X average, 1024x1024 resolution, and the appropriate laser power and gain were set for 405, 561, and 640 channels to avoid overexposure. Images were processed using ImageJ, and the maximum projection was applied to each of the stack images. To measure the stripe intensity of early embryos, multi-

channel images were first split into single channels and the stripe signal was highlighted and selected by the MaxEntropy thresholding method. For APR intensity measurements, the ROI tool was used to crop out the APR region of late-stage embryos. The cells with APR signal were also highlighted and selected by MaxEntropy thresholding. The particle measurement tool was used to measure the average intensity of all cells that had a signal. At the same time, the background signal (average intensity) was taken from cells without a signal in the same embryo. The relative intensity (signal to background) for each embryo was calculated using the stripe signal and background from the same embryo. To make comparisons between independent biological replicates, the average background signal of all embryos from each replicate was calculated. The relative intensity of each embryo from each replicate was normalized based on the average background signal of all embryos from same replicates. GraphPad Prism was used for data visualization and statistical analysis. To compare intensity from embryos in different groups, different signals from the same embryo (e.g., *lacZ* and *GFP*) were paired, and paired two-tailed t-tests were used to calculate p-values. All raw measurements and normalized data are included in Supplemental Data S2.

MicroC library construction

Embryos were collected on yeasted apple juice plates in population cages for 4 hours, incubated for 12hr at 25°C, then subjected to fixation as follows. Embryos were dechorionated for 2min in 3% sodium hypochlorite, rinsed with deionized water, and transferred to glass vials containing 5 mL PBST (0.1% Triton-X100 in PBS), 7.5 mL n-heptane, and 1.5mL fresh 16% formaldehyde. Crosslinking was carried out at room temperature for exactly 15min on an orbital shaker at 250rpm, followed by addition of 3.7 mL 2M Tris-HCl pH7.5 and shaking for 5min to quench the reaction. Embryos were washed twice with 15 mL PBST and subjected to secondary crosslinking. Secondary crosslinking was done in 10mL of freshly prepared 3mM final DSG and ESG in PBST for 45 min. at room temperature with passive mixing. The reaction was quenched by addition of 3.7mL 2M Tris-HCl pH7.5 for 5min, washed twice with PBST, snap-frozen, and stored at -80°C until library construction.

Micro-C libraries were prepared as previously described (Batut et al. 2022) with the following modifications: 50uL of 12-16hr embryos were used for each biological replicate. 60U of MNase was used for each reaction to digest chromatin to a mononucleosome:dinucleosome ratio of 4. Libraries were barcoded, pooled and subjected to paired-end sequencing on an Illumina Novaseq S1 100 nt Flowcell (read length 50 bases per mate, 6-base index read).

Micro-C data processing

MicroC data for *D. melanogaster* were aligned to custom genomes edited from the Berkeley Drosophila Genome Project (BDGP) Release 6 reference assembly (dos Santos et al. 2015) with BWA-MEM (Li and Durbin 2009) using parameters **-S -P -5 -M**. Briefly, the custom genomes are simply insertions of the transgenic sequence into the –142kb integration site, as predicted from perfect integration. These events were confirmed using PCR post-integration. The resultant BAM files were parsed, sorted, de-duplicated, filtered, and split with Pairtools (*https://github.com/mirnylab/pairtools*). We removed pairs where only half of the pair could be mapped, or where the MAPQ score was less than three. The resultant files were indexed with Pairix (*https://github.com/4dn-dcic/pairix*). The files from replicates were merged with Pairtools before generating 100bp contact matrices using Cooler (Abdennur and Mirny 2020). Finally, balancing and Mcool file generation was performed with Cooler's Zoomify tool.

Virtual 4C profiles were extracted from individual replicates using FAN-C (Kruse et al. 2020) at 400bp resolution. The values were summed across replicates and smoothed across three bins (1.2kb). Viewpoints were determined based on the most informative region for interpretation. Ultimately we decided to use 800bp regions in the gene body of either *GFP* or *lacZ*, moving downstream from the *eve* promoter. However, similar results were obtained using viewpoints

between transgenic *homie* and the promoters of either gene. For *homie* viewpoints, due to transgenic *homie* being masked, some loss of signal is expected at the viewpoint (**Figs. 7D** and **8**).

Data availability

Sequence data are available at GEO(GSE263229). All confocal images are available upon request.

Supplemental Figures

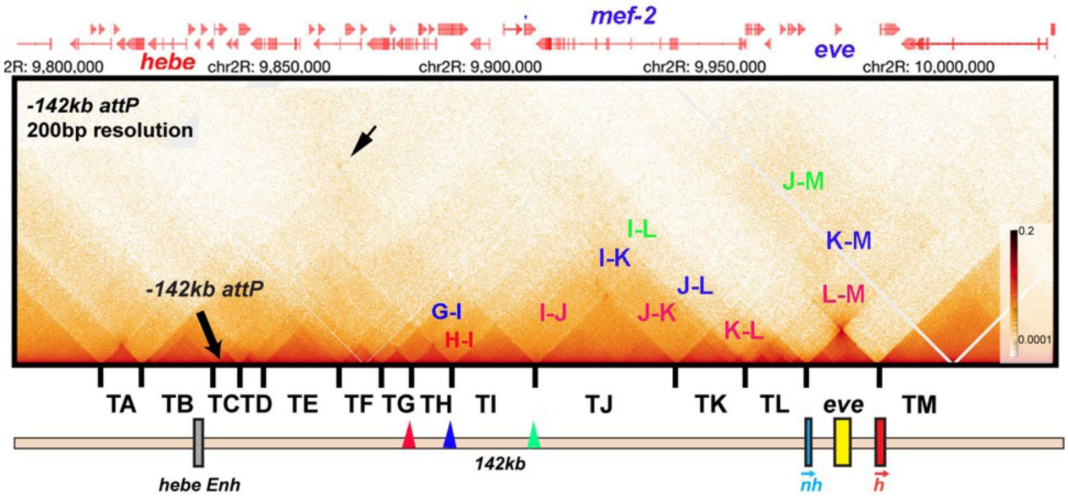

Figure Sup. 1.

TAD organization of the *even-skipped* gene and upstream region.

MicroC contact pattern for the chromosomal region spanning the attP site at –142 kb and the *eve* locus. Like the *eve* volcano, this contact pattern was generated using aggregated previously published NC14 data (Batut *et al*., 2022; Levo et al., 2022). The large black arrow indicates the –142kb locus where transgenes are integrated into the genome. LDC domains are labeled according to the interacting TADs: for example, H-I indicates crosslinking events generated by physical interactions between sequences in two neighboring TADs, TH and TI, while I-J indicates interactions between sequences in TI and TJ. Likewise, G-I indicates crosslinking events generated by physical interactions between sequences in TADs TG and TI, which are separated from each other by TAD TH. Note that unlike the TADs to the left of *eve*, the *eve* TAD does not interact frequently with its neighbors. The small black arrow indicates an interaction “dot” between the boundary marking the border of TA and TB and the boundary marking the border of TI and TJ. Red, blue and green arrowheads mark boundaries that may engage in partner switching (see text).

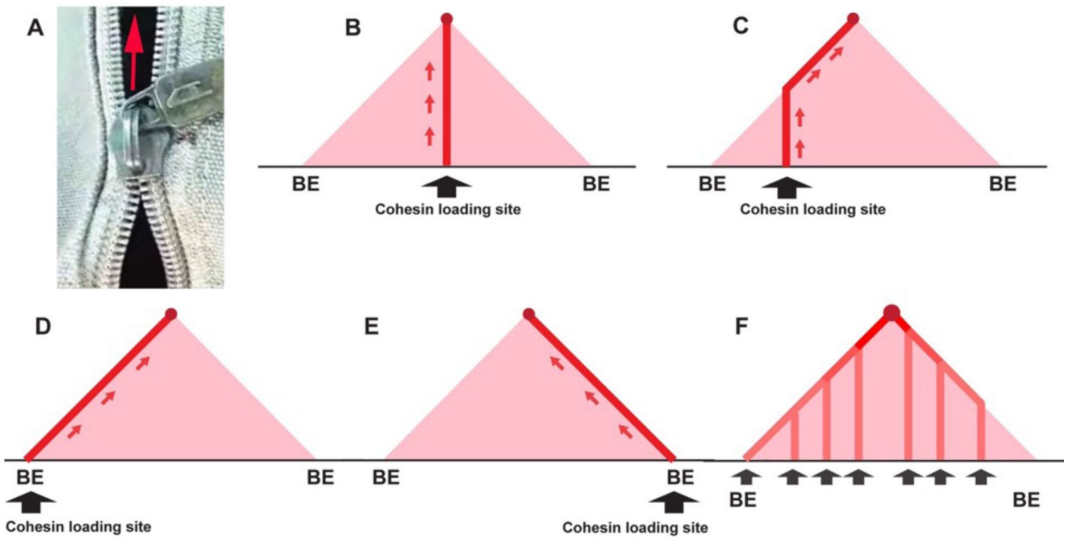

Figure Sup. 2.

Trouble ahead, trouble behind.

A) Illustration of a broken zipper—zipping in front but not closing behind. **B)** Crosslinking pattern expected for cohesin loading at a site very close to the middle of the TAD-to-be: The vertical line of linked DNA is generated when the two chromatin fibers in the TAD-to-be are transiently linked together as cohesin passes. When cohesin bumps into the CTCF roadblocks (BE) at the boundaries of the TAD, the vertical line stops. **C)** Cohesin loading site is offset from center of TAD-to-be to the left. The crosslinking initiates at the loading site and extends vertically until the cohesin complex bumps into the CTCF roadblock on the left. At that point, extrusion of the left chromatin fiber halts, while the chromatin fiber on the right continues to be extruded. The line of crosslinked sequences then extends at a 45° angle until cohesin comes to a halt at the CTCF roadblock on the right. **D and E)** The loading site is at or close to the boundary on the left (D) or the right (E). In this case extrusion will be unidirectional, and the DNAs crosslinked as cohesin passes will form a line that extends at a 45° angle until cohesin comes to a halt at the CTCF roadblock. **F)** Cohesin is able to load at multiple sites in the TAD-to-be. In this case, there will be a series of vertical lines whose intensity will be correlated with the relative frequency that a give loading site is used. The vertical lines will extend in each case until cohesin comes to a halt at the closest CTCF roadblock. At that point, the line of crosslinked DNA will extend at a 45° angle until that particular cohesin complex comes to halt at the CTCF roadblock that is farther away. The 45° line of crosslinked DNA will become progressive more intense as it approaches the apex of the triangle. These vertical lines might follow other paths if the loop extrusion is not at equal rates on the two sides, or if it varies stochastically, but even in these cases, the expectation that the 45° lines will be more intense toward the top of the “peak” is still valid.

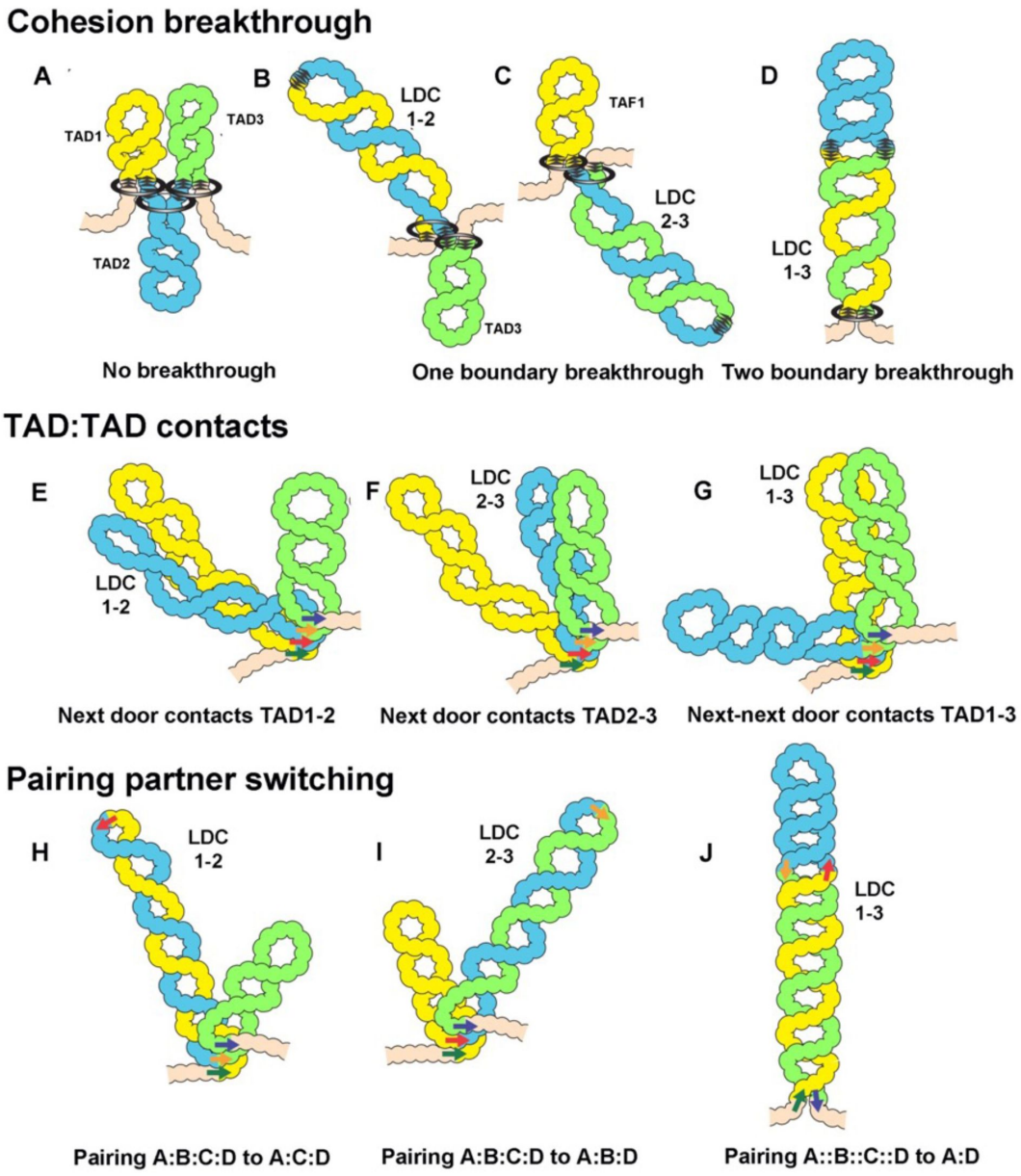

Figure Sup. 3.

Models for the formation of LDC domains.

Several different mechanisms could explain the LDC domains. **A-D)** In the loop-extrusion model, the LDC domains arise because cohesin sometimes breaks through a CTCF roadblock. In A) there are three primary TADs,: TAD1, TAD2, and TAD3. B and C) Occasional breakthrough events can generate a secondary TAD, LDC1:2 (TAD1+TAD2) or LDC2:3 (TAD2+TAD3). D) Even less frequent breakthrough events can generate a tripartite TAD (TAD1+TAD2+TAD3) that will correspond to LDC1:3. **E-G)** LDC domains arise because TADs sometimes bump into their neighbors. In E, TAD1 bumps into TAD2 to give LDC1:2, while in F, TAD2 bumps into TAD3 to give LDC2:3. G) Less frequently, TAD1 bumps into TAD3 to give LDC1:3. **H-J)** LDC domains arise because boundaries can switch partners. H and I) In H, boundary A (dark green arrow) skips boundary B (red arrow) and pairs with boundary C (tan arrow). This generates a new TAD, TAD1+TAD2, which corresponds to LDC domain 1:2. In I, boundary B (red arrow) skips boundary C (tan arrow) and pairs with D (dark blue arrow). This generates a new TAD, TAD2+TAD3, which corresponds to LDC domain 2:3. In J, boundary A (dark green arrow) skips both boundary B (red arrow) and boundary C (tan arrow), which don't pair with each other, and pairs with boundary D (dark blue arrow). This generates a new TAD, TAD1+TAD2+TAD3, which corresponds to LDC domain 1:3.

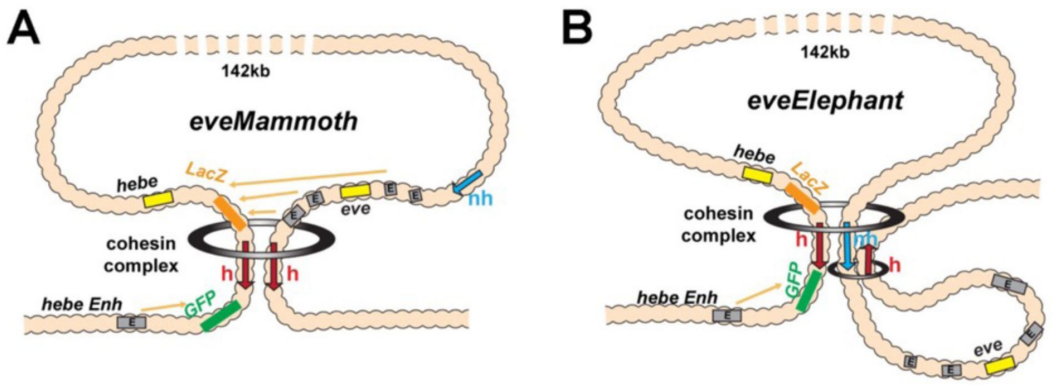

Figure Sup. 4.

Loop extrusion: *eveElephant*.

A) Loop extrusion orientation-dependent model for *LeimohG* transgene as the sketched in **Figure 3D**. This configuration generates *eveMa*. **B)** Instead of running through *nhomie*, the cohesin complex comes to a halt at the *nhomie* boundary, generating *eveElephant*. A second cohesin complex generates the *eve* TAD by loading at a site within the *eve* locus. Both *eveElephant* and the *eve* TAD are stem-loops. However, while this double stem-loop configuration would explain why the transgene *homie* makes contact with both *nhomie* and *homie*, the *eve* enhancers are isolated in their own loop and would not interact with the two reporters in the transgene.

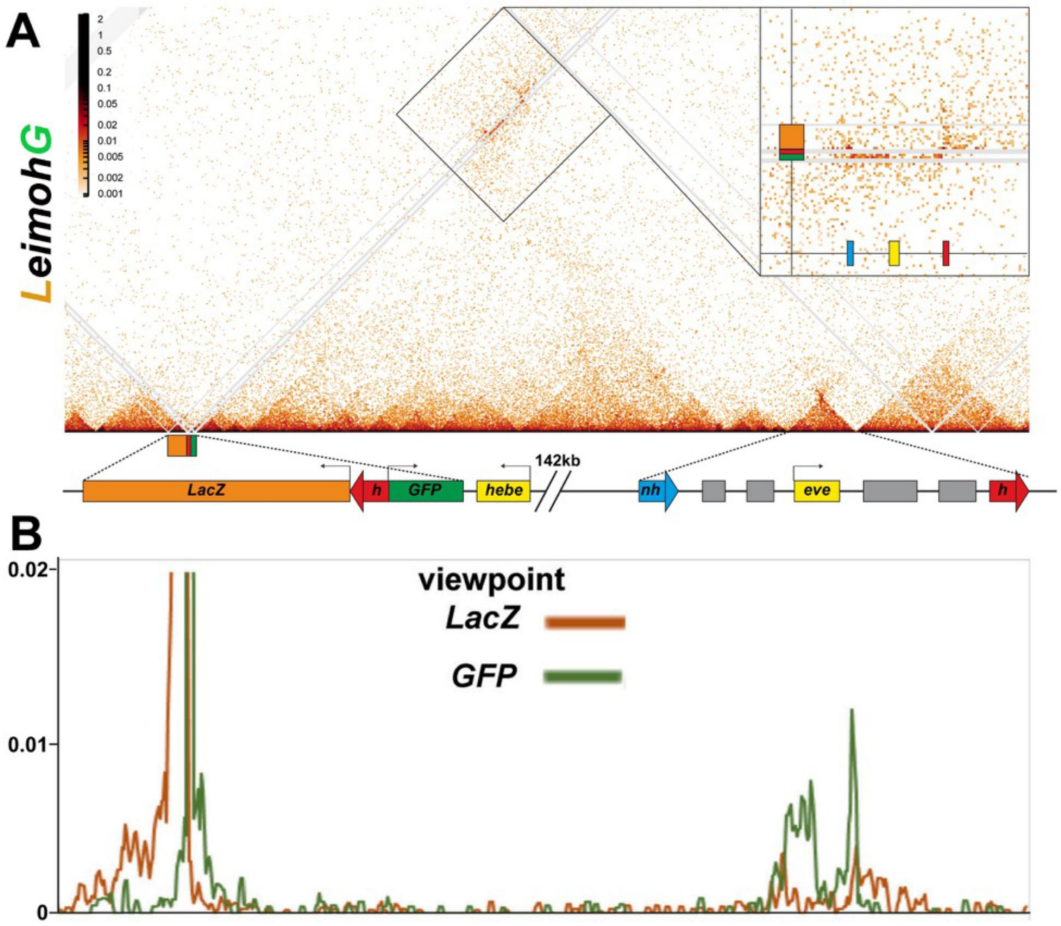

Figure Sup. 5.

MicroC of *LeimohG*.

A) MicroC contact map of *LhomieG*. Diagram below the contact map shows the *homie* transgene and the *eve* TADs. Gray lines are masked regions due to repeat sequences. Interactions between the transgene and the *eve* TAD are indicated by the boxed region, and this boxed region is also shown in the inset on the right. The inset shows that the physical interactions between *LhomieG* and sequences in the *eve* TAD are biased toward the *eve-gfp* reporter. **B)** "Virtual 4C" map from either the *lacZ* (brown line) or *GFP* (green line) gene body viewpoints.

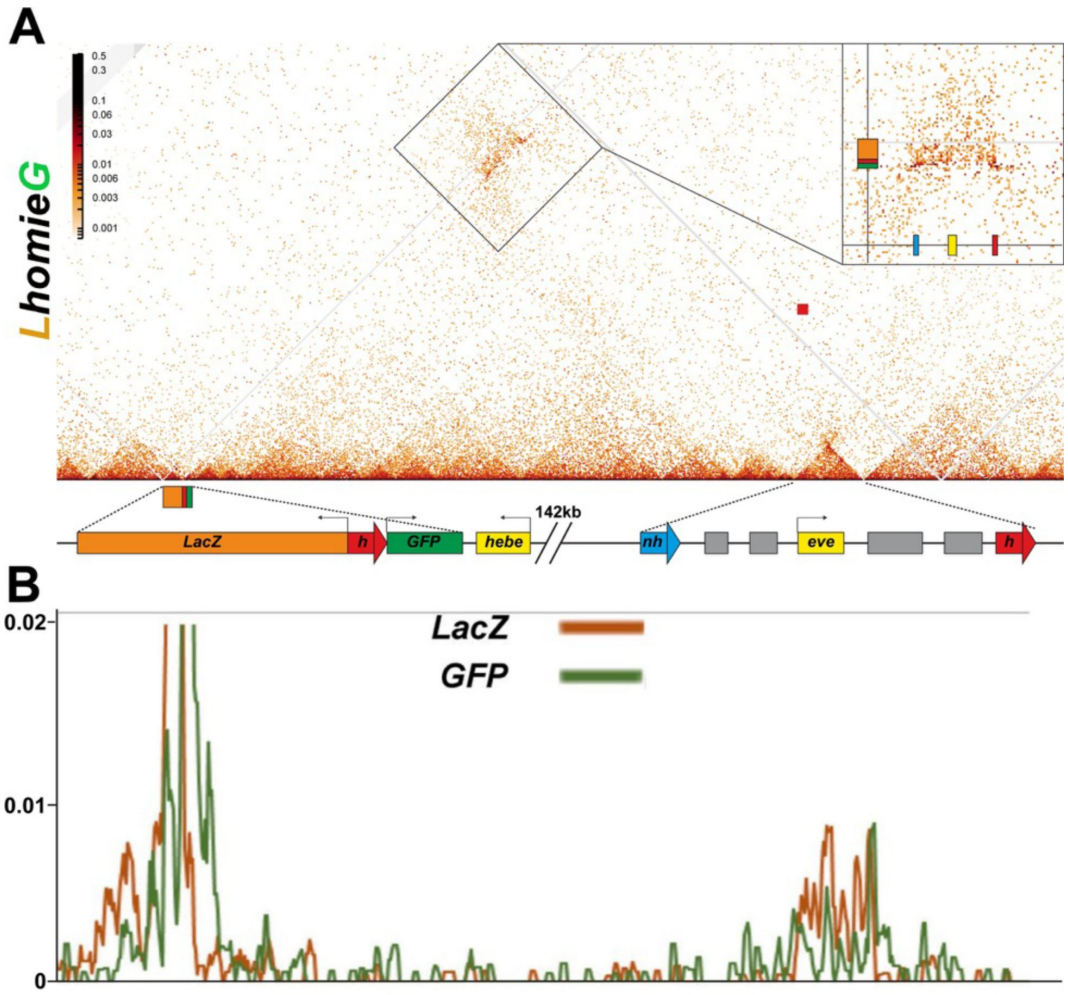

Figure Sup. 6.

Micro C of *LhomieG*.

A) MicroC map of *LhomieG*. Diagram below the contact map shows the *homie* transgene and the *eve* TADs. Gray lines are masked regions due to repeat sequences. The interaction pattern is the opposite of that observed for *LeimohG* (Figure Sup. 5). Instead of interacting with the *eve-gfp* reporter, sequences in the *eve* TAD interact preferentially with the *eve-lacZ* reporter. The relevant region is boxed, and also shown in the inset. **B)** "Virtual 4C" map from either the *lacZ* (brown line) or *GFP* (green line) gene body viewpoints.

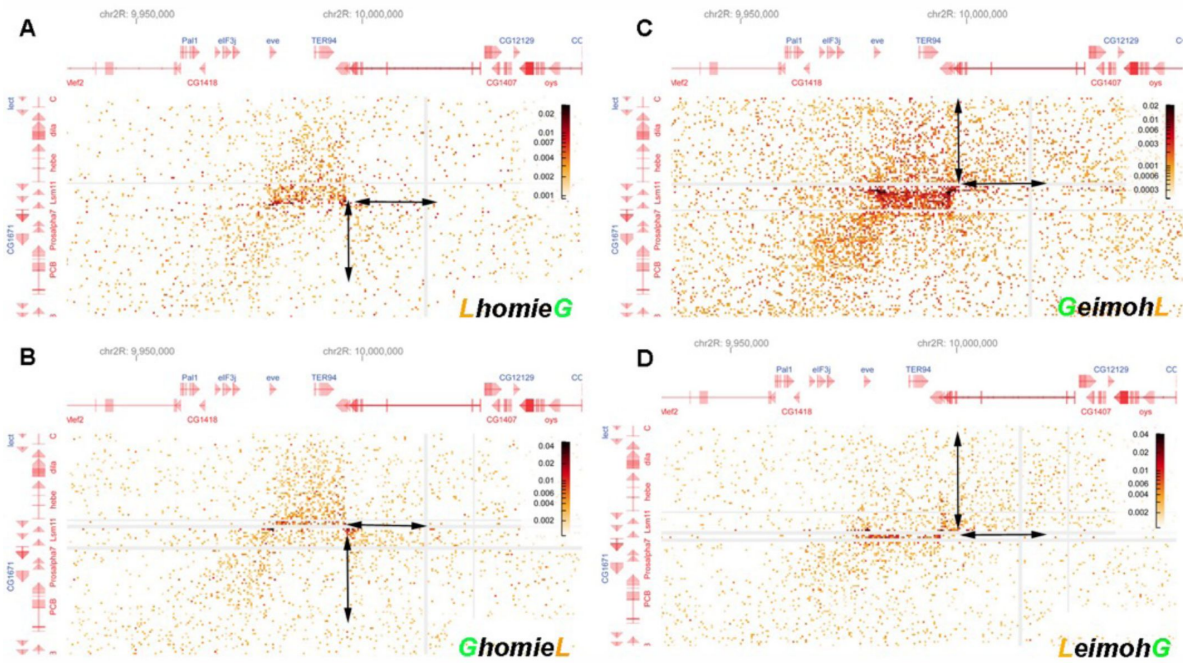

Figure Sup. 7.

Blowup of interactions generated by the transgene with sequences neighboring –142 kb and the *eve* TAD.

The interaction pattern indicates that the loop topology changes along with the orientation of transgenic *homie*. Blow-up of off-diagonal interactions, focused on the interaction between the endogenous *eve* locus and the –142kb transgenic insert with **A)** *LhomieG,* **B)** *GhomieL,* **C)** *GeimohL,* or **D)** *LeimohG*. Note the differences in preferential interaction between regions adjacent to transgenic *homie* and endogenous *homie* (indicated by the double arrow pairs). In the two cases where transgenic *homie* is in the same orientation in the genome as endogenous *homie* (A and B), regions downstream of the transgene preferentially interact with regions downstream of endogenous *homie* in the *TER94* TAD. In contrast, when transgenic *homie* is in the opposite orientation in the genome as endogenous *homie* (C and D), sequences upstream of the transgene preferentially interact with sequences downstream of endogenous *homie* in the *TER94* TAD.

Supplemental Tables

Table Supplemental #1. Statistical analysis of reporter expression in the *LeimohG* and *LhomieG* transgene inserts.

Table Supplemental #2. smFISH oligo probes.

Table Supplemental #3. Imaging data.

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Editors

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Reviewer #1 (Public Review):

Summary:

The authors addressed how long-range interactions between boundary elements are established and influence their function in enhancer specificity. Briefly, the authors placed two different reporters separated by a boundary element. They inserted this construct ectopically ~140 kb away from an endogenous locus that contains the same boundary element. The authors used expression patterns driven by nearby enhancers as an output to determine which enhancers the reporters interact with. They complemented this analysis with 3D DNA contact mapping. The authors found that the orientation of the boundary element determined which enhancers each reporter interacted with. They proposed that the 3D interaction topology, whether being circular or stem configuration, distinguished whether the interaction was cohesin mediated or through an independent mechanism termed pairing.

Strengths:

The transgene expression assays are built upon prior knowledge of the enhancer activities. The 3D DNA contacts confirm that transgene expression correlates with the contacts. Using 4 different orientations covers all combinations of the reporter genes and the boundary placement.

Weaknesses:

The interpretation of the data as a refusal of loop extrusion playing a role in TAD formation is not warranted, as the authors did not deplete the loop extruders to show that what they measure is independent. As the authors show, the single long DNA loop mediated by cohesin loop extrusion connecting the ectopic and endogenous boundary is clearly inconsistent with the results, therefore the main conclusion of the paper that the 3D topology of the boundary elements a consequence of pairing is strong. However, the loop extrusion and pairing are not mutually exclusive models for the formation of TADs. Loop-extruding cohesin complexes need not make a 140 kb loop, multiple smaller loops could bring together the two boundary elements, which are then held together by pairing proteins that can make circular topologies.

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Reviewer #2 (Public Review):

In Bing et al, the authors analyze micro-C data from NC14 fly embryos, focusing on the eve locus, to assess different models of chromatin looping. They conclude that fly TADs are less consistent with conventional cohesin-based loop extrusion models and instead rely more heavily on boundary-boundary pairings in an orientation-dependent manner.

Overall, I found the manuscript to be interesting and thought-provoking. However, this paper reads much more like a perspective than a research article. Considering the journal is aimed at the general audience, I strongly suggest the authors spend some time editing their introduction to the most salient points as well as organizing their results section in a more conventional way with conclusion-based titles. It was very difficult to follow the authors' logic throughout the manuscript as written. It was also not clear as written which experiments were performed as part of this study and which were reanalyzed but published elsewhere. This should be made clearer throughout.

It has been shown several times that Drosophila Hi-C maps do not contain all of the features (frequent corner peaks, stripes, etc.) observed when compared to mammalian cells. Considering these features are thought to be products of extrusion events, it is not an entirely

new concept that Drosophila domains form via mechanisms other than extrusion. That being said, the authors' analyses do not distinguish between the formation and the maintenance of domains. It is not clear to this reviewer why a single mechanism should explain the formation of the complex structures observed in static Hi-C heatmaps from a population of cells at a single developmental time point. For example, how can the authors rule out that extrusion initially provides the necessary proximity and possibly the cis preference of contacts required for boundary-boundary pairing whereas the latter may more reflect the structures observed at maintenance? Future work aimed at analyzing micro-C data in cohesin-depleted cells might shed additional light on this.

Additional mechanisms at play include compartment-level interactions driven by chromatin states. Indeed, in mammalian cells, these interactions often manifest as a "plume" on Hi-C maps similar to what the authors attribute to boundary interactions in this manuscript. How do the chromatin states in the neighboring domains of the eve locus impact the model if at all?

How does intrachromosomal homolog pairing impact the models proposed in this manuscript (Abed et al. 2019; Erceg et al., 2019). Several papers recently have shown that somatic homolog pairing is not uniform and shows significant variation across the genome with evidence for both tight pairing regions and loose pairing regions. Might loose pairing interactions have the capacity to alter the cis configuration of the eve locus?

In summary, the transgenic experiments are extensive and elegant and fully support the authors' models. However, in my opinion, they do not completely rule out additional models at play, including extrusion-based mechanisms. Indeed, my major issue is the limited conceptual advance in this manuscript. The authors essentially repeat many of their previous work and analyses. The authors make no attempt to dissect the mechanism of this process by modifying extrusion components directly. Some discussion of Rollins et al., 1999 on the discovery of Nipped-B and its role in enhancer-promoter communication should also be made to reconcile their conclusions in the proposed absence of extrusion events.

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Reviewer #3 (Public Review):

Bing et al. attempt to address fundamental mechanisms of TAD formation in Drosophila by analyzing gene expression and 3D conformation within the vicinity of the eve TAD after insertion of a transgene harboring a Homie insulator sequence 142 kb away in different orientations. These transgenes along with spatial gene expression analysis were previously published in Fujioka et al. 2016, and the underlying interpretations regarding resulting DNA configuration in this genomic region were also previously published. This manuscript repeats the expression analysis using smFISH probes in order to achieve more quantitative analysis, but the main results are the same as previously published. The only new data are the Micro-C and an additional modeling/analysis of what they refer to as the 'Z3' orientation of the transgenes. The rest of the manuscript merely synthesizes further interpretation with the goal of addressing whether loop extrusion may be occurring or if boundary:boundary pairing without loop extrusion is responsible for TAD formation. The authors conclude that their results are more consistent with boundary:boundary pairing and not loop extrusion; however, most of this imaging data seems to support both loop extrusion and the boundary:boundary models. This manuscript lacks support, especially new data, for its conclusions. Furthermore, there are many parts of the manuscript that are difficult to follow. There are some minor errors in the labelling of the figures that if fixed would help elevate understanding. Lastly, there are several major points that if elaborated on, would potentially be helpful for the clarity of the manuscript.

Major Points:

(1) The authors suggest and attempt to visualize in the supplemental figures, that loop extrusion mechanisms would appear during crosslinking and show as vertical stripes in the micro-C data. In order to see stripes, a majority of the nuclei would need to undergo loop extrusion at the same rate, starting from exactly the same spots, and the loops would also have to be released and restarted at the same rate. If these patterns truly result from loop extrusion, the authors should provide experimental evidence from another organism undergoing loop extrusion.
(2) On lines 311-314, the authors discuss that stem-loops generated by cohesin extrusion would possibly be expected to have more next-next-door neighbor contacts than next-door neighbor contacts and site their models in Figure 1. Based on the boundary:boundary pairing models in the same figure would the stem-loops created by head-to-tail pairing also have the same phenotype? Making possible enrichment of next-next-door neighbor contacts possible in both situations? The concepts in the text are not clear, and the diagrams are not well-labeled relative to the two models.
(3) The authors appear to cite Chen et al., 2018 as a reference for the location of these transgenes being 700nM away in a majority of the nuclei. However, the exact transgenes in this manuscript do not appear to have been measured for distance. The authors could do this experiment and include expression measurements.
(4) The authors discuss the possible importance of CTCF orientation in forming the roadblock to cohesin extrusion and discuss that Homie orientation in the transgene may impact Homie function as an effective roadblock. However, the Homie region inserted in the transgene does not contain the CTCF motif. Can the authors elaborate on why they feel the orientation of Homie is important in its ability to function as a roadblock if the CTCF motif is not present? Trans-acting factors responsible for Homie function have not been identified and this point is not discussed in the manuscript.
(5) The imaging results seem to be consistent with both boundary:boundary interaction and loop extrusion stem looping.
(6) The authors suggest that the eveMa TAD could only be formed by extrusion after the breakthrough of Nhomie and several other roadblocks. Additionally, the overall long-range interactions with Nhomie appear to be less than the interactions with endogenous Homie (Figures 7, 8, and supplemental 5). Is it possible that in some cases boundary:boundary pairing is occurring between only the transgenic Homie and endogenous Homie and not including Nhomie?
(7) In Figure 4E, the GFP hebe expression shown in the LhomieG Z5 transgenic embryo does not appear in the same locations as the LlambdaG Z5 control. Is this actually hebe expression or just a background signal?
(8) Figure 6- The LhomieG Z3 late-stage embryo appears to be showing the ventral orientation of the embryo rather than the lateral side of the embryo as was shown in the previous figure. Is this for a reason? Additionally, there are no statistics shown for the Z3 transgenic images. Were these images analyzed in the same way as the Z5 line images?
(9) Do the Micro-C data align with the developmental time points used in the smFISH probe assays?

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Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review)***:
>
> *Summary:*

> *The authors addressed how long-range interactions between boundary elements are established and influence their function in enhancer specificity. Briefly, the authors placed two different reporters separated by a boundary element. They inserted this construct ectopically ~140 kb away from an endogenous locus that contains the same boundary element. The authors used expression patterns driven by nearby enhancers as an output to determine which enhancers the reporters interact with. They complemented this analysis with 3D DNA contact mapping. The authors found that the orientation of the boundary element determined which enhancers each reporter interacted with. They proposed that the 3D interaction topology, whether being circular or stem configuration, distinguished whether the interaction was cohesin mediated or through an independent mechanism termed pairing.*
>
> *Strengths:*
>
> *The transgene expression assays are built upon prior knowledge of the enhancer activities. The 3D DNA contacts confirm that transgene expression correlates with the contacts. Using 4 different orientations covers all combinations of the reporter genes and the boundary placement.*
>
> *Weaknesses:*
>
> *The interpretation of the data as a refusal of loop extrusion playing a role in TAD formation is not warranted, as the authors did not deplete the loop extruders to show that what they measure is independent.*

(1.1) To begin with, our findings do not exclude the possibility that cohesin loop extrusion has some sort of role in the formation or maintenance of TADs in flies or other aspects of chromosome structure. On the other hand, it clearly is not determinative in defining the end-points of TADs or in generating the resulting topology (stem-loop or circle-loop). Our main point, which we feel we have established unequivocally, is that it can't explain many essential features of TADs or chromosome loops (see below) in Drosophila. This reviewer agrees with this point in their next paragraph (below). We also think that the loop extrusion model's general acceptance as THE driving force behind TAD formation in mammals is unwarranted and not fully consistent with the available data, as explained below.

As to the reviewer's specific point regarding depletion of loop extruders, we first note that completely eliminating factors encoding cohesin subunits in fly embryos isn't readily feasible. As cohesin is essential starting at the beginning of embryonic development, and is maternally deposited, knockdowns/depletions would likely be incomplete and there would always be some remaining activity. As long as there is some residual activity—and no disruption in TAD formation is observed—this experimental test would be a failure. In addition, any defects that are observed might arise not from a failure in TAD formation via loop extrusion but rather because the rapid mitotic cycles would be disrupted. A far better approach would be to deplete/knockdown cohesin subunits in tissue culture cells, as there is no requirement for the cells to undergo embryonic development. Moreover, since cell division is relatively slow, the depletion would likely eliminate much if not all of the activity before a checkpoint is reached.

While a drastic depletion of cohesin is not feasible in our model organism, we would draw the reviewer's attention to an experiment of this type which has already been done in mammalian tissue culture cells by Goel et al. (Goel et al. 2023). Unlike most Hi-C studies in mammals, the authors used region capture MicroC (RCMC). In contrast to published genome-wide mammalian MicroC experiments (c.f., (Hsieh et al. 2020; Krietenstein et al. 2020)) which require large bin sizes to visualize mammalian "TADs," the resolution of the experiments in Goel et al. (Goel et al. 2023) is similar to the resolution in our MicroC experiments (200-400

bp). A MicroC contact map from Goel et al. shows the *Pdm1g* locus on chromosome 5 before and after Rad21 depletion. The contact map visualizes a 250 kb DNA segment, which is only slightly larger than the ~230 kb DNA segment in Fig. 2C in our paper.

In this experiment, there was a 97% reduction in the amount of Rad21. However, as can be seen by comparing the contact profiles above and below the diagonal, there is little or no difference in TAD organization after cohesin depletion when individual TADs are visualized with a bin size of 250 bp. These results would indicate that mammalian TADs *do not* require cohesin.

Note also that the weak 45o stripes connecting different TADs (c.f. blue/green arrowheads) are still present after Rad21 depletion. In the most popular version of the loop extrusion model, cohesin loads at a site(s) somewhere in the TAD-to-be, and then extrudes both strands until it bumps into CTCF roadblocks. As illustrated in Figure Sup 2, this mechanism generates a vertical stripe originating at the cohesin loading site and extending until cohesin bumps into the left or right roadblock, at which point the stripe transitions into 45o stripe that ends when cohesin bumps into the other roadblock. While 45o stripes are visible, there is no hint of a vertical stripe. This suggests that the mechanism for generating stripes, if it is an active mechanism (rather than passive diffusion) may be quite different. The 45o stripes must be generated by a factor(s) that is anchored to one (blue arrowhead) or both (green arrowhead) boundaries. In addition, this factor, whatever it is, is not cohesin. The reason for this is that the 45o stripes are present both before and after Rad21 depletion. Moreover, if one were to imagine that the stripes represent a process involved in TAD formation, this process does not require cohesin (see Goel et al 2023).

It is worth noting another observation that is inconsistent with the cohesin loop extrusion/CTCF roadblock model for TAD formation/maintenance. CTCF is not found at all of the TAD boundaries in this 250 kb DNA region. This would suggest that there are other DNA binding proteins that have chromosomal architectural functions besides CTCF. In flies, many of the chromosomal architectural proteins are, like CTCF, polydactyl zinc finger (PZF) proteins (Bonchuk et al. 2021; Bonchuk et al. 2022; Fedotova et al. 2017). These include Su(Hw), CTCF, Pita, Zipic and CLAMP. The PZF family in flies is quite large. There are ~250 different PZF genes, and since only a handful of these have been characterized, it seems likely that additional members of this family will have architectural functions. Thus far, only one boundary protein, CTCF, has received attention in studies on mammalian chromosome architecture. As the mammalian genome is much larger and more complicated than the fly genome, it is difficult to believe that CTCF is the sole chromosomal architectural protein in mammals. In this respect, it is worth noting that there are ~800 members of the PZF family in mammalian genomes (Fedotova et al. 2017).

Goel et al. (Goel et al. 2023) did observe alterations in the contact profiles after Rad21 depletion when they visualized the *Ppm1g* region at much lower resolution (bin sizes of 5 kb and 1 kb). The 5 kb bin size visualizes a region of ~1.2 Mb, while the 1 kb bin size visualizes a region that spans ~800 kb. These large triangular units do not correspond to the individual TADs seen when Goel et al. visualized the *Ppm1g* locus at 250 bp resolution.

Nor do they correspond to TADs in Fig. 2 of our paper. Instead they represent TAD neighborhoods which, likely consist of 20-30 or more individual TADs. Consequently the alterations in contact patterns seen after Rad21 depletion are occurring at the level of TAD neighborhoods. This can be seen by comparing pixel density inside the blue lines before (above the diagonal) and after Rad21 depletion (below the diagonal) (Goel et al 2023). The more distant contacts between individual TADs within this neighborhood are preferentially reduced by Rad21 depletion (the region below and to the left of the double arrowhead). By contrast, the TADs themselves are unaffected, as are contacts between individual TADs and their immediate neighbors (see purple and light green asterisk). The other interesting feature is the loss of contacts between what appears to be partially overlapping neighborhoods. This

loss of neighborhood-toneighborhood contacts can be seen in the region located between the green and blue lines. The neighborhood that appears to partially overlap the *Ppm1g* neighborhood is outlined in purple.

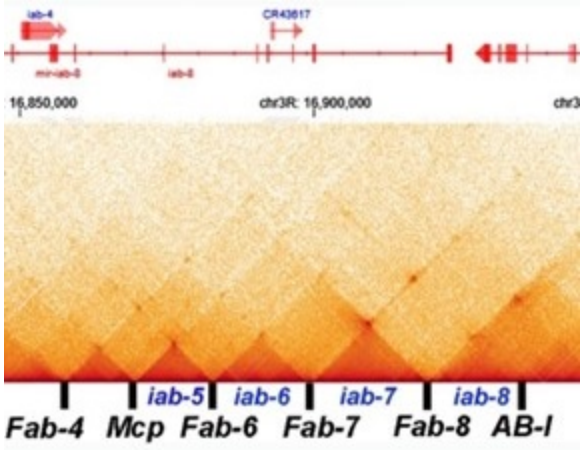
It worth noting that, with the exception of the high resolution experiments in Goel et al., all of the other studies on cohesin (and CTCF) have examined the effects on contact maps within (and between) large neighborhoods (bin sizes >1 kb). In most cases, these large neighborhoods are likely to be composed of many individual TADs like those seen in Goel et al. and in Fig. 2 of our paper. We also observe larger neighborhoods in the fly genome, though they do not appear to be as large as those in mammals. Our experiments do not address what role cohesin might have in facilitating contacts between more distant TADs located within the same neighborhoods, or between TADs in different neighborhoods, or whether loop extrusion is involved.

We would also note that the Drosophila DNA segment in Fig. 2C contains 35 different genes, while the mammalian DNA segment shown in Fig. 1 has only 9. Thus, in this part of the fly genome, Pol II genes are more densely packed than in the mammalian DNA segment. Much of the fly genome is also densely packed, and the size of individual TADs will likely be smaller, on average, than in mammals. Nevertheless, the MicroC profiles are not all that different. As is also common in flies, each TAD in the *Ppm1g* region only encompasses one or two genes. Note also that there are no volcano triangles with plumes as would be predicted for TADs that have a stem-loop topology.

In fact, as shown in Author response image 1, the high-resolution contact profile for the *Ppm1g* region shows a strong resemblance to that observed for the fly *Abd-B* regulatory domains. These regulatory domains are part of larger neighborhood that encompasses the *abd-A* and *Abd-B* genes and their regulatory domains.

Author response image 1.

Abd-B regulatory domains



> *As the authors show, the single long DNA loop mediated by cohesin loop extrusion connecting the ectopic and endogenous boundary is clearly inconsistent with the results, therefore the main conclusion of the paper that the 3D topology of the boundary elements a consequence of pairing is strong. However, the loop extrusion and pairing are not mutually exclusive models for the formation of TADs. Loop-extruding cohesin complexes need not make a 140 kb loop, multiple smaller loops could bring together the two boundary elements, which are then held together by pairing proteins that can make circular topologies.*

(1.2) In the pairing model, distant boundaries bump into each other (by random walks or partially constrained walks), and if they are “compatible” they pair with each other, typically in an orientation-dependent manner. As an alternative, the reviewer argues that cohesin need not make one large 140 kb loop. Instead it could generate a series of smaller loops (presumably corresponding to the intervening TADs). These smaller loops would bring *homie* in the transgene in close proximity to the *eve* locus so that it could interact with the endogenous *homie* and *nhomie* elements in the appropriate orientation, and in this way only one of the reporters would be ultimately activated.

There are two problems with the idea that cohesin-dependent loop extrusion brings transgene *homie* into contact with *homie/nhomie* in the *eve* locus by generating a series of small loops (TADs). The first is the very large distances over which specific boundary:boundary pairing interactions can occur. The second is that boundary:boundary pairing interactions can take place not only in *cis*, but also in *trans*.

We illustrate these points with several examples.

Fujioka et al. 2016, Fig 7 shows an experiment in which attP sites located ~2 Mb apart were used to insert two different transgenes, one containing a *lacZ* reporter and the other containing the *eve* anal plate enhancer (AP) (Fujioka et al. 2016). If the *lacZ* reporter and the AP transgenes also contain *homie*, the AP enhancer can activate *lacZ* expression (panel A,). On the other hand, if one of the transgenes has *lambda* DNA instead of *homie*, no regulatory interactions are observed (panel A,). In addition, as is the case in our experiments using the -142 kb platform, orientation matters. In the combination on the top left, the *homie* boundary is pointing away from both the *lacZ* reporter and the AP enhancer. Since *homie* pairs with itself head-tohead, pairing brings the AP enhancer into contact with the *lacZ* reporter. A different result is obtained for the transgene pair in panel A on the top right. In this combination, *homie* is pointing away from the *lacZ* reporter, while it is pointing towards the AP enhancer. As a consequence, the reporter and enhancer are located on opposite sides of the paired *homie* boundaries, and in this configuration they are unable to interact with each other.

On the top left of panel B, the *homie* element in the AP enhancer transgene was replaced by a *nhomie* boundary oriented so that it is pointing towards the enhancer. Pairing of *homie* and *nhomie* head-to-tail brings the AP enhancer in the *nhomie* transgene into contact with the *lacZ* reporter in the *homie* transgene, and it activates reporter expression. Finally, like *homie*, *nhomie* pairs with itself head-to-head, and when the *nhomie* boundaries are pointing towards both the AP reporter and the *lacZ* reporter, reporter expression is turned on.

Long distance boundary-dependent pairing interactions by the bithorax complex Mcp boundary have also been reported in several papers. Fig. 6 from Muller et al. (Muller et al. 1999) shows the pattern of regulatory interactions (in this case PRE-dependent “pairing-sensitive silencing”) between transgenes that have a mini-white reporter, the Mcp and scs’ boundaries and a PRE that is located close to Mcp. In this experiment flies carrying transgenes inserted at the indicated sites on the left and right arms of the 3rd chromosome were mated in pairwise combinations, and their trans-heterozygous progeny examined for pairing-sensitive silencing of the mini-white reporter.

Two examples of long-distance pairing-sensitive silencing mediated by *Mcp/scs’* are shown in Fig. 5b from Muller et al. 1999. The transgene inserts in panel A are *w#12.43* and *ff#10.5*. *w#12.43* is inserted close to the telomere of 3R at 99B. *ff10.5* is inserted closer to the middle of 3R at 91A. The estimate distance between them is 11.3 Mb. The transgene inserts in panel B are *ff#10.5* and *ff#11.102*. *ff#11.102* is inserted at 84D, and the distance between them is 11 Mb. Normally, the eye color phenotype of the *mini-white* reporter is additive: homozygyous inserts have twice as dark eye color as hemizygous inserts, while in _trans-_heterozygous

flies the eye color would be the sum of the two different transgenes. However, when a PRE is present and the transgene can pair, silencing is observed. In panel A, the t_rans-_heterozygous combination has a lighter eye color than either of the parents. In panel B, the _trans-_heterozygous combination is darker than one of the parents (*ff#10.5*) but much lighter than the other (*ff#11.102*).

All ten of the transgenes tested were able to engage in long distance (>Mbs) _trans_regulatory interactions; however, likely because of how the chromosome folds on the Mb scale (e.g., the location of meta-loops: see #2.1 and Author response image 3) not all of the possible pairwise silencing interactions are observed. The silencing interactions shown in Muller et.al. are between transgenes inserted on different homologs. *Mcp/scs'*-dependent silencing interactions can also occur in *cis*. Moreover, just like the *homie* and *nhomie* experiments described above, Muller et.al. (Muller et al. 1999) found that *Mcp* could mediate long-distance activation of *mini-white* and *yellow* by their respective enhancers.

The pairing-sensitive activity of the PRE associated with the *Mcp* boundary is further enhanced when the *mini-white* transgene has the *scs* boundary in addition to *Mcp* and *scs'*. In the experiment shown in Fig. 8 from Muller et al. 1999, the pairing-sensitive silencing interactions of the *Mcp/scs'/scs* transgene are between transgenes inserted on *different* chromosomes. Panel A shows pairing-sensitive silencing between *w#15.60*, which is on the X chromosome, and *w#15.102*, which is on the 2nd chromosome. Panel B shows pairing-sensitive silencing between the 2nd chromosome insert *w#15.60* and a transgene, *w#15.48*, which is inserted on the 3rd chromosome.

The long-distance *trans* and *cis* interactions described here are not unique to *homie*, *nhomie*, *Mcp*, *scs'*, or *scs*. Precisely analogous results have been reported by Sigrist and Pirrotta (Sigrist and Pirrotta 1997) for the *gypsy* boundary when the *bxd* PRE was included in the *mini-white* transgene. Also like the *Mcp*-containing transgenes in Muller et al. (Muller et al. 1999), Sigrist and Pirrotta observed pairing-sensitive silencing between _gypsy bxd_PRE *mini-white* transgenes inserted on different chromosomes. Similar long-distance (Mb) interactions have been reported for *Fab-7* (Bantignies et al. 2003; Li et al. 2011). In addition, there are examples of "naturally occurring" long-distance regulatory and/or physical interactions. One would be the regulatory/physical interactions between the p53 enhancer upstream of *reaper* and *Xrp1* which was described by Link et al. (Link et al. 2013). Another would be the nearly 60 meta-loops identified by Mohana et al. (Mohana et al. 2023).

Like *homie* at -142 kb, the regulatory interactions (pairing-sensitive silencing and enhancer activation of reporters) reported in Muller et al. (Muller et al. 1999) involve direct physical interactions between the transgenes. Vazquez et al. (Vazquez et al. 2006) used the *lacI*/*lacO* system to visualize contacts between distant *scs/Mcp/scs'*-containing transgenes in imaginal discs. As indicated in Vasquez et al. 2006, Table 3 lines #4-7, when both transgenes have *Mcp* and were inserted on the same chromosome, they colocalized in _trans-_heterozygotes (single dot) in 94% to 97% of the disc nuclei in the four pairwise combinations they tested. When the transgenes both lacked *Mcp* (Vasquez et al. 2006, Table 3 #1), co-localization was observed in 4% of the nuclei. When *scs/Mcp/scs'*-containing transgenes on the 2nd and 3rd chromosome were combined (Vasquez et al. 2006, Table 3 #8), colocalization was observed in 96% of the nuclei. They also showed that four different *scs/Mcp/scs'* transgenes (two at the same insertion site but on different homologs, and two at different sites on different homologs) co-localized in 94% of the eye imaginal disc nuclei (Vasquez et al. 2006, Table 3 #9). These pairing interactions were also found to be stable over several hours. Similar co-localization experiments together with 3C were reported by Li et al. (Li et al. 2011).

The *de novo* establishment of *trans* interactions between compatible boundary elements has been studied by Lim et al. (Lim et al. 2018). These authors visualized transvection (enhancer activation of a MS2 loop reporter in *trans*) mediated by the *gypsy* insulator, *homie* and *Fab-8* in NC14 embryos. When both transgenes shared the same boundary element,

transvection/physical pairing was observed in a small subset of embryos. The interactions took place after a delay and increased in frequency as the embryo progressed into NC14. As expected, transvection was specific: it was not observed when the transgenes had different boundaries. For *homie* it was also orientation-dependent. It was observed when *homie* was orientated in the same direction in both transgenes, but not when *homie* was orientated in opposite directions in the two transgenes.

While one could imagine that loop extrusion-dependent compaction of the chromatin located between *eve* and the transgene at -142 kb into a series of small loops (the intervening TADs) might be able to bring *homie* in the transgene close to *homie/nhomie* in the *eve* locus, there is no cohesinbased loop extrusion scenario that would bring transgenes inserted at sites 6 Mb, 11 Mb, on different sides of the centromere, or at opposite ends of the 3rd chromosome together so that the distant boundaries recognize their partners and physically pair with each other. Nor is there a plausible cohesin-based loop extrusion mechanism that could account for the fact that most of the documented long-distance interactions involve transgenes inserted on different homologs. This is not to mention the fact that long-distance interactions are also observed between boundarycontaining transgenes inserted on different chromosomes.

In fact, given these results, one would logically come to precisely the opposite conclusion. If boundary elements inserted Mbs apart, on different homologs and on different chromosomes can find each other and physically pair, it would be reasonable to think that the same mechanism (likely random collisions) is entirely sufficient when they are only 142 kb apart.

Yet another reason to doubt the involvement or need for cohesin-dependent loop extrusion in bringing the transgene *homie* in contact with the *eve* locus comes from the studies of Goel et al. (Goel et al. 2023). They show that cohesin has no role in the formation of TADs in mammalian tissue culture cells. So if TADs in mammals aren't dependent on cohesin, there would not be a good reason to think at this point that the loops (TADs) that are located between *eve* and the transgene are generated by, or even strongly dependent on, cohesin-dependent loop extrusion.

It is also important to note that even if loop-extrusion were to contribute to chromatin compaction in this context and make the looping interactions that lead to orientation-specific pairing more efficient, the role of loop extrusion in this model is not determinative of the outcome, it is merely a general compaction mechanism. This is a far cry from the popular concept of loop extrusion as being THE driving force determining chromosome topology at the TAD level.

> ***Reviewer #2 (Public Review):***
>
> *In Bing et al, the authors analyze micro-C data from NC14 fly embryos, focusing on the eve locus, to assess different models of chromatin looping. They conclude that fly TADs are less consistent with conventional cohesin-based loop extrusion models and instead rely more heavily on boundaryboundary pairings in an orientation-dependent manner.*
>
> *Overall, I found the manuscript to be interesting and thought-provoking. However, this paper reads much more like a perspective than a research article. Considering eLIFE is aimed at the general audience, I strongly suggest the authors spend some time editing their introduction to the most salient points as well as organizing their results section in a more conventional way with conclusion-based titles. It was very difficult to follow the authors' logic throughout the manuscript as written. It was also not clear as written which experiments were performed as part of this study and which were reanalyzed but published elsewhere. This should be made clearer throughout.*

> *It has been shown several times that Drosophila Hi-C maps do not contain all of the features (frequent corner peaks, stripes, etc.) observed when compared to mammalian cells. Considering these features are thought to be products of extrusion events, it is not an entirely new concept that Drosophila domains form via mechanisms other than extrusion.*

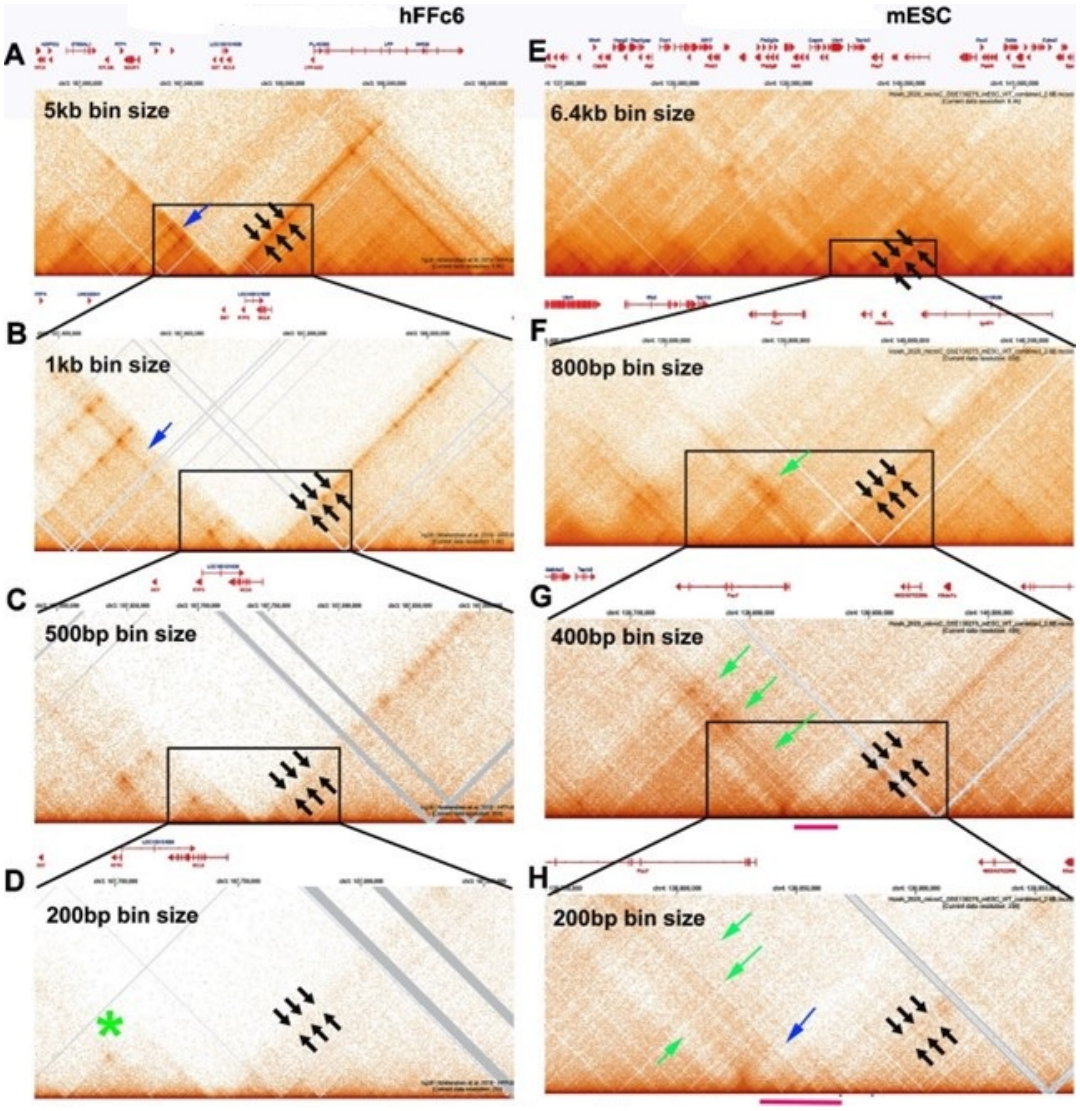
(2.1) While there are differences between the Hi-C contact profiles in flies and mammals, these differences likely reflect in large part the bin sizes used to visualize contact profiles. With the exception of Goel et al. (Goel et al. 2023), most of the mammalian Hi-C studies have been low resolution restriction enzyme-based experiments, and required bin sizes of >1 kb or greater to visualize what are labeled as "TADs." In fact, as shown by experiments in Goel et al., these are not actually TADs, but rather a conglomeration of multiple TADs into a series of TAD neighborhoods. The same is true for the MicroC experiments of Krietenstein et al. and Hsieh et al. on human and mouse tissue culture cells (Hsieh et al. 2020; Krietenstein et al. 2020). This is shown in Author response image 2. In this image, we have compared the MicroC profiles generated from human and mouse tissue culture cells with fly MicroC profiles at different levels of resolution.

For panels A-D, the genomic DNA segments shown are approximately 2.8 Mb, 760 kb, 340 kb, and 190 kb. For panels E-H, the genomic DNA segments shown are approximately 4.7 Mb, 870 kb, 340 kb and 225 kb. For panels I-L, the genomic DNA segments shown are approximately 3 Mb, 550 kb, 290 kb and 175 kb.

As reported for restriction enzyme-based Hi-C experiments, a series of stripes and dots are evident in mammalian MicroC profiles. In the data from Krietenstein et al., two large TAD "neighborhoods" are evident with a bin size of 5 kb, and these are bracketed by 45o stripes (A: black arrows). At 1 kb (panel B), the 45o stripe bordering the neighborhood on the left no longer defines the edge of the neighborhood (blue arrow: panel B), and both stripes become discontinuous (fuzzy dots). At 500 (panel C) and 200 bp (panel D) bin sizes, the stripes largely disappear (black arrows) even though they were the most prominent feature in the TAD landscape with large bin sizes. At 200 bp, the actual TADs (as opposed to the forest) are visible, but weakly populated. There are no stripes, and only one of the TADs has an obvious "dot" (green asterisk: panel C).

Author response image 2.

Mammalian MicroC profiles different bin sizes.

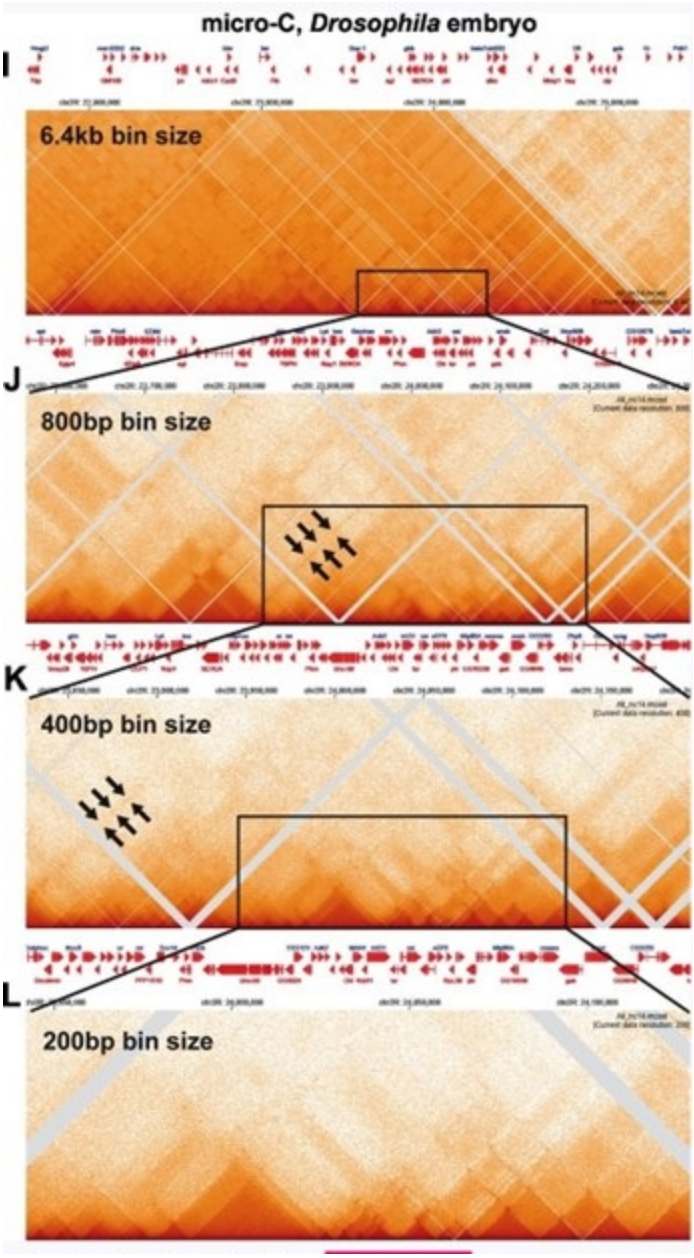


Large TAD neighborhoods bordered by stripes are also evident in the Hsieh et al. data set in Author response image 2 panels E and F (black arrows in E and F and green arrow in F). At 400 bp resolution (panel G), the narrow stripe in panel F (black arrows) becomes much broader, indicating that it is likely generated by interactions across one or two small TADs that can be discerned at 200 bp resolution. The same is true for the broad stripe indicated by the green arrows in panels F, G and H. This stripe arises from contacts between the TADs indicated by the red bar in panels G and H and the TADs to the other side of the volcano triangle with a plume (blue arrow in panel H). As in flies, we would expect that this volcano triangle topped by a plume corresponds to a stem-loop. However, the resolution is poor at 200 bp, and the profiles of the neighboring TADs are not very distinct.

For the fly data set, stripes can be discerned when analyzed at 800 bp resolution (see arrows in Author response image 3); however, these stripes are flanked by regions of lower contact, and represent TAD-TAD interactions. At 400 bp, smaller neighborhoods can be discerned, and these neighborhoods exhibit a complex pattern of interaction with adjacent neighborhoods. With bin sizes of 200 bp, individual TADs are observed, as are TAD-TAD interactions like those seen near *eve*. Some of the TADs have dots at their apex, while others do not—much like what is seen in the mammalian MicroC studies.

Author response image 3.

Mammalian MicroC profiles different bin sizes.



Stripes: As illustrated in Author response image 2 A-D and E-H, the continuous stripes seen in low resolution mammalian studies (>1 kb bins) would appear to arise from binning artefacts. At high resolution where single TADs are visible, the stripes seem to be generated by TAD-TAD interactions, and not by some type of “extrusion” mechanism. This is most clearly seen for the volcano with plume TAD in Author response inage 2 G and H. While stripes in Author response image 2 disappear at high resolution, this is not always true. There are stripes that appear to be “real” in Geol et al. 2023 for the TADs in the *Ppm1g* region, and in Author response image 1 for the *Abd-B* regulatory domain TADs. Since the stripes in the *Ppm1g* region are unaffected by Rad21 depletion, some other mechanism must be involved (c.f. (Shidlovskii et al. 2021)).

Dots: The high resolution images of mammalian MicroC experiments in Author response image 2D and H show that, like *Drosophila* (Author response image 3L), mammalian TADs don't always have a "dot" at the apex of the triangle. This is not surprising. In the MicroC procedure, fixed chromatin is digested to mononucleosomes with MNase. Since most TAD boundaries in flies, and presumably also in mammals, are relatively large (150-400 bp) nuclease hypersensitive regions, extensive MNase digestion will typically reduce the boundary element sequences to oligonucleotides.

In flies, the only known sequences (at least to date) that end up giving dots (like those seen in Author response image 1) are bound by a large (>1,000 kd) GAF-containing multiprotein complex called LBC. In the *Abd-B* region of BX-C, LBC binds to two ~180 bp sequences in *Fab-7* (dHS1 and HS3: (Kyrchanova et al. 2018; Wolle et al. 2015), and to the centromere proximal (CP) side of *Fab-8*. The LBC elements in *Fab-7* (dHS1) and *Fab-8* (CP) have both blocking and boundary bypass activity (Kyrchanova et al. 2023; Kyrchanova et al. 2019a; Kyrchanova et al. 2019b; Postika et al. 2018). Elsewhere, LBC binds to the *bx* and *bxd* PREs in the *Ubx* regulatory domains, to two PREs upstream of *engrailed*, to the *hsp70* promoter, the *histone H3-H4* promoters, and the *eve* promoter (unpublished data). Based on ChIP signatures, it likely binds to most PREs/tethering elements in the fly genome (Batut et al. 2022; Li et al. 2023). Indirect end-labeling experiments (Galloni et al. 1993; Samal et al. 1981; Udvardy and Schedl 1984) indicate that LBC protects an ~150-180 bp DNA segment from MNase digestion, which would explain why LBC-bound sequences are able to generate dots in MicroC experiments. Also unlike typical boundary elements, the pairing interactions of the LBC elements we've tested appear to be orientation-independent (unpublished data).

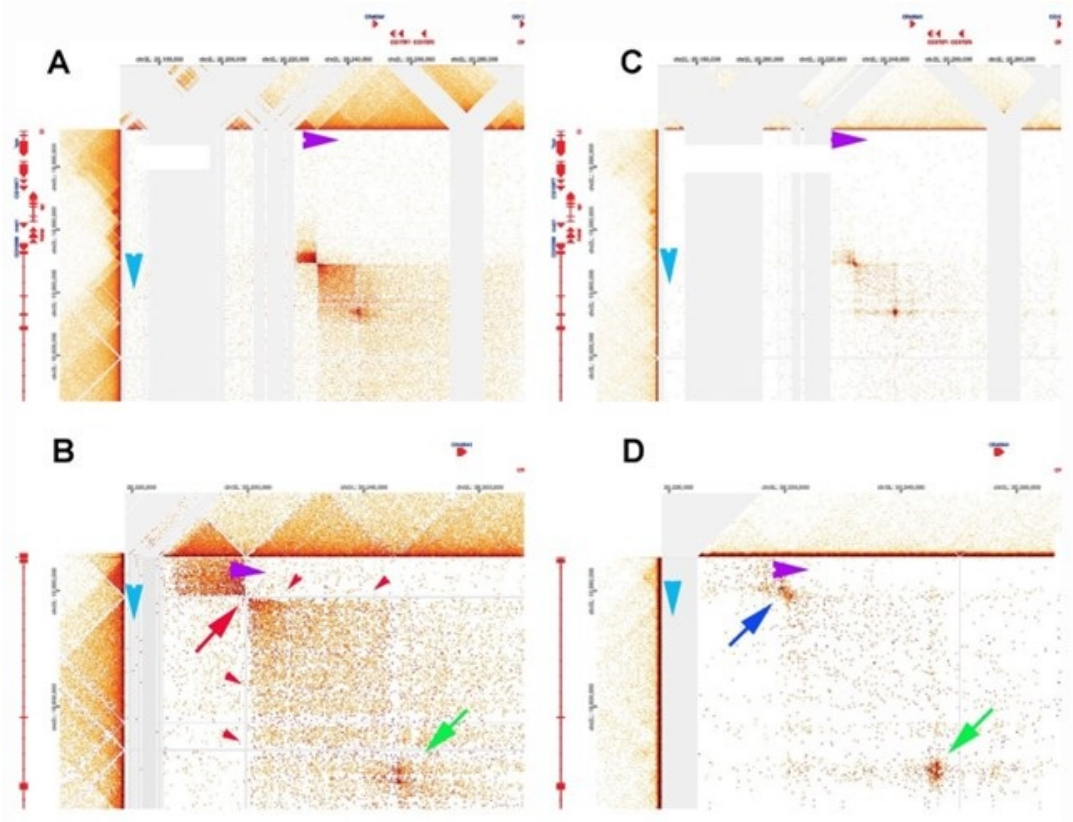
The difference in MNase sensitivity between typical TAD boundaries and LBC-bound elements is illustrated in the MicroC of the *Leukocyte-antigen-related-like* (*Lar*) meta-loop in Author response image 4 panels A and B. Direct physical pairing of two TAD boundaries (blue and purple) brings two TADs encompassing the 125 kb *lar* gene into contact with two TADs in a gene poor region 620 kb away. This interaction generates two regions of greatly enhanced contact: the two boxes on either side of the paired boundaries (panel A). Note that like transgene *homie* pairing with the *eve* boundaries, the boundary pairing interaction that forms the *lar* meta-loop is orientation-dependent. In this case the TAD boundary in the *Lar* locus pairs with the TAD boundary in the gene poor region head-to-head (arrow tip to arrow tip), generating a circle-loop. This circle-loop configuration brings the TAD upstream of the blue boundary into contact with the TAD upstream of the purple boundary. Likewise, the TAD downstream of the blue boundary is brought into contact with the TAD downstream of the purple boundary.

In the MicroC procedure, the sequences that correspond to the paired boundaries are not recovered (red arrow in Author response image 4 panel B). This is why there are vertical and horizontal blank stripes (red arrowheads) emanating from the missing point of contact. Using a different HiC procedure (dHS-C) that allows us to recover sequences from typical boundary elements (Author response image 4 panels C and D), there is a strong "dot" at the point of contact which corresponds to the pairing of the blue and purple boundaries.

There is a second dot (green arrow) within the box that represents physical contacts between sequences in the TADs downstream of the blue and purple boundaries. This dot is resistant to MNase digestion and is visible both in the MicroC and dHS-C profiles. Based on the ChIP signature of the corresponding elements in the two TADs downstream of the blue and purple boundaries, this dot represents paired LBC elements.

Author response image 4.

Lar metaloop. Panels A & bB: MicroC. Panels C & D: dHS-C



> *That being said, the authors' analyses do not distinguish between the formation and the maintenance of domains. It is not clear to this reviewer why a single mechanism should explain the formation of the complex structures observed in static Hi-C heatmaps from a population of cells at a single developmental time point. For example, how can the authors rule out that extrusion initially provides the necessary proximity and possibly the cis preference of contacts required for boundaryboundary pairing whereas the latter may more reflect the structures observed at maintenance?*

(2.2) The MicroC profiles shown in Fig. 2 of our paper were generated from nuclear cycle (NC) 14 embryos. NC14 is the last nuclear cycle before cellularization (Foe 1989). After the nuclei exit mitosis, S-phase begins, and because satellite sequences are late replicating in this nuclear cycle, S phase lasts 50 min instead of only 4-6 min during earlier cycles (Shermoen et al. 2010). So unlike MicroC studies in mammals, our analysis of chromatin architecture in NC14 embryos likely offers the best opportunity to detect any intermediates that are generated during TAD formation. In particular, we should be able to observe evidence of cohesin linking the sequences from the two extruding strands together (the stripes) as it generates TADs *de novo*. However, there are no vertical stripes in the *eve* TAD as would be expected if cohesin entered at a few specific sites somewhere within the TAD and extruded loops in opposite directions synchronously, nor are their stripes at 45o as would be expected if it started at *nhomie* or *homie* (see Figure Supplemental 1). We also do not detect cohesin-generated stripes in any of the TADs in between *eve* and the attP site at -142 kb. Note that in some models, cohesin is thought to be continuously extruding loops. After hitting the CTCF roadblocks, cohesin either falls off after a short period and starts again or it breaks through one or more TAD boundaries generating the LDC domains. In this dynamic model, stripes of crosslinked DNA generated by the passing cohesin complex should be observed throughout the cell cycle. They are not.

As for formation versus maintenance, and the possible involvement of cohesin loop extrusion in the former, but not the latter: This question was indirectly addressed in point #1.2 above. In this point we described multiple examples of specific boundary:boundary pairing interactions that take place over Mbs, in *cis* and in *trans* and even between different chromosomes. These long-distance interactions don't preexist; instead they must be established *de novo* and then maintained. This process was actually visualized in the studies of Lim et al. (Lim et al. 2018) on the establishment of *trans* boundary pairing interactions in NC14 embryos. There is no conceivable mechanism by which cohesin-based loop extrusion could *establish* the long or short distance *trans* interactions that have been documented in many studies on fly boundary elements. Also as noted above, its seems unlikely that it is necessary for long-range interactions in *cis*.

A more plausible scenario is that cohesin entrapment helps to stabilize these long-distance interactions after they are formed. If this were true, then one could argue that cohesin might also function to maintain TADs after boundaries have physically paired with their neighbors in *cis*. However, the Rad21 depletion experiments of Goel et al. (Goel et al. 2023) would rule out an essential role for cohesin in maintaining TADs after boundary:boundary pairing. In short, while we cannot formally rule out that loop extrusion might help bring sequences closer together to increase their chance of pairing, neither the specificity of that pairing, nor its orientation can be explained by loop extrusion. Furthermore, since pairing in *trans* cannot be facilitated by loop extrusion, invoking it as potentially important for boundary-boundary pairing in *cis* can only be described as a potential mechanism in search of a function, without clear evidence in its favor.

On the other hand, the apparent loss of contacts between TADs within large multi-TAD neighborhoods (Geol et al. 2023) would suggest that there is some sort of decompaction of neighborhoods after Rad21 depletion. It is possible that this might stress interactions that span multiple TADs as is the case for *homie* at -142, or for the other examples described in #1.2 above. This kind of involvement of cohesin might or might not be associated with a loop extrusion mechanism.

> *Future work aimed at analyzing micro-C data in cohesin-depleted cells might shed additional light on this.*

(2.3) This experiment has been done by Goel et al. (Goel et al. 2023) in mammalian tissue culture cells. They found that TADs, as well as local TAD neighborhoods, are not disrupted/altered by Rad21 depletion (see Geol at al. 2023 and our response to point #1.1 of reviewer #1).

> *Additional mechanisms at play include compartment-level interactions driven by chromatin states. Indeed, in mammalian cells, these interactions often manifest as a "plume" on Hi-C maps similar to what the authors attribute to boundary interactions in this manuscript. How do the chromatin states in the neighboring domains of the eve locus impact the model if at all?*

(2.4) Chromatin states have been implicated in driving compartment level interactions.

Compartments as initially described were large, often Mb sized, chromosomal segments that "share" similar chromatin marks/states, and are thought to merge via co-polymer segregation. They were visualized using large multi-kb bin sizes. In the studies reported here, we use bin sizes of 200 bp to examine a DNA segment of less than 200 kb which is subdivided into a dozen or so small TADs. Several of the TADs contain more than one transcription unit, and they are expressed in quite different patterns, and thus might be expected to have different "chromatin states" at different points in development and in different cells in the

organism. However, as can be seen by comparing the MicroC patterns in our paper that are shown in Fig. 2 with Fig. 7, Figure Supplemental 5 and Figure Supplemental 6, the TAD organization in NC14 and 12-16 hr embryos is for the most part quite similar. There is no indication that these small TADs are participating in liquid phase compartmentalization that depends upon shared chromatin/transcriptional states in NC14 and then again in 12-16 hr embryos.

In NC14 embryos, *eve* is expressed in 7 stripes, while it is potentially active throughout much of the embryo. In fact, the initial pattern in early cycles is quite broad and is then refined during NC14. In 12-16 hr embryos, the *eve* gene is silenced by the PcG system in all but a few cells in the embryo. However, here again the basic structure of the TAD, including the volcano plume, looks quite similar at these different developmental stages.

As for the suggestion that the plume topping the *eve* volcano triangle is generated because the TADs flanking the *eve* TAD share chromatin states and coalesce via some sort of phase separation:

This model has been tested directly in Ke et al. (Ke et al. 2024). In Ke et al., we deleted the *nhomie* boundary and replaced it with either *nhomie* in the reverse orientation or *homie* in the forward orientation. According to the compartment model, changing the orientation of the boundaries so that the topology of the *eve* TAD changes from a stem-loop to a circle-loop should have absolutely no effect on the plume topping the *eve* volcano triangle. The TADs flanking the *eve* TAD would still be expected to share the same chromatin states and would still be able to coalesce via phase transition. However, this is not what is observed. The plume disappears and is replaced by “clouds” on both sides of the *eve* TAD. The clouds arise because the *eve* TAD bumps into the neighboring TADs when the topology is a circle-loop.

We would also note that “compartment-level” interactions would not explain the findings presented in Muller at al. 1999, in Table 1 or in Author response image 4. It is clear that the long distant (Mb) interactions observed for *Mcp*, *gypsy*, *Fab-7*, *homie*, *nhomie* and the blue and purple boundaries in Author response image 4 arise by the physical pairing of TAD boundary elements. This fact is demonstrated directly by the MicroC experiments in Fig. 7 and Fig Supplemental 4 and 5, and by the MicroC and dHS-C experiments in Author response image 4. There is no evidence for any type of “compartment/phase separation” driving these specific boundary pairing interactions.

In fact, given the involvement of TAD boundaries in meta-loop formation, one might begin to wonder whether some of the “compartment level interactions” are generated by the specific pairing of TAD boundary elements rather than by “shared chromatin” states. For example, the head-tohead pairing of the blue and purple boundaries generates a *Lar* meta-loop that has a circle-loop topology. As a consequence, sequences upstream of the blue and purple boundary come into contact, generating the small dark rectangular box on the upper left side of the contact map. Sequences downstream of the blue and purple boundary also come into contact, and this generates the larger rectangular box in the lower right side of the contact map. A new figure, Fig. 9, shows that the interaction pattern flips (lower left and top right) when the meta-loop has a stem-loop topology. If these meta-loops are visualized using larger bin sizes, the classic “compartment” patchwork pattern of interactions emerges. Would the precise patchwork pattern of “compartmental” interactions involving the four distant TADs that are linked in the two meta-loops shown in Fig. 9 persist as is if we deleted one of the TAD boundaries that forms the meta-loop? Would the precise patchwork pattern persist if we inverted one of the meta-loop boundaries so that we converted the topology of the loop from a circle-loop to a stem-loop or vice versa? We haven’t used MicroC to compare the compartment organization after deleting or inverting a meta-loop TAD boundary; however, a comparison of the MicroC pattern in WT in Fig. 1C with that for the *homie* transgenes in Fig. 7 and Figs. Supplemental 5, 6 and 7 indicates a) that novel patterns of TAD:TAD interactions are generated by this *homie* dependent mini-meta-loop and b) that the patterns of TAD:TAD

interactions depend upon loop topology. Were these novel TAD:TAD interactions generated instead by compartment level interactions/shared chromatin states, they should be evident in WT as well (Fig. 1). They are not.

> *How does intrachromosomal homolog pairing impact the models proposed in this manuscript (Abed et al. 2019; Erceg et al., 2019). Several papers recently have shown that somatic homolog pairing is not uniform and shows significant variation across the genome with evidence for both tight pairing regions and loose pairing regions. Might loose pairing interactions have the capacity to alter the cis configuration of the eve locus?*

(2.5) At this point it is not entirely clear how homolog pairing impacts the *cis* configuration/MicroC contact maps. We expect that homolog pairing is incomplete in the NC14 embryos we analyzed; however, since replication of *eve* and the local neighborhood is likely complete, sister chromosomes should be paired. So we are likely visualizing the 3D organization of paired TADs.

> *In summary, the transgenic experiments are extensive and elegant and fully support the authors' models. However, in my opinion, they do not completely rule out additional models at play, including extrusion-based mechanisms. Indeed, my major issue is the limited conceptual advance in this manuscript. The authors essentially repeat many of their previous work and analyses.*

(2.6) In our view, the current paper makes a number of significant contributions that go well beyond those described in our 2016 publication. These are summarized below.

A) While our 2016 paper used transgenes inserted in the -142 kb attP site to study pairing interactions of *homie* and *nhomie*, we didn't either consider or discuss how our findings might bear on the loop extrusion model. However, since the loop extrusion model is currently accepted as established fact by many labs working on chromosome structure, it is critically important to devise experimental approaches which test the predictions of this particular model. One approach would be to deplete cohesin components; however, as discussed in #1.1, our experimental system is not ideal for this type of approach. On the other hand, there are other ways to test the extrusion model. Given the mechanism proposed for TAD formation—extruding a loop until cohesin bumps into CTCF/boundary road blocks—it follows that only two types of loop topologies are possible: stemloop and unanchored loop. The loop extrusion model, as currently conceived, can't account for the two cases in this study in which the reporter on the wrong side of the *homie* boundary from the *eve* locus is activated by the *eve* enhancers. In contrast, our findings are completely consistent with orientation-specific boundary:boundary pairing.

B) In the loop extrusion model, cohesin embraces both of the extruded chromatin fibers, transiently bringing them into close proximity. As far as we know, there have been no (high resolution) experiments that have actually detected these extruding cohesin complexes during TAD formation. In order to have a chance of observing the expected signatures of extruding cohesin complexes, one would need a system in which TADs are being formed. As described in the text, this is why we used MicroC to analyze TADs in NC14 embryos. We do not detect the signature stripes that would be predicted (see Figure Supp 2) by the current version of the loop extrusion model.

C) Reporter expression in the different -142 kb transgenes provides only an indirect test of the loop extrusion and boundary:boundary pairing models for TAD formation. The reporter expression results need to be confirmed by directly analyzing the pattern of physical interactions in each instance. While we were able to detect contacts between the transgenes and *eve* in our 2016 paper, the 3C experiments provided no information beyond that. By

contrast, the MicroC experiments in the current paper give high resolution maps of the physical contacts between the transgene and the *eve* TAD. The physical contacts track completely with reporter activity. Moreover, just as is the case for reporter activity, the observed physical interactions are inconsistent with the loop extrusion model.

D) Genetic studies in Muller et al. (Muller et al. 1999) and imaging in Vazquez et al. (Vazquez et al. 2006) suggested that more than two boundaries can participate in pairing interactions. Consistent with these earlier observations, viewpoint analysis indicates the transgene *homie* interacts with both *eve* boundaries. While this could be explained by transgene *homie* alternating between *nhomie* and *homie* in the *eve* locus, this would require the remodeling of the *eve* TAD each time the pairing interaction switched between the three boundary elements. Moreover, two out of the three possible pairing combinations would disrupt the *eve* TAD, generating an unanchored loop (c.f., the *lambda* DNA TAD in Ke et al., (Ke et al. 2024)). However, the MicroC profile of the *eve* TAD is unaffected by transgenes carrying the *homie* boundary. This would suggest that like *Mcp*, the pairing interactions of *homie* and *nhomie* might not be exclusively pairwise. In this context is interesting to compare the contact profiles of the *lar* meta-loop shown in Author response image 4 with the different 142 kb *homie* inserts. Unlike the *homie* element at -142 kb, there is clearly only a single point of contact between the blue and purple boundaries.

E) Chen et al. (Chen et al. 2018) used live imaging to link physical interactions between a *homie* containing transgene inserted at -142 kb and the *eve* locus to reporter activation by the *eve* enhancers. They found that the reporter was activated by the *eve* enhancers only when it was in “close proximity” to the *eve* gene. “Close proximity” in this case was 331 nM. This distance is equivalent to ~1.1 kb of linear duplex B form DNA, or ~30 nucleosome core particles lined up in a row. It would not be possible to ligate two DNAs wrapped around nucleosome core particles that are located 330 nM apart in a fixed matrix. Since our MicroC experiments were done on embryos in which the gene is silent in the vast majority of cells, it is possible that the *homie* transgene only comes into close enough proximity for transgene nucleosome: *eve* nucleosome ligation events when the *eve* gene is off. Alternatively, and clearly more likely, distance measurements using imaging procedures that require dozens of fluorescent probes may artificially inflate the distance between sequences that are actually close enough for enzymatic ligation.

F) The findings reported in Goel et al. (Goel et al. 2023) indicate that mammalian TADs don’t require cohesin activity; however, the authors do not provide an alternative mechanism for TAD formation/stability. Here we have suggested a plausible mechanism.

> *The authors make no attempt to dissect the mechanism of this process by modifying extrusion components directly.*

(2.7) See point #1.1

> *Some discussion of Rollins et al. on the discovery of Nipped-B and its role in enhancer-promoter communication should also be made to reconcile their conclusions in the proposed absence of extrusion events.*

(2.8) The reason why reducing *nipped-B* activity enhances the phenotypic effects of *gypsy*-induced mutations is not known at this point; however, the findings reported in Rollins et al. (Rollins et al. 1999) would appear to argue against an extrusion mechanism for TAD formation.

Given what we know about enhancer blocking and TADs, there are two plausible mechanisms for how the Su(Hw) element in the *gypsy* transposon blocks enhancer-promoter interactions in the *gypsy*-induced mutants studied by Rollins et al. First, the Su(Hw) element

could generate two new TADs through pairing interactions with boundaries in the immediate neighborhood. This would place the enhancers in one TAD and the target gene in another TAD. Alternatively, the studies of Sigrist and Pirrotta (Sigrist and Pirrotta 1997) as well as several publications from Victor Corces' lab raise the possibility that the Su(Hw) element in *gypsy*-induced mutations is pairing with *gypsy* transposons inserted elsewhere in the genome. This would also isolate enhancers from their target genes. In either case, the loss of *nipped-B* activity increases the mutagenic effects of Su(Hw) element presumably by strengthening its boundary function. If this is due to a failure to load cohesin on to chromatin, this would suggest that cohesin normally functions to *weaken* the boundary activity of the Su(Hw) element, i.e., disrupting the ability of Su(Hw) elements to interact with either other boundaries in the neighborhood or with themselves. Were this a general activity of cohesin (to *weaken* boundary activity), one would imagine that cohesin normally functions to disrupt TADs rather than generate/stabilize TADs.

An alternative model is that Nipped-B (and thus cohesion) functions to stabilize enhancerpromoter interactions within TADs. In this case, loss of Nipped-B would result in a destabilization of the weak enhancer:promoter interactions that can still be formed when *gypsy* is located between the enhancer and promoter. In this model the loss of these weak interactions in *nipped-b* mutants would appear to increase the "blocking" activity of the *gypsy* element. However, this alternative model would also provide no support for the notion that Nipped-B and cohesin function to promote TAD formation.

> ***Reviewer #3 (Public Review):***
>
> *Bing et al. attempt to address fundamental mechanisms of TAD formation in Drosophila by analyzing gene expression and 3D conformation within the vicinity of the eve TAD after insertion of a transgene harboring a Homie insulator sequence 142 kb away in different orientations. These transgenes along with spatial gene expression analysis were previously published in Fujioka et al. 2016, and the underlying interpretations regarding resulting DNA configuration in this genomic region were also previously published. This manuscript repeats the expression analysis using smFISH probes in order to achieve more quantitative analysis, but the main results are the same as previously published. The only new data are the Micro-C and an additional modeling/analysis of what they refer to as the 'Z3' orientation of the transgenes. The rest of the manuscript merely synthesizes further interpretation with the goal of addressing whether loop extrusion may be occurring or if boundary:boundary pairing without loop extrusion is responsible for TAD formation. The authors conclude that their results are more consistent with boundary:boundary pairing and not loop extrusion; however, most of this imaging data seems to support both loop extrusion and the boundary:boundary models. This manuscript lacks support, especially new data, for its conclusions.*

(3.1) The new results/contributions of our paper are described in #2.6 above.

Although there are (two) *homie* transgene configurations that give expression patterns that would be consistent with the loop extrusion model, that is not quite the same as strong evidence supporting loop extrusion. On the contrary, key aspects of the expression data are *entirely inconsistent* with loop extrusion, and they thus rule out the possibility that loop extrusion is sufficient to explain the results. Moreover, the conclusions drawn from the expression patterns of the four transgenes are back up by the MicroC contact profiles—profiles that are also not consistent with the loop extrusion model. Further, as documented above, loop extrusion is not only unable to explain the findings reported in this manuscript, but also the results from a large collection of published studies on fly boundaries. Since all of these boundaries function in TAD formation, there is little reason to think that loop extrusion makes a significant contribution at the TAD level in flies. Given the results reported by Goel et

al. (Goel et al. 2023), one might also have doubts about the role of loop extrusion in the formation/maintenance of mammalian TADs.

To further document these points, we've included a new figure (Fig. 9) that shows two meta-loops. Like the loops seen for *homie*-containing transgenes inserted at -142 kb, meta-loops are formed by the pairing of distant fly boundaries. As only two boundaries are involved, the resulting loop topologies are simpler than those generated when transgene *homie* pairs with *nhomie* and *homie* in the *eve* locus. The meta-loop in panel B is a stem-loop. While a loop with this topology could be formed by loop extrusion, cohesion would have to break through dozens of intervening TAD boundaries and then somehow know to come to a halt at the blue boundary on the left and the purple boundary on the right. However, none of the mechanistic studies on either cohesin or the mammalian CTCF roadblocks have uncovered activities of either the cohesin complex or the CTCF roadblocks that could explain how cohesin would be able to extrude hundreds of kb and ignore dozens of intervening roadblocks, and then stop only when it encounters the two boundaries that form the *beat-IV* meta-loop. The meta-loop in panel A is even more problematic in that it is a circle-loop--a topology that can't be generated by cohesin extruding a loop until comes into contact with CTCF roadblocks on the extruded strands.

> *Furthermore, there are many parts of the manuscript that are difficult to follow. There are some minor errors in the labelling of the figures that if fixed would help elevate understanding. Lastly, there are several major points that if elaborated on, would potentially be helpful for the clarity of the manuscript.*
>
> *Major Points:*
>
> *(1) The authors suggest and attempt to visualize in the supplemental figures, that loop extrusion mechanisms would appear during crosslinking and show as vertical stripes in the micro-C data. In order to see stripes, a majority of the nuclei would need to undergo loop extrusion at the same rate, starting from exactly the same spots, and the loops would also have to be released and restarted at the same rate. If these patterns truly result from loop extrusion, the authors should provide experimental evidence from another organism undergoing loop extrusion.*

(3.2) We don't know of any reports that actually document cohesion extrusion events that are forming TADs (TADs as defined in our paper, in the RCMC experiments of Goel et al. (Goel et al. 2023), in response #1.1, or in the high-resolution images from the MicroC data of Krietenstein et al (Krietenstein et al. 2020) and Hseih et al. (Hsieh et al. 2020). However, an extruding cohesin complex would be expected to generate stripes because it transiently brings together the two chromatin strands as illustrated by the broken zipper in Figure Supplemental 2 of our paper. While stripes generated by cohesin forming a TAD have not to our knowledge ever been observed, Fig. 4 in Goel et al. (Goel et al. 2023)) shows 45o stripes outlining TADs and connecting neighboring TADs. These stripes are visible with or without Rad21.

In some versions of the loop extrusion model, cohesin extrudes a loop until it comes to a halt at both boundaries, where it then remains holding the loop together. In this model, the extrusion event would occur only once per cell cycle. This is reason we selected NC14 embryos as this point in development should provide by far the best opportunity to visualize cohesin-dependent TAD formation. However, the expected stripes generated by cohesin embrace of both strands of the extruding loop were not evident. Other newer versions of the loop extrusion model are much more dynamic—cohesin extrudes the loop, coming to a halt at the two boundaries, but either doesn't remain stably bound or breaks through one or both boundaries. In the former case, the TAD needs to be reestablished by another extrusion event, while in the latter case LDC domains are generated. In this dynamic model, we should also be

able to observe vertical and 45o stripes (or stripes leaning to one side or another of the loading site if the extrusion rates aren't equal on both fibers) in NC14 embryos corresponding to the formation of TADs and LDC domains. However, we don't.

> *(2) On lines 311-314, the authors discuss that stem-loops generated by cohesin extrusion would possibly be expected to have more next-next-door neighbor contacts than next-door neighbor contacts and site their models in Figure 1. Based on the boundary:boundary pairing models in the same figure would the stem-loops created by head-to-tail pairing also have the same phenotype? Making possible enrichment of next-next-door neighbor contacts possible in both situations? The concepts in the text are not clear, and the diagrams are not well-labeled relative to the two models.*

(3.3) Yes, we expect that stem-loops formed by cohesin extrusion or head-to-tail pairing would behave in a similar manner. They could be stem-loops separated by unanchored loops as shown in Fig. 1B and E. Alternatively, adjacent loops could be anchored to each other (by cohesin/CTCF road blocks or by pairing interactions) as indicated in Fig. 1C and F. In stem-loops generated either by cohesin extrusion or by head-to-tail pairing, next-next door neighbors should interact with each other, generating a plume above the volcano triangle. In the case of circle-loops, the volcano triangle should be flanked by clouds that are generated when the TAD bumps into both next-door neighbors. In the accompanying paper, we test this idea by deleting the *nhomie* boundary and then a) inserting *nhomie* back in the reverse orientation, or b) by inserting *homie* in the forward orientation. The MicroC patterns fit with the predictions that were made in this paper.

> *(3) The authors appear to cite Chen et al., 2018 as a reference for the location of these transgenes being 700nM away in a majority of the nuclei. However, the exact transgenes in this manuscript do not appear to have been measured for distance. The authors could do this experiment and include expression measurements.*

(3.4) The transgenes used in Chen et al. are modified versions of a transgene used in Fujioka et al. (2016) inserted into the same attP site. When we visualize reporter transcription in NC14 embryos driven by the *eve* enhancers using smFISH, HCR-FISH or DIG, only a subset of the nuclei at this stage are active. The number of active nuclei we detect is similar to that observed in the live imaging experiments of Chen et al. The reason we cited Chen et al. (Chen et al. 2018) was that they found that proximity was a critical factor in determining whether the reporter was activated or not in a given nucleus. The actual distance they measured wasn't important. Moreover, as we discussed in response #2.6 above, there are good reasons to think that the "precise" distances measured in live imaging experiments like those used in Chen et al. are incorrect. However, their statements are certainly correct if one considers that a distance of ~700 nM or so is "more distant" relative to a distance of ~300 nM or so, which is "closer."

> *(4) The authors discuss the possible importance of CTCF orientation in forming the roadblock to cohesin extrusion and discuss that Homie orientation in the transgene may impact Homie function as an effective roadblock. However, the Homie region inserted in the transgene does not contain the CTCF motif. Can the authors elaborate on why they feel the orientation of Homie is important in its ability to function as a roadblock if the CTCF motif is not present? Trans-acting factors responsible for Homie function have not been identified and this point is not discussed in the manuscript.*

We discussed the "importance" of CTCF orientation in forming roadblocks because one popular version of the cohesin loop extrusion/CTCF roadblock model postulates that CTCF must be oriented so that the N-terminus of the protein is facing towards the oncoming cohesin complex, otherwise it won't be able to halt extrusion on that strand. When *homie* in

the transgene is pointing towards the *eve* locus, the reporter on the other side (farther from *eve*) is activated by the *eve* enhancers. One possible way to explain this finding (if one believes the loop extrusion model) is that when *homie* is inverted, it can't stop the oncoming cohesin complex, and it runs past the *homie* boundary until it comes to a stop at a properly oriented boundary farther away. In this case, the newly formed loop would extend from the boundary that stopped cohesin to the *homie* boundary in the *eve* locus, and would include not only the distal reporter, but also the proximal reporter. If both reporters are in the same loop with the *eve* enhancers (which they would have to be given the mechanism of TAD formation by loop extrusion), both reporters should be activated. They are not.

For the boundary pairing model, the reporter that will be activated will depend upon the orientation of the pairing interaction—which can be either head-to-head or head-to-tail (or both: see discussion of LBC elements in #2.1). For an easy visualization of how the orientation of pairing interactions is connected to the patterns of interactions between sequences neighboring the boundary, please look at Fig. 9. This figure shows two different meta-loops. In panel A, head-tohead pairing of the blue and purple boundaries brings together, on the one hand, sequences upstream of the blue and purple boundary, and on the other hand, sequences downstream of the blue and purple boundaries. In the circle loop configuration, the resulting rectangular boxes of enhanced contact are located in the upper left and lower right of the contact map. In panel B, the head-to-tail pairing of the blue and purple boundary changes how sequences upstream and downstream of the blue and purple boundaries interact with each other. Sequences upstream of the blue boundary interact with sequences downstream of the purple boundary, and this gives the rectangular box of enhanced interactions on the top right. Sequences downstream of the blue boundary interact with sequences upstream of the purple boundary, and this gives the rectangular box of enhanced contact on the lower left.

CTCF: Our analysis of the *homie* boundary suggests that CTCF contributes little to its activity. It has an Su(Hw) recognition sequence and a CP190 "associated" sequence. Mutations in both compromise boundary activity (blocking and -142 kb pairing). Gel shift experiments and ChIP data indicate there are half a dozen or more additional proteins that associate with the 300 bp *homie* fragment used in our experiments.

Orientation of CTCF or other protein binding sites: The available evidence suggests that orientation of the individual binding sites is not important (Kyrchanova et al. 2016; Lim et al. 2018)). Instead, it is likely that the order of binding sites affects function.

> *(5) The imaging results seem to be consistent with both boundary:boundary interaction and loop extrusion stem looping.*

It is not clear whether the reviewer is referring to the different patterns of reporter expression— which clearly don't fit with the loop extrusion model in the key cases that distinguish the two models—or the live imaging experiments in Chen et al. (Chen et al. 2018).

> *(6) The authors suggest that the eveMa TAD could only be formed by extrusion after the breakthrough of Nhomie and several other roadblocks. Additionally, the overall long-range interactions with Nhomie appear to be less than the interactions with endogenous Homie (Figures 7, 8, and supplemental 5). Is it possible that in some cases boundary:boundary pairing is occurring between only the transgenic Homie and endogenous Homie and not including Nhomie?*

Yes, it is possible. On the other hand, the data that are currently available supports the idea that transgene *homie* usually interacts with endogenous *homie* and *nhomie* at the same time. This is discussed in #2.6D above. The viewpoints indicate that crosslinking occurs more frequently to *homie* than to *nhomie*. This could indicate that when there are only pairwise

interactions, these tend to be between *homie* and *homie*. Alternatively, this could also be explained by a difference in relative crosslinking efficiency.

> *(7) In Figure 4E, the GFP hebe expression shown in the LhomieG Z5 transgenic embryo does not appear in the same locations as the LlambdaG Z5 control. Is this actually hebe expression or just a background signal?*

The late-stage embryos shown in E are oriented differently. For *GlambdaL*, the embryo is oriented so that *hebe*-like reporter expression on the ventral midline is readily evident. However, this orientation is not suitable for visualizing *eve* enhancer-dependent expression of the reporters in muscle progenitor cells. For this reason, the 12-16 hr *GeimohL* embryo in E is turned so that the ventral midline isn't readily visible in most of the embryo. As is the case in NC14 embyros, the *eve* enhancers drive *lacZ* but not *gfp* expression in the muscle progenitor cells.

> *(8) Figure 6- The LhomieG Z3 (*LeimohG*) late-stage embryo appears to be showing the ventral orientation of the embryo rather than the lateral side of the embryo as was shown in the previous figure. Is this for a reason? Additionally, there are no statistics shown for the Z3 transgenic images.*

Were these images analyzed in the same way as the Z5 line images?

The *LeimohG* embryo was turned so that the *hebe* enhancer-dependent expression of *lacZ* is visible. While the *eve* enhancer-dependent expression of *lacZ* in the muscle progenitor cells isn't visible with this orientation, *eve* enhancer-dependent expression in the anal plate is.

> *(9) Do the Micro-C data align with the developmental time points used in the smFISH probe assays?*

The MicroC data aligns with the smFISH images of older embryos: 12-14 hour embryos or stages 14-16.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *This was a difficult paper to review. It took me several hours to understand the terminology and back and forth between different figures to put it together. It might be useful to put the loop models next to the MicroC results and have a cartoon way of incorporating which enhancers are turning on which reporters.*
>
> *I also found the supercoiled TAD models in Figure 1 not useful. These plectoneme-type of structures likely do not exist, based on the single-cell chromosome tracing studies, and the HiC structures not showing perpendicular to diagonal interactions between the arms of the plectonemes.*

We wanted to represent the TAD as a coiled 30nM fiber, as they are not likely to resemble the large loops like those shown in Fig. 1 A, D, and G.

> *There are no stripes emerging from homies, which is consistent with the pairing model, but there seem to be stripes from the eve promoter. I think these structures may be a result of both the underlying loop extruders + pairing elements.*

There are internal structures in the *eve* TAD that link the upstream region of the *eve* promoter to the *eve* PRE and sequences in *nhomie*. All three of these sequences are bound by

LBC. Each of the regulatory domains in BX-C also have LBC elements and, as shown in Author response image 1, you can see stripes connecting some of these LBC elements to each other. Since the stripes that Goel et al. (Goel et al. 2023) observed in their RCMC analysis of *Ppm1g* didn't require cohesin, how these stripes are generated (active: e.g, a chromatin remodeler or passive: e.g., the LBC complex has non-specific DNA binding activity that can be readily crosslinked as the chromatin fiber slides past) isn't clear.

> *The authors say there are no TADs that have "volcano plumes" but the leftmost TAD TA appears to have one. What are the criteria for calling the plumes? I am also not clear why there is a stripe off the eve volcano. It looks like homie is making a "stripe" loop extrusion type of interaction with the next TAD up. Is this maybe cohesin sliding off the left boundary?*

The reviewer is correct, the left-most TAD TA appears to have a plume. We mentioned TA seems to have a plume in the original text, but it was inadvertently edited out.

Two different types of TADßàTAD interactions are observed. In the case of *eve*, the TADs to either side of *eve* interact more frequently with each other than they do with *eve*. This generates a "plume" above the *eve* volcano triangle. The TADs that comprise the *Abd-B* regulatory domains (see Author response image 1) are surrounded by clouds of diminishing intensity. Clouds at the first level represent interactions with both next-door neighbors; clouds at the second level represent interactions with both next-next-door neighbors; clouds at the third level represent interactions with next-next-next door neighbors. The *Abd-B* TADs are close to the same size, so that interactions with neighbors are relatively simple. However, this is not always the case. When there are smaller TADs near larger TADs the pattern of interaction can be quite complicated. An example is indicated by the red bar in Author response image 2

> *The authors state "In the loop-extrusion model, a cohesin complex initiating loop extrusion in the eve TAD must break through the nhomie roadblock at the upstream end of the eve TAD. It must then make its way past the boundaries that separate eve from the attP site in the hebe gene, and come to a halt at the homie boundary associated with the lacZ reporter." Having multiple loops formed by cohesin would also bring in the 142kb apart reporter and homie. Does cohesin make 140 kb long loops in flies?*

A mechanism in which cohesin brings the reporter close to the *eve* TAD by generating many smaller loops (which would be the intervening TADs) was discussed in #1.2.

> *Figure 5 title mistakes the transgene used?*

Fixed.

> *In figure 6, the orientation of the embryos does not look the same for the late-stage panels. So it was difficult to tell if the eve enhancer was turning the reporter on.*

Here we were focusing mainly on the AP enhancer activation of the reporter, as this is most easily visualized. It should be clear from the images that the appropriate reporter is activated by the AP enhancer for each of the transgene inserts.

> *It is not clear to me why the GFP makes upstream interactions (from the 4C viewpoint) in GhomileLZ5 but not in LhomieGZ5? Corresponding interactions for Fig Supp 5 & 6 are not the same. That is, LacZ in the same place and with the same homie orientation does not show a similar upstream enrichment as the GFP reporter does.*

We are uncertain as to whether we understand this question/comment. In GhomieLZ5 (now *GhomieL*, the *lacZ* reporter is on the eve side of the homie boundary while *gfp* is on the *hebe* enhancer side of the homie boundary. Since homie is pointing away from gfp, pairing interactions with *homie* and *nhomie* in the *eve* locus bring the *eve* enhancers in close proximity with the *gfp* reporter. This is what is seen in Fig. 7 panel D—lower trace. In LhomieGZ5 (now *GeimohL*) the *lacZ* reporter is again on the *eve* side of the *homie* boundary while *gfp* is on the *hebe* enhancer side of the *homie* boundary. However, in this case *homie* is inverted so that it is points away from *lacZ* (towards *gfp*). In this orientation, pairing brings the *lacZ* reporter into contact with the *eve* enhancers. This is what is seen in the upper trace in Fig. 7 panel D.

The orientation of the transgene is switch in Fig. Supp 5 and 6. For these "Z3) transgenes (now called *LeimohG* and *LhomieG* the *gfp* reporter is on the *eve* side of *homie* while the *lacZ* reporter is on the *hebe* enhancer side of *homie*. The interactions between the reporters and *eve* are determined by the orientation of *homie* in the transgene. When *homie* is pointing away from *gfp* (as in *LeimohG*), *gfp* is activated and that is reflected in the trace in Supp Fig. 5. When *homie* is pointing away from *lacZ*, *lacZ* is activated and this is reflected (though not as cleanly as in other cases) in the trace in Supp Fig. 6.

> *I did not see a data availability statement. Is the data publicly available? The authors also should consider providing the sequences of the insertions, or provide the edited genomes, in case other researchers would like to analyze the data.*

Data have been deposited.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Minor Points:*
>
> *(1) There is an inconsistency in the way that some of the citations are formatted. Some citations have 'et al' italicized while others do not. It seems to be the same ones throughout the manuscript. Some examples: Chetverina et al 2017, Chetverina et al 2014, Cavalheiro et al 2021, Kyrchanova et al 2008a, Muravyova et al 2001.*

Fixed

> *(2) Pita is listed twice in line 48.*

Fixed

> *(3) Line 49, mod(mdg4)67.2 is written just as mod(mdg4). The isoform should be indicated.*

This refers to all Mod isoforms.

> *(4) Homie and Nhomie are italicized throughout the manuscript and do not need to be.*

This is the convention used previously.

> *(5) The supplemental figure captions 1 and 2 in the main document are ordered differently than in the supplemental figures file. This caused it to look like the figures are being incorrectly cited in lines 212-214 and 231-232.*

Fixed

> *(6) Is the correct figure being cited in line 388-389? The line cites Figure 6E when mentioning LlambdaG Z5; however, LlambdaG Z5 is not shown in Figure 6.*

Fixed

> *(7) Section heading 'LhomieG Z5 and GhomieL Z5' could be renamed for clarity. GhomieL Z5 results are not mentioned until the next section, named 'GhomieL Z5'.*

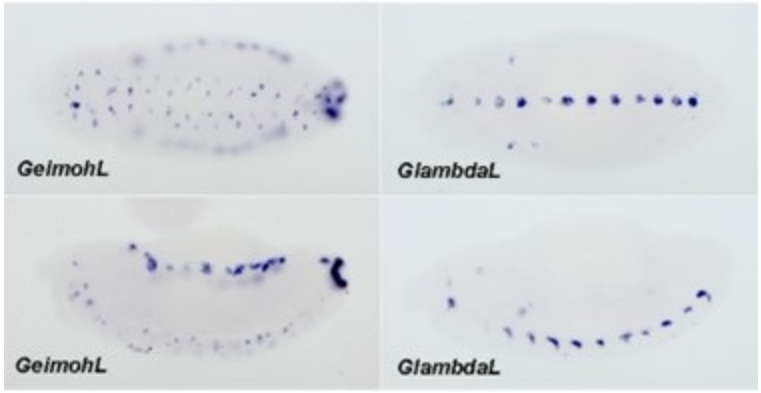
Fixed

> *(8) Can the authors provide better labeling for control hebe expression? This would help to determine what is hebe expression and what is background noise in some of the embryos in Figures 4-6.*

Author response image 5 shows expression of the *lacZ* reporter in *GeimohL* and *GlambdaL*. For the *GlambdaL* transgene, the *hebe* enhancers drive *lacZ* expression in 1216 hr embryos. Note that *lacZ* expression is restricted to a small set of quite distinctive cells along the ventral midline. *lacZ* is also expressed on the ventral side of the *GeimohL* embryo (top panel). However, their locations are quite different from those of the *lacZ* positive cells in the *GlambdaL* transgene embryo. These cells are displaced from the midline, and are arranged as pairs of cells in each hemisegment, locations that correspond to *eve*-expressing cells in the ventral nerve cord. The *eve* enhancers also drive *lacZ* expression elsewhere in the *GeimohL* embryo, including the anal plate and dorsal muscle progenitor cells (seen most clearly in the lower left panel).

Author response image 5.

lacZ expression in *Giemohl* and *Glambdal* embryos



> *(9) The Figure 5 title is labeled with the wrong transgene.*

Fixed

> *(10) Heat map scales are missing for Figures 7, supplemental 5, and supplemental 6.*

Fixed

> *(11) Did the authors check if there was a significant difference in the expression of GFP and lacZ from lambda control lines to the Homie transgenic lines?*

Yes. Statistical analysis added in Table Supplemental #1

> *(12) The Figure 7 title references that these are Z3 orientations, however, it is Z5 orientations being shown.*

Fixed

> *(13) The virtual 4C data should include an axis along the bottom of the graphs for better clarity. An axis is missing in all 4C figures.*

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