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**Chromatin boundary elements organize genomic architecture and developmental gene regulation in Drosophila *Hox* clusters**

Ma Z *et al.* Genomic architecture and developmental gene regulation

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**Abstract**

The three-dimensional (3D) organization of the eukaryotic genome is critical for its proper function. Evidence suggests that extensive chromatin loops form the building blocks of the genomic architecture, separating genes and gene clusters into distinct functional domains. These loops are anchored in part by a special type of DNA elements called chromatin boundary elements (CBEs). CBEs were originally found to insulate neighboring genes by blocking influences of transcriptional enhancers or the spread of silent chromatin. However, recent results show that chromatin loops can also play a positive role in gene regulation by looping out intervening DNA and “delivering” remote enhancers to gene promoters. In addition, studies from human and model organisms indicate that the configuration of chromatin loops, many of which are tethered by CBEs, is dynamically regulated during cell differentiation. In particular, a recent work by Li *et al* has shown that the SF1 boundary, located in the Drosophila *Hox* cluster, regulates local genes by tethering different subsets of chromatin loops: One subset enclose a neighboring gene *ftz*, limiting its access by the surrounding *Scr* enhancers and restrict the spread of repressive histones during early embryogenesis; and the other loops subdivide the *Scr* regulatory region into independent domains of enhancer accessibility. The enhancer-blocking activity of these CBE elements varies greatly in strength and tissue distribution. Further, tandem pairing of SF1 and SF2 facilitate the bypass of distal enhancers in transgenic flies, providing a mechanism for endogenous enhancers to circumvent genomic interruptions resulting from chromosomal rearrangement. This study demonstrates how a network of chromatin boundaries, centrally organized by SF1, can remodel the 3D genome to facilitate gene regulation during development.

**Key words:** Chromatin boundary element; Insulator; *CTCF*; Chromatin loop domains;Drosophila; *Hox* genes

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**Core tip:** Genomic organization in higher eukaryotes needs to fulfill at least three distinct functions: gene compaction, gene insulation and gene regulation. Chromatin loops appear to be a common structural unit that serves all these functions. A recent study has characterized a series of chromatin boundary elements (CBEs) in the Drosophila *Hox* cluster. Selective and dynamic interactions between these CBEs tether chromatin loops that not only insulate neighboring genes, but also organize enhancer traffic to regulate gene expression during development.

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**Introduction**

Transcriptional regulation plays a pivotal role in controlling gene activity during development, physiological responses and diseases. The current paradigm of eukaryotic transcriptional regulation emphasizes the assembly of activator complexes at distal regulatory DNA elements called enhancers[1-4]. Studies have shown that communication between these enhancers and their target promoters also constitutes a critical and highly regulated step towards transcription activation. Although the mechanisms of such communication are not fully understood, studies have shown that distal regulatory elements looping to gene promoters can trigger transcriptional activation or repression[5-7]. Mounting evidence suggests that configuration of chromatin fibers in the three-dimensional (3D) space can profoundly affect the access of regulatory sequences to genes during cell differentiation (Figure 1)[6,8-12].

**role of chromatin boundaries in organizing 3D chromatin architecture**

The mechanisms that regulate chromatin loop formation are poorly understood. Evidence converges on a type of specialized regulatory DNA called chromatin boundary elements (CBEs), also known as insulators. These elements were originally identified as DNA sequences that separate neighboring genomic domains[13,14]. They also interrupt enhancer-promoter communications without affecting the activities of these elements *per se*[9,15-20]. Boundary-like elements have been found from yeast to humans, often between divergently expressed genes. This is consistent with their functional role in maintaining independent domains of gene regulation. The best-characterized CBEs include the vertebrate beta-globin insulator, the Drosophila Gypsy insulator, and the boundaries from the Drosophila *Hox* loci[21-27]. The initial clue that CBEs may function by pairing with each other and tether chromatin loops came when their enhancer-blocking activity was found to depend on the position, orientation and arrangement of these elements[28-37]. In addition, the loop domains tethered by CBEs in the Drosophila *Hox* complexes, including Fab-7, Fab-8 and SF1, have also been shown to defined domains of distinct histone modifications that correlate with local gene activity[27,38,39]. Importantly, CBEs can play multi-faceted roles in gene regulation by either blocking or promoting enhancer-promoter interactions, depending on the topology and configuration of these loops[27,36,40-42]. The recent advent of genome-wide chromosomal-capture technology (3C, 4C, 5C and Hi-C, Figure 2) and protein association (ChIP) methods has provided powerful tools for assessing the spatial organization of the chromatin fibers and genomic conformation *in* *vivo*[8,43-51]. Recent reports indicate that CBE sequences, such as CTCF binding sites, correlate well with borders of Topological Associating Domains (TADs), which are megabase-sized units of genomic interaction domains[8,10,36,48,51-53]. These findings suggest that the interactions among CBEs/insulators may tether long-range chromatin loops that underlie much of the genomic architecture.

Diverse DNA sequences and protein factors have been associated with CBE function and genomic architecture. The most-conserved and best-characterized CBE/architectural protein is CTCF, a zinc-finger DNA-binding protein[18,19,48,50,54-56]. It mediates enhancer-blocking activity and underpins long-range chromatin loops in both Drosophila and vertebrates. Cohesin and condensin complexes, in conjunction with other CBE factors, are also well-conserved machinery that mediates long-range chromatin interactions from yeast to humans[50,57-59]. Additional classes of CBE complexes have been identified in Drosophila, anchored by the SuHw, BEAF, GAGA and ZW5 DNA-binding factors, respectively (for recent reviews see[60]). The Drosophila centrosomal protein CP190 associates with these complexes and contributes to their boundary and architectural function[59-63]. In addition, gene promoters, retrotransposons, and house keeping genes have also been shown to associate with CBE or architectural functions[48,64-66].

**Regulated *vs* static chromatin architecture**

Maps of global genomic interactions (Hi-C) have been generated from numerous mammalian and Drosophila cell lines. These maps indicate that the TAD organization is a pervasive feature of the interphase nuclei[8,10,47,48,60]. Further, megabase-sized TAD domains appear to be relatively stable across different cell lineages, even conserved across species[48]. Consistent with this, constitutive and robust binding of CBE/architectural proteins collocalizes with TAD borders[51]. In contrast, dynamic reconfiguration of chromatin loops at sub-TAD (inter-TAD) scale appears to underlie much of the regulatory interactions[5,6,8,67-69]. Such dynamic reorganization may be critical for establishing distinct gene activity and nuclear organizations that are cell fate specific[60,68,70]. A recent work on the Drosophila *Hox* gene regulation revealed that transient and cell-specific chromatin loops coincide with domains of enhancer and promoter access, as well as domains of repressive histone modifications[27].

An important question that now arises is how to distinguish the static structural roles of chromatin loops in genomic architecture from the regulated chromatin loops that are tissue- and developmental stage-specific. It is unclear whether the two types of architecture utilize different cis- and trans-acting components. Significantly, most of CBE/architectural proteins identified so far are ubiquitous and constitutively expressed, raising questions about their roles in regulation.

**Developmentally regulated chromatin loop domains in the *Drosophila* *Hox* cluster**

A recent manuscript byLi *et al*[27] provided evidence that developmentally regulated chromatin loop domains are involved in gene regulation in the Drosophila *Hox* cluster.*Hox/HOM* genes control animal segment identity along the anterior-posterior body axis. The complexes represent the most conserved gene family, not only in gene function, gene regulation, but also in gene organization. Indeed, the striking “collinear” relationship between the order of *Hox* genes on the chromosome and the order of their expression domains along the body axis has often been cited as the defining feature of the animal kingdom. In a previous publication, the group has reported the identification of SF1, a CBE located within the Drosophila *Hox* cluster between *Sex* *comb reduced* (*Scr*) and its neighbor *fushi tarazu* (*ftz*, Figure 3A[26,27,71]. SF1 was shown to contain a strong enhancer-blocking activity in the early Drosophila embryos. It also exhibits a strong activity in protecting the transgenic miniwhite reporter against the influences of neighboring chromatin in late development. In the current study, the authors used the 3C technique to probe for points of contact that SF1 makes in the surrounding genomic regions. The search had lead to the identification of several novel CBEs, located downstream of *ftz*[27]. In particular, SF1 pairs transiently but strongly with an SF2 CBE to enclose *ftz* in a chromatin loop in the 4-8 h embryos (Figure 3B)[27]. This *ftz* loop coincides with a domain of chromatin depleted in repressive histone marks including H3K27Me3 and H3K9Me3 (grey circles, Figure 3B). The loop also corresponds to a domain of restricted access to the *ftz* promoter by the surrounding *Hox* enhancers (curved arrows, Figure 3B). In the 12-16h embryos, SF1 dissociates from SF2, resulting in a spread of repressive marks into the *ftz* domain and a dramatic increase in interference to the *ftz* promoter by the neighboring regulatory sequences[27,72]. These results suggest that the loop tethered by SF1 and SF2 plays a key role in insulating both *Scr* and *ftz* regulation. Importantly, pairing of SF1 and SF2 in tandem allows a distal enhancer to “bypass” the block by both CBEs, providing a mechanism for the *Scr* enhancers located downstream of *ftz* to “leap” over *ftz,* andcommunicate with the *Scr* promoter (green ovals, Figure 3B[45,73-75]. Besides the SF1-SF2 pairing, SF1 also anchors other chromatin loops, which may subdivide and facilitate the *Scr* early and late regulatory elements[27]. Furthermore, evidence was provided that the activities of some of these novel CBEs are tissue restricted (for details see[27]. These results suggest that attributes of chromatin conformation can be regulated in a tissue-, stage- and gene-specific fashion to direct transcriptional outcome. Interestingly, the SF1-SF2 interval represent an evolutionarily conserved genomic block (Powell Conserved Region) that houses the entire *ftz* gene and is in an inverted orientation in several *Drosophila* species (Grey arrows, bottom of Figure 3B[76]). These observations suggest that chromatin loops may insulate the communications between genes and their regulatory sequences from the chromosome rearrangements, resulting in intermingling and interdependence of the genomic “modules” during evolution.

The above study has provided strong evidence that CBE-tethered chromatin loops can direct enhancer traffic during development. However, several questions remain to be addressed. First, in order to demonstrate the in vivo function of SF1 and SF2-tethered chromatin loops in ftz and *Scr* regulation, it is critical to examine the chromatin configuration and ftz and *Scr* expression patterns in mutant animals where SF1 or SF2 is deleted. In particular, since the chromatin loop tethered by SF1 and SF2 was hypothesized to restrict enhancer access to the ftz promoter and impede the spread of silent chromatin into the active ftz domain, one would expect to see increased capture of the ftz promoter by the neighboring *Scr* enhancers, and a higher level of repressive histone marks within the ftz region in mutant animals. Consequently, reduced or ectopic expression of the *ftz* gene may be observed. Similarly, the SF1-SF2 loop was postulated to facilitate the *Scr* distal enhancers to their promoter. Deletion of SF1 or SF2 would disrupt the loop configuration and the *Scr* enhancer-promoter interactions, leading to changes in the *Scr* expression.

In the above study the authors also noted that the SF1-SF2 chromatin loop coincides with the inverted ftz region in several Drosophila species. Furthermore, several genomic rearrangements in the Antennapedia *Hox* complex besides the ftz domain also coincide with potential CBE-tethered chromatin loops. The authors hypothesized that chromatin loops tethered by boundary elements may promote genomic crossover and at the same time insulate any potential deleterious impacts of these rearrangements to the surrounding genes. The hypothesis can be tested from several angles. Demonstration of corresponding loop domains in these Drosophila species, validation of boundary activity that flank the loop domains, and general correlation between genomic rearrangements and CBE-tethered chromatin loops would go a long way proving this hypothesis.

**Major gaps in knowledge and future studies**

Despite recent advances in our understanding of chromatin structure and genomic organization, how key features of our genetic material re regulate development, physiological and pathological responses remain poorly understood. Below are three major gaps in our understanding of themechanisms and function of genomic architecture.

***Animal model studies***

Although conformation capture technologies have provided a critical means for assessing the genomic interactome and architecture, the methods have so far been largely applied to cultured cell lines. This is due to the need of quantities of homogeneous cell population for using the mapping technology. These studies can provide important information into aspects of nuclear and genomic organization. However, the maps do not faithfully reflect the characteristics and dynamics of animal cells and tissues during development. On the other hand, maps have been generated from mixed tissues, such as those from whole embryos. Those are not ideal either, as they reflect an average of the interactions from many cell types, and may suffer from low signal to noise ratio[77]. Future studies using cells from authentic animal models with highly defined tissue and developmental identity would be essential. Purification methods that can be applied to diverse cell and tissue types, and the use of single cell technologies in mapping genomic interactions should provide critical insights into the organization and function of genomic organization and nuclear architecture in a more physiologically relevant setting.

***Causal relationship to gene regulation***

Another gap in our knowledge is in the causal relationship between chromatin configuration and gene regulation. Much of the current studies correlate global TAD structures with domains of active or silent chromatin. Numerous studies have also correlated chromatin loop organization with local enhancer-promoter interactions during gene activation[27,78,79]. However, to demonstrate that these changes in chromosomal configuration play a primary role in regulating enhancer-promoter interactions or in organizing chromatin domains, rather than simply correlating with these events, it would be essential to examine these interactions and domains in mutants where the corresponding genomic configurations are disrupted. In addition, future studies should also aim to provide a more quantitative description how changes in chromatin configuration instruct or permit gene regulation events.

***Mechanism of tissue-specific regulation***

The protein factors that control developmental stage- and tissue-specific chromatin loops are poorly characterized. In particular, majority of the currently known CBE/architectural proteins are constitutively and broadly expressed in animal tissues. Therefore, regulated chromatin conformation may require novel factors that are more restricted in their temporal or spatial distribution. Recent studies have suggested that such factors may associate with chromatin and with each other weakly, and loop within a shorter range[27,78,79]. As a result, their association and function may only be revealed from ChIP experiments and conformation captures of higher reading depth, and possibly from purified or enriched source tissues. Combinations of molecular genetic and biochemical approaches may be required to identify these proteins.

**conclusion**

The human genome is highly organized in the three dimensional space. CBEs represent an important mechanism that organizes such complex architecture. Functionally, these elements separate neighboring genes to ensure their independent regulation. Boundary elements also tether chromatin loops to promote communications between distant genetic elements. As such, chromatin boundary activity is essential for proper genomic function including transcriptional regulation. Despite such critical roles, how CBE activities and chromatin loop formation are regulated, especially in a developmental context, is still poorly documented and poorly understood. Current gaps in our knowledge are in their roles in authentic animal models, in their instructing rather permitting genomic interactions during transcriptional regulation, and in their cis and trans- components that can mediate tissue- and stage-specific genomic architecture. Future studies that combine single cell technology, live imaging and other genomic, cell biological and biochemical approaches should mend these gaps and further elucidate this novel mechanism that control our genomic output.

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**Figure 1 The roles of chromatin boundary elements in organizing genomic and nuclear architecture.** Chromatin boundary element (CBE) complexes (brown ovals) may interact with each other (left), nuclear matrix (middle) or nuclear envelope (right) to tether chromatin loops that modulate enhancer-promoter interactions (curved arrows), and block the spread of silent chromatin (grey forbidden circles). Green and orange arrows indicate promoters of hypothetical Genes X and Y, respectively. Green and orange boxes, enhancer elements for Genes X and Y, respectively.



**Figure 2 Key steps in chromosomal conformation capture protocols (adapted from Stadhouders *et al*[46], 2013).** Bold blue upper case letters denote capture protocols and black lower case phrases denote the key steps. Steps 1 and 2 (cell crosslinking, chromatin preparation and fragmentation) are common to all capture protocols. Following these, the ends of captured DNA fragments are ligated under highly diluted condition (Step 3A, Proximity ligation), and junctions of captured DNAs are detected by PCR using pray- and bait-specific primers (Step 4A, 3C). Alternatively, capture events can be selected by immunoprecipitation using antibody against specific architectural proteins (Step 3B for ChIA-PET). Capture events can be also enriched by pull-down of Biotin-labeled ligation junction (Step 3C for Hi-C). After purification, libraries of junction DNAs are amplified and sequenced by next-generation-sequencing (NGS). After Step 3A, library of bait- and pray-specific primers can be used in conjunction (Step 4B for 5C) to generate libraries of selected capture junctions for NGS sequencing. Bait-specific primers can also be used to generate a capture library (Step 4C for 4C or Multiplex 4C).



**Figure 3 Hypothetical function of the SF1-SF2 chromatin loop in *Scr* and *ftz* gene regulation (adapted from *Li et al*[27], 2015).** A: Segmental expression patter of a Drosophila *Hox* gene *Scr* (green). A neighbor non-*Hox* gene *ftz* is expressed in pair-rule stripes (red); B: A model for developmentally regulated SF1-SF2 chromatin loop in modulating enhancer-promoter communications and preventing spread of silent chromatin in the Drosophila *Hox* complex. The chromatin loop anchored by the interaction between SF1 and SF2 (green and red vertical ovals, respectively) during early *Drosophila* embryogenesis can restrict the access of *ftz* and *Scr* enhancers (red and green ovals, respectively) to neighboring gene promoters (curved block signs). The SF1-SF2 loop also prevents the spreading of the repressive H3K9Me3 and H3K27Me3 histone modification (grey forbidden circles) into the active *ftz* gene. Pairing of SF1 and SF2 may facilitate “enhancer bypass”, connect the *Scr* with its distal enhancers (green ovals) in 3D in the early Drosophila embryo. The *ftz* gene domain is present in opposite orientation in various Drosophila species (grey long arrows, bottom). FD: *Ftz* distal enhancer; F1/5: *Ftz* stripe 1/5 enhancer; *Antp*: *Antennapedia,* a *Hox* gene located distal to *ftz*.