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Function and regulation of chromatin insulators in dynamic genome organization

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Abstract

Chromatin insulators are DNA-protein complexes that play a crucial role in regulating chromatin organization. Within the past two years, a plethora of genome-wide conformation capture studies has helped reveal that insulators are necessary for proper genome-wide organization of topologically associating domains, which are formed in a manner distinct from that of compartments. These studies have also provided novel insights into the mechanics of how CTCF/cohesin-dependent loops form in mammals, strongly supporting the loop extrusion model. In combination with single-cell imaging approaches in both *Drosophila* and mammals, the dynamics of insulator-mediated chromatin interactions are also coming to light. Insulator-dependent structures vary across individual cells and tissues, highlighting the need to study the regulation of insulators in particular temporal and spatial contexts throughout development.

Keywords

chromatin; insulator; CTCF; cohesin; genome organization

Introduction

Primarily over the past decade, imaging combined with genome-wide chromatin conformation capture techniques have revealed with increasing resolution, key features of 3D chromatin organization in eukaryotic cells. In metazoa, each chromosome occupies specific regions within the nucleus called chromosome territories. Genomic intervals along each chromosome further spatially segregate into distinct transcriptionally active (A-type) or inactive (B-type) regions termed compartments that can form by interaction among sequences distributed across large linear distances [1]. An additional form of chromatin

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Declaration of Interest

The authors declare no conflict of interest.

organization is the topologically associating domain (TAD), which is a sub-megabase region of high self-interaction that displays limited interaction outside the domain (reviewed in [2]). Inherent to TAD organization are chromatin loops that form between distant loci within the TAD, and loops between sequences at TAD boundaries have also been observed. These interacting loci and TAD boundaries are often occupied by insulator proteins (also referred to as architectural proteins), which have well-established roles in loop formation and control of *cis*-regulatory interactions at specific loci. However, how insulators affect higher order chromatin structure and resultant gene expression on a genome-wide level remained unclear due to their essential function in cell viability and organismal development.

In this review, we summarize recent studies that provide a refined understanding for how insulator proteins contribute to genome-wide chromatin organization on different levels. At the coarsest scale, mammalian insulators are needed for TAD formation and insulation between TADs, and the basic mechanics of large loop formation have begun to be revealed. Studies in both mammalian cells and *Drosophila* address the dynamics of insulator activity and finer-scale local loop formation on the cellular as well as organismal level. Finally, we explore the regulation of insulators in a cell type-specific manner using the nervous system as an example.

Mammalian CTCF and cohesin play key roles in large loop formation and TAD insulation but not compartmentalization

Recent Hi-C studies demonstrate that the mammalian zinc-finger CTCF insulator protein and interacting cohesin ring complex contribute to large loop formation and TAD insulation genome-wide. Earlier work found that TAD borders are enriched for CTCF binding [3] and that a subset of these boundary sites form loops with one another [4], leading to the hypothesis that CTCF may be involved in TAD formation by promoting loop formation. Deletion of a TAD boundary or CTCF site within the boundary resulted in fusion of two adjacent TADs and loss of insulation from enhancer activity [5–9]. Incomplete depletion of CTCF by RNAi showed limited decreases in both intra-TAD interactions as well as TAD insulation [10]; however, recent use of the auxin-inducible degron (AID) system to efficiently deplete CTCF from mouse embryonic stem cells resulted in dramatic loss of both looping and TAD insulation [11, 12]. Similarly, AID depletion or deletion of maternal cohesin in the early mouse zygote, or deletion of the cohesin loader NIPBL, led to virtually complete loss of all loop domains and TADs [12–15]. Intriguingly, a new super-resolution imaging study of single cells using a novel tracing technique to label large segments of the genome verified that TAD-like structures exist in individual cells [16]. The genomic positions of these structures vary across individual cells, but the borders display a higher frequency of coincidence with CTCF and cohesin sites on average (Figure 1). AID depletion of cohesin did not disrupt these structures but randomized their position, suggesting that cohesin is not actually required for TAD formation and underscoring the importance of high-resolution single cell analyses.

Importantly, depletion of either CTCF or cohesin does not eliminate organization of A/B chromatin compartments. Instead, loss of cohesin or NIPBL actually strengthens genome

compartmentalization [12–15], indicating that these two levels of chromatin organization are not hierarchical and further suggesting that TADs can force interactions between active and repressed chromatin states that would not otherwise associate (Figure 2). Transcriptional state is highly correlated with compartmentalization and appears to be a major driver of compartment formation [17]. It is possible that the self-associating properties of certain chromatin-associated proteins, such as HP1 or Polycomb group complexes, could also play a role. Central questions that remain are how compartments form and to what extent they drive genome organization and feed back on gene expression.

Mechanics of CTCF/cohesin-dependent loop formation

Since the discovery that cohesin contributes to CTCF insulator activity during interphase, numerous models for how the tripartite cohesin ring contributes to loop formation have been proposed. The key finding that CTCF sites are oriented oppositely at loop domain anchors genome-wide [4] provided the basis for the currently favored loop extrusion model (reviewed in [18]). In this model, cohesin pinches a small DNA loop and extrudes it by pulling until it reaches a chromatin-bound CTCF molecule oriented in the proper direction (Figure 3). Consistent with this model, deletion or depletion of the cohesin release factor Wapl or its cofactor Pds5 cause cohesin-dependent chromatin loops and TADs to increase in size [12, 15, 19]. Furthermore, ultra-deep Hi-C and Hi-ChIP of cohesin revealed “stripes” or “extrusion lines”, evidence of progressive contact between a loop anchor and an entire domain that is indicative of the extrusion process [20, 21]. These signatures are often visible on only one side of the domain, suggesting that the extrusion process can occur with one cohesin subunit being stalled at a CTCF boundary with the other sliding across the domain in order to extend the loop. Finally, Vian et al. found that recovery of loops after depletion of cohesin and auxin washout is dependent on ATP, and once formed, loops remain stable without further energy input. It would be ideal to be able to directly visualize the loop extrusion process by cohesin and CTCF, as has been recently performed for the related SMC complex condensin [22].

Limited gene expression changes result from loss of TADs and loops

Given the striking changes in genome-wide chromatin organization, loss of CTCF or cohesin leads to surprisingly modest changes in gene expression. RNA-seq analysis revealed that only 370 genes significantly changed in expression after one day of CTCF depletion, and the magnitude of changes observed were relatively small [11]. Since expression analysis was performed on a population of cells, it is possible that larger changes in gene expression in individual cells due to variable effects on TADs and/or their positions are averaged out across many cells. Consistent with CTCF functioning to insulate TADs, upregulated genes tend to be located next to a TAD border that separates the gene from a neighboring enhancer. Downregulated genes were generally not located near TAD borders, but CTCF occupancy was observed slightly upstream of the transcription start site. Curiously, the orientation of the CTCF site matches the direction of transcription, suggesting a more local looping-related function such as promotion of enhancer-promoter communication. Depletion of cohesin or Nipbl resulted in somewhat larger changes in gene expression than that of CTCF, particularly downregulation of genes near super enhancers [13, 14] as well as widespread

upregulation of intergenic or antisense transcription from preexisting active promoters or enhancers [14]. These results suggest that cohesin may play a larger role than CTCF in proper enhancer-promoter communication within TADs, especially near super enhancers. Consistent with this view, recent work showed that cohesin but not CTCF binding is associated with hormone-dependent changes in chromatin looping and gene expression [23]. Although mild, these cumulative gene expression differences could easily cause a large deleterious impact on cell viability and function.

Dynamics of insulator-dependent chromatin organization

Recent work to address chromatin dynamics demonstrated rapid formation and re-organization of chromatin structure at the organismal level as well as within individual cells. In mammals, TADs, loops, and compartments are visible as early as the 1-cell stage [15], and these structures progressively strengthen during subsequent cell divisions [15, 24, 25]. In response to lymphocyte activation, mouse B cells form thousands of new short-range CTCF-dependent loops and domains that may result from increased cohesin loading [26]. Similarly, heat stress applied to human ES cells changes CTCF occupancy and CTCF/cohesin-dependent looping on the local level to form new interactions within TADs [21]. Single-molecule tracking and fluorescence recovery after photobleaching (FRAP) experiments in mouse cells quantified the average residence time of CTCF on chromatin as 9–120 sec, whereas cohesin stays bound approximately 22 min [26, 27]. Once released, CTCF searches and rebinds in approximately 1 min while cohesin takes 33 min, suggesting that loops are frequently dissolved and reconstructed instead of remaining stable as previously assumed. Why CTCF and cohesin display such different dwell times on chromatin is unclear; nevertheless, these data suggest that the two factors do not form stable complexes while bound to chromatin. Notably, re-introduction of cohesin into fully depleted cells results in TAD formation in as little as 20 min [13]. Time course analysis showed that recovery of loops varied considerably across the genome, with transcriptionally active regions, particularly those containing super enhancers, being faster to reform compared to regions marked by repressive histone marks. This result might indicate that loop extrusion is preferentially nucleated at super enhancers. Finally, periodic versus infrequent loop extrusion may help increase the likelihood of distant enhancers and promoters within the loop to stably pair and activate gene expression.

Lessons on chromatin organization and insulator dynamics from the fly

Recent high resolution Hi-C studies in *Drosophila* have revealed several key differences in coarse-scale genome organization compared to mammals, as well as the role of CTCF. In *Drosophila* early embryonic development, TADs do not begin to form until cell cycle 14 [28, 29], which also corresponds to the onset of zygotic transcription. Deletion of both maternal and zygotic CTCF permits development until the pharate adult stage, well after TAD formation is established in the embryo [30]. Although reasonably well-conserved between mammals and *Drosophila*, neither *Drosophila* CTCF nor cohesin are found to be highly enriched at high-resolution TAD borders [17, 31–33]. Instead, other arthropod-specific insulator proteins [34], particularly CP190 and BEAF-32, are observed at TAD borders [17, 32, 33, 35]. It is important to note that the comparison of various Hi-C studies is

complicated not only by differences in resolution but also differences in parameters used for TAD calling [36]. Depletion of BEAF-32 was not observed to affect chromosome conformation [33], consistent with the possibility that a variety of insulator proteins serve redundant roles in controlling chromatin organization [37]. Chromatin loops can be observed between CTCF sites; however, these do not display any orientation bias [17]. The mechanics of how *Drosophila* and other non-mammalian TADs and loops form is an important question that remains to be answered.

Single-cell *in vivo* imaging studies have allowed real-time visualization of insulator-dependent chromosome movements to facilitate enhancer-promoter interactions. These studies utilized MS2/PP7 stem-loop-based labeling to simultaneously monitor the nuclear locations of a distant enhancer versus promoter and quantify their transcription activity in embryos [38, 39]. Chen et al. examined the looping of a distant enhancer with a promoter on the same chromosome (Figure 4) while Lim et al. monitored the pairing of two homologous chromosomes with enhancer-promoter interaction *in trans*. Both studies found that transcription requires sustained enhancer-promoter interaction, which is stabilized by self-pairing of Homie and/or *gypsy* insulator sequences. These chromatin movements and subsequent transcriptional activation occur on the scale of minutes. It will be of great interest to examine how insulator-dependent chromatin movements differ across cell types throughout development.

Tissue-specific regulation of *Drosophila* insulator activity in the nervous system

The first evidence for tissue-specific insulator regulation came with the identification of a nervous system-specific insulator antagonist in *Drosophila*. The Shep RNA-binding protein, which is required for proper neuronal remodeling through regulation of gene expression [40–43], interacts on chromatin with the core *gypsy* insulator components, Su(Hw), Mod(mdg4)67.2, and CP190 [44]. Shep negatively regulates *gypsy* barrier activity as well as insulator nuclear localization specifically in the central nervous system (CNS). Interestingly, Su(Hw) represses neuronal gene expression in nonneuronal cell types and is not expressed in neurons [45], suggesting that functional interaction between Shep and Su(Hw) may be restricted to glial and/or precursor cells. It remains to be determined whether Shep may regulate other insulator proteins in neurons, such as CTCF, which is required for proper *Abd-B Hox* gene expression in the CNS [30, 46].

Regulation of mammalian CTCF to control chromatin organization, development, and function of the nervous system

Although mammalian CTCF is required for viability in all cell types thus far tested, the nervous system may be particularly sensitive to CTCF function. This topic has been reviewed in detail in two recent review articles [47, 48], and we focus here on CTCF regulation in the CNS. Briefly, mouse CTCF displays higher expression in the nervous system compared to other tissues [49] and associates with a large number of brain-specific genomic loci [50]. Although aggregate TAD borders are generally constant across different

tissue types, smaller intra-TAD regions of high local interaction are observed close to genes expressed in a tissue-specific manner, including nervous system-specific genes [51]. These frequently interacting regions (FIREs) are conserved across human and mouse nervous tissue, and CTCF depletion decreases FIRE interaction frequencies. CTCF occupancy sharply decreases during the transition between multipotent to neural progenitor cells (NPCs) [52], and CTCF is required for survival of neural progenitor cells, as well as prevention of their premature differentiation [53]. Finally, conditional knockout of CTCF in neurons at later stages of development or at the adult stage can lead to profound deficits in learning and memory, motor coordination and social behaviors in adults [49, 54, 55]. Consistent with these studies, AID depletion studies of CTCF in NPCs versus cells differentiated into resting post-mitotic astrocytes showed that CTCF is required for TAD insulation in both cell types; however, a weaker effect was observed in differentiated cells [11]. Notably, restoration of CTCF levels rescued insulation in NPCs but not resting astrocytes, suggesting either that progression through the cell cycle is required or that a differential regulatory mechanism for CTCF function exists in astrocytes.

Key targets of CTCF regulation that may explain several of the above mutant phenotypes are the protocadherin (Pcdh) genes, which are required for proper formation and function of neural circuits. Intriguingly, the Pcdh genes reside in a large TAD that is conserved in humans and mice, and deletion of the histone H3K9 methyltransferase SETDB1 in postnatal mouse neurons causes this TAD to collapse, allowing ecotpic activation of the cPcdh locus [56]. This effect is concomitant with loss of H3K9 and DNA methylation as well as an increase in CTCF occupancy at the Pcdh locus, suggesting that SETDB1 acts as an antagonist of CTCF binding to repress Pcdh genes by controlling topology of the locus. Identification of additional factors that regulate CTCF binding and/or activity in the nervous system as well as other specific cell types are important avenues of future research.

Conclusion

A veritable explosion of high resolution Hi-C studies combined with imaging approaches over recent years have yielded a better understanding of 3D genome organization and the specific role of chromatin insulators. It will be important to test these refined models on the single cell level using both high resolution conformation capture and recently developed imaging methods. Due to their large size and highly flexible nature, lack of structural information about chromatin insulator proteins and their interactions with DNA remains a large blind spot in the field. Finally, further examination of insulator activities including their dynamics and regulation in the *in vivo* context will ultimately provide a more complete picture of their biological importance.

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These results are consistent with ensemble Hi-C studies but provide additional insights on the single cell level.

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neuronal target of CTCF is the cPcdh locus. This study identified a histone methyltransferase as a neuron-specific repressor of CTCF. SETDB1 prevents excess CTCF binding at the cPcdh locus to control chromatin topology and gene expression.

Highlights

- CTCF and cohesin are required for genome-wide organization of TADs
- TADs and compartments are not hierarchical forms of chromatin organization
- Strong molecular evidence supports the loop extrusion model in mammals
- Live imaging in *Drosophila* shows that insulators are needed for stability of pairing
- Insulators are specifically regulated in the nervous system of *Drosophila* and mammals

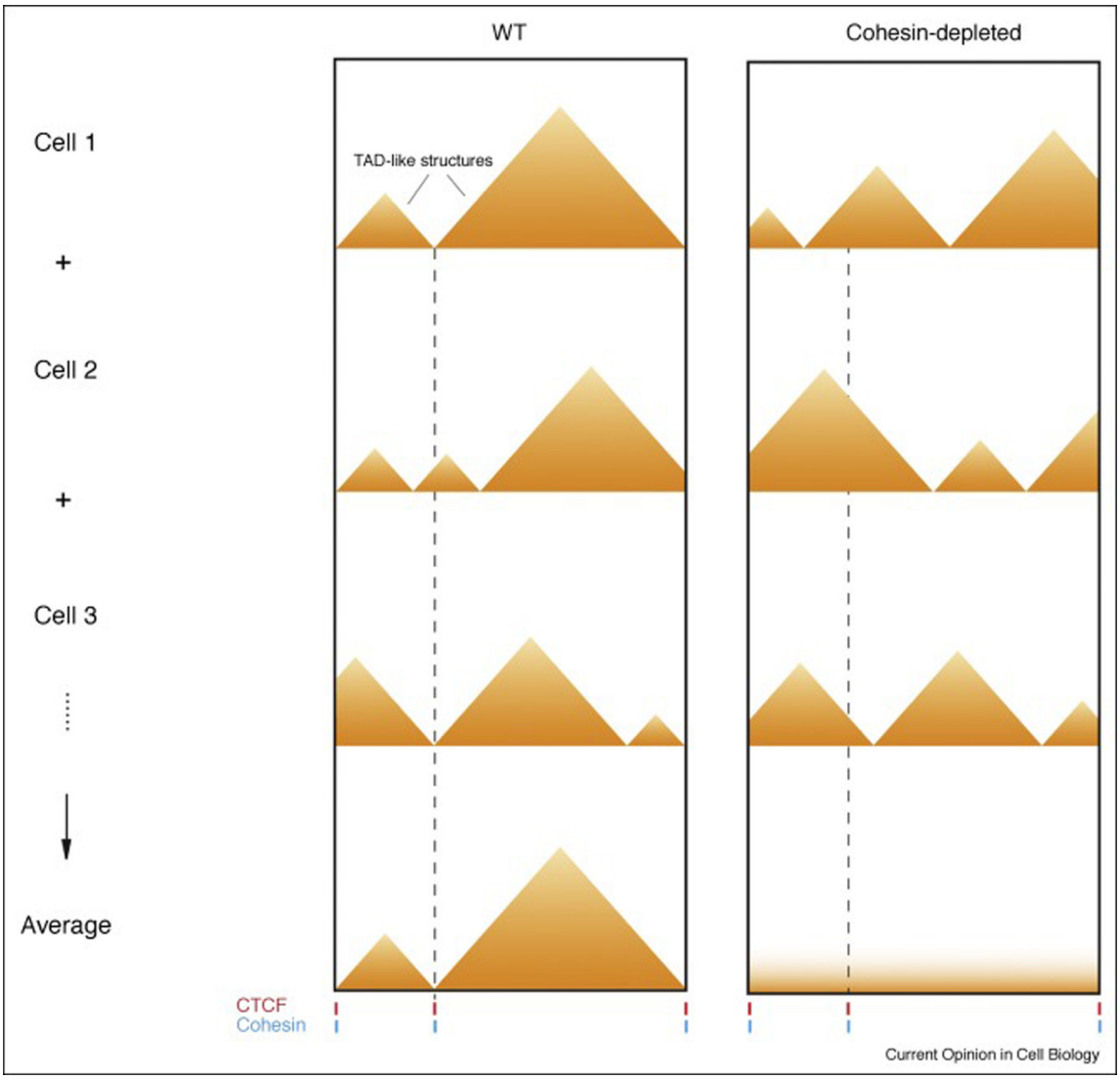

Figure 1.

Cohesin is required to position TAD boundaries at CTCF/cohesin-occupied loci. Variable locations of TAD-like structures and their boundaries are observed using high resolution imaging across individual cells, with the highest frequency of boundaries at CTCF/cohesin-occupied sites. Upon cohesin depletion, the location of TAD boundaries is randomized. This effect would be visualized as overall loss of TADs upon cohesin depletion in ensemble Hi-C studies.

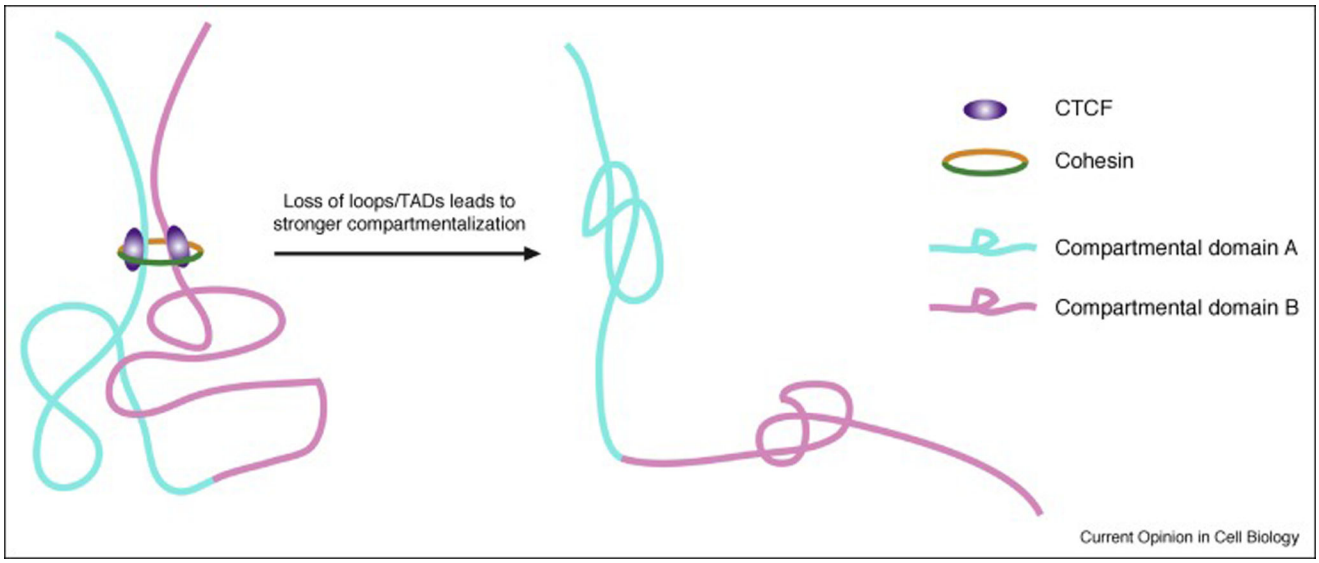

Figure 2.

TADs and compartments are not hierarchical structures. CTCF/cohesin-dependent TADs can force interactions between different compartment types. Loss of TADs strengthens compartments genome-wide.

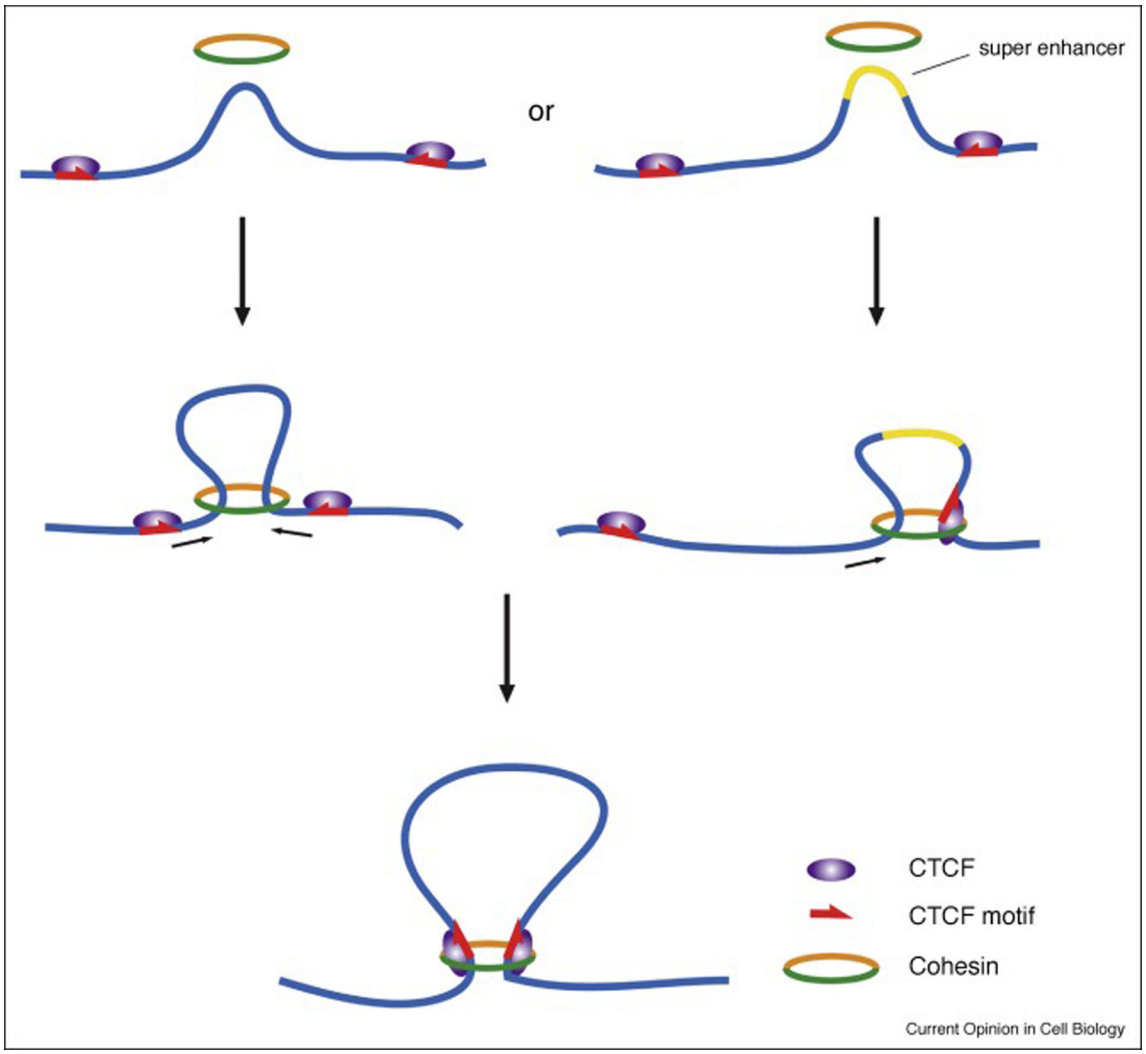

Figure 3.

The loop extrusion model underlying CTCF/cohesin-mediated chromatin organization. The cohesin complex loads onto chromatin, perhaps preferentially at superenhancers, and progressively pushes chromatin through its ring-like structure to extend the loop until the complex encounters a CTCF molecule positioned in the correct orientation. Extrusion can occur in one or both directions. This process requires ATP, and cohesin ATPase activity may be involved.

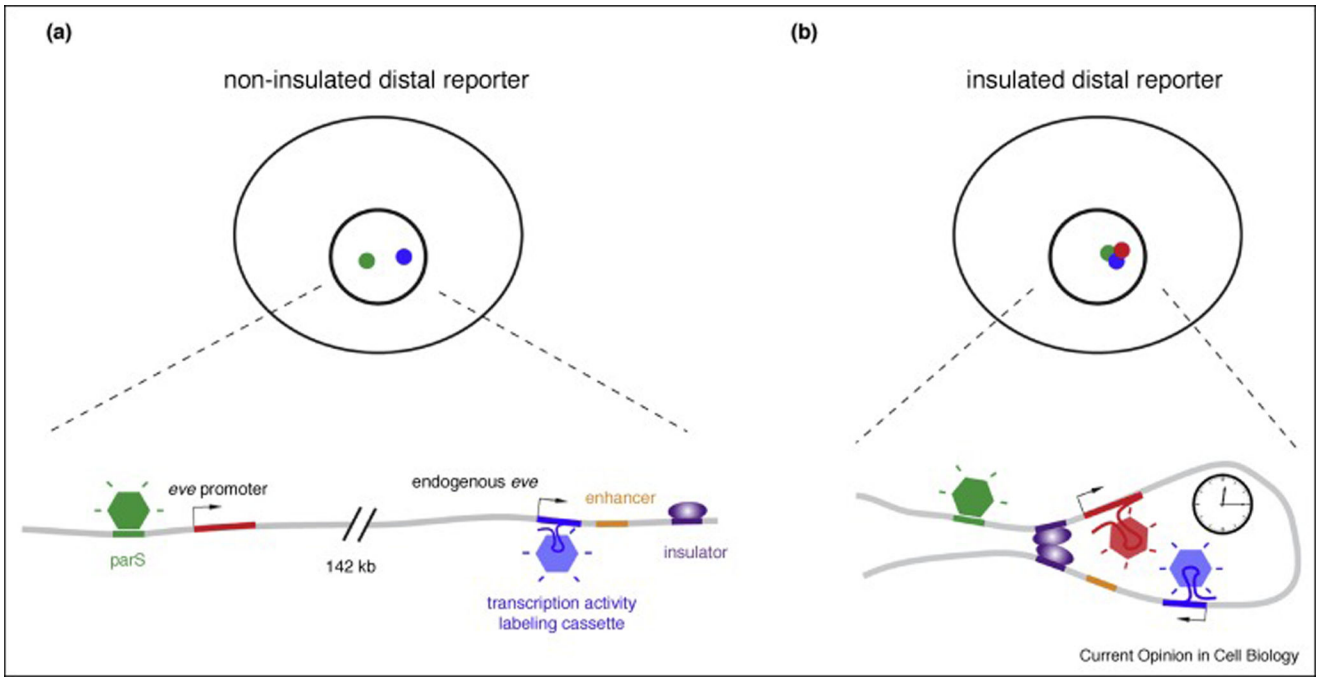

Figure 4.

In vivo imaging at the single-cell level to visualize insulator-mediated regulation of transcription activity. A) Without an insulator, the distal reporter (green spot) remains transcriptionally inactive. Transcriptional activity (blue spot) at the endogenous *eve* locus shows spatial separation. B) Presence of an insulator sequence increases the stability of the loop and pairing of the reporter with the endogenous locus. Sustained proximity is required to activate transcription of the reporter (red spot).