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Insulators, long range interactions, and genome function

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Summary

Insulators are DNA-protein complexes that can mediate interactions in cis or trans between different regions of the genome. Although originally defined on the basis of their ability to block enhancer–promoter communication or to serve as barriers against the spreading of heterochromatin in reporter systems, recent information suggests that their function is more nuanced and depends on the nature of the sequences brought together by contacts between specific insulator sites. Here we provide an overview of new evidence that has uncovered a wide range of functions for these sequences in addition to their two classical roles.

Introduction

Insulators have now been found in most eukaryotes, from yeast to humans [1]. They were originally described in *Drosophila* and this organism remains noteworthy for the number of characterized insulators (Figure 1A). *Drosophila* insulators share two proteins, Mod(mdg4) and CP190, which interact with a variety of DNA binding proteins whose function appears to be limited to the recruitment of the shared components to different genomic locations, where they may play distinct roles [2]. Insulators in *S. cerevisiae* and *S. pombe* are mostly limited to RNA polymerase III promoter sequences containing binding sites for TFIIC [3–5]. In vertebrates, the most widely studied insulator is CTCF, which requires cohesin for functionality and also associates with other co-factors, although their general requirement for CTCF function is not clear (Figure 1B) [6–8]. SINE elements and their associated Pol III promoters have been also shown to act as insulators in mammals [9], but the relevance of this initial observation had not been pursued in detail until recently.

Results accumulated in the last two years suggest that insulators participate in numerous nuclear processes. In addition to blocking enhancer-promoter interactions, insulators can also direct enhancers to the appropriate promoters. Insulators can not only block the spreading of heterochromatin but they can also demarcate the boundaries between a variety of epigenetic states. Furthermore, the effect of insulators on genome biology goes beyond their involvement in transcription processes as they are also involved in regulating V(D)J recombination. Here we review recent results underscoring the variety of functional outcomes that arise as a consequence of the unique ability of insulators to mediate intra- and inter-chromosomal interactions between different regions of the genome. Although the name “insulator” may be counterintuitive in the context of these new functions, we will continue to use this name given its widespread use in the literature.

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Regulation of enhancer-promoter interactions

Transcriptional enhancers have the ability to act over long distances to activate gene expression. This property gives enhancers great functional flexibility, being able to activate promoters of non-adjacent genes over distances of up to 1 Mb. But the same properties that are at the core of enhancer function are also a potential problem. How do enhancers find their target promoters and how is their activity confined to a specific gene? The CTCF insulator has been shown to confine enhancer action to a specific promoter by inhibiting its interaction with a second promoter in what is the archetypal example of endogenous insulator function. In the imprinted *H19/Igf2* locus, endodermal enhancers present downstream of *H19* are inhibited from activating the *Igf2* gene by a CTCF insulator located between the two genes [10,11]. A similar function has now been described for the *Drosophila* dCTCF insulator [12]. This protein requires CP190 for proper function. In normal Kc cells, a CTCF site present between an enhancer and promoter of the *Eip75B-RB* gene is inactive due to the absence of CP190. When cells are treated with the hormone ecdysone, the *Eip75B-RB* promoter is turned on. After 3 hr of hormone treatment, CP190 is recruited to the dCTCF site, the insulator is activated, and the *Eip75B-RB* gene is turned off. In addition to demonstrating the effect of insulators on enhancer-promoter interactions, this example illustrates the principle that insulator activity can be modulated by recruitment of other protein components. In these two cases, the effect of the insulator on enhancer-promoter interactions is mediated by the formation of loops between individual insulator sites. As a consequence, the enhancer and promoter are partitioned to separate loops, precluding activation of transcription by mechanisms still unclear.

The ability of insulators to bring together sequences located far apart in the linear genome underlies their role in targeting enhancers to specific promoters. One interesting example is the use of CTCF for promoter selection and control of the latency cycle of the Epstein-Barr virus (Figure 2). In the genome of this virus, the enhancer OriP is shared by two downstream promoters, Cp and Qp. Cp determines the type III gene expression pattern for latency cycle III and Qp determines the type I expression program for latency cycle I [13–15]. CTCF binds both upstream of Cp and Qp. The default selection for OriP is the proximate promoter Cp. When Cp is active, deletion of either of the CTCF sites or depletion of CTCF does not affect the activity of Cp [13–15]. However, when CTCF-dependent interactions bring the distal OriP enhancer close to the Qp promoter, Cp is turned off. Mutations in either of the CTCF binding sites disrupt the interaction between OriP and Qp and lead to reactivation of Cp transcription. Thus, the insulator-mediated 3D structure of the chromatin contributes to promoter selection, probably by increasing the ability of Qp to compete out Cp. When Qp is not competent, Cp is selected by default [15].

A similar logic underlies the expression of genes that regulate insulin synthesis and secretion, which are tightly controlled in the β -cells of the pancreas [16]. Functionally related genes in the genome of prokaryotes are organized into operons in order to co-regulate the synthesis of proteins that function in the same pathway. Eukaryotes may accomplish a similar end result by using insulators to bring together regulatory sequences of genes that participate in a common pathway but are located far away in the genome. The *Insulin (INS)* gene is located 300 kb away from the *SYT8* gene, which is an important regulator of insulin secretion. CTCF sites are present next to *INS* and *SYT8*, and mediate long-range interactions between both genes. Glucose treatment increases the expression of *INS* and *SYT8*, and it enhances the interaction between the two genes. Depletion of CTCF attenuates the interaction as well as *SYT8* expression [16]. Therefore, CTCF-mediated interactions can bring distal regulatory sequences located in the *INS* gene close to *SYT8* to activate expression of this gene with the same tissue specificity as *INS*. The generality of this conclusion is supported by results from a genome-wide study of CTCF dependent intra-

and inter-chromosomal interactions in mouse ES cells [17]. A total of 1,480 *cis*- and 336 *trans*-interacting loci were identified in this study. Using p300 as a mark for the location of enhancer sequences, the results show that CTCF-mediated loops bring these enhancers in close proximity to the promoters they regulate in mouse ES cells.

Establishment of chromatin domains

Loops formed by contacts between insulator sites can not only determine interactions between regulatory sequences but they can also establish distinct domains of chromatin structure defined by the presence of specific histone modifications. Genome-wide studies of CTCF distribution have uncovered a subset of CTCF sites localized at boundaries between active and repressive chromatin domains marked by acetylated histone H2A in Lys5 (H2AK5Ac) and trimethylated histone H3 in Lys27 (H3K27me3), respectively [18]. These regions are different between HeLa and CD4+ T cells, suggesting a possible role for CTCF-delimited domains in establishing lineage-specific gene regulation. This conclusion is supported by results from a the genome-wide study of CTCF dependent interactions mentioned above [17]. Alignment of CTCF-mediated loops with the location of histone modifications led to the finding of five different classes of loops, some of which demarcate chromatin domains with different epigenetic features (Figure 3). Category I loops are enriched in active histone modifications such as H3K4me1, H3K4me2 and H3K36me3, whereas the repressive marks H3K9me3, H3K20me3 and H3K27me3 are depleted inside but present outside of the loops. Category II loops show the opposite distribution of histone modifications, with H3K9me3, H3K20me3 and H3K27me3 inside of the loops, indicating the formation of repressive chromatin domains delimited by the presence of CTCF sites. Category III loops contain nucleosomes enriched in mono, di and trimethylated H3K4 at the boundaries of the loops, whereas the active transcription H3K36me3 and repressive H3K27me3 modifications are observed outside the loops on opposite sides [18].

The functional significance of the chromatin domains demarcated by CTCF is beginning to emerge from studies of specific loci. In human lung fibroblasts IMR90 cells the *HOXA9-13* genes are silenced but other genes in the *HOXA* locus are transcriptionally active [19]. Consistent with this transcription pattern, the region containing *HOXA9-13* is rich in H3K27me3 while the adjacent region containing the active *HOXA1-7* genes is marked by H3K4me2, H3K4me3 and H3K36me3. The distribution of the repressive marks depends on the interaction between two CTCF insulators flanking *HOXA9-13*. Knockdown of CTCF results in the disruption of loop formation and spreading of silencing histone modifications, which causes downregulation of the adjacent *HOXA6-7* genes [19].

A second example of CTCF-delimited chromatin domains in the control of gene expression and the establishment of cell fates is that of the Wilm's tumor protein (Wt1) in the regulation of epithelial-mesenchymal transitions during the development of the epicardium, and the kidney (Figure 4). These processes are controlled by Wt1 through the regulation of the expression of the *Wnt4* gene [20]. Wt1 binds to the promoter of *Wnt4* in both tissues with opposite outcomes. In kidney cells, Wt1 recruits the CBP/p300 coactivators and turns on transcription of *Wnt4*. In epicardial cells, Wt1 recruits the Bsp1 co-repressor to silence *Wnt4*. These Wt1-induced changes in *Wnt4* expression correlate with changes in chromatin structure. Kidney cells, in which the *Wnt4* gene is active, contain H3K4me3, H3K9ac and H3K14ac in the *Wnt4* locus. Epicardial cells, where *Wnt4* is silenced, contain H3K27me3 instead. The Wt1-dependent changes of chromatin marks in the *Wnt4* locus are confined to a region of the genome that is delimited by CTCF. The formation of a loop enclosing the *Wnt4* locus may be the basis for the functional chromatin domain established by Wt1. In the absence of CTCF, the chromatin domain created by Wt1 spreads outside of its normal boundaries and alters the transcription of neighboring genes [20].

Regulation of recombination

The effects of insulators on genome function are not limited to transcription processes. At the *Igh* locus, insulators regulate V(D)J recombination through promoter selection to transcribe non-coding RNA from the proper promoter and by bringing together distant sequences to undergo specific patterns of recombination. DNA rearrangements in the *Igh* locus of pro-B cells are under temporal and spatial regulation during B cell development. The process begins with D_H to J_H rearrangement followed by rearrangement of a V_H gene segment to D_HJ_H . In mice, there are more than 100 V_H genes spanning a 2.5 Mb region, whereas the J_H genes occupy 2 kb. How do all the V_H genes access the small J_H cluster in the *Igh* locus? Insulators are critical for the execution of this complex pattern of DNA rearrangements. There are about 60 CTCF sites throughout the V_H region and 2 clusters within other parts of the *Igh* locus in pro-B cells [21,22]. Of the two CTCF clusters not in V_H , one is next to D_H and the other is at the 3' regulatory region of J_H . These two sites interact strongly in pre-pro-B and pro-B cells, but only minimally in murine embryonic fibroblasts, promoting the selection of D_H promoters over V_H promoters before D_H - J_H combination takes place. These insulators also interact with an intronic enhancer (E_{μ}), which is required for the antisense transcription of D_H . Antisense transcription through the D_H locus precedes D_H - J_H rearrangement and has been proposed to make the D_H region accessible for subsequent recombination [21–23]. Thus, CTCF-mediated interactions select the D_H over the V_H promoters for antisense transcription as well as to first bring together D_H and J_H instead of V_H . Later, in pro-B cells, the locus compacts to bring V_H genes close to the D_H - J_H region through interactions that also depend on CTCF [21,22]. As a consequence, insulator-mediated chromosome interactions regulate V(D)J recombination both spatially and temporally.

A twist on this effect of insulator-mediated chromatin architecture on DNA recombination is that described for the T-cell receptor α locus (*Tcra*) [24]. The formation of loops by CTCF requires the presence of cohesin, which can also mediate interactions between different sequences on its own to regulate the transcription of various genes. In mouse thymocytes, cohesin binding sites flank the TEA promoter and the E_{α} enhancer of *Tcra*. Deletion of the gene for the Rad21 component of cohesin in these cells at the time when the locus is rearranged leads to a defective chromatin architecture in the region. Cohesin is required enhancer-promoter interactions necessary for *Tcra* transcription and deposition of H3K4me3. This histone modification then recruits the recombination machinery, which causes *Tcra* rearrangements. Therefore, long-range interactions mediated by cohesin indirectly control rearrangements in the *Tcra* region by regulating the transcriptional state of the locus [24].

The TFIIC insulator: more general than we thought?

Work carried out in *S. cerevisiae* and *S. pombe* indicates that RNA Polymerase III promoters can act as barriers and insulators. This property has been traced to the TFIIC transcription factor [25]. In mammals, TFIIC binds to human Alu sequences and murine SINE elements, both of which can act as insulators [9]. New results now suggest that Pol III promoters in human tDNA genes can act as both enhancer-blocking and barrier insulators [26]. It is not clear at this point whether this property can be exclusively assigned to the presence of TFIIC. Insulator function of these sequences correlates with their ability to mediate interactions with other tDNAs in the genome. One possible interpretation of these results is that these interactions mediate the formation of Pol III transcription factories. TFIIC sites are often found adjacent to CTCF sites in the genome, suggesting that the two proteins may function together to establish long-range interactions [27,28]. Interestingly,

TFIIIC colocalizes with condensin in budding and fission yeast chromosomes, pointing to an interesting similarity to the interaction of CTCF with cohesin [29].

Concluding remarks

It is becoming increasingly clear that the role of insulators is to mediate inter- and intra-chromosomal interactions between different regions of the genome. In so doing, insulators can establish a specific three-dimensional organization of the genome within the nucleus of eukaryotic cells. An important question is whether this structure is a consequence or an effector of genome function. Loop structures created by some regulatory sequences, such as enhancers and promoters, are a consequence of their function. In the cases reviewed above, insulators mediate intra- and inter-chromosomal interactions in order to elicit a specific functional response. Thus, it is possible that the three-dimensional structure of the genome determines its function. As a consequence, insulators may be responsible for the establishment of patterns of three-dimensional architecture of the chromatin fiber that are cell-type specific and responsible for distinct blueprints of gene expression during development. Insulator-mediated 3D structure may carry epigenetic information that needs to be maintained during mitosis. Examination of these possibilities remains one of the main challenges for future research in the field.

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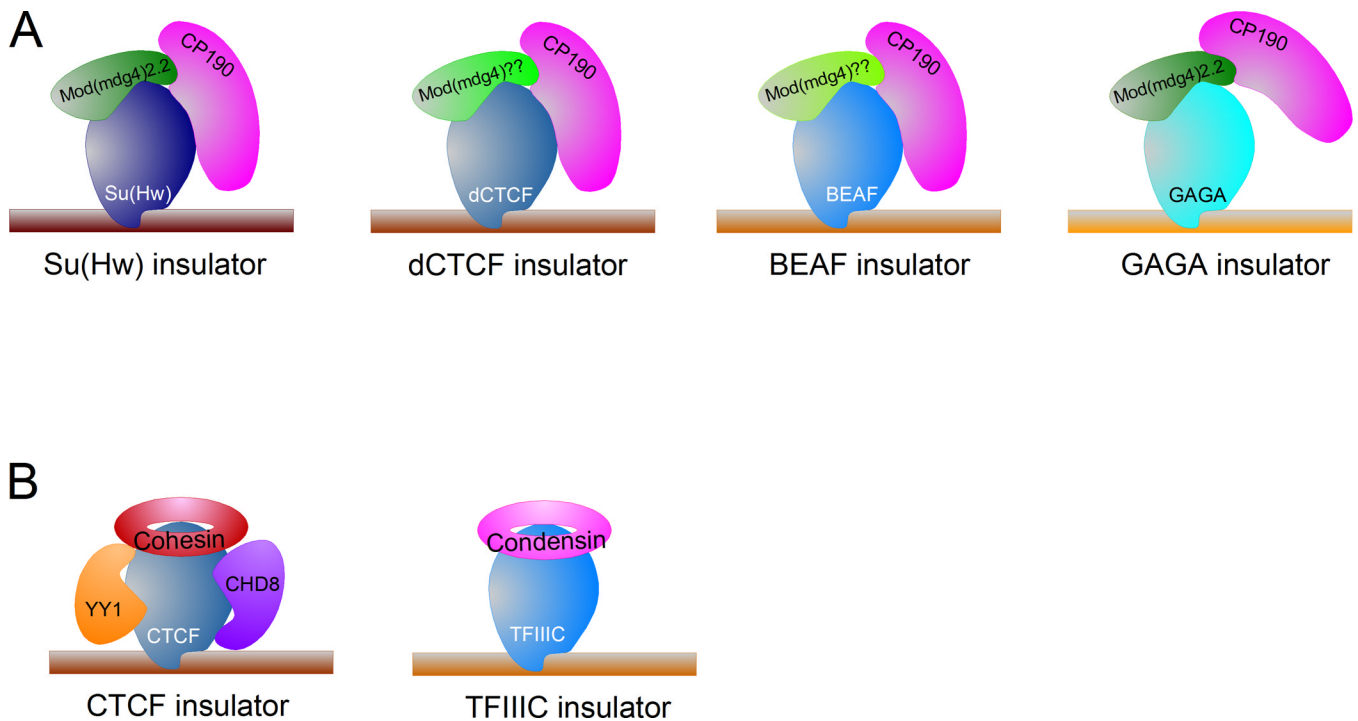
**Figure 1.**

Diagram showing the structure of different *Drosophila* and vertebrate insulators. **A.** Each *Drosophila* insulator subclass contains a different binding protein that may define the specific function of the corresponding subclass. All insulators share the common protein CP190, although the role of this protein in the function of the GAGA insulator has not been demonstrated experimentally. In addition, all subclasses may also have one Mod(mdg4) isoform. The gypsy/Su(Hw) insulator contains Mod(mdg4)2.2. The dCTCF and BEAF insulators lack this isoform but contain a different variant of Mod(mdg4). GAGA has been shown to interact with Mod(mdg4)2.2. **B.** Structure of the vertebrate CTCF and TFIIC insulators. Indicated are factors associated with CTCF such as cohesin, CHD8 and YY1

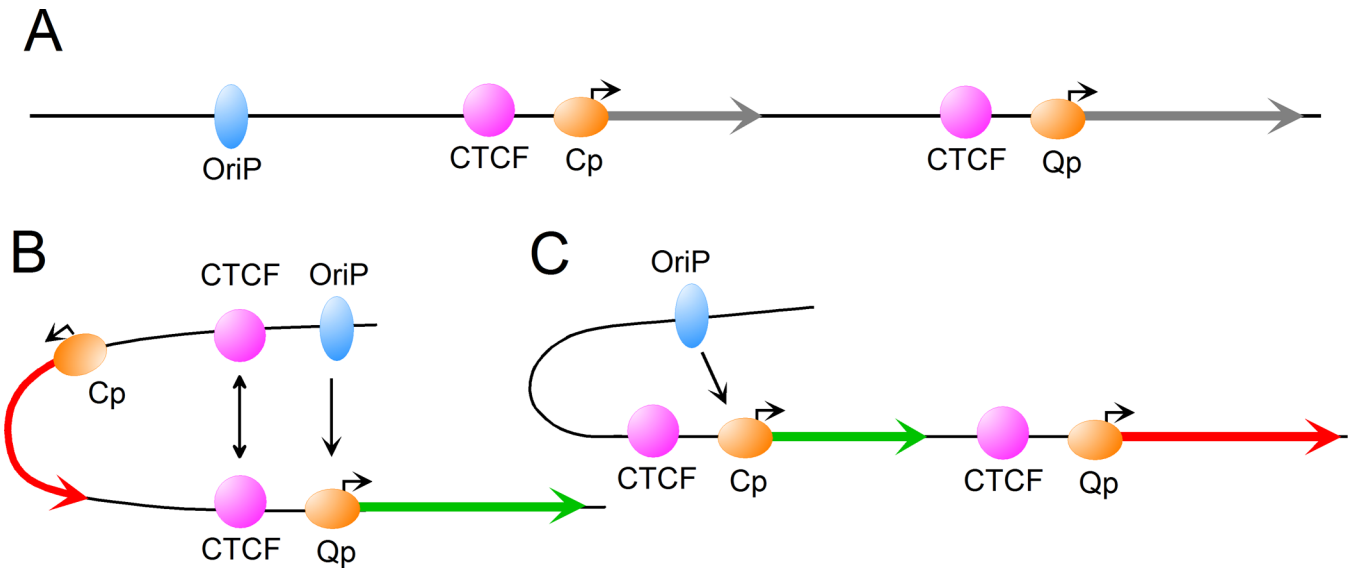


Figure 2. CTCF regulates enhancer-promoter interactions to control latency types of Epstein-Barr virus. **A.** linear organization of the EBV genome. **B.** Model showing CTCF-mediated looping that brings the OriP enhancer into contact with the Qp promoter in type I cells. **C.** In type III cells, the OriP enhancer interacts directly with the Cp promoter.

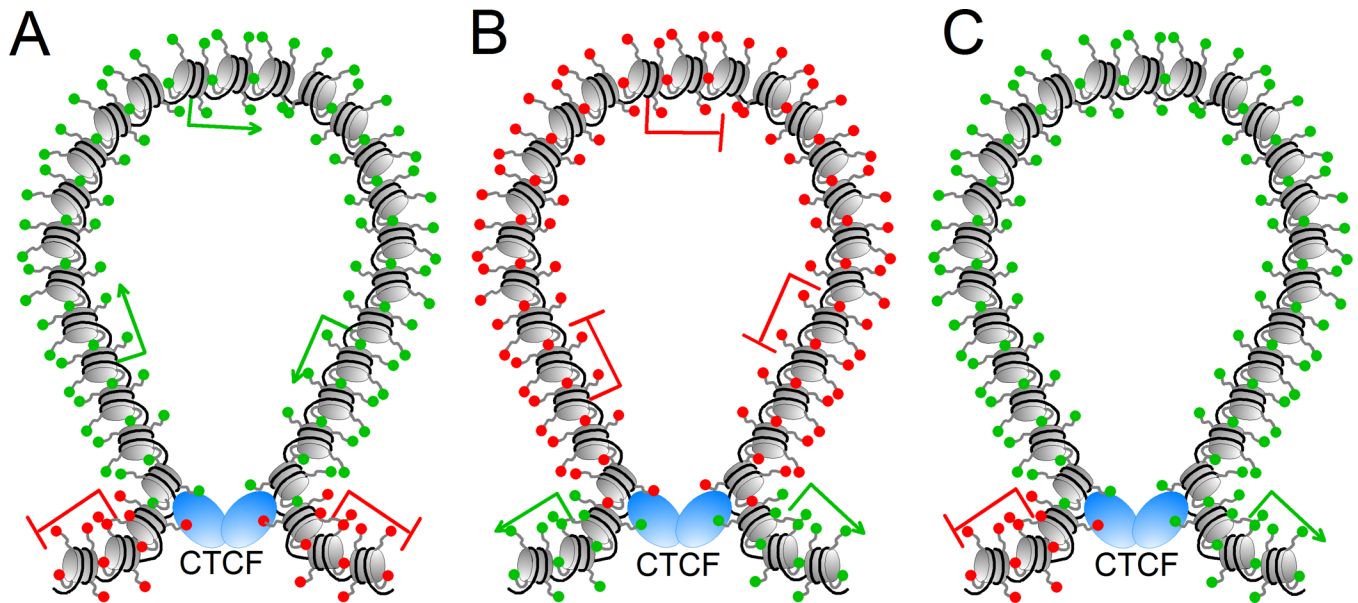


Figure 3.

Structure of some of the domains created by interactions between CTCF insulators in mouse embryonic stem cells. Actively transcribed genes are represented by a green arrow and silenced genes by a red line; nucleosomes and the histone tails are represented in grey, with active histone modifications indicated as green spheres and repressive modifications as red spheres. DNA is represented in black and CTCF as blue ovals. **A.** CTCF forms a loop to separate a domain containing active histone modifications and transcribed genes from repressive marks and silenced genes. **B.** CTCF forms a loop to separate a domain containing repressive histone modifications and silenced genes from active marks and transcribed genes. **C.** CTCF forms a loop containing nucleosomes enriched in mono and dimethylated H3K4, and trimethylated H3K4 at the boundaries of the loops, whereas the active transcription modification H3K36me3 and repressive H3K27me3 mark are observed outside the loops on opposite sides.

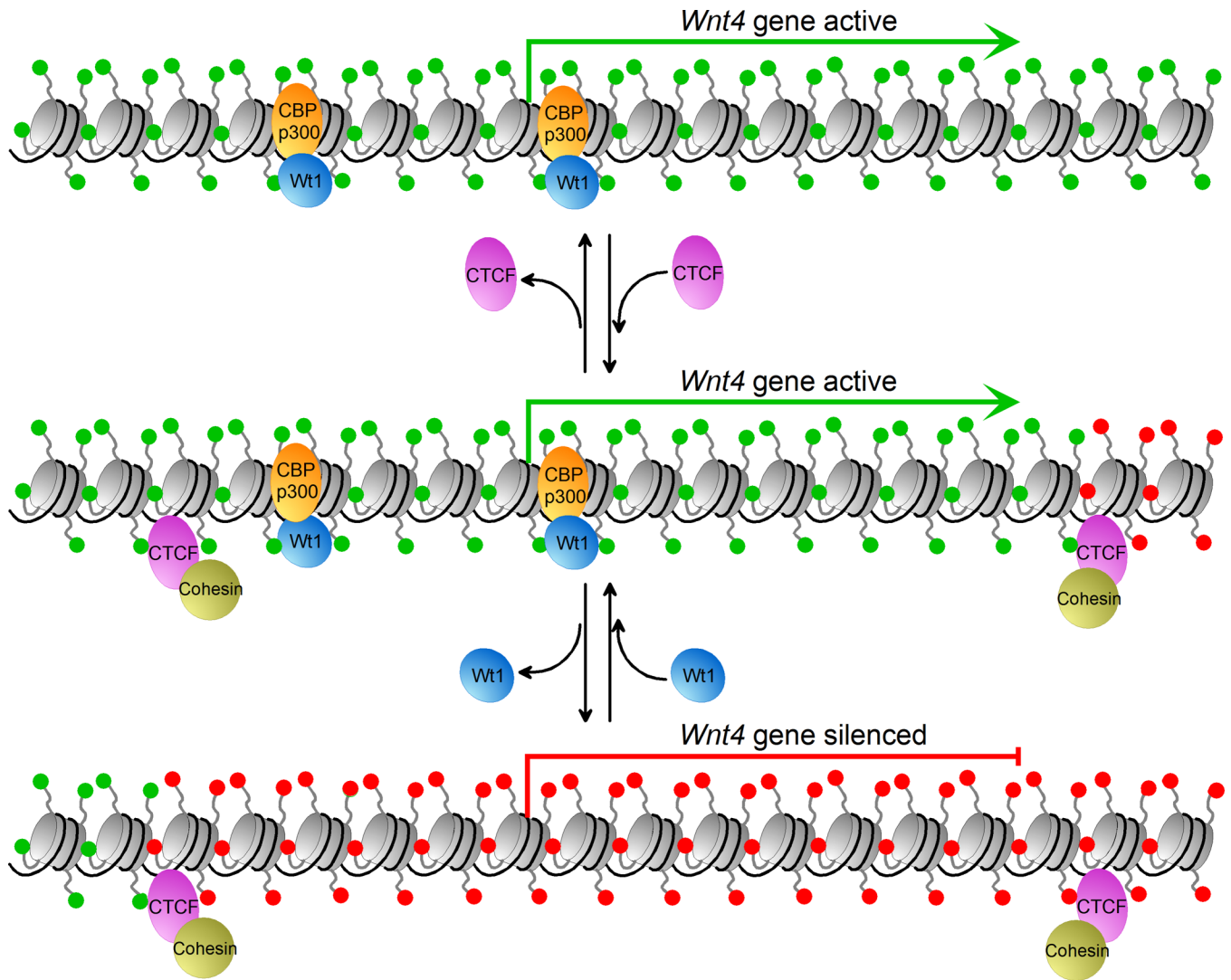


Figure 4. CTCF forms boundaries at the *Wnt4* Locus. The *Wnt4* gene is flanked by two CTCF sites (middle row). The gene is active in mesenchymal kidney cells where it is bound by the transcription factor Wt1, which recruits the co-activators CBP/p300. The activate histone marks (green) are present in the domain defined by CTCF. Mutation of the Wt1 transcription factor in these cells causes repression of the *Wnt4* gene and enrichment of repressive chromatin marks (red) within the CTCF domain (bottom row). The reverse effect is seen in epicardial cells (not shown). CTCF is critical for the establishment/maintenance of the chromatin domain by Wt1 at the *Wnt4* locus. Loss of the CTCF protein causes the spreading of the active chromatin past the boundary (top row) and can result in the transcriptional activation of neighboring genes.