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Insulators and domains of gene expression

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The genomic organization into active and inactive chromatin domains imposes specific requirements for having domain boundaries to prohibit interference between the opposing activities of neighbouring domains. These boundaries provide an insulator function by binding architectural proteins that mediate long-range interactions. Among these, CTCF plays a prominent role in establishing chromatin loops (between pairs of CTCF binding sites) through recruiting cohesin. CTCF-mediated long-range interactions are integral for a multitude of topological features of interphase chromatin, such as the formation of topologically associated domains, domain insulation, enhancer blocking and even enhancer function.

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Introduction

The concept of inactive and active chromatin domains was suggested quite early on as a way to interpret compact and less dense chromatin packaging in diploid interphase nuclei or in polytene chromosomes. The existence of such domains specifically requires the presence of domain boundaries to insulate the opposite activities of neighbouring domains. Such shielding elements, known as insulators, have been functionally identified by a position-independent high-level expression of a transgene in mice and flies [1,2]. In contrast to this barrier effect of an insulator, another shielding activity was called enhancer blocking [3], since it interferes with the action of an enhancer on a specific promoter when the insulator is positioned between the two.

Following the discovery of several *Drosophila*-specific insulator binding proteins (IBPs), such as BEAF32 [4], Su(Hw) [5,6] and Zw5 [7], the vertebrate factor CTCF [8,9] was shown to mediate insulation [10]. Later, the high conservation of chromatin insulation was demonstrated by the identification of CTCF in *Drosophila* (dCTCF) [11–13] and by comparing shared features (Table 1). Here, we summarize recent results on the genome-wide binding of these and more recently discovered insulator factors, and the projection of these binding sites onto the three-dimensional chromatin structure. These observations and results from high throughput analyses and functional tests are discussed with respect to a unifying mechanism for insulator-mediated barrier function and enhancer blocking activity.

CTCF: inhibitor and facilitator of enhancer function

Enhancer blocking activity of an insulator depends on its arrangement, that is, it has to be situated between the enhancer and promoter. This fact alone implies that the enhancer blocking activity is achieved by interfering with the chromatin looping required for enhancer/promoter contact. Detailed analysis of three-dimensional looping and the role played by the insulator protein CTCF revealed that CTCF not only possesses interference (enhancer blocking) activity, but also additionally mediates chromatin contacts or loops required for enhancer function. Examples for such bivalent consequences of loop formation are discussed below.

Bioinformatics evaluation of genome-wide chromatin interaction data led to the construction of a genome-wide interaction map of regulatory elements, which indicated that enhancer–promoter interactions are highly cell-type specific. Key interacting components are CTCF and cohesin [14]. This is exemplified by the MHC-II locus, which is active in B cells and bound by CTCF at 15 sites. In plasmablasts, this locus is inactive and only one third of the CTCF sites are bound. This correlates with the finding that CTCF is required for the cell type specific three-dimensional architecture of the locus and for maximal MHC-II gene expression in B cells [15•].

Another example is where CTCF/cohesin organizes a loop pattern that includes the promoter of the PTGS2 gene such that the PTGS2 gene is activated. In cancer cells the CpG island at the PTGS2 promoter is methylated and the gene is turned off. This silencing mechanism is in part caused by the methylation-induced loss of CTCF binding, which results in a change in chromatin looping and abrogation of gene activity [16].

Regulation of dCTCF binding in *Drosophila* development is seen at the homeotic gene *Ultrabithorax* (*Ubx*), which is activated by Ubx enhancer elements in the third thoracic leg imaginal disc. Here, a dCTCF site at the enhancer generates a loop with the gene promoter. In inactive tissues

Table 1

Insulator components with conserved features in vertebrates and *Drosophila*.

Factor	Organism	Description	References
CTCF	*H. sap.*	Enhancer blocking activity of the chicken beta-globin insulator	[10]
dCTCF	*D. mel.*	Enhancer blocking of Fab-8 insulator	[13]
GAGA	*D. mel.*	Enhancer blocking of the eve promoter	[77]
Th-POK	*M. mus.*	Binding to enhancer-blocking elements in murine Hox clusters	[78]
Cohesin	*H. sap.*	Cohesin is required for enhancer blocking of the H19 ICR	[79]
	D. mel.	Enriched at TAD borders	[54••]
TFIIIC	*H. sap.*	Loss of binding to tDNA promoters reduces their enhancer blocking activity	[80]
	D. mel.	Binding to borders of topological domains (ChIP-seq)	[54••]
Condensin	*M. mus.*	Binding correlates with enhancer blocking capacity of TAD borders	[54••]
	D. mel.	Enriched at TAD borders	[54••]
Rm62	*D. mel.*	Interacts with CP190 and mutations affect gypsy-mediated insulation in ct and y2-loci	[81]
p68	*H. sap.*	Along with SRA required for CTCF to perform proper insulation	[82]
PARP1	*D. mel.*	Modifies insulator functions	[83]
	H. sap.	Prevents DNA methylation of CTCF target sites. Controls circadian transcription	[84,85••]
dMes-4	*D. mel.*	BEAF-32 co-factor, involved in gene regulation	[86]
PRDM5	*M. mus.*	Interacts and overlaps with CTCF and Cohesin; recruits G9a (HMT)	[87]
Nurf-301	*D. mel.*	Regulates Fab-8 enhancer blocking activity	[72•]
Bptf	*H. sap.*	Interacts with CTCF; regulates nucleosomal arrays around CTS	[74]
TGF-β signal-ling	*D. mel.*	Genome-wide overlap with and dependency, to some extend, on dCTCF	[88]
	H. sap.	CTCF physically interacts with Smad3 and recruits Smad to H19 ICR	[89]
CP190	*D. mel.*	enhancer-blocking activity, mediates long-range interactions	[25,37]
Kaiso	*H. sap.*	Similar to BTB domain of CP190; mediates enhancer-blocking activity: physically interacts with CTCF	[90]

this dCTCF site is not occupied and enhancer/promoter interaction is lost [17], clearly demonstrating that this dCTCF site is a facilitator of enhancer action.

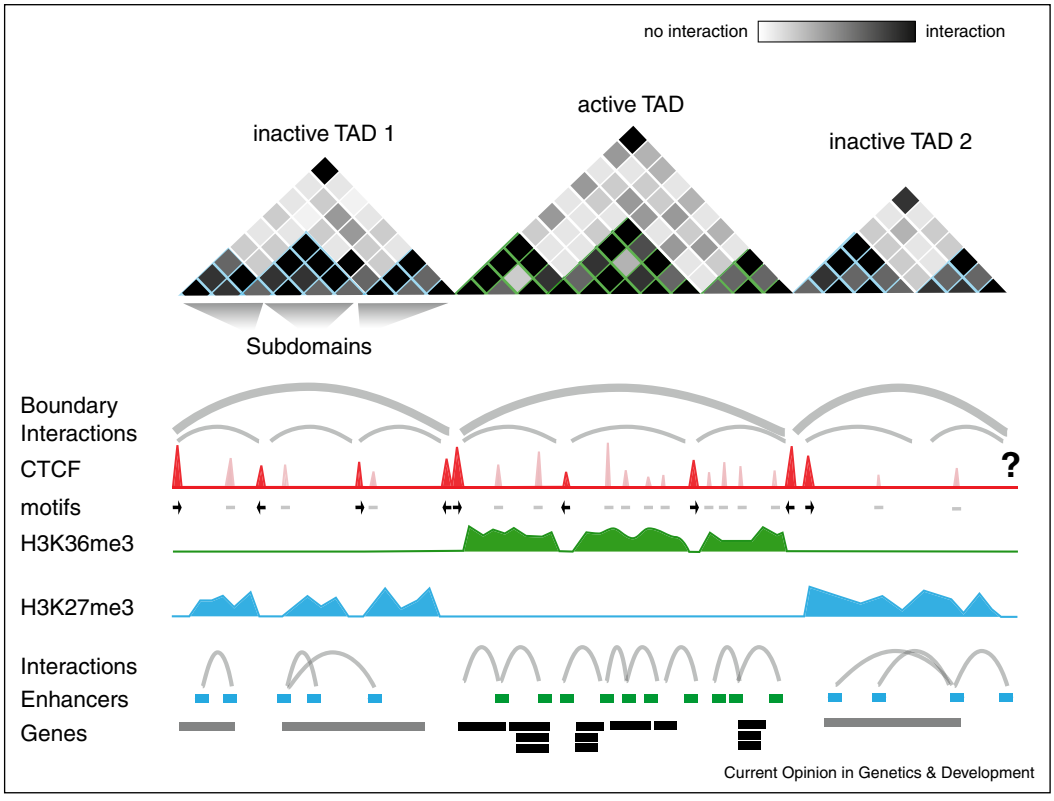
Not only can enhancer/promoter interactions be facilitated by CTCF/cohesin, but also other 3D-interactions may depend on CTCF sites. For example, CTCF-dependent enhancer/enhancer clustering in the nucleus was observed in thymocytes. Targeted 3C analysis demonstrated that interactions between the *Cd3* super-enhancers as well as with other enhancers were significantly weakened in cohesin-deficient thymocytes [18•]. Furthermore, CTCF/cohesin dependent inter-chromosomal contacts control enhancer inhibition in case of the Sox-2 and Sox-17 genes [19••]. Similarly, enhancer inhibition and facilitation was observed in erythroid cells, where together with other factors, CTCF bound to several sites mediates an intra-chromosomal interaction on chromosome 1 between the TAL1 promoter and its downstream enhancer, allowing for regulated TAL1-expression. However, in T-cell acute lymphoblastic leukemia, these interactions are altered, resulting in aberrant expression of the TAL1 oncogene [20••].

Barrier function and topologically associated domains

The identification of chromatin domains with either active histone marks or silencing modifications led to the concept of barriers that prevent one domain interfering with the neighbouring one. Loss of barrier function has often been related to inactive marks spreading into the active domain [21–29], although activation of inactive domains is possible as well (see below). Initial analyses revealed CTCF binding and loop formation at barrier sites flanked by opposite chromatin states [30–32]. In *Drosophila*, depletion of dCTCF results in a small change in H3K27me3 spreading [33], when testing genome-wide effects. Additional architectural proteins are also present at chromatin barriers and may compensate for the loss of CTCF-dependent barrier functions (see below). Therefore, only a few barrier sites, which are primarily dependent on CTCF, showed an expansion of the H3K27me3 mark into the flanking region [34–38]. As discussed below, the role, played by insulators and CTCF in barrier function, is further supported by the analysis of homeotic genes, and illustrated by the concept of topologically associated domains [39] (TADs) (Figure 1).

Homeotic genes are expressed during development in a cell type, and stage specific manner. The collinear genomic arrangement and expression of the gene clusters specify the segmental identities along the body axis of *Drosophila* and mammals. Thus, in a given cell type or specific developmental stage one group of Hox genes may be turned off by Polycomb function, resulting in H3K27me3 modification of the respective gene locus.

Figure 1



Insulators, chromatin domains and topologically associated domains (TADs). Interaction matrix representing a virtual Hi-C experiment (top). The grey scale above indicates interaction frequencies. Interactions occur predominantly within TADs (*e.g.* enhancer–promoter interactions), which are often grouped in subdomains. Interactions between TAD boundaries are thought to depend on the binding of CTCF (shown as a schematic ChIP-seq track in red) to its cognate DNA-binding motif (black arrows). CTCF sites not involved in binding to TAD boundaries are shown in pale red and grey motifs, respectively. Motifs involved in long-range chromatin interactions show an inverted repeat orientation (see Figure 2). As not all TAD boundaries are bound by CTCF it is likely that additional factors may be involved in their function (indicated by question mark). TADs are often co-incident with chromatin domains represented by a schematic ChIP-seq track for an active (H3K36me3; green) and a repressive (H3K27me3; blue) histone modification. Active TADs are gene-rich (black bars for active genes) in contrast to gene-poor repressed domains (grey bars).

In both mammals and *Drosophila*, *Hox* gene clusters are marked by CTCF/dCTCF binding at the borders between individual regulatory elements of the *Hox* genes [12,40–42]. In *Drosophila*, developmental expression is specific for each parasegment. Chromatin purified from single parasegments revealed a 'step-wise' pattern of acetylated H3K27 (active gene domain) or of H3K27me3 (inactive gene domain) with sharp, dCTCF-bound boundaries at the bithorax complex (BX-C) regulatory domains [43••]. This suggests that functional boundaries associated with dCTCF binding restrict H3K27me3 or H3K27 acetylation to one domain, preventing spreading into the neighbouring domain.

A similar situation is found with the mouse and human *HoxA* genes. Kinetic analyses of myelomonocytes differentiating into monocytes/macrophages revealed a dynamic change in HoxA cluster topology [44]. *HoxA* expression in ES cells is silenced by H3K27me3, whereas differentiation into neuronal cells is marked by activation of the rostral group of the *HoxA* cluster, while the caudal group of genes remain silenced [45,46]. Again, the pattern of gene activity is associated with a 'step-wise' pattern of H3K27 modification [47••] with H3K27me3 enriched at silent genes. To test the requirement for CTCF at functional boundaries, the CRISPR/Cas technique was used to delete a CTCF binding site separating the active gene groups from the repressed genes within the *HoxA* cluster. CTCF loss at these sites resulted in spreading of H3K4 methylation, an active chromatin modification, into the repressed region, thereby activating a caudal *Hox* gene [46,47••]. This clearly shows that CTCF acts as a barrier, in this case for active marks spreading into a silenced region.

Analysis of *Hox* genes suggests that barrier function is linked with TAD organization (Figure 1). In wildtype, *Hox* gene expression and H3K27 modification correlate with two TADs in motor neurons. Deletion of the CTCF site at the boundary not only removes the barrier, but also

shifts the TAD boundary further into the caudal TAD, up to the next CTCF site [47••]. Upon removing this site as well, the barrier and TAD boundary shifted even further into the caudal gene region. Thus, both barrier function and TAD boundary function are controlled by CTCF and are probably two features of the same phenomenon.

How do these structural units relate to genomic functions such as control of transcription? In one study, ChIA-PET was used to generate a map of enhancer–promoter interactions in ES cells. Genes controlled by super-enhancers were found to reside within a super-enhancer domain structure with the flanking, protein-bound CTCF/cohesin sites forming a loop. Consequently, these loops generate insulated neighbourhoods that are preserved in multiple cell types. Similarly, Polycomb repressed genes are organized in insulated neighbourhoods flanked by CTCF/cohesin, thereby forming a Polycomb domain [48••].

Another strategy to identify functional domains was to insert regulatory sensor transposons into hundreds of sites within the mouse genome. The enhancers identified in this screen acted along broad regions that correlated strongly with TADs [49••]. This suggests that three-dimensional enhancer action is restricted to the genomic region defined by TADs, and therefore the functional domain structure concurs with the topological features identified by 3D mapping.

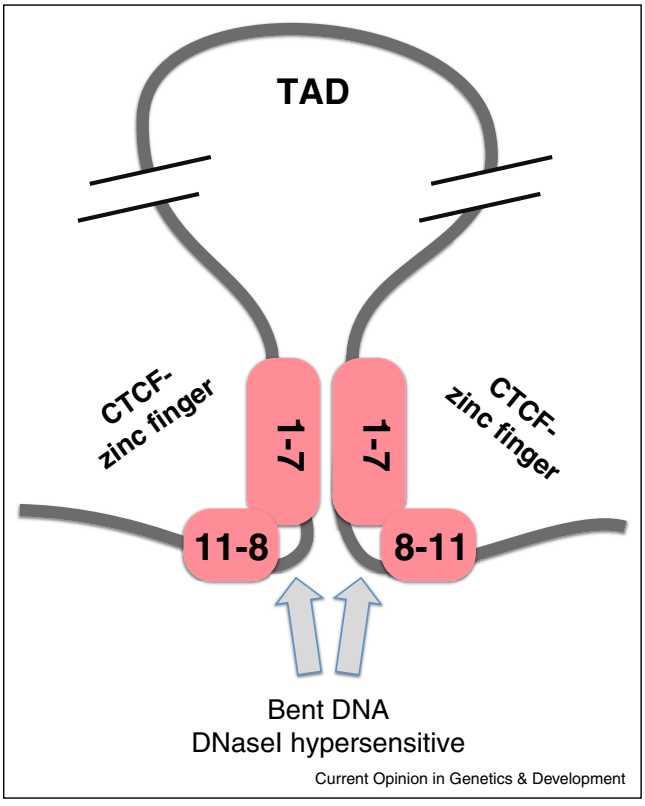
Which features of TAD domain boundaries are required for TAD formation? There is good evidence that interactions within TADs contribute to boundary function [50]. In addition, TAD domain boundaries are strongly enriched for CTCF/Cohesin binding. The importance of CTCF/Cohesin for the structural and functional integrity of TADs has been documented in several cases. The consequence of CTCF loss for the 3D structure of chromatin was tested by depleting CTCF [51,52•]. Some changes were observed, such as a mild reduction in intra-domain interactions as well as a gain in inter-domain interactions. Nevertheless, the overall organization and long distance interaction remained. This, and the fact that many more CTCF sites exist that are not located at TAD boundaries (Figure 1), argues for additional factors involved in the 3D landscape of chromatin. In fact, besides CTCF/dCTCF, many architectural factors have been found at TAD borders. These factors are cohesin components SMC3 and RAD21, TFIIIC subunits, condensin subunits and PRDM5, a SET domain protein [53••,54••]. Furthermore, there is evidence that CTCF is an RNA binding protein and that RNA is involved in CTCF recruitment and long-range interaction [55]. Another *Drosophila* IBP, Su(Hw) has been shown to interact with RNA as well [56].

The functional importance of the TAD organization becomes evident when TAD borders are deleted. Human families with rare limb malformations show rearrangements in the extended *WNT6/IHH/EPHA4/PAX3* region. Comparable rearrangements were generated in mice using the CRISPR/Cas technique [57••]. These mutations resulted in disease-relevant changes in interactions between promoters and non-coding DNA as well in aberrant gene expression. Furthermore, these mice developed digital malformations similar to phenotypes observed in patients. These changes in chromatin interaction and function only occurred if the rearrangement disrupted a CTCF-associated TAD boundary [57••].

The relevance of TAD organization is further underscored by its evolutionary conservation. A group of homeobox genes, called the *Six* cluster, is highly conserved from sea urchins and zebrafish to mice and humans. Similarly, the TAD organization is conserved with two largely independent regulatory landscapes contained within two adjacent TADs [58•]. Interestingly, CTCF binding sites at the TAD borders are found in opposite orientations, also a highly conserved feature. CTCF sites divergent between species correlate with divergence of an internal domain structure. Comparing genomes and domain structures of mouse and dog revealed insertions, inversions and duplications. Interestingly, in each case the rearrangement occurred at the border between two TADs [59••].

In *Drosophila*, functional tests revealed that inverted insulators form loops more efficiently than insulators in identical orientation [60]. Similarly, in vertebrates it became obvious that the direction of CTCF binding sites plays a major role in determining which combinations of CTCF binding sites are compatible for interacting and subsequently generating loops. First, the orientation of CTCF binding motifs is strongly conserved across evolution [59••]. Second, genome-wide analysis revealed that 72% or 48% of the mouse or human TAD borders, respectively, contain a pair of convergent CTCF sites [53••,58•]. This suggests a functional role of pairing between CTCF bound TAD borders, and that the selection of sites involved in pairing may be driven by the orientation of the CTCF binding sequences. Indeed, CRISPR/Cas mediated inversion of one of the CTCF binding sites in the *Pcdh* and *beta-globin* gene clusters induced directional switching of genome topology or partial merging of neighbouring chromatin domains [61••]. But how can the direction of CTCF binding motifs influence pairing between insulators often separated by several hundred kilobases of DNA? A hypothetical model includes the biophysical ability of CTCF to bend DNA by 90° [62]. This causes a structure with an orientation that may be more accessible to pairing with another CTCF molecule bound to an inverted binding site (Figure 2). Furthermore, such a three-dimensional arrangement may have sterical consequences for nucleosome formation and for binding of cohesin and additional factors. Physical modelling suggested a loop extrusion model explaining why loops tend not to overlap and why

Figure 2



The structure and orientation of the CTCF/DNA complex may guide pairing of TAD boundaries. A hypothetical model includes the biophysical ability of CTCF to bend DNA by 90° [62]. When DNA binding motifs are convergent, this may facilitate homodimeric CTCF interaction and formation of the bent conformation at the bottom of the loop. The DNA bend is found at the DNA spacer between zinc finger groups 1 to 7 and 8 to 11, which has been identified by DNase I hypersensitivity [91]. Physical modelling supports a loop extrusion model in the context of paired CTCF binding sites in convergent orientation [63••].

the CTCF-binding motifs at pairs of loop anchors lie in the convergent orientation [63••]. This model was nicely supported by genome editing altering CTCF-binding sites. In every case the convergent rule correctly predicted loop formation [63••].

The specific role of *Drosophila* CP190

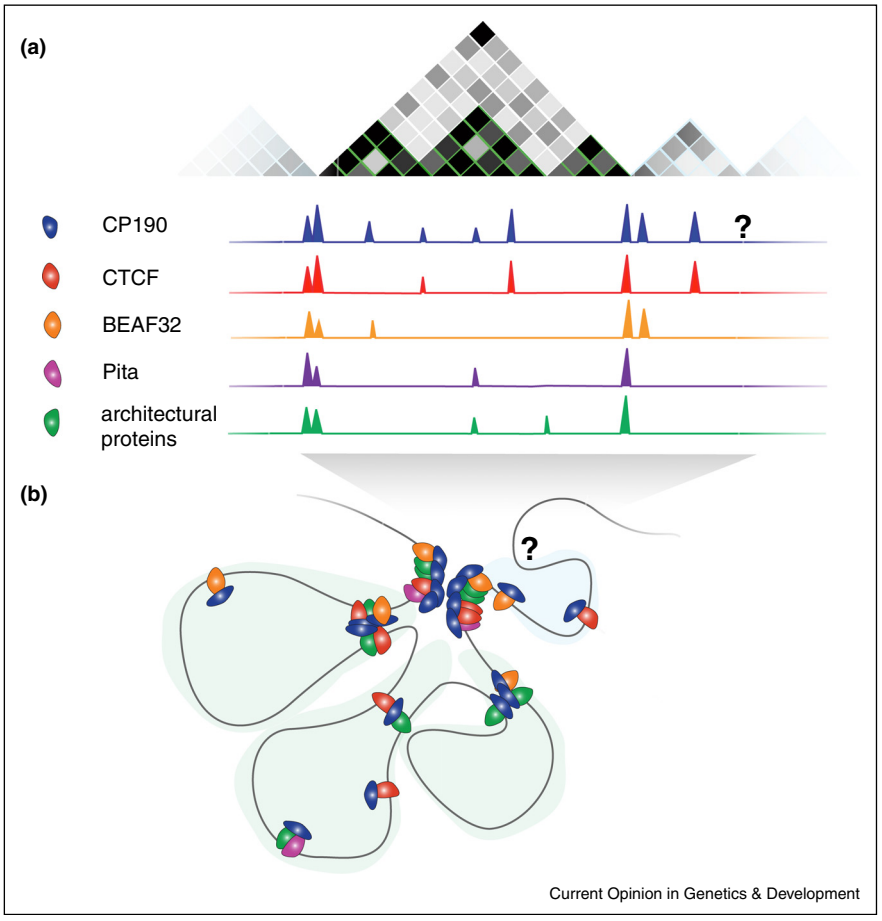
As described above, the general functions of insulator factors are highly conserved between vertebrates and *Drosophila* (Table 1). Nevertheless, an insect specific factor crucial for insulator function is the centrosomal protein 190 (CP190). Although identified in the context of centrosomes [64], a functional role was found in insulation mediated by Su(Hw) [65]. Subsequently, other IBPs have been identified that also bind CP190, for example BEAF-32, GAF, Zw5 and dCTCF, which frequently co-localize with CP190 throughout the *Drosophila* genome [33]. From these data it became evident that a class of potential insulator sites was bound by CP190 in the absence of any known DNA binding factors.

Recent searches for additional, DNA binding and CP190 interacting factors identified insulator binding factors 1 and 2 (Ibf1, Ibf2) [66], a zinc finger protein interacting with CP190 (ZIPIC) and Pita [67•]. All four factors mediate enhancer blocking of transgenes in *Drosophila*. Genome-wide binding was frequently found to be clustered with other IBPs and with TAD borders [54••]. In addition to this correlation, many IBP binding sites are found within TADs and many TAD boundaries are not associated with IBPs (Figure 3). Mapping of the CP190 protein revealed separate interaction domains with Pita and ZIPIC [67•]. This suggests that CP190 has a bridging function, simultaneously contacting several proteins. Such a feature was implicated when deletion of the CP190 interaction domain of BEAF-32 [68•] resulted in BEAF-32 located at distant sites failing to interact with GAF or dCTCF bound promoters. The biophysical capacity of CP190/BEAF-32 to mediate long-range interactions *in vitro* further supports the bridging function of CP190 [69].

Assuming that CP190 is a bridging factor and that IBPs are frequently clustered [33,54••,67•,70], one can envision a concept of several IBPs targeting CP190 more efficiently (Figure 3). On the other hand, this multitude of clustered and CP190 interacting factors causes some kind of redundancy. This is evident from a dCTCF mutant lacking the CP190 interaction domain, which is still able to function similarly to wildtype dCTCF [71]. Based on synergistic recruitment one would expect CP190 binding to scale with IBP binding, which indeed could be shown [67•]. Furthermore, insulator function and topological domain border strength both correlate with IBP protein occupancy [54••].

The search for additional architectural proteins involved in insulator function revealed many more factors contributing to insulator strength, such as TFIIIC, Rad21 (cohesin), Chromator, DREF, L(3)mbt and condensin factors CAP-H2 and Barren [54••]. Occupancy and clustering of these factors to individual sites correlates with enhancer-blocking activity and TAD border strength. Thus, more architectural factors binding to insulators increase the insulator function. When re-analysing binding and Hi-C data from mouse and human ESCs and IMR90 fibroblasts, a similar conclusion could be drawn: mammalian TAD borders are enriched for the architectural factors CTCF, TFIIIC, cohesin and condensin components and binding correlates with topological structure and regulatory potential [54••]. Thus, a highly conserved molecular mechanism for TAD boundary function and insulation (Table 1) arises from the binding strength of factors connected by protein/protein interactions mediated by CP190, cohesin and condensin and possibly many others.

Figure 3



Drosophila CP190 recruitment and strength of TAD boundaries/insulators correlate with combinatorial binding of architectural proteins. **(a)** The interaction matrix represents TADs. Boundaries between TADs are often marked by CP190 binding (schematic ChIP-seq track, blue). CP190 is recruited to chromatin by a wide variety of insulator binding factors (IBPs, as exemplified by CTCF, BEAF32 and Pita in schematic ChIP-seq tracks). Frequently, different insulator binding factors cluster together, suggesting a cooperative recruitment mode for targeting CP190 to chromatin. Combinatorial recruitment of CP190 to TAD boundaries may be functionally important since high occupancy of IBPs and other architectural proteins such as cohesin, condensin and TFIIIC predict the strength of insulator function as well as TAD borders [54••]. It should be noted that not all TAD boundaries are bound by known IBPs (?) and that many IBP binding sites are found within TADs. **(b)** The physical DNA string model summarizes the contact and binding data illustrated in (a).

In addition to the architectural and looping functions, an enzymatic activity in nucleosomal depletion was postulated due to the finding that dCTCF/CP190 binding sites show reduced nucleosomal occupancy, whereas dCTCF sites devoid or depleted of CP190 are loaded with nucleosomes [37]. A functional siRNA screen identified NURF and dREAM complexes binding to CP190 and being required for enhancer blocking [72•,73]. Probably, the nucleosomal remodelling activity of ISWI, a component of NURF, causes nucleosomal depletion at CP190/dCTCF sites. Interestingly, a NURF and CTCF connection has also been found in vertebrates (Table 1) [74].

Testing chromatin conformation at a synthetic cluster of hundreds of binding sites for a LacI-CP190 fusion revealed a general opening and expansion of chromatin in *Drosophila* cells [75]. A similar function was mediated by vertebrate CTCF in vertebrate cells [36,76]. Analysis of chromatin before and after CTCF recruitment revealed active removal of the H3K27me3 mark, likely by incorporating the H3.3 variant [36]. This variant is often associated with unstable nucleosomes and may explain that insulators are depleted of nucleosomes, and that flanking nucleosomes are free of the repressive histone mark H3K27me3.

When comparing vertebrate and *Drosophila* in respect to chromatin domains and insulation, many observations are comparable, as discussed above. Nevertheless, there are many more IBPs in *Drosophila*, not found in vertebrates as is CP190. Potentially, the demand for efficient, and maybe locus-specific insulation may be much higher in

case of the very compact *Drosophila* genome. This diversity of IBPs seems to be functionally merged by CP190.

Conclusions and perspectives

Recent advances in determining the three-dimensional folding and interaction of chromatin at high resolution have highlighted the impact of higher-order chromatin structure on genome function. This is supported by the emerging concept of topologically associated domains separating the genome into conserved chromosomal neighbourhoods encompassing blocks of similarly regulated genomic regions. Architectural proteins, including CTCF, are the determinants for the strength of TAD formation and insulator function. The selection of interacting regions, in the case of CTCF, is dictated by the binding site orientation. It is obvious that CTCF and its orientation only partly account for the determinants selecting and mediating proper interactions. About 30 000 sites in the vertebrate genome are bound by CTCF, but only a fraction is found at TAD borders. What are the factors or combinations of factors determining the specificity of interacting elements? Furthermore, not all TAD borders have CTCF sites. Which factors or features are mediating the boundary function in these cases?

Despite the fact that many more insulator proteins are known in *Drosophila* than in vertebrates, the general features and many of the components are highly conserved (Table 1). Does this mean there are many more vertebrate factors involved in insulation that are yet to be found in vertebrates? And if so, will they help in solving the specificity problem?

In addition to long-range interaction and looping functions, characteristic chromatin modifications are found at insulators and are required for insulator activity. Furthermore, RNA molecules are involved in CTCF function. It remains to be seen whether these activities are fundamental to insulator function, or whether they support efficient binding of the architectural proteins, thereby maintaining long-range interactions.

Acknowledgements

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