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Roles of chromatin insulator proteins in higher-order chromatin organization and transcription regulation

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Eukaryotic chromosomes are condensed into several hierarchical levels of complexity: DNA is wrapped around core histones to form nucleosomes, nucleosomes form a higher-order structure called chromatin, and chromatin is subsequently compartmentalized in part by the combination of multiple specific or unspecific long-range contacts. The conformation of chromatin at these three levels greatly influences DNA metabolism and transcription. One class of chromatin regulatory proteins called insulator factors may organize chromatin both locally, by setting up barriers between heterochromatin and euchromatin, and globally by establishing platforms for long-range interactions. Here, we review recent data revealing a global role of insulator proteins in the regulation of transcription through the formation of clusters of long-range interactions that impact different levels of chromatin organization.

Introduction

The proper organization of eukaryotic chromosomes determines the manner in which the DNA sequence is interpreted in a large number of cellular processes, including DNA replication, repair and transcription.[1-6] In particular, a gene transcriptional activation state depends on its position in the genome and on its local chromatin structure.[7] In addition, three-dimensional loops have been implicated in all levels of chromatin organization ranging from kb-size loops to larger intrachromosomal loops hundreds of kb in size.[6,8,9] Both kinds of loops were shown to regulate several biological processes, such as X chromosome inactivation,[10] and developmentally regulated transcription[11] or repression.[12] The connection between transcription regulation and higher-order nuclear organization of chromatin has been a long-standing question in modern biology, and the mechanisms responsible for this connection are just starting to emerge.

Chromatin insulators may be key determinants of the proper organization of eukaryotic chromosomes. These DNA sequences were originally identified in *Drosophila melanogaster* (hereafter Drosophila) and chicken as distinct cis-regulatory elements that block the action of distant enhancers[13-15] and that might define the boundaries of chromatin domains to demarcate the distinction between heterochromatin and euchromatin.[16-21] Recent progresses in the field have provided strong support for both types of models, leading to the identification of two major classes of insulators: 'enhancer blocking' insulators (EB insulators) block communications between adjacent regulatory elements in a position-dependent manner (e.g., prevent distant enhancers from activating a promoter when placed between them) (**Fig. 1B**), and 'barrier insulators' prevent the silencing of euchromatic genes by blocking the spreading to nearby heterochromatin[20,22] (**Fig. 1A**). These insulation activities either trigger the activation or repression of transcription in a locus-dependent manner. The persistence of transcriptionally active genes throughout cell division implies the existence of regulatory elements or epigenetic mechanisms that enforce and maintain the distinction between heterochromatin and euchromatin.

Distinct families of insulators have been described to date, defined by the insulator binding protein (IBP) that is essential for their activity.[21,23] In *Saccharomyces cerevisiae* and *Saccharomyces pombe*, TFIIIC was found to be an evolutionary conserved barrier insulator factor.[24-26] Extensive studies in Drosophila have identified five insulator families including those bound by: Suppressor of Hairy-wing [Su(Hw)], boundary element-associated factor (BEAF), Zeste-white 5 (Zw5),[27] the GAGA factor (GAF),[21] and dCTCF,[28] a distant sequence homolog of mammalian CTCF (see below). In vertebrates, CCCTC-binding factor (CTCF) is the only insulator protein that has been characterized,[29-31] although putative homologs of Drosophila and yeast insulator proteins were also recently identified.[32,33]

Despite this large variety, insulator proteins share common molecular mechanisms. Four general properties have been

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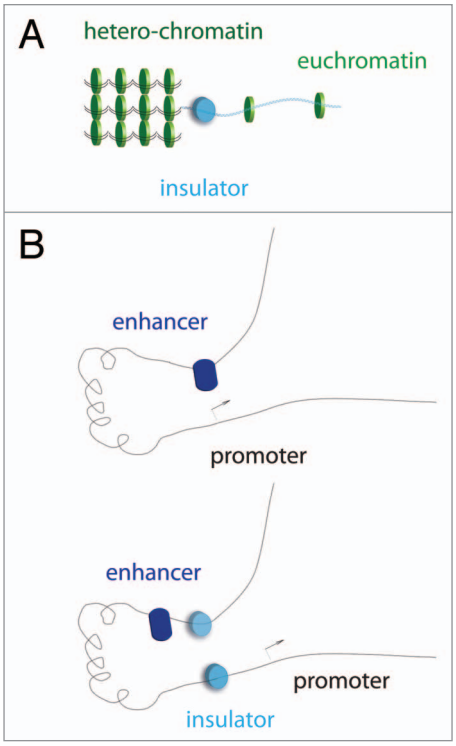

Figure 1. Insulator mechanisms. (A) Barrier insulators (cyan cylinder) physically confine heterochromatin regions (condensed green discs, left) to avoid their spreading to parts of the genome that have to be actively expressed (sparse green discs, right); (B) enhancer-blocker insulators (cyan cylinder) positioned between an enhancer (blue) and a promoter (arrow) affect gene expression by avoiding that these two regions get in close contact (top: enhancer contacts promoter, bottom: insulator prevents enhancer-promoter interactions).

repetitively associated with the activity of the known insulators and their binding proteins, from yeast to humans:

(1) their activity may be associated to chromatin accessibility, e.g., by interacting or recruiting specific transcription or remodeling factors to position, evict, exchange or modify nucleosomes/histones in their vicinity[20,34-39] or by preventing DNA methylation.[40]

(2) unlike desilencing activities, the activity of insulators more specifically involves directionality and the establishment of long-range interactions with distant elements and/or the clustering of multiple insulators into 'bodies'.[7,15,25,41-43]

(3) insulator binding proteins might derive from transposons and/or ancient transcription factors, yet it remains unclear if they might function as derivative of promoter elements or promoter decoys/sink.[26,44]

(4) more generally, insulators may be involved in the compartmentalization of the interphasic chromatin, involving interactions with specific nuclear compartments such as lamina or nuclear pore complexes at the nuclear periphery, or other nuclear structures to regulate the activation states of genes through positioning of chromatin.[12,42,45-47]

Each insulator binding protein interacts with a unique network of partners that is often defined by the chromatin context and genetic location of the insulator element, as well as by the cell cycle stage and cell type. The composition of this network ultimately determines the specific activity of the insulator at that locus. To be able to draw general mechanistic conclusions, it is thus of upmost importance to identify the different factors composing specific insulator elements, to understand the role of these factors, and to characterize their interactions with components of the transcription machinery (e.g., RNA polymerase, transcription factors, enhancers), chromatin remodelers, histone modifiers (e.g., de-/acetylases, de-/methylases) and nuclear architectural proteins (e.g., histones, cohesin, lamina). The use of Chromosome Conformation Capture (3C) [48] and derivative technologies[49] is also likely to enable a better understanding of the degrees to which insulators compartmentalize interphasic chromatin with respect to, for instance, chromosome territories,[50] as predicted for CTCF.[51]

In this review, we will focus on recent data highlighting three different aspects of insulator binding proteins that support their role as key regulators of transcription, local chromatin accessibility as well as long-range interactions.

Insulator-Binding Proteins: the First Layer Toward Insulator Formation?

BEAF was originally identified as an essential factor for the EB activity of the *scs*' element,[19] the first insulator element reported in reference 18, beaf encodes for two alternative spliced forms: BEAF-32A and 32B. BEAF-32B was shown to be necessary and sufficient for EB activity[23,52] and to represent the majority (~99%) of DNA-bound BEAF.[53] BEAF-32 harbors an N-terminal BED finger domain, a C-terminal domain that is involved in protein-protein interactions and self-interaction properties,[19,54] and a middle, coiled-coil region that was suggested to be required to determine its cellular localization.[55] As for Su(Hw), or Zw5, there are no sequence homologs of BEAF-32 in vertebrates, although the structural characterization of these proteins and the biochemical analysis of their interaction partners may result in the future identification of potential homologs in vertebrates, as recently suggested for GAF.[32] An interesting hypothesis is that the different functional or structural modules in insulator proteins may be encoded in multiple factors in vertebrates. Actually, BED finger domains are found in one or more copies in regulatory factors and transposases from plants, animals and fungi. BED fingers are 50–60 amino acid long, and contain a characteristic motif with two highly conserved aromatic positions and a shared pattern of cysteines and histidines that are predicted to form a zinc finger that belongs to the WRKY-GCM1 zinc-finger superfamily of DNA-binding factors.[56] Additional members of this family include transcription factors like ATF2 or DREF[43] as well as numerous human zinc-finger BED proteins of unknown function. Biochemical experiments showed that BEAF-32B (hereafter called BEAF) binds specific DNA sequences mostly as a trimer. Each BEAF subunit targets one CGATA motif, while point mutations within this consensus motif abolish binding and insulation.[54] Clusters of 3–4 CGATA motifs create high-affinity (k_D ~ 10 pM) binding sites[23] that are often organized in a pair-wise configuration ('dual-core' element) to separate

head-to-head gene pairs[35] although additional arrangements were also found.[38] Importantly, BEAF binding sites are preferentially distributed in pairs separated by ~10 kb, suggesting that long-range BEAF/BEAF interactions could lead to the formation of long-range DNA contacts. BEAF binding sites partially overlap with dCTCF binding sites,[38,39] suggesting partial redundancies and/or interplay among various EB activities.

Su(Hw) is one of the most extensively studied insulator binding proteins in Drosophila. Su(Hw) possesses EB[57] as well as barrier activity[58,59] and binds specifically to a sequence motif that was first identified in the gypsy retrotransposon.[60] It was earlier suggested that different domains of the protein are involved in either EB or barrier function probably by the recruitment of protein interaction partners.[59] Su(Hw) contains of 12 zinc fingers and acidic domains at its N- and C-terminal. In addition, it contains a leucine zipper motif close to the C-terminal end of the protein[61] that is involved in the interaction with Modifier of mdg4 [Mod(mdg4)].[62] A 12 bp YRY TGC ATA YYY (Y-Pyrimidin, R-Purine) Su(Hw) consensus DNA binding motif was first identified in the gypsy insulator region.[60] Further biochemical and genome-wide DNA binding studies of Su(Hw) resulted in hundreds of new non gypsy binding sites from which a 20 bp consensus motif was derived that allows variations in the TGCATA core binding region.[63,64]

Zw5 belongs to the zf-AD (zinc-finger associated domain) superfamily and sequence analysis results in 8 Cys2-His2-type (CH2)-type Zn-fingers (smart.embl-heidelberg.de). Zw5 is awaiting biochemical characterization.

CTCF was originally discovered as a transcription factor regulating c-myc.[30] Full length CTCF and dCTCF contain two highly conserved domains: an 11 zinc finger central DNA-binding domain and an N-terminal domain. Recent studies mapping the genome-wide distribution of human CTCF found that it occupies a 11–15 bp core consensus sequence and binds in vitro to a specific 12 bp variation of this consensus with very high-affinity (K_D = 0.1 nM).[65-67] Biochemical assays have indicated that CTCF can in fact use various combinations of the zinc-finger domains to bind different DNA target sequences that may be influenced by post-transcriptional modifications (see below).[68] Mass spectrometry, yeast two-hybrid and biochemical methods showed that CTCF interacts with itself by forming dimers and higher oligomers,[69,70] suggesting a mechanism by which CTCF could mediate long-range interactions. dCTCF was originally discovered as the insulator protein responsible for activity of the Fab-6 and Fab-8 insulator elements in Drosophila.[28,71] An 11 bp consensus sequence was identified as the binding motif of dCTCF and represents a subset of the human CTCF consensus. Full-length dCTCF (818 amino-acids) was shown to bind an instance of this motif (CAG GCG GCG C) in vitro with high-affinity.[71]

Finally, GAGA factor (GAF) was first identified as a key regulator of homeotic genes[72] that binds to the DNA consensus motif GA(CT) in the Ultrabithorax promoter,[73] later defined as GAGAGAG.[74] GAF was shown to possess an N-terminal BTB domain involved in oligomerization and a Cys2-His2-type Zn-finger that is essential for specific DNA binding.[75,76] The C-terminal, glutamine-rich domain of GAF is involved in transcriptional activation.[77] Recently, the role of GAF as an IBP has been questioned due to its low EB activity,[22] and was rather proposed to function as a 'de-silencer'[42] (see below).

Post-transcriptional modifications (e.g., phosphorylation, PAR-ylation and SUMO-ylation) are involved in the regulation of insulator protein activities and will be discussed in Section 6. Such modifications, the chromatin context and the presence of co-factors may in turn regulate or affect the DNA binding patterns of insulator proteins, as suggested by recent genome-wide studies showing that the binding sites occupied by insulator proteins are not solely defined by their consensus sequences.

IBPs are often necessary but not sufficient to ensure insulation activity at a specific locus, and several insulator co-factors have been shown to be additionally required. One example is the gypsy insulator, which in addition to Su(Hw) requires Mod(mdg4) [78,79] and Centrosomal Protein 190 (CP190),[80] a protein originally described for its ability to bind to the centrosome during mitosis.[81] Recent studies suggest that CP190 plays a crucial role in the insulation function of various IBPs (see below).

Genome-Wide Studies: Highlighting the Role of Insulators in Chromatin Organization

Genome-wide analysis of the binding of insulator proteins across eukaryotic genomes challenged classic insulator models by unveiling their distribution with respect to multiple annotations, and highlighting their important functions in situ.[35,38,39,53,65,66,71,82,83] In particular, these binding sites were found to be near the promoters of thousands of active genes and/or at the border of heterochromatin domains, supporting their functions as barrier or EB insulators.

Genome-wide studies in human, mouse and chicken cell types showed that CTCF binds to ~30,000 binding sites that are located in inter-genic regions (~50%) and often close to promoters (~20–30%).[65,66] In Drosophila, genome-wide studies in cultured cells and embryos showed that dCTCF, Su(Hw) and BEAF possess partially redundant localization patterns[35,38,39] that strongly correlate with regions of distinct transcriptional activity.[38,39,53,65,71] BEAF and dCTCF binding sites were found to be specifically enriched close to promoters, and to some extent to transcription start sites (TSS) and transcription end sites. The binding profiles of BEAF and dCTCF vary according to distinct cell types, suggesting a role in transcription, cell identity, development and regulation of specific gene ontologies including the cell cycle.[35,38,53] In contrast, Su(Hw) binding sites were found to be predominantly enriched in or near heterochromatic regions.[35,38,39,53,71] Recently, these distinct genomic distributions were used to define a division of Drosophila IBPs into two major classes depending on the enrichment of their binding sites close to promoters (Class I: BEAF, dCTCF) or in gene-poor regions (Class II: Su(Hw)).[39] Several BEAF and dCTCF binding sites have since been validated as true EB insulators using classical reporter gene assays.[22] However, other studies have also shown that binding sites for BEAF, dCTCF and CTCF are also found to be enriched at the borders of repressed domains,[37,82] suggesting that IBPs may function both as barrier and EB insulators, depending on additional co-factors and/or genomic environment.

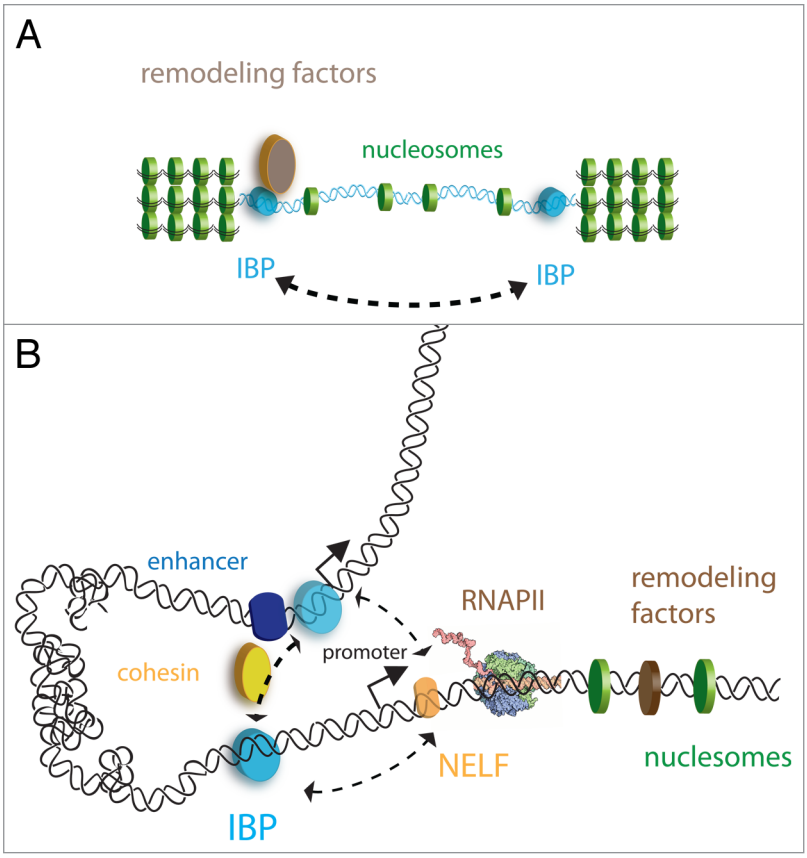

Figure 2. Enhancer-blocking activity and long-range interactions. (A) The barrier activity of insulators depend on local chromatin structure (nucleosomes, green disks) and on interactions of IBPs with chromatin remodeling factors (brown disk). (B) The enhancer blocking activity may depend on long-range interactions between insulators, mediated by insulator binding proteins (IBP, cyan cylinders). Alternatively, long-range interactions may occur between insulator sequences and RNAPII complexes (atomic model) that are paused upon NELF (orange circle) recruitment. These interactions may involve co-factors such as i.e., cohesin (yellow circle).

Initially, the molecular mechanism of chromatin insulation was tightly linked to chromatin accessibility which is generally coupled to gene activation (reviewed in ref. 84). The first insulator elements were identified based on the sensitivity to DNase I treatment of chromatin at insulator sites, which correspond to hypersensitive sites (HS).[16] Chromatin accessibility is not a specific property of insulators, but has been repetitively observed for the characterized insulators and more recently for BEAF, dCTCF and GAF binding sites that mark the most prominent nucleosome-free regions (NFR) genome-wide.[23,35,38,39,53,66,71] Chromatin opening is essential for desilencing but not sufficient for barrier activity, as suggested using reporter assays in yeast.[42] Such reporter systems could distinguish desilencing activities (that open chromatin to favor the expression of genes independently of directionality) from the barrier activity of IBPs that depends on long-range interactions with other distant cis-regulatory elements (and thereby of directionality; see section below). The full activity of insulator barriers may thus also depend on their interactions with distant elements, not solely on chromatin accessibility. Recent simulations of chromatin folding may suggest a role of histone depletion (and by extension chromatin remodeling) in facilitating the formation of these chromatin loops,[85] arguing for a function of de-silencing activities in regulating long-range interactions.

Experimentally, it was shown that barrier insulators may actually involve interactions with chromatin remodelers[20,34,66,82] in order to impede the propagation of repressive marks[34,36,86] but also to control de novo deposition of marks in the active region insulated (**Fig. 2A**). The identification of co-factors has been essential to better understand the molecular mechanisms of insulator barriers[31] (see section 6 for a discussion of insulator co-factors). In yeast, the Remodel the Structure of Chromatin (RSC) complex was shown to play a key role in the insulation activity of TFIIIC.[36] In Drosophila, it is interesting that dCTCF, BEAF, Su(Hw) and GAF all bind to putative insulator sites that are also bound by CP190,[38] an essential factor involved in nuclear organization and in restricting the spreading of the heterochromatin histone mark K27me3.[37] In vertebrates, the remodeling activity at the HS4 insulator of the chicken *β-globin* locus could be distinguished from the EB insulating activity of CTCF. In this case, barrier activity actually depends on USF (Upstream Stimulatory Factor), a factor that binds to a distinct region of HS4 to mediate the deposition of active histone modifications,[40] highlighting that barrier and EB functions could be uncoupled. In addition, HS4 also harbors a third region that is recognized by VEZF1 (Vascular Endothelial Zinc Finger 1) [87] which independently mediates protection from DNA methylation. Insulator barriers in vertebrates were thus proposed to play a role both in restricting DNA methylation and in recruiting histone modifiers, with functions being required to regulate the establishment of an epigenetically stable silent chromatin state.[40] Long-range communication among distant insulators may also be more efficient if the intervening sequences are 'silenced' as shown for the yeast HMR locus.[86] The genomic context of insulators may thus be important to consider for insulating activities as the final impact of an insulator will depend on the multiple interactions among other distant elements.[44]

Interestingly, the Levine's group[88] demonstrated that paused RNAPII, stalled downstream of a TSS, possesses enhancer blocking activity that is dependent on NELF, a key player of transcriptional pausing by RNAPII. Additionally, it was suggested that the mechanism behind this EB activity involves interactions between paused RNAPII complexes and IBPs, which will prevent the activation of RNAPII by proximal enhancers[88] (**Fig. 2B**). Furthermore, other studies showed that a significant fraction of GAF- and BEAF-associated genes interact with NELF.[53,89-91] Taken together, these data suggest that this mechanism may be widespread, and further raise the possibility that IBPs may be directly involved in the process of transcriptional pausing. Recent

work has suggested alternative strategies for the local organization of chromatin by transcription through the production of non coding RNAs (ncRNAs), involving the chromatin remodeling and elongation factors spt16 and FACT.[92] Intergenic ncRNAs might actually provide an interplay in the function of insulators (reviewed in ref. 93) and potential feedback loops between insulator-mediated RNAPII binding and the subsequent re-organization of chromatin should be addressed.

The common IBPs and mechanisms shared between chromatin barriers and EB insulators strongly argue that their activities depend on their interaction with a variety of co-factors including key regulators of chromatin structure and on their genomic contexts (distribution with respect to cis-regulatory elements including enhancers, promoters or repressive elements). It is worth noting that the resolution of Chip-chip and Chip-seq experiments is often of the order of ~100–1,000 bp. Thus, the resolution of a given experiment sets an upper limit for the maximum precision with which the relative localization of binding sites of IBPs and those of other factors/cis-regulatory elements can be established. Higher resolution genome-wide, and locus-specific studies will be needed in future to validate and further understand the global roles of IBPs.

Long-Range Interactions

A key feature of insulators is their ability to occasionally or temporarily form clusters. Recently, novel methods including 3C[48] and several variants have allowed for the first time to experimentally test whether insulator activity requires the formation of long-range DNA contacts.

One of the first proofs of the existence of insulator-mediated long-range contacts in Drosophila came from studies on the *scs* and *scs'* insulators. Co-immunoprecipitation studies and 3C experiments showed that *scs* and *scs'*, which are bound by Zw5 and BEAF, physically interact with each other at a distance of 15 kb.[94] Further characterization of such hetero-typic long-range interactions involving distinct IBPs may provide a better understanding of how insulators may in turn regulate gene expression, as recently shown.[44] Such long-range interactions among EB insulators can also lead to the enforcement of gene silencing, as illustrated by the Drosophila chromatin remodeling proteins of the Polycomb group (PcG). PcG proteins bind to polycomb response element (PRE) sequences and later recruit a further protein complex called PRC2 that is responsible for gene silencing of the hox cluster.[95] It was recently demonstrated that long-range trans interactions between two PcG-binding regulatory elements do not depend on PRE elements as previously thought but rather on the presence of insulators.[96]

In vertebrates, CTCF plays a major role at regulating inter- and intra-chromosomal long-range interactions. Indeed, Hi-C and genome-wide data showed that up to ~35% of the total identified long-range interactions carry CTCF binding sites.[51] Notably, CTCF binding sites are present at ~60% of the inter-chromosomal and ~20% of the intra-chromosomal long-range interactions. Several genetic regions involved in insulator-mediated long-range interactions by CTCF were studied in detail.

One of the best characterized regions is the chicken β-globin locus. Four genetic insulators are found at its 5'-end (5'HS4) whereas only one is found at its 3'-end (3'HS1). The binding of CTCF to 5'HS4 and 3'HS1 and its EB activity were shown to block transcription of the *β-globin* locus.[26,97] Recent data have shed light into the factors involved in CTCF-mediated long-range interactions. CTCF purification studies from HeLa cell nuclei resulted in co-purification of many sub-nuclear architectural proteins, with nucleophosmin being one of the main factors.[69]

The other region studied in detail for CTCF's EB activity is the imprinting control region (ICR) that is located between the *Igf2* (insulin like growth factor) gene and the *H19* locus. The ICR consists of four CTCF insulator binding sites whose occupation depends on their methylation state. For the maternal allele it was shown in embryonal tissue that the ICR is not methylated and CTCF binds to it. Under these conditions, CTCF blocks the long range interaction between the *Igf2* promoter and its enhancer region, and the *H19* gene locus is active. On the paternal allele, the ICR is methylated and CTCF binding to the ICR is blocked.[98] This allows enhancer-promoter communication and *Igf2* is expressed, whereas the expression of the H19 region is blocked by the binding of its repressor.[26] Downregulation of CTCF, Chip-chip-analysis and 3C-experiments further showed that the long-range interaction between ICR and the *Igf2* promoter depends on CTCF.[26,98] Finally, a modified 3C technique was used to reveal that mouse CTCF is also able to promote inter-chromosomal or trans interactions between the ICR from the *Igf2*/*H19* loci on chromosome 7 and *Wsb1*/*Nf1* on chromosome 11.[99] Interestingly, long-range interactions at the ICR were also shown to be affected by non-coding RNAs.[100] Further studies will be needed to unveil whether ncRNAs play a more general role at the regulation of long-range contacts among enhancer, promoters and insulators, and whether this regulation feedbacks into the production of such ncRNAs.[101]

Studies at several different loci revealed that CTCF interacts with cohesin in the regulation of long-range interactions and the expression of gene clusters. Cohesin is a complex formed by Smc1 and Smc3 heterodimer, the Rad21 (Mcd1, Scc1) kleisin protein family, and Stromalin (SA, Scc3),[102] that was first described for its key role in the structural organization of chromosomes during replication and is essential for sister chromatid cohesion after DNA replication in S-Phase.[103] More recently, cohesin was shown to be involved in gene regulation in yeast[24] and Drosophila[104] through the formation of long-range chromatin interactions.[105] Genome-wide studies in humans showed that ~71% of CTCF and cohesin binding sites overlap, with CTCF being required for the specific localization pattern of cohesin.[106] More detailed 3C studies of the apolipoprotein gene region (APO), which contains three CTCF- and three cohesin-enriched sites that result in the formation of two chromatin loops, showed that knockdown of either CTCF or cohesin leads to a disruption of long-range loops and to significant changes in APO expression.[107] Similarly, the depletion of cohesin causes an enhanced *Igf2* expression and a decrease in *H19* expression levels, suggesting that cohesin is part of the enhancer-blocking activity of CTCF.[108] More recently, in situ 3D-FISH experiments at the *Igh* locus showed

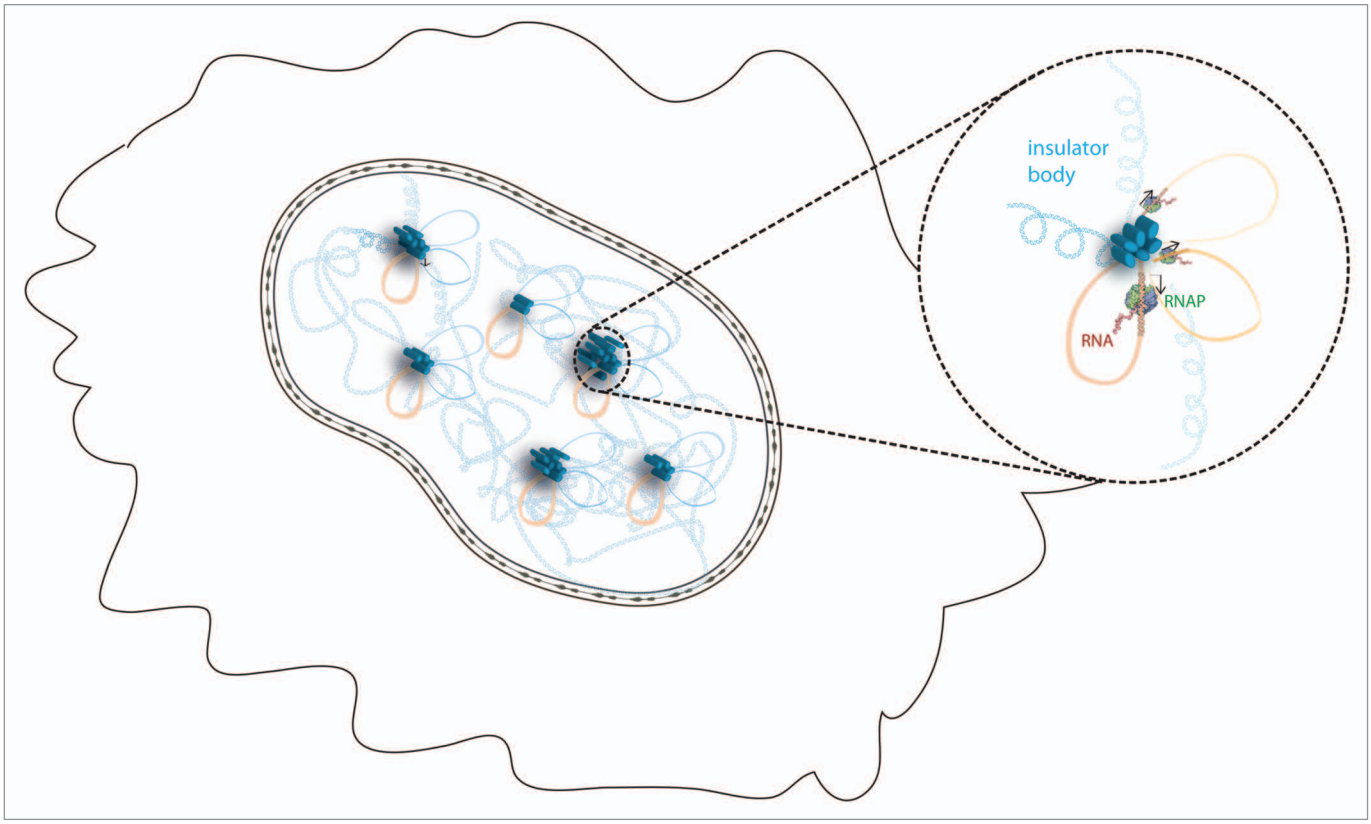

Figure 3. Insulator bodies. Several insulator bodies are assembled in the nucleus (solid line) of a cell (membrane as thin wiggly line). An insulator body (top right) consists of a clustering of chromatin loops held together through a network of interactions between IPBs and related factors and involve the formation of higher-order long-range interactions and the co-regulation of multiple genes. Different insulator bodies may regulate distinct gene ontologies, and they may thus vary in size (represented in figure by a different number of IBPs).

CTCF depletion leads to only minimal changes in long-range distances,[109] suggesting that CTCF is not the only factor responsible for the general architecture of the locus but is rather required to mediate and stabilize long-range interactions. Taken together, these data show that CTCF is a key factor in the organization of gene clusters through the formation of long-range DNA contacts that are essential for transcription.

Microscopy Studies of Insulator Proteins

In previous sections, we have shown evidence that: (1) IBPs bind genome-wide to thousands of sites in intergenic and promoter regions, (2) different IBPs may regulate the expression of different gene families, (3) IBPs are necessary for the formation of long-range interactions, and their disruption leads to perturbations in transcription. These observations could be explained by a model in which many insulators cluster together in spatially defined regions of the nucleus. These clusters have been named 'insulator bodies' and represent nuclear bodies in which co-regulated genes come together in spatially localized regions through the formation of long-range interactions (**Fig. 3**). Interestingly, insulator bodies may be spatially or functionally related to transcription factories, which have been experimentally defined as protein-rich foci where resources are shared to optimize the process of transcription,[3] although no such link has been experimentally proved.

Fluorescence microscopy provided the first support of the existence of insulator bodies. Images of fluorescently labeled Su(Hw) and dCTCF[41,43,110] show that IBPs gather in a small number of fluorescent clusters per cell (~20–50). In addition to Su(Hw) and dCTCF, CP190 was also shown to localize into nuclear bodies,[111] suggesting that CP190 could be a common regulator of distinct types of insulator clusters (characterized by each insulator protein subclass).

When considered together, the microscopy images reported in literature show a heterogeneous scenario in which insulator bodies greatly differ in size within single cells and number when comparing different individual cells and different cell types. This heterogeneity may actually reflect their functional role. First, different gene families could be regulated at each insulator body, resulting in clusters of different sizes and compositions that are IBP specific. Second, different insulators could regulate specific epigenetic programs in different cell types,[112] resulting in cell-type specific distributions of insulator cluster numbers and sizes. This possibility is tempting as it suggests that cells use insulators to maintain certain epigenetic programs throughout cell division and differentiate by regulating the assembly of distinct classes of insulator bodies. Third, insulator bodies need to be dynamic. At

short time scales (~ms to seconds), molecular processes, such as the structural dynamics of chromatin and higher-order contacts, the kinetics of association/dissociation typical of protein-protein and protein-DNA interactions, and most importantly the process of transcription will define time fluctuations in insulator body statistics. In fact, a recent paper by Akbari et al. showed by time-lapse fluorescence microscopy that CP190 insulator bodies are highly dynamic. On a longer time-scale (~hrs), the dynamic stability of insulator body numbers and sizes will be regulated by cellular factors that vary over the cell cycle and due to external conditions. Intriguingly, CP190 cycles between its inter-phasic binding sites in G_1, overlapping with Su(Hw), BEAF-32, GAF and dCTCF binding sites, and its binding to centrosomes in mitosis.[113] Similarly, the clustering of TFIIIC sites into bodies in inter-phasic chromatin[25] also cycles to bind to centromeres during mitosis,[114] involving potentially conserved mechanisms. It remains unclear to what extent the clustering of insulators depends on local remodeling activities, DNA methylation and/or histone modifications that might in turn favor long-range interactions.

Quantitative and higher resolution fluorescence microscopy methods will be needed to gain more information on the role of static and dynamic heterogeneity of insulator clusters and their role in insulator function and transcription regulation, as well as in the identification of factors associated to insulator bodies.

Structural Components of an Insulator

Although the molecular mechanisms involved in the formation of insulator clusters is still not fully understood, it is clear that in addition to IBPs many insulator co-factors will be required to (1) make the physical contacts required for the cohesion of insulators, (2) organize chromatin through higher-order long-range interactions and (3) anchor insulators to nuclear structures (such as the nuclear envelope).

The insulator proteins themselves are the first candidates for the protein-protein interactions needed for insulator body cohesion.[35,38,115] CTCF, BEAF and Su(Hw) contain zinc-finger binding domains that allow them to bind DNA specifically, as well as other domains (C-terminus for BEAF, N-terminus for CTCF) that have been implicated in their ability to self-oligomerize.[19] Self-interactions might be important, for instance as shown for BEAF whose ability to 'cluster'/bind cooperatively to its multiple binding sites is abolished upon deletion of its C-terminal domain. Characterization of the number of IBPs present in insulator bodies may thus be important to better understand their architecture and/or the minimal/maximal number of IBP molecules needed for DNA binding and/or for clustering. Steric hindrance among multiple chromatin-bound IBPs, or other mechanisms, could for example limit insulator clustering. It remains to be demonstrated whether IBPs can make loops between multiple (more than two) insulator binding sites. Current technologies, such as 3C/Hi-C, are only able to directly detect bi-partite interactions (two insulator binding sites only). HiC frequencies are often too small and thus predicted 3-way interactions drop into small, insignificant probabilities. Further methods will need to be developed and used to address this important question.

Insulator proteins, however, are not the only factor that could provide the protein-protein contacts necessary to hold insulator-mediated long-range interactions together. First, ChIP[37,38,43] and microscopy experiments[41,43,110] identified CP190 and Mod(mdg4) as additional cofactors possibly contributing to the cohesion of long-range contacts. Not only these proteins co-localize with dCTCF,[43,116] Su(Hw) [80] and BEAF[38] but are essential to the activity of insulators.[80,112,117] Moreover, these forms of regulation of insulator body assembly can be responsible for changes in the expression pattern or play some important role in development by maintaining specific epigenetic programs that induce cell differentiation.[112] It may be important to study the relative distribution of these factors throughout the cell cycle, as CP190 specifically associates with centrosomes at mitosis.

Second, cohesin could play a major role in providing the physical contacts required to maintain insulator-mediated long-range interactions. A body of evidence shows that CTCF interacts with cohesin.[106,108,118,119] This interaction involves the C-terminal domain of CTCF and the SA2 subunit of cohesin, which is proposed to bridge chromatin regions bound by CTCF and encircled by the other cohesin subunits.[120] CTCF mediates gene regulation not only by forming intra-chromosomal loops, but also by establishing and regulating specific contacts between different chromosomes,[99,121] whose specificity may require other factors in addition to cohesin.

Third, insulator proteins have been shown to associate to structural components of the nucleus. CTCF associates with the nuclear matrix[69,122] through direct interactions with nucleophosmin.[69] Further ChIP experiments showed that the presence of intact CTCF binding sites is required to recruit CTCF to the outer surface of the nucleolus.[69] In analogy to the CTCF-nucleophosmin interaction, Su(Hw) is able to induce the formation of chromatin loops by tethering DNA to the nuclear lamina. Fluorescence microscopy experiments have shown that Su(Hw) localizes preferentially to the nuclear periphery[41] while immunoprecipitation assays have unveiled a network of interactions between Su(Hw), Mod(mdg4) and dTopors, the Drosophila homolog of the Topoisomerase I binding arginine-serine rich (RS) protein, that tethers Su(Hw) to the nuclear membrane through direct interactions with lamin.[123] These findings suggest that insulator are tethered to the nuclear periphery by clusters of proteins held together by a vast network of interactions.[20]

The confinement of genetic material near the nuclear envelope is a shared feature between several insulators. Genome-wide DNA adenine methyltransferase identification (DamID) [124] on Drosophila[46] and human cell lines[45] showed how large portions of the two genomes are found to reside along the nuclear envelope. These lamina associated domains (LADs) are characterized by low gene-expression levels and display enriched binding of Su(Hw) and human CTCF at their borders. Other insulator proteins in Drosophila, however, are depleted within LAD regions,[45] consistent with their association with actively transcribed genes. It seems likely that in these cases the main function of Su(Hw) and CTCF is to prevent the spreading of chromatin repression to other regions of the genome. Taken together, the interactions of IBPs with the nuclear envelope[42] or with the lamina[45,46] may also

reflect the potential activation state of genes and/or their positions relative to chromosome territories. Upon activation, genes may often move away from the interior of chromosome territories, and the role of IBPs and co-factors remains to be determined in this context as IBPs bind mostly to inter-genic regions.

Post-transcriptional modifications. Insulator proteins often display post-translational modifications (PTMs) that change during the cell cycle and that can modulate their functions by directly affecting their interactions with co-factors and their DNA binding properties. For instance, in the gypsy insulator, SUMOylation (Small-Ubiquitin-like Modifier) of Mod(mdg4) and the regulatory protein CP190 are known to interfere with the barrier activity of Su(Hw) but not to influence its DNA-binding capacity.[125] CTCF display a large variety of post-transcriptional modifications: it is PAR(Poly-ADP-Ribose)-ylated and SUMOylated at its N-terminus and phosphorylated at its C-terminus. Similary to what was observed at the gypsy insulator, PAR-ylation of CTCF was shown to be involved in a positive regulation of insulator activity but not to influence its DNA-binding properties.[115] These examples suggest that post-translational modifications are involved in the regulation of protein-protein interactions that mediate long-range interactions.[126]

PTM have been also implicated in the regulation of the binding pattern of insulator proteins. The most notable example is that of CTCF, in which PTMs induce different conformational changes in the protein that lead to different combinations of zinc-fingers being used to bind distinct consensus sequences.[68,127]

Finally, other PTMs have been described that affect the function of IBPs by an unknown mechanism. CTCF phosphorylation and SUMO-ylation are involved in its repressive transcriptional function,[127,128] whereas phosphorylation of BEAF is involved in its association to the nuclear matrix.[55]

Discussion

Future technological advances and their impact in the chromatin field. Recent advances in the chromatin field have been made possible through the combination of high-throughput sequencing used in combination with previous technological breakthroughs including ChIP and/or 3C and derivative technologies.[48] However, current genome-wide methods have two inherent caveats. First, these methods require large sample sizes to increase precision by averaging, which works well for homogeneous populations. Thus, special care must be taken in the interpretation of results that may be derived from heterogeneous samples, as in this case averaging will not increase precision but rather decrease accuracy: for example a sample with two cell populations, one with a strong and the other with a weak protein binding peak at a specific locus, cannot be discerned from a single homogeneous population with a moderate peak. Second, fast dynamic processes are blurred: for instance, the binding 'peaks' of a given factor involved in a rapid process might only be apparent at the limited number of loci where this process is slow. Novel methodologies, such as single-cell genomics will certainly play an important role in the future, as they may allow for the genome-wide detection of protein binding sites, protein-protein interactions, and long-range contacts while not only avoiding sample heterogeneity, but rather providing a full measure of the degree of heterogeneity in a cell population. In particular, important advances in gene sequencing and measurement of protein levels in single-cells have already been achieved,[129] and further extensions to Hi-C and ChIP technologies may be around the corner.

Fluorescence microscopy-based methods naturally avoid heterogeneous sampling and permit the observation of dynamical events. These methods have, currently, several limitations which considerably weaken this advantage. First, light diffraction limits the maximum resolving power of conventional microscopes to ~200–300 nm, making it impossible to determine the inner architecture of nuclear structures smaller than this resolution limit (e.g., replication/transcription factories or PcG bodies), or to determine whether different factors spatially co-localize at distances shorter than this limit. For instance, a recent publication clearly demonstrates that only at higher spatial resolutions can double layer invaginations of the nuclear membrane be detected and different components of the nuclear pore complex be differentially localized:[130] proteins that in conventional images, due to the low resolution, seem to be bound to the nuclear envelope are in reality separated by almost 150 nm. Recent advances in fluorescence microscopy will surely drive important developments in the chromatin field. On the one hand, several methods referred to as super-resolution microscopies, have allowed for resolutions as high as 20 nm to be achieved.[131] These methods will be important to determine the internal architecture of diffraction-limited nuclear structures (such as bodies), to unveil the spatial interrelationship between different nuclear structural components, and to more accurately pinpoint interactions between cofactors. On the other hand, fluorescence fluctuation methods, such as fluorescence cross-correlation spectroscopy, allow for the direct quantification of interactions between factors, a measure that is impossible by co-localization methods.

Second, current microscopy methods are limited in their ability to detect fast dynamics. Many of the factors involved in chromatin organization and transcription regulation rapidly fluctuate between a low mobility state (when they are associated to DNA or nuclear structures) and a fast diffusing, unbound state. Current microscopy methods are not well suited for these kind of studies (as signals from both fractions are mixed and can lead to artifactual interpretations) but current and future developments in single-particle tracking methods using photo-activable markers[132] may provide the means of exploring the dynamics of chromatin-associated enzymes during their different activities. Finally, further advances in the development of automated microscopy methods[133] will allow for higher throughput, more reliability through better statistics and standardization, and improved precision through the development of novel image treatment methods.

Roles of chromatin insulators in cell cycle and development. Classical models have postulated that insulators work either as heterochromatin barriers or enhancer blockers. However, recent studies have shown that these definitions may be promiscuous: barrier activities often necessitate long-range interactions and enhancer blocking may require chromatin remodeling functions.

In addition, insulator binding proteins seem to be able to confer both activities depending on the chromatin context and interaction partners, and to couple chromatin organization at the level of nucleosomes with higher-order chromatin organization. Ultimately, it is clear that further mechanistic insights into the roles played by IBPs and insulators in transcription regulation and chromatin architecture will go hand in hand with new developments in the transcription field.

In addition to their role in transcription regulation, insulators may be important in remodeling chromosome architecture during stages of the cell cycle other than interphase. In addition to being key factors regulating the activity of insulators in interphase, IBPs and cohesin are present during S phase and mitosis.[4,102,118] Recent work has actually shown that long-range interactions in S phase requires cohesin to cluster the DNA replication origins within factories.[134] Given the link between cohesin binding and CTCF, this result further raises the possibility that IBPs might play a role in subsequent cell cycle phases. In addition to the well known binding pattern of cohesin to centromere regions,[135] CTCF has also been found at these sites during metaphase,[118] prompting a possible role of IBPs during chromosome organization during cell division. Biochemical and genetic identification of the distinct factors interacting with IBPs, as well as their binding patterns at different cell cycle stages will be key to clarify the possible global roles of IBPs in chromosome organization.

The gene activation and repression patterns induced by insulators might survive mitosis to maintain the epigenetic programs from one cell generation to the next but may also depend on development stage.[136] By the interaction with cofactors like cohesin, insulator binding proteins are the first layer in creating inter- or intrachromosomal long range connections that influence gene expression.[106,108] Interestingly, transcript levels of dCTCF and BEAF show large variations during development in Drosophila[137] (see also www.fymine.org). dCTCF and BEAF are upregulated during early embryonic stages, and downregulated in the larval state. Changes in the transcription levels of insulator proteins during different developmental stages could reflect their involvement in eventually regulating important developmental programs, as suggested by Bushey et al.[38] Future experiments, investigating both the binding patterns and networks of interaction partners of IBPs as well as their ability to organize chromatin by clustering, at different stages of development will be necessary to fully understand the role of insulator bodies in the maintenance of cell identity and differentiation.

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