Cell. Mol. Life Sci. (2014) 71:4089–4113
DOI 10.1007/s00018-014-1672-6

Cellular and Molecular Life Sciences

REVIEW

Expanding the roles of chromatin insulators in nuclear architecture, chromatin organization and genome function

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Received: 31 January 2014 / Revised: 31 May 2014 / Accepted: 23 June 2014 / Published online: 11 July 2014

Abstract Of the numerous classes of elements involved in modulating eukaryotic chromosome structure and function, chromatin insulators arguably remain the most poorly understood in their contribution to these processes in vivo. Indeed, our view of chromatin insulators has evolved dramatically since their chromatin boundary and enhancer blocking properties were elucidated roughly a quarter of a century ago as a result of recent genome-wide, high-throughput methods better suited to probing the role of these elements in their native genomic contexts. The overall theme that has emerged from these studies is that chromatin insulators function as general facilitators of higher-order chromatin loop structures that exert both physical and functional constraints on the genome. In this review, we summarize the result of recent work that supports this idea as well as a number of other studies linking these elements to a diverse array of nuclear processes, suggesting that chromatin insulators exert master control over genome organization and behavior.

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Keywords Chromatin insulators · Insulator evolution · Architectural proteins · Genome organization · Chromatin looping · Gene transcription

Abbreviations

NAPs	Nucleoid associated proteins
BTB	Bric-a-Brac/Tramtrack/Broad Complex
CTCF	CCCTC-binding factor protein
BEAF-32	Boundary element-associated factor of 32kD
Su(Hw)	Suppressor of Hairy wing
CP190	Centrosomal Protein 190kD
Mod(mdg4)67.2	Modifier of mdg4
GAF	GAGA Factor
Dwg/Zw5	Deformed wings
HSV-1	Herpes simplex virus 1
EBV	Epstein–Barr virus
KSHV	Kaposi's sarcoma-associated herpesvirus
SINE	Short Interspersed Element
AS1/AS2	Asymmetric leaves 1 and 2
L(3)mbt	Lethal(3) malignant brain tumor
TADs	Topologically associating domains
ESCs	Embryonic stem cells
NPCs	Neural progenitor cells
LCR	Locus control regions
PRE	Polycomb Response Element
eve	Even skipped
ORC	Origin recognition complex proteins
pre-RC	Pre-replication complex
SBS	Su(Hw) binding site
PARylation	Poly (ADP-ribosyl)ation
PARP	Poly (ADP-ribose) polymerase
SUMO	Small ubiquitin-like modifier

Introduction

Much of what we understand about chromosomes, their dynamic behavior throughout the cell cycle and role in heredity can be traced back to the meticulous work of the German anatomist Walther Flemming in the late 1800s. Based on his observation of tissue stained with basophilic dyes from the fire salamander, *Salamandra salamandra*, he first described the pattern of thread-like filaments that he termed chromatin, or 'stainable material' [1]. At its simplest biochemical definition, chromatin consists of an assembly of DNA and associated proteins that form the basic structural component of chromosomes, exerting both structural and functional influences on the genome. Chromatin has traditionally been regarded as a eukaryotic specialty, thought to have evolved from the need to satisfy the structural constraints required to package large and complex genomes within the reduced three-dimensional space of the nucleus and ensure proper chromosome segregation during cell division. However, bacterial and archaea genomes are also packaged into chromatin-like structures by the action of DNA-associated proteins. Both archaea and eukaryotes possess histone proteins that assemble into octamers and wrap DNA to form nucleosomes, an organization that is further refined into higher-order structures by the action of other DNA and chromatin-associated proteins [2–4]. Bacteria and mitochondria, either having lost or lacking homologs to many of these proteins, utilize the action of nucleoid associated proteins (NAPs) to promote DNA bending, compaction and looping to package their genome into higher-order chromatin structures in a manner analogous to their eukaryotic counterparts [5–7].

The existence of a similar means by which DNA molecules are packaged into higher-order chromatin-like structures across the three domains of life suggests an ancient and critical role for this process in modulating genome behavior, organization and fidelity during cell division. This is attributed to the dual nature of chromatin itself—the topological constraints imposed by higher-order structures required for packaging can directly impart significant functional influences upon the genome as well. These include processes that control the nuclear distribution of chromosomes into distinct territories during interphase, chromatin compaction during mitosis, DNA replication, DNA repair and gene expression [8–12]. Transcriptional regulation, for example, occurs at multiple levels that include recruitment of transcription factors and RNA Pol II, controlling enhancer–promoter communication and influencing both splicing and termination events. This is achieved via nucleosome position, density and posttranslational histone modifications coupled to long-range physical looping contacts that act to coordinate these events within the proper nuclear compartments. As a result, chromatin can exert master control over the expression of thousands of genes in a cell type-specific manner (reviewed in [13–17]).

However, our understanding of how chromatin achieves such control over genome behavior within the context of the nucleus remains incomplete. This is primarily due to the fact that the in vivo function of many of the chromatin elements involved in coordinating these processes remains poorly defined. Chromatin insulators represent one class of elements whose in vivo functions are only beginning to be elucidated. These were first identified in *Drosophila melanogaster* as distinct DNA sequences that could buffer the negative influence of repressive chromatin on gene expression and restrict enhancer–promoter communication in a directional manner in transgenic reporter assays [18–21]. Both properties are conferred by a number of proteins, some of which recognize specific DNA motifs within the insulator sequence by virtue of multiple zinc finger domains. Others do not bind to DNA directly and instead possess well-defined protein interacting domains (such as the Bric-a-Brac/Tramtrack/Broad Complex (BTB) family) that allow them to interact with other insulator proteins. All insulators identified to date in *Drosophila* require the action of these multi-protein complexes, which is thought to be true of the less well-characterized mammalian and yeast insulators as well.

Chromatin insulators have been identified in a handful of model and non-model organisms, yet our thinking of how these elements function in vivo has been shaped, and in some cases dominated, by extensive genetic characterization using transgenic phenotypic reporter assays in *Drosophila melanogaster*. Although the contributions from these studies have been absolutely critical in elucidating many aspects of chromatin insulator behavior, recent high-throughput biochemical-based assays more suited to analyzing their behavior within their defined genomic context has revealed a complex insulator landscape in vivo whose functional role(s) and impact on genome behavior are only beginning to be understood. ChIP-Seq studies have revealed thousands of individual sites bound by insulator proteins scattered throughout eukaryotic genomes that in the case of *Drosophila* display a high degree of combinatorial binding. This is further augmented by an increased enrichment in looping contacts between these sites in both flies and mammals as revealed by chromosome conformation capture techniques such as Hi-C [22–29].

In a practical sense, these studies have revealed that our traditional view of chromatin insulators as enhancer blockers and boundary elements that establish independent domains of gene expression is too narrow, failing to account for a number of recent observations. First, some insulator protein binding sites in *D. melanogaster* cannot support either of these properties [30–32], while evidence

from humans suggests that not all insulator sites located between gene-regulatory enhancers and promoters disrupt communication between these elements [33]. Additionally, chromatin insulators might also facilitate enhancer–promoter communication or interact with promoters directly to stimulate gene expression [34–36]. These recent findings, which appear to contradict those observed in transgenic assays, leads to an important question: Does a unifying mechanism of insulator function exist that can account for all in vivo and in vitro observations?

Indeed, the global emerging view is that chromatin insulators facilitate the organization of the chromatin fiber into higher-order looping structures across multiple spatial scales by mediating long-range contacts between distant genomic sites. As a result, these elements exert both topological/physical and functional constraints on the genome. This process is dependent upon the ability of insulator proteins to interact with one another while remaining physically bound to their cognate insulator DNA sequence, hence leading to the formation of chromatin loop structures. These loops serve architectural roles, dictating the organization of the chromatin fiber within the nucleus. Furthermore, depending on where insulators are located with respect to each other and to gene-regulatory elements, these loops can also facilitate, block, or exert no significant influence on enhancer–promoter communication. By design, this means that 'insulation' is a general property derived from the higher-order organization of the chromatin fiber within the nucleus, not restricted solely to the activity of chromatin insulators per se. Nonetheless, the ability of chromatin insulators to mediate these higher-order structures can account for both original properties observed in transgenic assays as well as their in vivo behavior, and as a result the formation of chromatin loops has become the predominant model for how these elements function mechanistically in vivo (Fig. 1).

Although the canonical view of insulator function involves mediating chromatin loops in space, recent studies have also suggested the possibility of a number of non-canonical roles for these elements that may or may not be linked to their chromatin looping behavior. These include involvement in the stress response, modulating the behavior of Polycomb elements and a more direct role in transcriptional regulation, in addition to high-throughput data suggesting a potential link with DNA replication, repair and mRNA stability. In this review, we summarize the results of recent work that has lead to the need to expand our view of the nuclear function of these elements and their contribution to chromosome organization, spatial positioning within the three-dimensional space of the nucleus and overall genome control. We focus specifically on their evolutionary history, basic themes that have emerged from studies devoted to analyzing these elements within their native genomic contexts, explore potential non-canonical roles for these elements in other aspects of nuclear biology and how their activity is regulated to direct specific outcomes.

Chromatin insulators: origin, distribution and function in eukaryotic genomes

DNA sequences that confer one or both of the classic chromatin insulator properties identified from transgenic assays in *Drosophila melanogaster* have been found in yeast, nematodes, plants, sea urchins and most vertebrates, either by direct characterization of these elements or using computational motif analysis [37–48]. In other eukaryotes,

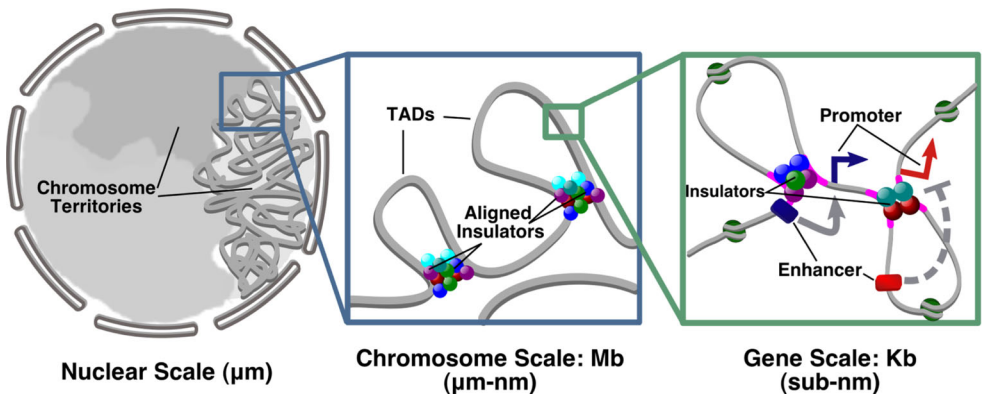

Fig. 1 Chromatin insulators contribute to nuclear and genome organization by mediating long-range looping contacts across multiple spatial scales. At the nuclear scale (μm), interphase chromosomes are organized into discrete nuclear territories. Aligned insulators, bound by nearly all known insulator proteins (*colored spheres*), preferentially interact with one another to establish topologically associating domains (TADs) at the chromosome scale (Megabase, Mb) (*blue inset, middle panel*). These domains are then further refined by the action of other classes of insulators at the gene scale (kilobase, kb) to direct specific gene-regulatory outcomes, both facilitating and disrupting enhancer–promoter communication depending on the genomic context (*green inset, right panel*)

insulator DNA sequences have not been identified or characterized directly, but nonetheless possess orthologs of CTCF and are thus expected to possess chromatin insulators as well. In *Drosophila*, there are thousands of endogenous insulator sites distributed throughout the genome that bind different combinations of insulator proteins, while mammalian genomes with larger *C* values possess tens of thousands of binding sites for the CCCTC-binding factor protein (CTCF) [23, 28, 49–52]. These simple findings alone suggest that insulators likely played a key role in shaping genome organization and behavior during the radiation of the eukaryotic lineage. Here, we discuss their evolutionary history and summarize recent work that supports a role for chromatin insulators as key drivers in genome evolution.

Role of chromatin insulators in shaping genome organization and behavior

Genome organization is tightly coupled to its functional behavior. This is best exemplified by the placement of gene-regulatory elements, such as enhancers, with respect to their cognate gene promoters. In higher eukaryotes, enhancers can be located many kilobases from their target promoters, and often interspersed by other gene loci of unrelated function. The promiscuous nature of enhancers dictates that other regulatory mechanisms exist to prevent misexpression of non-target genes. This is less of a concern in prokaryotes, in which most genes are organized into operons—clusters of genes involved in a specific biochemical pathway that are under the collective control of a single regulatory element located near promoters.

However, some eukaryotes, such as certain species of nematodes and plants, also organize parts of their genome into operons [53, 54]. Interestingly, these genomes also lack all of the canonical insulator proteins found in *D. melanogaster* and vertebrates, such as CTCF. The most striking example is found within the nematode clade, in which CTCF has been lost in a subset of species (such as *Caenorhabditis elegans*), yet retained in others [48]. In nematodes possessing CTCF, operons have not yet been identified. On the other hand, the *C. elegans* genome organizes a fraction of its genes (>17 %) in operons that utilize a single regulatory element/promoter to control multiple genes of related function [55]. This difference in genome organization might reflect the need to compensate for the loss of CTCF to establish and maintain the correct regulatory circuitry that would otherwise be under CTCF control. A similar situation might also be true for some species of plants that possess clusters of operon-like genes involved in secondary metabolite biosynthesis [54]. Finally, recent computational work has revealed that CTCF and its binding sites are restricted to bilaterian phyla with significant conservation between binding sites located in Hox clusters across both vertebrates and invertebrates, indicating that the origination of CTCF may have been responsible for the emergence of the bilaterian body plan as a result of its influence on Hox cluster organization [47].

The influence of insulator binding sites on genome organization and function is not limited to CTCF, however. Binding sites for the *Drosophila melanogaster* insulator protein, boundary element-associated factor of 32kD (BEAF-32) are unique to the *Drosophila* clade and show a strong enrichment within promoters and 5′ UTRs of distinct classes of genes located in a head-to-head fashion along the chromosome, a pattern not observed in other closely related insect species such as the mosquito [56, 57]. Furthermore, the gain or loss of insulator binding sites observed in different *Drosophila* species correlate with changes in genome organization that could possibly lead to alterations in complex traits [58]. These data suggest that the emergence of new insulator binding sites can have a significant impact on the genome, establishing or rewiring existing gene-regulatory circuits that lead to diverse phenotypic outcomes.

Taken collectively, the correlation between clusters of operon-like genes and the absence of CTCF in certain eukaryotes, coupled with the influence that species-specific insulator proteins may exert on genome architecture, suggests that these sequences and their cognate proteins might be a key driver in genome evolution. Whether such genome organization principles might reveal clues as to the presence, distribution and influence of insulator sequences in less well-studied eukaryotic genomes remains to be seen, but nonetheless might provide an intriguing method for predicting the presence or absence of insulators in silico.

Origins and distribution of chromatin insulators

An important component of insulator evolution that is often overlooked involves their origins and how they became distributed throughout the genome to exert their organizational and functional influences. Some have proposed that insulator sequences evolved from other gene-regulatory elements, such as promoters, a hypothesis put forth given their similar biochemical signatures (low nucleosome density, overlap with other transcription factors and flanking histone modifications) [59]. Many binding sites for BEAF-32, CP190 and dCTCF (but less so for Su(Hw) or Mod(mdg4)67.2) map within or near promoters in *D. melanogaster*, suggesting a potential shared function between some, but not all classes of insulators and gene promoters [23, 28]. This behavior is also observed for human CTCF, where its binding sites are enriched at a

number of promoters that overlap with other transcription factor binding sites [60, 61]. Additionally, tDNA promoters in both yeast and humans can function as barriers to the spread of repressive chromatin [62–64], while recent evidence suggests insulators can interact directly with promoters to facilitate transcription [33, 36, 65].

An alternative explanation, supported by an increasing amount of computational evidence, suggests that at least some insulator sequences may have a viral origin and/or spread to new genomic locations as a result of mobile DNA transposition. Such a mechanism has also been suggested to account for the expansion of transcription factor binding sites in eukaryotes [66–69]. A number of *Drosophila* retroviruses and retrotransposons, such as *gypsy*, *Idefix* and *ZAM*, harbor insulator sequences in their LTRs [70, 71], while dCTCF contributes to *R1* and *R2* retrotransposon-dependent silencing of ribosomal genes by binding directly to sequences within these mobile elements [72]. Additionally, a number of plant matrix attachment regions (MARS) that possess insulator activity are also derived from plant-specific retroelements [41, 73, 74]. Mammalian SINE (Short Interspersed Element) transposons also possess insulator activity and contain binding sites for both CTCF and TFIIIC, the RNA Pol III general transcription factor responsible for imparting insulator activity to yeast and human tDNA promoters [62–64, 75–77]. Finally, herpes simplex virus 1 (HSV-1), Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) all contain CTCF-binding motifs which play a key role in regulating the latency state during viral infection [78–83]. Taken collectively, these data suggest a relationship between mobile DNA and chromatin insulators that could potentially account for the horizontal transfer of insulator sequences and the proteins that bind to them into new genomes, as well as their subsequent radiation to new sites following colonization.

Indeed, highly conserved CTCF-binding sites flanked by degenerate transposon-derived repeat sequences were identified at numerous sites throughout mammalian genomes, indicative of ancient insertion events that occurred prior to divergence of the mammalian clade [69]. Also, lineage-specific expansion of unique CTCF-binding sites are associated with SINEs, suggesting that mobile elements have played a significant role in distributing insulator sequences throughout the mammalian genome [69]. It will be extremely important for future computational work to take advantage of "molecular paleontology" methods to determine whether mobile DNA played a significant role in shaping the complex insulator landscape in *Drosophila* and other eukaryotes, with the nematode genomes in particular providing a powerful means of comparison based on the known presence and absence of CTCF in different species of this clade [48, 69].

Phylogenetic distribution of chromatin insulator proteins

Surprisingly, although the pervasive presence of chromatin insulators in eukaryotic genomes suggests a critical role in genome organization and behavior, the majority of insulator proteins responsible for recognizing insulator sequences and conferring insulator activity do not appear to be broadly conserved among these taxa. Of the seven primary *Drosophila* proteins that significantly contribute to insulator function, including Suppressor of Hairy wing (Su(Hw)), Centrosomal Protein 190kD (CP190), Modifier of mdg4 (Mod(mdg4)67.2), GAGA Factor (GAF), Deformed wings (Dwg/Zw5) and the aforementioned dCTCF and BEAF-32 proteins, only dCTCF and GAGA Factor (c-Krox/Th-POK) are found outside of arthropods and remain the only mammalian insulator proteins identified to date [46, 47, 84, 85]. However, the amino acid sequences are poorly conserved, with only the central cluster of DNA-binding zinc fingers in dCTCF and the BTB domain (in addition to a single zinc finger domain) of GAGA Factor displaying mild sequence conservation between flies and mammals. Furthermore, two of these proteins (BEAF-32 and Dwg/Zw5) appear to be specific to the *Drosophila* lineage [57, 86]. None of these proteins are found in yeast or plants, and only a handful of nematodes possess CTCF [47, 48]. TFIIIC is perhaps the most highly conserved insulator protein in eukaryotes, although this is most likely the result of the critical role it plays in recruiting RNA Pol III for proper transcription of tDNA genes. Only two plant-specific DNA-binding proteins have been suggested to regulate *KNOX* target gene expression in a manner analogous to the enhancer blocking property of insulators, asymmetric leaves 1 and 2 (AS1/AS2) [40]. However, the *gypsy* insulator from *Drosophila* can function in a heterologous manner as a boundary element to facilitate transgene expression in *Arabidopsis thaliana* when *su(Hw)* is present [87]. This finding is somewhat unusual, given the apparent absence of two other proteins required for full *gypsy* insulator activity, Mod(mdg4)67.2 and CP190 [88, 89]. Since these two proteins are not conserved in plants, it remains to be seen whether proteins exist in *Arabidopsis* that are functionally analogous to their *Drosophila* counterparts to support Su(Hw)-dependent insulator function.

Nonetheless, it is obvious that our knowledge of the proteins possessing or contributing to insulator function remains largely incomplete, even within well-studied model organisms such as yeast and *Drosophila.* A growing number of partners have been shown to interact genetically by altering insulator behavior, found associated with insulator proteins at a handful of sites across the genome as a result of ChIP-Seq or have been identified after large-

scale protein interaction mapping studies [90–93]. In *Drosophila*, mutations in *Ey(2)* (*Sus1* in yeast), a highly conserved component of the SAGA chromatin remodeling/histone acetyltransferase complex, alter the boundary activity of *gypsy*, but not its enhancer blocking ability [94]. *dTopors*, a ubiquitin ligase, *Top2*, the fly homolog of Topoisomerase II and a number of RNAi machinery genes (the *Rm62* RNA helicase, *aubergine* (*aub*) and *piwi*) also show genetic interactions with *mod*(*mdg4*)*67.2* and *su*(*Hw*) that result in altered *gypsy* enhancer blocking capability [95–97]. Additionally, a growing number of potential partners have been identified by ChIP-Seq, including components of the exosome, responsible for mRNA decay [99]; Myc, an oncogenic transcriptional activator [101]; lethal(3) malignant brain tumor (L(3)mbt), a chromodomain-containing tumor suppressor protein [93]; SAGA, a histone acetyltransferase [100]; Brahma, a chromatin remodeler [100]; and certain isoforms of the double bromodomain protein Fs(1)h (the *Drosophila* ortholog of vertebrate Brd4) [98]. However, little functional data exist outside of these correlative findings. A common thread among most of these components identified by ChIP is that they colocalize at some, but not all insulator sites, in addition to being found at additional sites not bound by insulator proteins, suggesting that these factors impart a specific functionality upon the insulator sequences they associate with. Finally, a new insulator complex termed Elba was recently identified in *Drosophila*, which consists of three proteins of previously unknown function: Elba1, Elba2 and Elba3 [102]. These proteins cooperate as a trimer to bind DNA and differ from other known insulator proteins in that their expression and DNA-binding behavior are developmentally regulated, adding yet another layer of complexity to the function of these elements in vivo.

In other eukaryotes, particularly mammals, the cohesin complex has been shown to not only interact directly with CTCF, but also to be required for its function as an insulator [103–106]. This is thought to derive from the ability of the cohesin ring complex to stabilize CTCF-mediated loops in *cis* in a manner analogous to their role in holding sister chromatids together until their segregation during M-phase [24, 107, 108]. However, cohesin does not appear to be generally required for CTCF function, as cohesin-independent CTCF sites also exist, although whether these sites function as insulators by mediating long-range contacts has yet to be rigorously tested [109]. Interestingly, the CTCF/cohesin connection is not conserved in *Drosophila*, suggesting that this interaction was either lost in flies or appeared prior to diversification of the vertebrate lineage [110].

In addition to cohesin, however, only a few other vertebrate proteins have been identified that may contribute to insulator function through cooperation with CTCF. Prdm5, a SET domain-containing histone methyltransferase is enriched at CTCF-binding sites in mouse embryonic stem cells and physically interacts with CTCF, cohesin and TFIIIC [111]. Furthermore, work from the Human ENCODE project identified significant overlap between CTCF and the vertebrate-specific transcription factors ZNF143 and SIX5, which interestingly was only observed in one of the cell types tested (GM12878) [112]. However, like most novel associations identified via ChIP-Seq, the functional consequences of these associations remain unknown—whether they influence the insulator function of CTCF or its ability to function as a traditional transcription factor will require additional investigation.

Finally, a new insulator protein (COMPASS-like) specific to the ambulacraria clade (echinoderms and hemichordates), identified in the sea urchin *Paracentrotus lividus*, was shown to be responsible for the enhancer blocking property of the *sns5* insulator located in the early histone locus [44, 113]. Furthermore, an urchin-specific homolog of the Imitation SWI (ISWI) chromatin remodeling complex protein contributes to the insulator function of the *arylsulfatase* (ArsI) insulator from *Hemicentrotus pulcherrimus* [114, 115]. Interestingly, ISWI has been shown to bind directly and modulate the behavior of the Fab7 and SF1 insulators in *D. melanogaster* as well [116], while ArsI can function in a heterologous manner in both plants and mammals to protect against transgene-silencing position effects [117–120], a property also observed for the *Drosophila* gypsy insulator in *Arabidopsis* [87].

These findings highlight an important facet of insulator biology. It is obvious that our understanding of how these elements function in vivo is limited by the fact that they have been understudied outside of *D. melanogaster*. This is due, in part, to the lack of conservation of many of these proteins outside of CTCF and the lineage specificity for others (BEAF-32, Zw5). Therefore, it is tempting to conclude that there are many other lineage-specific proteins that either contribute to the insulator function of CTCF in these species, or function as novel insulator complexes themselves. The future identification of new proteins possessing or contributing insulator properties, many of which are likely to be lineage or species-specific, will most certainly continue as the field progresses.

Taken collectively, the data presented above leads to a number of important considerations regarding the evolutionary pressures operating on these elements and their impact on genome organization and behavior as a result. First, the presence of insulator sequences in both single- and multicellular eukaryotes coupled with the lineage specificity of many insulator proteins, with the exception of CTCF, suggests that selection has primarily targeted insulator *function*, rather than amino acid conservation of a core set of insulator proteins. We favor this idea for the

simple reason that such a mechanism would allow for all eukaryotic organisms to share a common architectural organization of their genomes involving long-range looping contacts at the megabase level. This would be the most efficient means by which to package their genomes, thus satisfying the topological and physical constraints imparted by the small volume of the nucleus. This basic organization, likely orchestrated by the broadly conserved CTCF, would then be further refined at the kilobase level by the action of species-specific insulator proteins having undergone rapid selection once arising within a particular lineage. The functional consequences resulting from the action of these lineage-specific insulator proteins might then be responsible for, or at least contribute to, the complex phenotypes and differential traits observed between species. This reduced stringency of selection, targeting the ability to form long-range contacts while allowing for the diversification of proteins that can modulate this behavior, ensures that genomes contain sufficient plasticity to generate a myriad of phenotypic outcomes while sharing a conserved means by which to package themselves in the nucleus. This idea is supported by a number of recent high-throughput studies showing a similar topological organization of yeast, fruit fly and human genomes, which we discuss in the next section.

Chromatin insulators and higher-order chromatin structure

The original hypothesis of chromatin insulators as generators of higher-order loop structures was derived from the need to provide a unifying mechanism to explain how both properties observed in transgenic assays—directional enhancer blocking and barrier/boundary activity—might be achieved within the nucleus [121]. Insulator bodies were originally thought to be the physical manifestations of such chromatin looping, although recent evidence strongly suggests this is not the case [89, 122–125]. The first indirect evidence for chromatin loop formation came from analysis of transgenes carrying two *gypsy* insulators between an enhancer and a promoter, which nullified the enhancer blocking effect observed with either a single intervening insulator or a promoter flanked by insulators [126, 127]. These studies suggested that such "insulator bypass" was the topological consequence of the two intervening insulators interacting with one another to form a chromatin loop that would then bring the upstream enhancer in close proximity to the downstream promoter to allow for transgene activation. Development of biochemical methods to measure contacts between genomic sites allowed for direct testing of this hypothesis [128], and it was later shown that the *Drosophila scs* and *scs'* insulators located at the 87A7 heat shock locus could physically interact with one another to generate a chromatin loop, a process thought to be dependent on the ability of BEAF-32 (bound to *scs'*) and Zw5 (bound to *scs*) to physically interact with one another while remaining bound to DNA [22]. This was later directly confirmed in mammals where conditional knockout of CTCF led to a reduction in chromatin looping at the β-globin locus in mice [129], while ectopic addition of a single CTCF insulator within the same locus in human cells induced the formation of alternate loops that disrupted communication between β-globin gene promoters and its locus control region (LCR) [26].

The nuclear consequences associated with these looping contacts between insulators are numerous, from directing the physical organization of the chromatin fiber at the megabase level, to facilitating or excluding communication between regulatory elements by altering looping contacts at the kilobase level. Below, we summarize recent findings regarding the role of chromatin looping by insulators in genome organization and function.

Chromatin insulators as architectural modulators of the genome

Of the major biological discoveries that have been elucidated within the last 5 years, arguably the most significant is the collective finding that eukaryotic genomes possess conserved global folding principles that allow them to package themselves within the nucleus in a highly ordered manner that reflects its underlying functional state [25, 29, 130–134]. The picture that has emerged from these studies is that genomes are organized into a hierarchy of loop structures across multiple spatial scales, with large demarcations at the megabase (Mb) level and smaller, spatially restricted looping contacts occurring preferentially within these domains at the kilobase (kb) level. Loops occurring at the Mb level correspond to the physical or topological organization of the chromatin fiber that are thought to serve an architectural role in folding interphase chromosomes within the nucleus, while the contacts occurring at the kb level are thought to dictate the functional behavior of the genome by controlling gene activity.

The large Mb-scale demarcations, first identified in mammals using high-resolution Hi-C and better known as 'topologically associating domains' (TADs), are largely conserved (60–70 %) not only across multiple cell types, including stem and differentiated cells, but also between species [130]. The borders of these TADs are enriched in CTCF, constitutively active housekeeping genes, tRNA genes and SINE retrotransposons, suggesting that these elements might collectively drive the demarcation of the genome into these large architectural domains. Interestingly, three of the four factors (CTCF, tRNA genes and

SINEs) are associated in some way with insulator activity, suggesting that these elements have played a key role in shaping this organization. Furthermore, despite the modest conservation of TADs between different cell types, dynamic changes in spatially restricted looping contacts were observed within each domain, possibly a reflection of the distinct transcriptional profiles responsible for generating cell identity following differentiation [130]. However, it is important to point out that the spatially restricted looping contacts occurring within these TADs could not be examined at sufficient resolution to accurately test this hypothesis.

Such organization, interestingly, is not a mammalian specialty. The *Drosophila* genome, despite being separated from its mammalian counterparts by roughly 990 million years of evolution [135], possesses similar organization principles. Sexton and colleagues identified well-defined, long-range contacts between distant genomic sites at large spatial scales in *D. melanogaster* embryonic nuclei, termed 'physical domains' [29], equivalent to mammalian TADs [130]. Interestingly, the borders of these domains were also found to be enriched in specific combinations of insulator proteins, such as CP190, CTCF and BEAF-32 as well as histone marks corresponding to active transcription. Su(Hw) was not associated with borders and instead localized within these domains, suggesting that the hierarchical organization of the genome might be established by distinct classes of insulators [29]. These findings were later confirmed and extended by Hou and colleagues [25] who showed that *D. melanogaster* TAD borders are enriched in specific combinations of insulator proteins, although Su(Hw) was also found at a subset of borders in combination with CP190, CTCF, BEAF-32 and Mod(mdg4)67.2. These so-called 'aligned insulators' corresponding to strong enrichment of these insulator proteins within a 300 bp window, also correlate with the boundaries of H3K37me3 domains where CTCF appears to be important for their maintenance [32].

In addition to aligned insulators, gene density and active transcription also correlate with TAD borders, with higher gene density located near borders and lower densities within domains. Interestingly, analysis of P-element insertions revealed a distinct bias toward domain borders and higher levels of reporter expression, which gradually decreased the further from a domain border the insertion occurred irrespective of active or repressive histone marks [25]. This suggests that the topological organization of the chromatin fiber is not only required for architectural purposes, but also exerts significant functional influence on the genome as well.

Taken collectively, the existence of large, Mb-scale demarcations across eukaryotes suggests that these interactions underlie a conserved folding principle responsible for packaging the chromatin fiber within the nucleus. Although insulators are enriched at their borders, the pervasive presence of active transcription at these sites as well suggest that other factors cooperate with these elements to establish genome architecture in space.

Cell/tissue-specific looping by insulators and generation of diverse functional outcomes

Although previous work demonstrating the demarcation of the genome into large Mb-scale TADs was critical for our understanding of how the chromatin fiber is folded at large spatial scales, interactions occurring at smaller scales within these domains were not readily detectable. This was primarily due to cost-based limitations in the amount of sequencing reads that would need to be generated to achieve sub-Mb resolution, particularly for an entire mammalian genome. Thus, although these sub-Mb-scale looping contacts were hypothesized to be dynamic and reflect the underlying functional state of the genome, likely in a cell type-specific manner as a result of distinct transcriptional networks, this has not been rigorously tested in detail until recently.

Phillips-Cremins and colleagues [136] generated chromatin interaction maps at seven genomic regions containing key genes involved in stem cell renewal (*Oct4, Nanog, Sox2, Olig1–2* and *Klf4*) for mouse embryonic stem cells (ESCs) and neural progenitor cells (NPCs) at unprecedented resolution ($\sim$4 kb). Within each TAD identified in a previous work, a number of smaller subtopologies (sub-TADs) were identified. Unlike their TAD counterparts, these subtopologies were found to be highly variable between ESCs and NPCs, with 83 ES-cell-specific interactions lost upon differentiation and 165 NPC-specific interactions generated following differentiation compared to 260 interactions conserved between the two cell types. Importantly, these interactions occurred over multiple spatial scales, from >1 Mb to <1 kb, and were mediated by distinct combinations of chromatin proteins, including CTCF, cohesin and the transcriptional coactivator Mediator, depending on the distance. CTCF alone or CTCF/cohesin was found to mediate large (>1 Mb) interactions and represented the majority of the 260 conserved interactions across cell types, in agreement with previous work outlining CTCF as being enriched near the borders of invariant TADs [130]. Interactions occurring between 100 and 300 kb were enriched in all three proteins, while those <1 kb were enriched in Mediator only and tended to represent the majority of ESC-specific interactions. Perhaps not surprisingly, these Mediator/cohesin sites appear to mediate ES-cell-specific looping contacts between regulatory elements and promoters of genes required for pluripotency, as loss of either protein leads to a reduction

in expression of these genes in ESCs. Taken collectively, these findings provide the first convincing evidence that the organization of the chromatin fiber into a hierarchy of relatively stable (TADs) and dynamic (sub-TADs) loop structures, mediated by insulators and other architectural proteins across multiple spatial scales, is critical for generating cell type-specific gene-regulatory networks [136].

Other studies have also highlighted the importance of chromatin insulators in establishing cell type-specific regulatory networks and controlling inducible gene expression. In *Drosophila*, CP190 is recruited to chromatin to direct specific looping contacts required for proper expression of inducible genes in response to the hormone ecdysone [137], while recent work in mammals has shown that CTCF and cohesin contribute to cell type-specific looping at the β-globin locus in human erythroid K562 and fibroblast 293T cells despite identical CTCF-binding profiles [24]. Computational polymer modeling of chromatin based on Hi-C data at this locus revealed that CTCF interactions in erythroid cells, which facilitate contacts between β-globin gene promoters and the LCR to stimulate expression, fold the chromatin into a compact globule with these elements located in close spatial proximity near the periphery. In non-erythroid cells where the β-globin genes are silent, differential CTCF interactions drive the β-globin genes away from the LCR in the periphery [138]. This suggests that CTCF contacts mediated in a cell-specific context can drive the topological folding of the chromatin fiber to direct specific gene-regulatory networks.

Importantly, although these studies have been critical to our understanding of how alternative loop formation by chromatin insulators defines specific regulatory outcomes in a cell or tissue-specific manner, how this is achieved mechanistically (through other tissue-specific factors or the action of posttranslational modifications) remains the most important point to address regarding alternate chromatin looping by insulators in vivo.

Chromatin looping as a means to control polycomb behavior

Although looping contacts mediated by insulators across smaller and more restricted spatial scales function to facilitate or exclude contacts between enhancers and promoters, they have also been shown to control the behavior of Polycomb group (PcG) proteins. These proteins are best known for their role in epigenetic silencing of developmental genes, mediated through the action of Polycomb Response Elements (PREs) located in the underlying DNA sequence. These elements recruit large PcG complexes that direct repressive H3K27me3 marks in the surrounding chromatin environment, which are then bound by Polycomb to promote silencing.

The interplay between insulators and PREs has been well established. A single copy of the *gypsy* insulator can block the spread of PcG repression from a flanking PRE, whereas the placement of two intervening insulators results in restoration of repression in a manner analogous to that observed for insulator bypass in enhancer–promoter communication [126, 127, 139–141]. This suggested that chromatin looping mediated by insulators might control the spread of PcG repression along the chromatin fiber. This was later confirmed using a high-resolution 3C method that showed not only the interaction between the two intervening *gypsy* insulators, but also direct contact between the PRE and promoter and enrichment of H3K27me3, which was not observed following removal of a single insulator [142]. Importantly, removal of the PRE did not alter looping contacts within the transgene, suggesting that insulators are required for long-range, localized repression by PREs, which has also been confirmed in other studies examining the *Mcp* and *Fab7* regulatory regions in the bithorax complex [142, 143].

Recent high-throughput studies have also outlined the link between chromatin insulators and Polycomb behavior. *Drosophila* aligned insulators, consisting of binding sites for CTCF, CP190, Mod(mdg4)67.2, BEAF-32 and Su(Hw), have been shown to border domains of H3K27me3, where intriguingly, CTCF appears to be responsible for their maintenance [32]. Furthermore, the numerous long-range intrachromosomal contacts observed between PcG domains throughout the *Drosophila* genome are likely to be mediated by insulators as well [29].

High-throughput methods offer the advantage of detailing the interplay between PREs and insulators in their native genomic contexts, although a recent study in *D. melanogaster* utilizing a large transgene containing both the *even skipped* (*eve*) and *TER94* genes plus all regulatory elements has provided significant insight into how insulators control PRE behavior and modulate enhancer–promoter communication in vivo [144]. The *eve* locus is a traditional PcG domain, kept in the off state by Pc-dependent silencing in specific tissues by a PRE located at its 3′ end, flanked by an aligned insulator known as *Homie* that has previously been shown to mediate extremely long-range contacts in the genome and function as a hotspot for P-Element insertions [25, 32, 145]. At its endogenous position, *Homie* performs three main functions that appear to be derived from its ability to mediate long-range interactions between other insulator sites: blocking the spread of H3K27me3 and Pc from the PRE into the downstream *TER94* locus that would otherwise lead to silencing, prevent misexpression of *TER94* by *eve* 3′ regulatory elements, and also facilitate contacts between these *eve* regulatory elements and its promoter. Within the context of the transgene, removal of *Homie* led to an increase in

H3K27me3 levels throughout the *TER94* locus and subsequent silencing, although it should be noted the deletion also removed sequence just upstream of the *TER94* promoter that might be required for full transcription. However, both of these effects were suppressed by removal of the PRE in the absence of *Homie*, suggesting the PRE is primarily responsible for repression and that *Homie* restricts the action of the PRE into neighboring domains. Furthermore, although deletion of *Homie* led to disruption of *TER94* expression in its normal pattern by Pc spreading, weak *TER94* expression was also observed in the traditional *eve* stripe pattern, suggesting that *Homie* also prevents *eve* enhancers from acting on the *TER94* promoter. Interestingly, removal of the PRE at the same time as *Homie* disrupted this weak *TER94* stripe expression, suggesting that certain enhancers, such as those located within Pc domains, may be able to partially function in a restricted environment and hence require the action of other elements, such as insulators, for full inactivation. Alternatively, PREs might be able to target enhancers to promoters, depending on the chromatin conformation. Finally, *Homie* also facilitates interactions between 3′ *eve* regulatory elements and its promoter, stimulating transcription. Removal of *Homie* weakened expression from the *eve* promoter, which did not appear to be due to promoter competition from the downstream *TER94* promoter as its removal did not increase *eve* expression, although the authors did not rule out whether other promoters in the vicinity of the transgene insertion site might also be targeted by the *eve* enhancers [144]. Taken collectively, these findings suggest that the ability of *Homie* to modulate PRE and enhancer behavior at the *eve* locus is likely a result of chromosome topology driven by *Homie*-mediated contacts. It would be of interest for future work to identify these interactions and how they might be altered in cells where *eve* expression is activated compared to the repressed (PcG silenced) state.

In addition to these findings, another important consideration was revealed in this study regarding insulator behavior in vivo. Insulator swapping experiments, in which *Homie* was replaced by other well-known insulators such as *gypsy, Fab-7, Fab-8* and *scs'*, showed variable effects on PRE-blocking and facilitation of *eve* expression. *Fab-7, Fab-8* and *gypsy* could all robustly substitute for *Homie* in both these regard, although in some cases a mild dependence on orientation was observed. This was particularly true for *scs'*, which was only able to partially compensate for the PRE-blocking function in a strict orientation manner and could not facilitate *eve* expression [144]. This lack of interchangeability for some types of insulators is in agreement with previous work demonstrating that swapping the *Fab-7* and *Fab-8* insulators within the bithorax complex leads to partial homeotic transformations [146]. These findings suggest that insulator placement in the genome is not random, and that specific functions bestowed upon different classes of insulators are likely dependent on the types of proteins present. Given the insulator protein composition of the aligned insulator *Homie* (BEAF-32, CP190, dCTCF, Su(Hw), Mod(mdg4) and GAF), it most certainly functions as a TAD border whose properties are clearly distinct from those established by other classes of insulators. It is therefore not surprising that non-aligned insulators (such as *scs'* and Su(Hw)-only insulators), bound by only a subset of proteins and which operate within repressive regions far from TAD borders [25], cannot fully rescue *Homie* activity in vivo.

Non-canonical roles for insulators

The data outlined in the previous section strongly supports a role for chromatin insulators in mediating long-range chromatin looping contacts between distant genomic sites. However, recent studies have also suggested a link with a number of other nuclear processes, including the stress response, DNA replication and repair, and a more direct role in transcriptional regulation, suggesting that chromatin insulators may play a more general or pervasive role in nuclear function than previously thought. Although some of these relationships are directly associated with the chromatin looping ability of chromatin insulators, it remains to be seen whether others might be the result of other functions of these proteins not attributable to looping. Given the central role higher-order chromatin structure plays in modulating virtually all aspects of nuclear behavior, perhaps it is not too surprising that these elements have been linked to a number of broad and seemingly disparate biological processes. It is likely that as the focus of high-throughput studies shifts from correlative to causative and computational methods improve to supplement traditional wet-lab approaches, many more will be identified. Below, we summarize the results of recent work that have revealed these connections and speculate on other nuclear processes in which these elements might be involved.

Insulators under stress

Much focus has been placed on understanding the general architecture of the chromatin fiber in the nucleus and the functional implications of this organization under what are assumed to be normal, homeostatic cellular conditions. However, relatively little focus has been placed on how these are altered under conditions of stress. Given that the earliest single-cell ancestors were likely to be under constant assault from a barrage of environmental insults,

including heat shock, oxidative, genotoxic and osmotic stress, these external cues likely played a major role in shaping the evolution of the eukaryotic lineage. In addition, practically all stress responses rely on a number of signal transduction pathways whose activation of specific transcription factors leads to altered genome behavior through modulation of specific gene-regulatory circuits. Collectively, this suggests an intimate link between environmental stress and nuclear function, with insulators and their loops likely to play a key role in this process.

The first evidence that chromatin insulators might be involved in the stress response came from heat shock studies in *Drosophila*, in which elevated temperatures lead to a global reduction in the amount of chromatin-bound CP190, but not BEAF-32 or CTCF, from polytene chromosomes. However, the functional consequences of this behavior were not addressed and it remains unclear whether this removal was a direct response targeted to alter insulator function specifically, or some other aspect of CP190 behavior [137]. Recently, we identified a definitive link between chromatin insulators and stress by showing that the formation of insulator bodies, punctate nuclear foci consisting of colocalized Su(Hw), CP190, dCTCF and Mod(mdg4)67.2 protein first identified more than a decade and half ago, only occur under conditions of osmotic stress [89, 122–125]. These structures were originally hypothesized to be the physical manifestations of chromatin looping, the result of multiple protein-bound insulators interacting with one another to organize the chromatin fiber within the nucleus, hence forming the basis for insulator function in vivo. Previous work had suggested that these structures might not represent functional insulators, instead acting as storage sites for the pool of insulator proteins poised for activity [147, 148]. Surprisingly, the formation of these structures correlated with a dramatic reduction in chromatin-bound Su(Hw) and a corresponding alteration in long-range looping contacts in the *mbl* locus, suggesting that these structures were not localized foci of DNA-bound insulator protein interactions required for chromatin loop formation. Instead, these findings suggested that removal of insulator proteins from chromatin under conditions of osmostress leads to dramatic changes in its higher-order structure and nuclear architecture [125].

Taken collectively, these findings suggest an involvement of insulators in the stress response, with the intriguing possibility of a chromatin-based sensing and adaptation mechanism to osmostress orchestrated by these elements. Although the relationship between stress and the activation of specific transcription factors is well established, the link between chromatin and stress per se remains relatively unexplored despite the obvious epigenetic implications of such insults [149, 150]. The observation that many global chromatin regulators from yeast affect the transcriptional kinetics of inducible/repressible genes but not steady state transcriptional levels during diamide stress suggests that many chromatin factors may have evolved to specifically modulate environmental and other inducible expression cues, which would be necessary to help the cell cope with the stressor [151]. Whether *Drosophila* insulators may have also evolved as a means to moderate the osmostress response or other inducible environmental cues remains to be elucidated. However, given that inducible changes in gene expression by ecdysone hormone treatment alters the recruitment of CP190 and other insulator proteins to specific genomic sites to establish/stabilize distinct chromatin loops, it is likely that the underlying principles regarding chromatin function in response to environmental cues is conserved in many eukaryotes [137].

Nonetheless, a number of intriguing questions remain regarding insulator body formation under conditions of osmostress. Understanding their physiological significance, if any, remains a key component. Also, it is clear that such a rapid and dramatic alteration in insulator protein behavior and nuclear architecture is likely to be tightly regulated, making elucidating the mechanism by which these structures form a priority. Additionally, a complete genome-wide picture of the effects that osmostress and insulator body formation impose on DNA occupancy and looping contacts, particularly for different classes of insulators, will be critical for understanding how insulators might ensure survival under such conditions or drive recovery once osmostress is alleviated. Our limited ChIP analysis suggests the intriguing possibility that certain insulators might function as landmarks to restore or maintain the 'default' chromatin architecture under such conditions. Although all insulator sites tested showed a significant decrease in Su(Hw) occupancy under conditions of osmostress, the *Homie* insulator showed less of a reduction than the other two classes of insulators tested [125]. As mentioned above, *Homie* is an aligned insulator that likely functions as a TAD border, which might suggest that despite extensive chromosome condensation, the global folding architecture of the chromosome within the nucleus remains intact as a result of these insulators remaining bound to chromatin under these conditions. The maintenance of the architectural organization of the chromosomes might ensure that once the osmostress is alleviated and other insulator proteins migrate back to chromatin, only the functional status of the cell has to be restored, allowing for rapid recovery. Interestingly, a recent study reported that the insulator proteins that remain bound to tightly condensed mitotic chromosomes belong to the aligned insulator class at domain borders, which were suggested to help maintain chromosome architecture throughout the cell cycle by persisting throughout mitosis [101]. Taken collectively, this may suggest a novel mechanism by which insulators

function to maintain chromosome architecture despite extensive chromosome condensation (Fig. 2).

Chromatin insulators in DNA replication

DNA replication is a tremendous molecular feat that involves a myriad of protein complexes responsible for ensuring error-free synthesis of the genome only once each cell cycle. Chromatin plays a central role in regulating this process, where the same challenges it poses during transcription for transcription factor binding, physical contact between enhancers and promoters, and barriers to elongating RNA pol II, must be overcome by the replication machinery [8]. This brings up an often overlooked or ignored aspect of chromatin insulators—if their ability to form higher-order structures can directly modulate the behavior of RNA pol II by restricting access within particular loop domains, might these same structures also pose a similar restriction during DNA replication? Or, might other properties of these elements help facilitate this process, depending on the cell cycle stage?

DNA replication relies on the presence of replication origins in the underlying DNA sequence, which are recognized by origin recognition complex proteins (ORC) in G1 to form the pre-replication complex (pre-RC), responsible for licensing the origin for subsequent firing during S-phase and recruitment of the holoenzyme complex. Interestingly, these replication origins are notoriously difficult to predict based on underlying DNA sequence features, and instead appear to share the common feature of

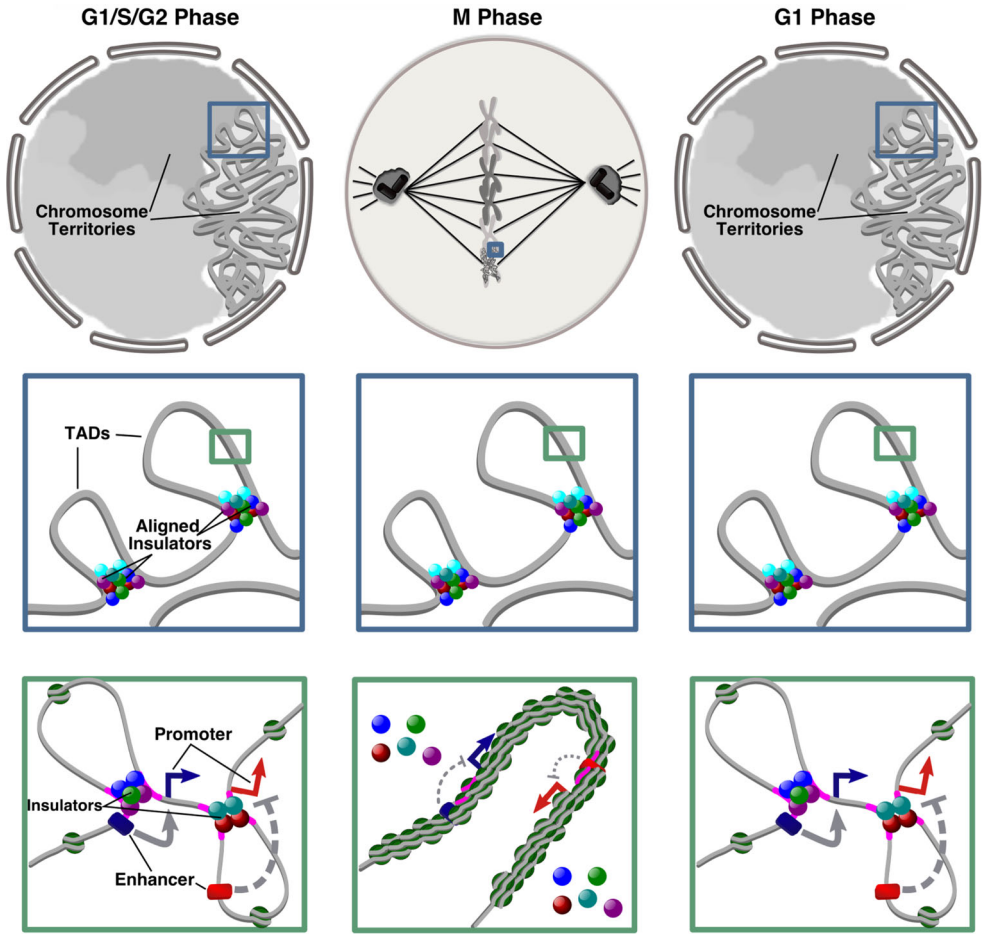

Fig. 2 Aligned insulators might function to maintain the 'default' architectural organization of the chromatin fiber during periods of chromosome condensation. During G1/S/G2 phase of the cell cycle, interphase chromosomes are organized in the nucleus by insulators across multiple spatial scales as described in Fig. 1. During M-phase when chromosomes undergo a large amount of condensation, the topologically associating domains (TADs) established by aligned insulators during interphase are maintained as a result of these proteins remaining bound to chromatin under such a state (*middle panel, blue inset*). However, functional domains established by other classes of insulators are lost as a result of protein removal, leading to a global alteration in transcriptional profiles (*middle panel, green inset*). Following cytokinesis, chromosome territories are quickly reestablished in G1, aided by the maintenance of the TADs throughout mitosis (*right panel, blue inset*), while functional domains are reestablished as the other pool of insulator proteins migrate back to their cognate binding sites (*right panel, green inset*)

being nucleosome depleted [152–154]. Chromatin insulators are also nucleosome free, both in *Drosophila* and mammals, suggesting that these elements might contribute to the formation and/or recognition of replication origins during G1 [28, 50, 155].

Recently, such a link between chromatin insulators and DNA replication was reported in *Drosophila* [100]. Using available computational data, a subset of Su(Hw) binding sites in the *Drosophila* genome were found to overlap with ORC proteins and the MCM2-7 helicase complex, with ORC2, ORC3 and ORC6 showing strong enrichment on or near Su(Hw) binding sites. Co-immunoprecipitation experiments revealed weak interactions between Su(Hw) and ORC3, while CDC45, a protein recruited to pre-RC complexes to license the origin for firing, was also detected on a subset of Su(Hw) binding sites. This behavior appears to depend on the ability of Su(Hw) to recruit the histone acetyltransferase SAGA and the BRAHMA chromatin remodeling complex, which cooperate to ensure low nucleosome density levels at Su(Hw) binding sites and thus a favorable chromatin environment for ORC binding. However, not all Su(Hw) binding sites recruit ORC components, and the ones that do constitute only $\sim$6 % of all replication origins in the genome. The remaining 94 % of origins associate with other known nucleosome-free regions, such as active gene promoters, that also tend to be enriched in the insulator proteins GAF, BEAF-32 and CTCF [23, 56, 100]. In support of this finding, it was recently shown that the transcription factor Myc associates with ORC2 during G1, which also overlap with a subset of insulators possessing distinct combinations of proteins [101]. While this data supports a positive role for insulators in the early steps of DNA replication during G1, more work will be required to understand if insulators play a direct role in establishing replication origins, or whether origins form simply by exploiting these nucleosome free regions established for other purposes, such as transcriptional activation.

Furthermore, it remains poorly understood whether insulators might influence DNA replication during S-phase, a particularly important consideration given the significant barrier higher-order loop structures might exert on elongating DNA polymerases. In yeast, tDNA promoters (presumably bound by TFIIIC) enhance replication fork pausing [156]. In mice and human epithelial cells, displacement of CTCF from the *E-cadherin* locus by overexpression of the licensing factor cdc6 led to gene silencing followed by a concomitant increase in adjacent replication origin firing [158], and RNAi-knockdown of CTCF also led to an increase in origin activity near a CTCF-binding site within the *INK4/ARF* locus [157]. Furthermore, CTCF controls the differential replication patterns at the *H19/Igf2* imprinted locus in mice, with mutations in the binding site normally bound by CTCF on the maternal allele resulting in a late to early switch in replication timing [159]. Finally, chromatin-bound CTCF also reduced replication efficiency at the *dystrophia myotonica 1* (*DM1*) locus [160]. This further supports the idea that mammalian CTCF negatively influences DNA replication during S-phase, likely through modulation of local chromatin structure such as nucleosome position and density, the formation of chromatin loops or simply acting as a barrier itself to a progressing DNA polymerase. However, if the formation of chromatin loops due to long-range contacts between CTCF sites acts antagonistically to elongating DNA polymerases, this is likely to depend on the genomic context, since cohesin, which overlaps with a large fraction of CTCF-binding sites, is enriched at replication origins and is required for stable association of chromatin fiber loops with replication factories. These cohesin-dependent chromatin loops are necessary for proper DNA synthesis, yet do not require the activity of CTCF [161].

Taken collectively, these data suggest that insulators might play an inhibitory role in DNA replication during S-phase, and a positive role during G1. It will be critical for future work in *Drosophila*, yeast and vertebrates to distinguish between these possibilities, which will likely require cell cycle stage-specific (G1, S, G2, M) binding profiles for insulator, ORC and licensing proteins coupled with the corresponding spatial conformation maps in addition to follow-up phenocopy studies, a feat likely easier said than done. Nonetheless, such data would highlight how the architecture of the chromatin fiber changes as a result of insulator behavior to allow for distinct nuclear processes to occur, in a manner analogous to such data collected under conditions of stress.

Chromatin insulators and DNA repair

DNA repair, yet another nuclear function heavily influenced by chromatin structure, is critical for ensuring that all forms of DNA damage are repaired prior to replication to prevent potentially deleterious mutations from being passed to daughter cells. Unlike DNA replication, in which a core set of proteins performs a single, specific function, there are many different types of DNA repair mechanisms, mediated by a large number of different proteins. Chromatin proteins play a key role in marking damaged regions and recruiting these different repair proteins to the lesion [162]. Might chromatin insulators also be involved in this process? Recently, BRD4, a bromodomain-containing protein implicated primarily in transcriptional control, was shown to be involved in the DNA damage signaling response in mammals [163]. Its *Drosophila* homolog, *Fs(1)h*, was recently shown to colocalize at insulator

binding sites, particularly mirroring that of Mod(mdg4)67.2 while physically interacting with GAF, CP190, Su(Hw) and Mod(mdg4)67.2 [98]. Although it remains to be determined whether the same BRD4-mediated DNA damage response attenuation observed in mammals is conserved in *Drosophila*, insulators might play a role in this process as well. Also, mutations in *su*(*Hw*) appear to increase DSB repair efficiencies in the *Drosophila* germline, suggesting that a conserved function of insulators might involve DNA repair [164].

Chromatin insulators as gene-specific transcription factors

The idea that insulator proteins might operate as 'traditional' transcriptional activators and repressors for a particular subset of genes is not a novel concept, nor is such activity likely to be mutually exclusive from its ability to mediate chromatin loops. However, it has suffered from little supporting evidence until recently, and the lack of a clear agreement in whether insulation can be broadly included in the many properties that define a transcriptional activator/repressor. Insulators have traditionally been treated as distinct gene-regulatory elements with their own properties, a view that has been further compounded by their pervasive presence throughout the genome and prevailing hypothesis that they function as general organizers of higher-order chromatin loop structures. This simplistic idea posits that their effect on transcription would have a tendency to be more global, rather than confined to individual genes.

However, evidence from *Drosophila* and vertebrates indicates that for some insulator proteins, their role extends beyond just architectural and they appear to be responsible for controlling specific gene-regulatory circuits, perhaps in a tissue-specific fashion. Vertebrate CTCF was originally shown to possess both transcriptional repressor and activator behavior before its role as an insulator was elucidated [37, 165–167]. A number of vertebrate CTCF-binding sites also overlap with RAD21 and SMC3, which are members of the cohesin complex that associate with a number of transcription factors and the Mediator complex to regulate gene expression [24, 168, 169], in addition to the vertebrate-specific transcription factors ZNF143 and SIX5 in GM12878 cells [112].

Furthermore, a number of target genes were identified in the *Drosophila* ovary that were specifically misregulated in a *su*(*Hw*) mutant background [170]. Nearly all *su*(*Hw*) mutant alleles, in addition to showing a *gypsy* insulator phenotype, also show an oogenesis phenotype, resulting from extensive egg chamber apoptosis that ultimately leads to female sterility [171]. However, the oogenesis phenotype does not appear to be a result of altered insulator function, as certain *su*(*Hw*) alleles, such as $su(Hw)^f$, disrupt *gypsy* insulator function, yet do not show oogenesis defects [172, 173]. $su(Hw)^f$ is the result of a point mutation that disrupts one of the 12 zinc fingers (ZnF) that mediate the ability of Su(Hw) to bind DNA. However, a comparison of the binding profiles for wild-type and $Su(Hw)^f$ proteins in the ovary revealed that a third of Su(Hw) binding sites (SBS) observed in wild type were still occupied in the $Su(Hw)^f$ mutant. This suggested that these sites retain functional Su(Hw) activity that is required for oogenesis, but is not insulator dependent [174]. Indeed, microarray profiles of wild type and mutant ovaries identified a number of genes whose transcripts levels were significantly altered in the mutant background, with an overwhelming proportion showing elevated expression. Furthermore, roughly 35 % of these misregulated genes contained an SBS within or near their promoters, supporting a role for Su(Hw) in direct transcriptional control primarily as a repressor. Interestingly, more than half of these target genes are expressed primarily in the brain with reduced expression in the wild-type ovary and were upregulated in *su*(*Hw*) mutant wing discs, suggesting that Su(Hw) might be responsible for repression of neural genes in non-neural tissue. Importantly, this repression does not appear to require CP190 or Mod(mdg4)67.2, further supporting the idea that Su(Hw) has additional functions as a transcriptional repressor that might not rely on insulator properties [170].

In addition to Su(Hw), Mod(mdg4)67.2 has also been implicated in direct transcriptional control. Mutations in the tumor suppressor gene *lethal* (*3*) *malignant brain tumor* (*l*(*3*)*mbt*) lead to overproliferation of neuroepithelium cells and subsequent tumor formation as a result of derepression of genes involved in the Salvador–Hippo–Warts pathway, which plays a key role in controlling cell proliferation and tissue size. Genome-wide analysis of L(3)mbt revealed a significant overlap with a subset of insulator binding sites, particularly those containing Mod(mdg4)67.2. Interestingly, reduction of *mod*(*mdg4*) expression, but not other insulator components, phenocopied mutations in *l*(*3*)*mbt* and resulted in the upregulation of a Salvador–Hippo–Warts reporter, suggesting that Mod(mdg4)67.2 contributes to repression of SHW pathway genes through cooperation with L(3)mbt to control neuronal tissue size and proliferation [93].

These experiments outline two key aspects of insulator behavior that we are only beginning to understand: their role as transcription factors and the importance of cell or tissue specificity in their regulatory properties. The data given above in addition to a number of recent studies suggest that Su(Hw) and other *gypsy* components might play a critical role in specific aspects of neural biology. Recently, Bayesian profiling of chromatin proteins in

Drosophila classified Su(Hw) as being associated with transcriptional repression and neuronal processes [175]. Furthermore, *su(Hw)* was found to be highly upregulated in neuroblasts (NBs) and placed within a hypothetical transcriptional network responsible for NB self-renewal [176], suggesting that it might be responsible for regulating a defined set of genes needed for neuronal maintenance through interactions with other factors. An intriguing candidate is the neural-specific RNA binding protein *alan shepard* (*shep*), which has been shown to influence the behavior of Su(Hw)- and Mod(mdg4)67.2-containing insulators within the CNS and also directly interacts with *gypsy* proteins in vitro [91]. The overall genome-wide binding profile for Shep correlates strongly with regions of active transcription, such as RNA pol II enrichment and active histone marks, while also localizing to transcriptional active puffs on polytene chromosomes, suggesting that Shep might be involved in transcriptional activation of neural genes. Interestingly, colocalization between all three proteins was observed at 271 sites within CNS-derived BG3 culture cells, suggesting that these insulator sites might be involved in transcriptional activation imparted by the Shep protein [91]. This is further supported by the finding of Soshnev et al. [170] that the small percentage of Su(Hw)-target genes that showed a positive dependence on Su(Hw) were also dependent on CP190 and Mod(mdg4)67.2, unlike the majority of Su(Hw)-repressed genes in which mutations in *CP190* and *Mod(mdg4)67.2* did not alter expression.

Taken collectively, these data suggest that chromatin insulators may play a key role in regulating tissue-specific gene regulatory networks in a manner more reminiscent of direct transcriptional control than traditional insulator function. In the context of the prevalent hypothesis that different classes of insulators contribute to chromosome architecture across multiple spatial scales that direct the topological organization at the highest level and the functional status of the genome at the lowest level, perhaps it is not surprising that a subset of insulator binding sites, such as those located far from domain borders and unlikely to contribute to architectural organization, would be tasked with controlling specific genes in these spatially restricted environments deep within these domains. This could explain why insulators bound by Su(Hw) only, Mod(mdg4)67.2 only or a combination of the two appear to have a more significant effect on transcription of specific genes—these proteins on their own are rarely found near TAD borders and instead tend to localize within them in more repressive environments [25, 29]. In this case, other non-insulator chromatin proteins, such as Shep and L(3)mbt cooperate with these classes of insulators at a subset of sites to control transcription in a gene-specific manner, either in response to inducible cues or other cell type-specific factors to establish cell identity (Fig. 3). This hypothesis further supports the idea that distinct classes of insulators, bound by different combinations of proteins, perform distinct functions over multiple spatial scales. Collectively, these classes are thus responsible for establishing both the architectural and functional organization of the chromatin fiber within the nucleus, thereby exerting master control over the genome.

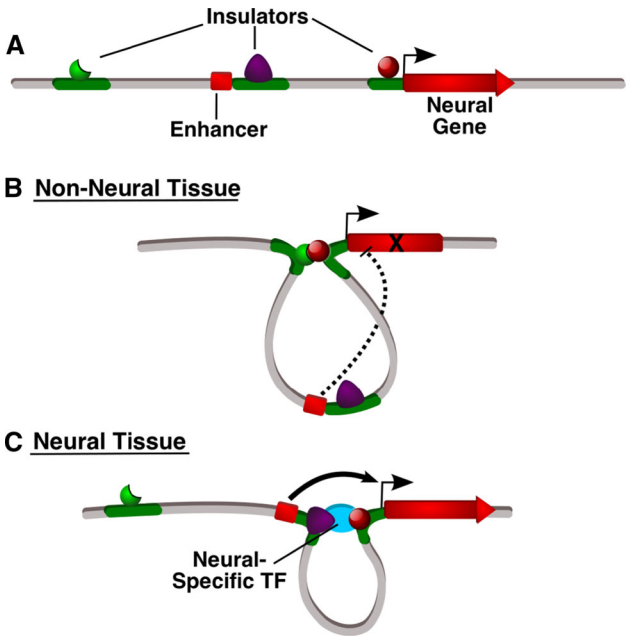

Fig. 3 A hypothetical model for how insulators might contribute to transcriptional regulation for a subset of genes in a tissue-specific manner. **a** An enhancer and neural gene promoter is flanked by two protein-bound insulators (*purple* and *red colored shapes*), in addition to an upstream element. **b** In non-neural tissue, preferential interactions between the upstream and promoter insulators establish a chromosome conformation that spatially separates the enhancer from the promoter, resulting in repression. **c** In neural tissue, this interaction is lost in favor of a higher-affinity interaction between a tissue-specific transcription factor and the flanking insulators, establishing a chromosome conformation that brings the enhancer in close proximity to the promoter and subsequent gene activation

Regulation of insulator activity

Of the many facets of chromatin insulator biology that remain poorly understood, how insulator activity is regulated remains the most glaringly deficient. How are the correct looping contacts established between insulator sites and how are they altered in response to specific stimuli? Is their DNA-binding ability regulated, and if so, how? It is surprising that such little effort has been put forth regarding these key questions, as a better understanding of how these are accomplished is likely to provide significant insight toward our understanding of how these elements control

genome behavior. To date, no specific signal transduction pathways have been linked to alterations in insulator behavior, although a number of posttranslational modifications to the proteins themselves have been reported in both vertebrates and *Drosophila.* Although in many cases the functional consequences of these modifications remain poorly understood, it is feasible to predict that such changes could alter the behavior of insulator proteins by creating or masking protein interacting domains, or alter the DNA-binding affinity of zinc finger domains in either a direct or allosteric manner. Below, we summarize the different posttranslational modifications and other novel regulatory mechanisms that have been linked to these proteins.

Poly (ADP-ribosyl)ation (PARylation)

Poly (ADP-ribosyl)ation (PARylation), catalyzed by the poly (ADP-ribose) polymerase (PARP) has been linked to both vertebrate and *Drosophila* CTCF, where such activity appears to be tightly coupled to both insulator and nucleolar function. PARP copurifies with CTCF in mammalian cells along with other nucleolar proteins, such as nucleophosmin, and PARylated CTCF localizes specifically to the nucleolus where it appears to negatively influence nucleolar transcription [177, 178]. Furthermore, a similar behavior was also observed in *Drosophila*, where CTCF was shown to localize to the nucleolus and bind to specific sites within the R1 transposon [72]. Reduction of CTCF by RNAi lead to an increase in nucleolar transcription in addition to pronounced nucleolar fragmentation, a phenotype also observed after loss of PARP [72]. It is important to note, however, that in addition to the distinct nucleolar population of CTCF observed in these studies, a separate general nucleoplasmic population was also present, which might suggest that PARylated CTCF is only required for its nucleolar role rather than for its function as an insulator elsewhere in the genome.

However, this does not appear to be the case, as PARylated CTCF has been detected at other genomic sites and has been shown to directly influence insulator behavior. In mice, modified CTCF has been detected at the *H19/IGF2* imprinted locus, where it contributes to silencing of the maternal allele [179]. This silencing was lost upon inhibition of PARylation, while the insulator activity of a transgenic reporter was also reduced. Interestingly, the levels of PARylated CTCF differ in a tissue-specific manner, indicating that this mark might contribute to the ability of CTCF to establish or maintain cell identity in a differential manner. Most intriguingly, however, is that PARylation of CTCF does not appear to alter its DNA-binding behavior. CTCF still remains bound in either state (PAR ON/OFF), suggesting that PARylation alters the protein interaction profile of CTCF and in turn its looping behavior, to control insulator action [179].

Such behavior has also been observed in *Drosophila*, suggesting that control of CTCF activity by PARylation might underlie an ancient and evolutionarily conserved regulatory mechanism for insulator function. Recently, it was shown that CP190, CTCF, Mod(mdg4)67.2 and Su(Hw) are PARylated both in vitro and in vivo, which is lost upon PARP inhibition [180]. Mutations in *Parp* partially suppressed *gypsy*-induced phenotypes of both y^2 and ct^6 mediated by Su(Hw), Mod(mdg4)67.2 and CP190, while a CP190 mutant unable to be PARylated (K566A) also showed a similar behavior. This is also true for the CTCF-dependent insulator within the *Fab-8* element, suggesting that PARylation is needed for *Drosophila* insulator function. As observed in mammals, this regulation appears to globally target the protein interaction profile rather than the DNA-binding ability of Su(Hw) or CTCF, as inhibition of PARylation led to a reduction in the amount of CTCF that associated with CP190, a behavior also observed for the un-PARylatable CP190 mutant (K566A) and reduced the population of insulator proteins that copurify with the nuclear matrix fraction. ChIP-Seq verified that the DNA-binding profile between PARP-inhibited and control cells did not globally differ, with the exception of a subset of sites, a profile that was also mirrored in the K566A CP190 mutant (loss occurred at only 10 % of CP190 sites, 22 % of CTCF, 0.4 % of Su(Hw), and 5 % of Mod sites). This suggests that although PARP is not generally sufficient to alter the DNA and chromatin binding ability of these proteins, the presence (or absence) of other factors at these affected sites in combination with PARP can influence these associations and therefore might impart specific functions upon them [180].

In accordance with this hypothesis, many of the affected sites were found to be located far (>2 kb) from aligned insulator sites corresponding to TADs, suggesting that they might not alter the physical or topological organization of the chromatin fiber at the Mb scale, but instead act on the sub-Mb and kb scale to control functional interactions [180]. This idea is supported by the fact that spatially restricted looping contacts mediated by PARP-affected sites were reduced in PARP-inhibited cells, while looping contacts were maintained within the *Bithorax* complex whose insulator binding profile was not altered under the same conditions. This suggests that PARP only contributes to a subset of looping interactions mediated by insulators. However, it is important to note that not all possible interactions were lost from PARP-affected sites, although there appears to be a mild correlation between the combinations of insulator proteins present (particularly CP190) at these sites and the severity of looping contact loss. Taken collectively, although this data supports the notion

that PARP can stabilize insulator contacts to contribute to insulator function, clearly other factors are acting on insulators in parallel to orchestrate the complex organization of the chromatin fiber dictated by these elements [180].

Small ubiquitin-like modifier (SUMO)

The small ubiquitin-like modifier (SUMO), a small peptide structurally similar to ubiquitin, has been shown to be involved in a number of biological processes and chromatin insulation is no exception. Human CTCF contains two strong SUMOylation motifs, one near each terminus, and can be modified by three SUMO isoforms (1, 2 and 3) both in vitro and in vivo, but much like PARylation, SUMOylation does not appear to alter its DNA-binding ability [181]. Interestingly, this SUMOylation enhanced CTCF's repressive function at the c-*myc2* promoter in much the same way that PARylation enhanced the repressive function of CTCF at the maternal *H19/Igf2* allele, indicating that different posttranslational modifications can function redundantly, or specify distinct outcomes depending on the combinations of modifications present, primarily through modulation of protein–protein interactions [181].

In *Drosophila*, SUMOylation has also been shown to alter insulator behavior, although how this is achieved and whether it might function positively or negatively remains debatable. The E3 ligase *dTOPORs*, an enzyme possibly responsible for attaching SUMO to target proteins, interacts genetically with *mod*(*mdg4*)*67.2* and alters *gypsy* insulator function [95]. Also, both Mod(mdg4)67.2 and CP190 are modified by SUMO in vitro and in vivo which appears to negatively influence insulator function. SUMO colocalizes with a subset of CP190 and Mod(mdg4)67.2 sites on polytene chromosomes, although it does not appear to be required for chromatin binding at these sites [182]. This suggests that much like PARylation, SUMO might only target a subset of insulator sites, although rather than positively influencing insulator function through looping stabilization to dictate specific regulatory outcomes, SUMO instead disrupts insulator activity by destabilizing these loops.

It is important to point out that whether SUMO actually attenuates insulator function by reducing looping, or might promote looping at a subset of insulator sites, remains to be tested using high-throughput methods. Increased levels of SUMOylation were reported to disrupt the formation of insulator bodies [182], which at the time were thought to be the physical manifestations of chromatin looping that dictated insulator function. Conversely, reduced levels of SUMOylation increased insulator activity in transgenic reporter assays. These findings suggested that SUMO negatively regulates insulator function [182]. However, recent work has shown that SUMOylation appears to be required for the formation of insulator bodies, in contrast to previous findings. Knockdown of either SUMO or CP190 disrupted insulator body formation in S2 cells, while loss of SUMO did not appear to alter the interaction between CP190 and Mod(mdg4)67.2 [148]. Furthermore, we found that CP190 undergoes a rapid and reversible cycle of SUMOylation and deSUMOylation that correlates nicely with the dynamics of insulator body formation and disassembly. CP190 becomes heavily SUMOylated during osmostress and subsequent insulator body formation, followed by rapid de-SUMOylation in the order of seconds following recovery in isotonic media and the disappearance of insulator bodies [125].

However, the role that a SUMOylated CP190 might be playing in insulator body formation remains poorly understood. SUMO plays a key role in the formation and maintenance of other nuclear bodies such as PML and PcG bodies, although how this is regulated is not known [183]. In our case, we favor the simple idea that SUMOylation of CP190 (and perhaps other insulator proteins/interacting partners) under conditions of osmostress creates new protein interacting platforms that are not present under normal physiological conditions, which in turn alters the binding profile among these proteins and leads to the formation of the structurally organized insulator bodies. It should be noted that nearly all insulator proteins have computationally predicted SUMO interacting motifs (SIMs) that might mediate these interactions with SUMOylated CP190 and other modified proteins under these conditions. Whether SUMOylation under these conditions might also contribute to the massive removal of these proteins from DNA during osmostress remains to be elucidated, although such a system now provides a suitable means to study how such control is achieved. Clearly, much work remains to be done in understanding how SUMO contributes to not only insulator body formation, but insulator function as well.

Other forms of regulation

Although SUMOylation and PARylation of insulator proteins constitute the primary focus of how insulator activity can be regulated, other mechanisms have been identified that contribute to this process as well. Specifically, mammalian CTCF can undergo phosphorylation by casein kinase II (CK2), an evolutionarily conserved protein kinase in eukaryotes linked to metabolism, transcription and DNA repair. Phosphorylation on Ser612 converts CTCF from a transcriptional repressor into a transcriptional activator at the *c-myc* promoter [184, 185], a finding opposite to the repressive effect that CTCF SUMOylation had on *c-myc* [181]. This suggests that CTCF activity might be distinctly controlled via combinatorial or antagonistic function of

different types of modifications. Whether phosphorylation might also influence the behavior of *Drosophila* CTCF remains to be elucidated, although the evolutionary conservation of CK2 and other kinase networks favors this possibility, much like that observed for SUMOylation and PARylation.

In addition to posttranslational modifications, might other regulatory mechanisms also contribute to insulator activity? In mammals, CpG DNA methylation has been linked to CTCF occupancy and insulator function at the *H19/Igf2* and *DM1* loci [186–190], with the methyl modification within the CTCF sequence motif acting to disrupt DNA recognition. Interestingly, recent high-throughput analysis revealed that such a regulatory mechanism is more prevalent than previously thought. Analysis of CTCF-binding profiles combined with bisulfite sequencing of 19 different human cell types (Human ENCODE consortium) revealed marked plasticity among CTCF occupancy, nearly half (41 %) of which were linked to variable patterns of DNA methylation at those binding sites [52]. Immortalized cell lines showed increased amounts of methylation, leading to altered CTCF-binding profiles compared to normal controls. Intriguingly, *CTCF* appeared to be upregulated in these immortalized lines [52], suggesting a potential feedback loop between DNA methylation, CTCF occupancy and *CTCF* expression that might play a key role in tumorigenesis. Although such a mechanism is unlikely to control insulator behavior in species that lack widespread DNA methylation (such as *S. cerevisiae, D. melanogaster* and *C. elegans*) [191], the ability to control the DNA-binding ability of insulator proteins at both the DNA and protein level would allow for a powerful means to fine-tune their behavior to achieve specific cellular outcomes.

In addition to DNA methylation, RNA might also contribute to regulation of insulators. Both coding and noncoding RNAs have been shown to nucleate formation of nuclear bodies and control nuclear organization [192]. Although components of the RNAi machinery, such as the Rm62 helicase and Argonaute 2 (AGO2) have been shown to either interact genetically with insulator components or physically colocalize to similar sites throughout the genome, these appear to be independent of the catalytic function of these RNAi proteins [92, 96], suggesting that their small RNAs may not contribute to regulating insulator function. Recently, however, mRNAs corresponding to *mod*(*mdg4*)*67.2* and *su*(*Hw*) were detected in insulator protein complexes [193]. Expression of untranslatable versions of these mRNAs, particularly *su*(*Hw*), led to an increase in wing margin notching in the *gypsy*-induced ct^6 background sensitized with mod^{u1}, indicating an enhancement of enhancer blocking in the presence of these transcripts. Thus, mRNAs are likely to play a direct role in regulating insulator behavior, although it will be important for future work to identify whether similar behavior is observed for non-*gypsy* components in *Drosophila* (CTCF, BEAF-32, GAF, Zw5) and vertebrate CTCF. This will be critical to determine whether this regulatory mechanism is a general feature of all insulator subclasses in *Drosophila*, and whether it might constitute an ancient and conserved mechanism of regulating insulator function in eukaryotes.

Conclusions and future perspectives

With the advent of high-throughput, genome-wide biochemical methods to probe the behavior of these elements in vivo, the role of chromatin insulators as general facilitators of chromatin loop structures is well supported. As such, our knowledge of how insulators contribute to genome organization within the three-dimensional space of the nucleus and the functional implications of such organization has advanced rapidly within the last few years. As the resolution of such techniques continues to approach the single base pair level, our knowledge of how these elements function across multiple spatial scales to dictate everything from the global folding principles of eukaryotic chromosomes to looping between regulatory elements that control gene regulatory circuits will continue to expand, perhaps in novel directions. Although insulators have been primarily studied in *Drosophila*, yeast and vertebrates, it is certain that other eukaryotes utilize insulator-like (architectural) proteins to drive chromosome and genome organization. As the field continues to expand into other organisms, our knowledge of how these proteins accomplish this feat will provide a powerful means to examine the evolutionary constraints imposed upon genome control. Importantly, as utilization of high-throughput methods in the field continues to progress from correlative to causative, the functional consequences of such organization will continue to provide novel insight into numerous aspects of nuclear biology. However, traditional genetic and biochemical approaches will continue to provide a solid foundation for the most poorly understood, but arguably most important facet of chromatin insulator biology—how the activities of these proteins are differentially regulated to establish both the topological organization of the genome within the three-dimensional space of the nucleus and its functional state in a cell- and tissue-specific manner.

Acknowledgments We would like to thank the current and former members of the Labrador Lab, including Hyuck-Joon Kang, Heather Wallace, Shaofei Zhang, Srilalitha Kuruganti, Maria P. Plata, Shih-Jui Hsu, Ran An, Ryan Rickels and Josh Barrios for collaboration and discussion. Work in the Labrador Lab was supported by Grants NIH GM78132-2 and NSF MCB-0616081 plus additional support from the College of Arts and Sciences, the Department of Biochemistry and Cellular and Molecular Biology and the Office of Research at the

University of Tennessee, Knoxville. TS was supported by a Graduate Research Fellowship from the National Science Foundation.

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