Defining Functionally Relevant Spatial Chromatin Domains: It is a TAD Complicated

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Abstract

Chromosome conformation capture and orthologous methods uncovered the spatial organization of metazoan chromosomes into autonomously folded substructures, often termed topologically associated domains (TADs). There is a striking correlation between TAD organization and hallmarks of genome function, such as histone modifications or gene expression, and disruptions of specific TAD structures have been associated with pathological misexpression of underlying genes. However, complete disruption of TADs seems to have mild effects on the transcriptome, raising questions as to the importance of chromatin topology in regulating the expression of most genes. Furthermore, despite a growing number of genetic perturbation studies, it is still largely unclear how TAD-like domains are defined, maintained, or potentially reorganized. This perspective article discusses the recent work exploring the complexity of the relationship between TADs and transcription, arguing that it is not satisfactorily explained by any of the "rules" that have been previously described.

A brief history of TADs

Based on the descriptions of heterochromatin from microscopy studies since the early 20th century, it has been appreciated that the genome is a heterogeneous and organized structure, long proposed to contribute to the regulation of its functions, such as transcription, replication, and repair. The advent of chromosome conformation capture methods, coupled to high-throughput sequencing (Hi-C), has expanded the coverage and resolution of complementary microscopy studies, uncovering an apparent hierarchical folding regime of the genome [1] (Fig. 1). In particular, some of the first Hi-C (and other variants) studies uncovered the organization of metazoan chromosomes into discrete, sub-megabase domains, often termed TADs (topologically associated domains), whereby intradomain interactions are stronger than those spanning the borders between TADs [2–4]. The TAD organization closely mirrors the functional demarcation of chromatin regions according to transcriptional activity [2,4,5], histone modifications [2–4,6], and replication timing [7], implying that genome structure and function may be mechanistically coupled. However, TAD organization was unchanged in mutant cell lines disrupting the deposition of specific histone modifications [3]. Further, despite extensive transcriptomic and epigenomic differences between cell types [8], initial Hi-C studies found that the TAD organization was largely invariant [9], even when comparing syntenic regions across different species [10]. TADs thus appearing to be "hard-wired," research efforts have since mostly focused on the fundamental mechanisms by which they are formed, and if or how such organization impinges on genome function.

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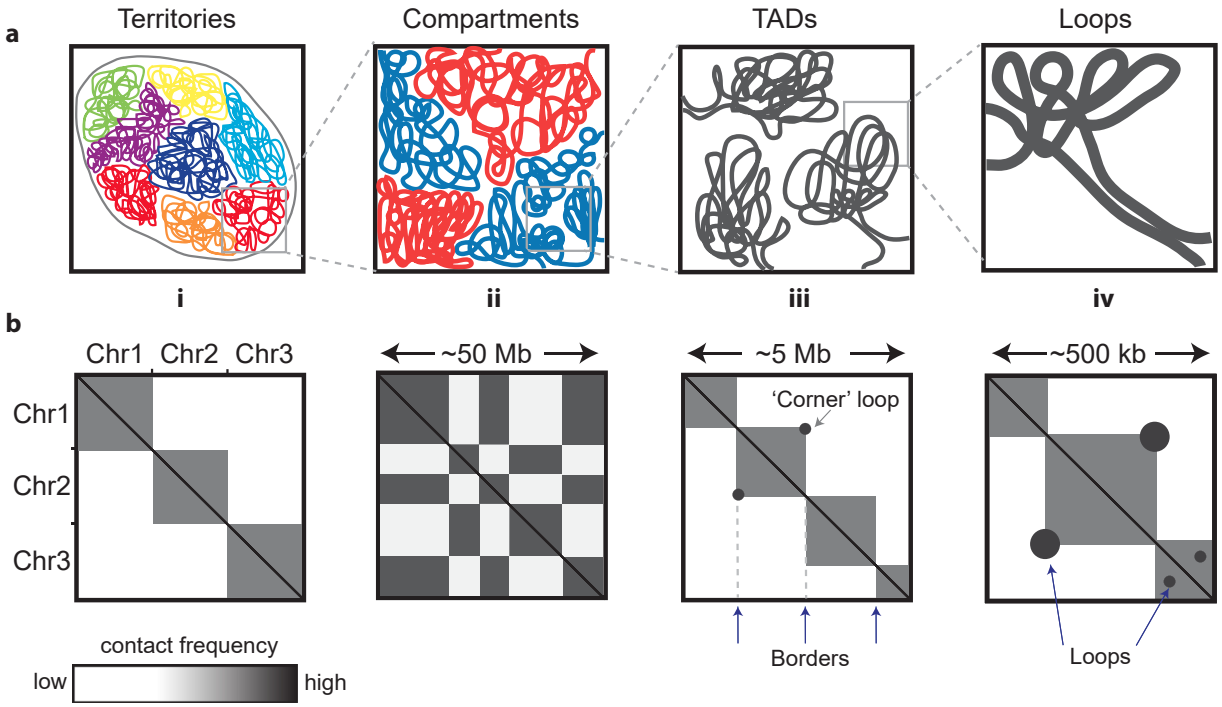

Fig. 1. Multiscale chromatin folding. a) Schema of the features of chromatin folding at different scales, and b) the appearance of the underlying Hi-C contact map that allows deduction of the topological feature. Contact maps have the chromosomal coordinates of the two interacting regions in the x and y axes, with their corresponding interaction strength denoted by the color of the heatmap. They invariably have a strong diagonal, corresponding to very frequent interactions between regions that are adjacent on the linear chromosome fiber. i) Chromosomes (denoted by different colors in a) fold into distinct territories, with limited intermingling between chromosomes. In the Hi-C contact map, most signals are restricted to the squares on the matrix corresponding to intrachromosomal interactions. ii) Chromosomal regions tend to coalesce into two different compartments (red or blue), whereby chromatin of the same "color" tends to co-associate, with reduced interactions between chromatin regions of opposite "color." This is inferred from the patchwork pattern of the Hi-C contact map, caused by alternating regions of increased and reduced interactions. iii) Chromosomes tend to fold into discrete TADs, whereby interactions within a domain are stronger than interactions between domains, represented by squares on the diagonal of Hi-C contact maps. The "edges" of the squares correspond to TAD borders. TADs often have foci of strong interactions between borders ("spots" on the corners of the squares; see also Fig. 4). iv) Looping interactions are inferred as foci or "spots" on the contact map, whereby the interaction between two elements is stronger than interactions between flanking regions. In Hi-C maps, the loops at TAD borders are usually the easiest to discern, but others, for example arising from promoter-enhancer interactions, may be observed within TADs.

TADs: an equilibrium of loop formation, growth, and dissociation?

Analysis of TAD boundaries revealed a high enrichment for active genes across metazoans [2,4,11,12], and co-binding of the insulator protein CTCF and the cohesin complex in mammalian cells [2,13]. Whereas cohesin is not enriched at Drosophila TAD borders, many fly-specific insulator proteins are present in addition to CTCF [4,12,14]. CTCF [15] (and other insulators in Drosophila [16,17]) and cohesin [18,19] are implicated in the formation of chromatin loops via the pairing of bound loci. It was proposed that stable anchoring of paired boundaries in this manner could organize the intervening chromatin into a TAD. Indeed, higher-resolution Hi-C maps in human cells found that a significant fraction of domains were demarcated by interaction foci at the "corners," implying an anchoring loop [6] (see also Fig. 1). Such loops are strongly enriched in CTCF and cohesin; even more strikingly, almost exclusively when the CTCF-binding DNA sequence motifs are in a convergent orientation [6,10]. The importance of the motif orientation was further demonstrated by the loss of specific chromatin loops when one of the motifs was experimentally inverted [20–22]. CTCF binding to the inverted site is unaffected, so how can the orientation of a twenty-nucleotide sequence have such a drastic effect on an interaction that can span a megabase, where there should be no torsional constraints on a simple protein-protein pairing event? An explanation can be provided by the loop extrusion model [22,23], whereby chromatin loops are constantly being

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extruded at many positions along the genome with a certain processivity before disassembling. The encounter (and presumed direct interaction) of two convergent CTCF complexes somehow forms a roadblock for further extrusion of any loops, resulting in an equilibrium of TADs demarcated by a seemingly stabilized loop interaction between the boundaries (Fig. 2). Cohesin has been proposed as the loop extruding factor based on different lines of evidence. First, acute degradation of cohesin protein or deletion of its loading factor, *Nipbl*, completely removes TADs [24–26]. More precisely, cohesin mutants deficient in ATP hydrolysis have a greatly reduced TAD organization [27], suggesting that cohesin-mediated loop extrusion is an active process. Second, perturbations of factors that unload cohesin from chromatin enhance a longer range CTCF-CTCF loop and TAD formation [26,28], presumably as more processive loops can now extend beyond their usual boundaries. Third, while still to be demonstrated for cohesins, ATP-dependent DNA loop extrusion by the closely related condensin complex was directly visualized *in vivo* [29]. Loop extrusion has been incorporated into different polymer physics models of chromosome folding and is currently the only model that predicts a requirement for convergent CTCF sites [22,23,30,31]. However, it should be noted that many features of Hi-C contact maps can be fitted by alternative physical models [32,33].

A simplistic view of TADs, taken together, has been built up whereby a pair of bound convergent CTCF sites can predict much of a chromosome's topology via cohesin-mediated loop extrusion. However, this model breaks down on many occasions. First, not all TAD borders contain CTCF [2,11], in line with the disruption of many, but not all, TADs on acute CTCF degradation [34] (see also Fig. 3). Conversely, the majority of CTCF-bound sites do not appear to form TAD borders, suggesting that other, unknown principles determine whether chromatin looping and/or domain organization is mediated by CTCF pairs. As noted examples, inversion of specific CTCF sites does indeed disrupt chromatin loops [20–22]; however, *de novo* loop formation between the inverted site and a now convergent CTCF-bound site in the opposite direction to the "wild-type" interaction is almost never observed. Similarly, interactions were detected for less than half of the "potential" convergent CTCF-bound motif pairs in one of the highest-resolution human Hi-C datasets to date [6] (and possibly, much fewer; see Note). Thus extra, unknown principles appear to need to be added to the "convergent rule" for predicting CTCF-mediated loop formation, let alone their possible role in organizing TADs.

Looking beyond CTCF … and beyond loop extrusion?

The other major class of TAD boundaries, often independent of CTCF binding, is active genes [2,4,11,12]. Although the loading and tracking of RNA polymerase during transcription causes topological changes on the underlying DNA [35], the link to TAD organization is much less clear. Bound transcriptional machinery may conceivably block cohesin loop extrusion, and cohesin is indeed enriched at CTCF-negative TAD borders containing active genes [11]. Further, in the absence of CTCF, cohesin is predominantly found at active promoters [36], although it is unclear whether this represents stalled loop extrusion points or the initial cohesin

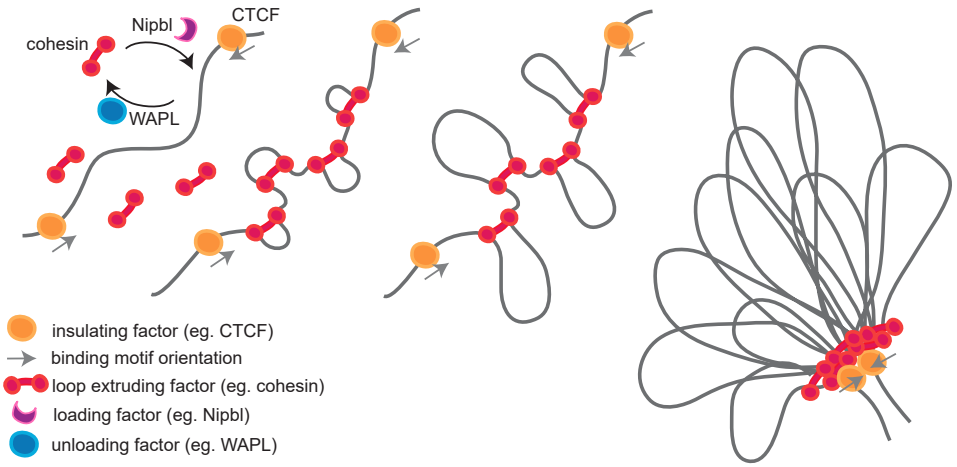

Fig. 2. Loop extrusion as a model for TAD formation. Cohesin is continuously loaded and unloaded from interphase chromatin, and bidirectionally extrudes loops while engaged on chromatin. Elements forming a roadblock to extrusion, such as the collision of convergent CTCF-bound sites, become a more stable barrier for the extruded TAD.

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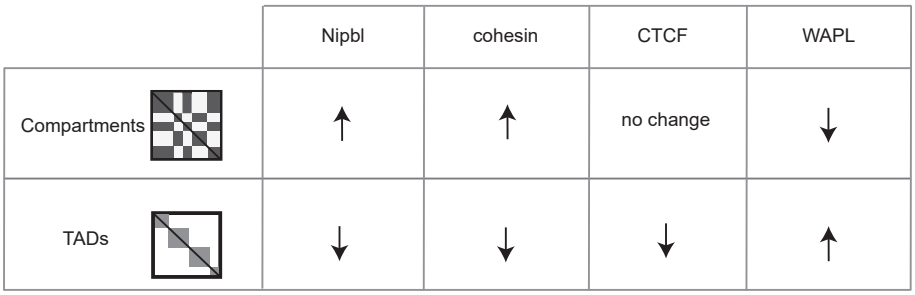

Fig. 3. TADs and compartments seem to be competing chromosome topologies. Summary of the effect on compartments or TADs on knockout or acute depletion of different factors involved in cohesin-mediated loop extrusion.

loading sites themselves. A direct link between transcription and loop extrusion has still to be demonstrated. Interestingly, the relatively small subset of TAD borders, which are altered during cell differentiation, corresponds with developmental gene expression changes, whereby borders are stronger at induced genes [11]. Similarly, progressive collinear activation of *Hox* gene clusters is associated with one repressive TAD-like domain splitting to a growing, active domain and a shrinking, repressive domain, with the border tracking to the transition point between active and silent genes [37,38]. An even more striking correlation between transcription and "contact insulation" (i.e., TAD border strength) is observed in early embryogenesis. Both mammalian and Drosophila zygotic chromosomes have no or very weak TADs, which are progressively strengthened throughout embryogenesis, particularly when the genome is activated and zygotic transcription starts [39–41]. It is important to note, however, the mounting evidence pointing against a simple causal link between transcription and TAD organization. First, when comparing developmental stages, the numbers of differentially expressed genes is far greater than the numbers of altered TAD borders. Second, pre-zygotic transcription embryos have very weak TADs in the same positions as later stages, suggesting that their borders are defined independently of ongoing transcription. Third, the TAD organization in transcriptionally shut-down spermatocytes is essentially the same as in somatic cells [41]. Fourth, transcription elongation inhibition with drugs only has mild effects on the TAD organization of early embryos [39–41] or apparent cohesin loop extrusion [27]. Finally, ectopic induction of a gene in embryonic stem cells, which forms a TAD border concomitant with gene expression during neural differentiation, had no effect on local TAD organization [11]. Therefore the link between transcription and TADs appears to be indirect and complex.

The very first Hi-C maps had insufficient sequence coverage to characterize TADs, but identified large chromosome regions, termed compartments, with a peculiar organization in that chromatin belonging to a particular compartment type preferentially formed homotypic interactions, and were preferentially localized away from different compartment types (see Fig. 1) [42]. Although greater numbers of compartment types can be identified from close Hi-C analysis [6,43], much of the interaction landscape can be explained by partitioning the genome into two compartments, with one predominantly containing transcriptionally active chromatin, and the other containing gene deserts and repressed genes. Developmental transitions are accompanied by large-scale changes in compartment identity, intimately linked to underlying gene expression [9]. Until recently, the link (if any) between compartments and TADs was unclear. Drosophila TADs appear to form homotypic contacts at the level of entire TADs, consistent with a higher-order organization defined by compartment identity [4]. Such a hierarchy is less evident within mammalian Hi-C maps but has been reported on closer analysis [44]. However, TADs and compartments were subsequently found to be mechanistically decoupled (see also Fig. 3); CTCF abrogation weakened most TADs, but compartment organization was left intact [34]. More strikingly, experiments disrupting (perturbing cohesin or cohesin-loading factors) [24–26] or enhancing (perturbing factors unloading cohesin) [28] loop extrusion caused bolstering or dampening, respectively, of compartments, suggesting that the mechanism organizing compartmentalization may actually compete with cohesin/CTCF-mediated TAD organization. Ultra-high resolution Hi-C studies of Drosophila, and eukaryotic species lacking CTCF, found that seemingly coarse compartments can actually be resolved to very small "compartmental domains," entirely explained by alternating runs of gene activity and inactivity [45]. This fine-scale organization is less apparent in large mammalian genomes but was also observed. Very recently, a Hi-C variant able to give pairwise interaction maps at nucleosome resolution (MicroC), was reported to uncover "micro-TADs" at the level of single gene units [46]. Overall, these findings suggest that very local

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chromatin state, particularly transcriptional activity, can influence local domain structures, perhaps autonomously. Unclear mechanisms, which may involve self-organizing principles (for example, genomic loci sharing bound factors which co-associate are more likely to be stabilized by the exchange of factors between them [1,47]) and/or phase separation causing aggregation of RNA polymerase-bound loci [48], can then promote homotypic interactions between active or inactive small domains in the formation of compartments. Somehow, this "ground state" of the folded chromosome may have an extra organization imposed upon it in mammalian cells by loop extrusion principles, with the boundaries of these TADs largely (but not completely) defined by CTCF. Although these principles can be recapitulated in physical models [31], more work needs to be done to explore their generality. For example, it is unclear whether transcriptionally active regions act additionally as TAD borders by acting as roadblocks for cohesin-mediated loops, whether additional promoter-enhancer looping interactions disrupt or reinforce other spatial organization principles, and/or whether the TAD "border" of an active gene is actually a very small active compartment, disrupting the organization of flanking repressive compartments.

TADs as genuine but extremely heterogeneous physical domains

Conventional Hi-C reports the average interaction map of millions of fixed nuclei, raising the question as to whether TADs are the genuine physically folded domains proposed from initial studies, or are instead just a statistical phenomenon. More recent adaptations of the Hi-C method to single cells revealed a large heterogeneity in chromosome structure and nuclear organization [49–51]. Interestingly, although their exact fitted structures can be highly variable, TADs were consistently identified in nearly all nonmitotic cells. Complementary high-throughput and/or super-resolution microscopy studies, labeling DNA sequences in individual fixed cells by *in situ* hybridization, showed a similar prevalence of TAD structures with a very large heterogeneity [52–54]. Closer analysis of numerous pairwise interactions further showed that TADs appear to be made up of a plethora of low-frequency interactions, with no one specific looping interaction appearing to define a TAD in every cell [55,56]. Interestingly, TAD-like domains were observed in individual cells by microscopy studies after cohesin depletion but were no longer delimited by CTCF/cohesin sites [52]. Since the domain borders were highly variable from cell to cell on cohesin depletion, population-averaged Hi-C approaches could not detect these structures. Thus, chromosomes appear to form genuine physically folded domains, in many but not all cases corresponding well to TADs called by Hi-C methods, but with a large degree of cell-to-cell variability. Two related open questions are whether different domain structures observed within cell populations correlate with transcriptional status, and how dynamic domain structures can be within the cell cycle of a particular cell. Cutting-edge microscopic techniques can now incorporate the high-coverage *in situ* hybridization of DNA and nascent RNA molecules [57,58]. Initial studies show a slight correlation between chromatin substructure and transcription of underlying genes, although chromatin structure is not necessarily a good predictor of gene activity. Addressing the second question requires the extension of live chromatin imaging techniques to entire domains, an ambitious but exciting prospect.

Are TADs functionally important?

Due to their link to insulator proteins, TADs have been proposed to delimit functionally "autonomous" parts of the genome, in particular by restricting the

Table 1. Nonexhaustive summary of genetic perturbation studies performed at TAD borders around model developmental genes and their consequences on ectopic gene expression.

Locus	Genetic perturbation	Expression phenotype	Ref
Xist	58 kb border deletion	Yes	[3]
Xist	40 kb border inversion	Yes	[55]
Xist	70 kb border (plus *Xite*) inversion	Mild	[55]
Epha4/Pax3/Wnt6	1.67 Mb border deletion	Yes	[49]
Epha4/Pax3/Wnt6	1.47 Mb near-border deletion	No	[49]
Epha4/Pax3/Wnt6	~600 kb border deletion	Yes	[77]
Epha4/Pax3/Wnt6	493 kb near-border deletion	No	[49]
Epha4/Pax3/Wnt6	1.05 Mb border inversion	Yes	[49]
Sox9/Kcnj2	400 kb intra-TAD duplication	Yes	[54]
Sox9/Kcnj2	1.6 Mb inter-TAD duplication	No	[54]
Sox9/Kcnj2	Deletion of four CTCF sites at border and five intra-TAD CTCF sites	No	[51]
Sox9/Kcnj2	Inversion of border (four CTCF sites)	Yes	[51]
Hoxa	Single CTCF deletions	Yes	[57]

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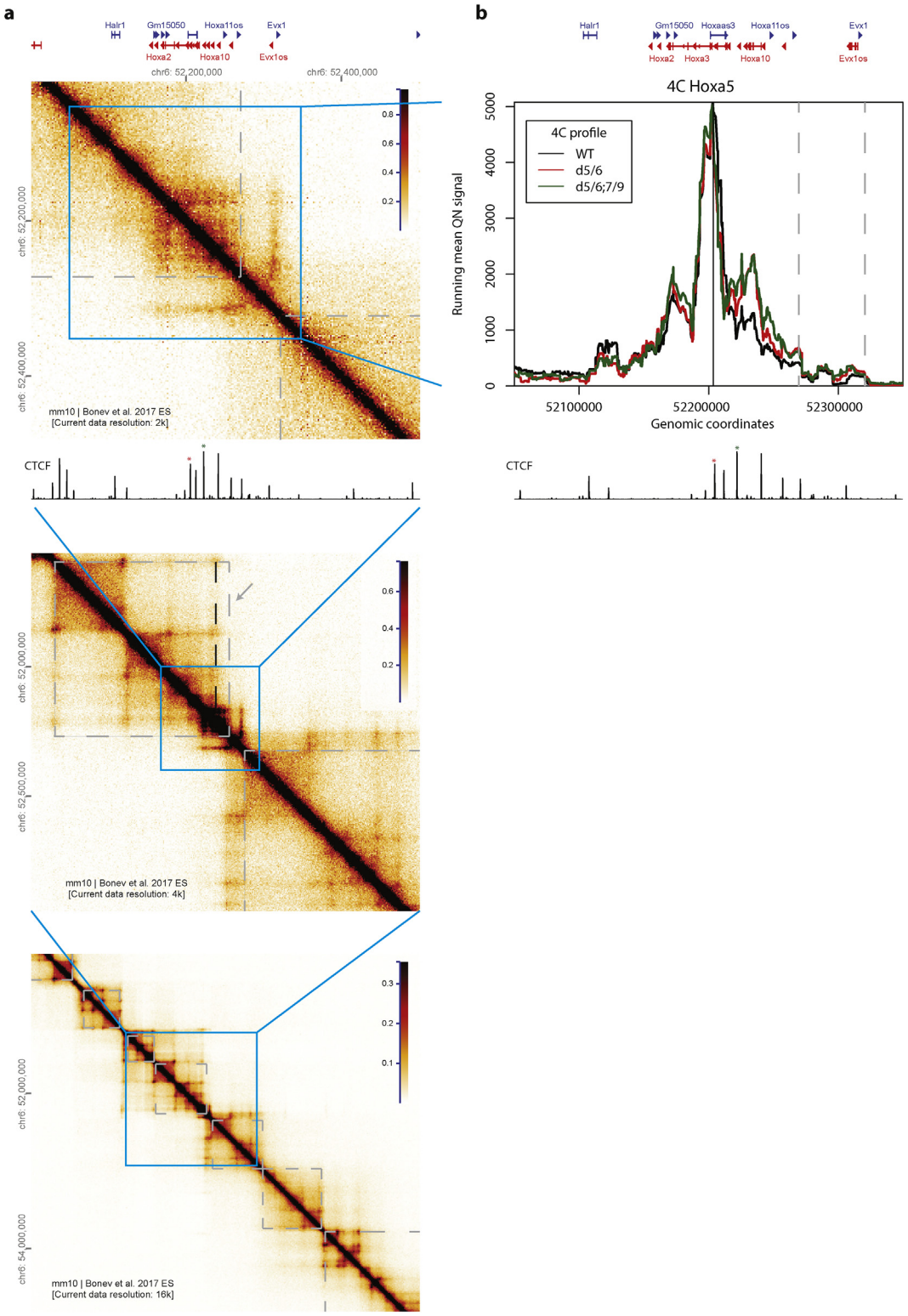

Fig. 4. Different chromatin structure-function relationships are observed at different scales, suggesting that analyses need to take all scales into account. a) High-resolution Hi-C maps from mouse embryonic stem cells [11], visualized with HiGlass [75], depict different zoomed-in views centered on the *Hoxa* locus. Blue squares indicate the region plotted in the subsequent zoom, going from bottom to top. At larger window sizes (bottom), hierarchical TAD structures are apparent as ‘‘squares within squares’’ on the map diagonal, representing more frequent intradomain than interdomain contacts. Foci of stronger Hi-C signal at the ‘‘corners’’ of these squares are CTCF-mediated anchors of the ‘‘loop domains’’ proposed to be

operational range of distal regulatory elements such as enhancers [1]. Well-characterized enhancers were frequently found in the same TAD as their target gene. More formally, enhancer trap assays found that reporters falling within the same TAD had largely the same expression patterns [59], and genome-wide analysis of promoter-enhancer chromatin loops found a strong predominance of intra-TAD contacts [60]. In addition to preventing aberrant interactions between promoters and inappropriate regulatory elements, the TAD organization may also make cognate promoter-enhancer interactions more efficient by reducing the search space of the two loci [61]. However, the aforementioned perturbation studies that removed TADs had only mild effects on the transcriptome [24,25,34], suggesting that domain insulation is largely not required for appropriate gene expression regulation. Recent elegant genome engineering studies within key developmental loci have found that altered TAD structures can indeed deregulate the expression of genes under complex control from multiple regulatory elements. However, the results from just this handful of model loci suggest the phenotypic consequences of altered TADs are context-dependent and difficult to predict from any "rules" (see Table 1 for an overview). In the X-inactivation locus and the *Wnt6/Ihh/Epha4/Pax3* region associated with many genetic diseases affecting limb formation, quite large deletion events (58 kb to > 1 Mb) at a TAD border cause fusion of the flanking TADs and ectopic expression of certain genes due to increased contacts with aberrant enhancers [3,62]. In some cases, this gene deregulation is sufficient for the underlying genetic disease. Interestingly, slightly smaller deletions omitting the CTCF sites presumed to define the TAD border did not cause TAD fusion or ectopic transcription [62]. Similarly, somatic copy number alterations in cancers have also been found to disrupt TADs and create ectopic promoter-enhancer interactions [63]. At other loci, such as *Hoxd* or the *Kcnj2/Sox9* region linked to sex determination, TAD structures were resilient to even quite large "border" deletions [64,65]. In the case of *Kcnj2/Sox9*, TAD fusion could only be achieved by making compound deletions of the CTCF sites at the border and of multiple intra-TAD CTCF sites [64]. The dependence of TAD structures on architectures within the domains has been previously described [33], but has been largely overlooked by current models of loop extrusion, which only require a barrier at the borders. Even more interestingly, the TAD fusion at *Kcnj2/Sox9* had no transcriptomic or pathological consequences, although inversions or duplications at the same border produced very strong sex determination and/or limb development phenotypes [64,66]. Thus in this locus, the TAD organization is not required for an enhancer to efficiently find its cognate gene, nor do ectopic enhancer-promoter contacts necessarily lead to aberrant transcription. Instead, *Sox9* expression is reduced by an inappropriate dosage of *cis*-regulatory elements (in duplications, assuming that the whole regulatory environment remains in the same TAD [66]) or by being isolated from its enhancers (in inversions) [64]. Similar to the latter case, inversions within the X-inactivation locus can swap the *cis*-regulatory environment of the competing genes *Xist* and *Tsix*, leading to ectopic activation of the former in male cells [67]. Larger inversions, which move local regulatory elements along with the gene, have milder phenotypes. These case study experiments greatly enrich and refine the conclusions drawn from genome-wide studies on global abrogation of *trans*-acting factors, and are necessary to move from simplistic models to a fuller understanding of any links between the chromatin architecture and transcriptional control.

A question of scale?

Conversely to some of the phenotypic resilience described above, deletions of just one or two CTCF sites within the *Hoxa* locus alters the chromatin boundaries delimiting repressive histone modifications during neural development and can generate homeotic transformations [68,69]. Although these sites clearly define functional domains, they are not identified as TAD borders in extremely high-resolution Hi-C maps in embryonic stem cells, neural precursors, or neurons [11] (see Fig. 4). Altered contact profiles with the *Hoxa5* gene are observed by 4C (a 3C variant identifying all interactions with one bait of interest) when these CTCF sites are deleted in embryonic stem cells (Fig. 4) and become even more pronounced during neural differentiation [68,69], suggesting that potentially important

formed by loop extrusion [6,22,23]. A zoomed-in view of the *Hoxa* locus (middle) actually shows the whole gene locus forms a separate domain, whose rightmost border (gray dashed line with arrowhead) does not exactly coincide with the conventional CTCF-bound loop domain defining a conventional TAD (black dashed line). Specific CTCF sites whose deletions cause distinct phenotypes [68,69] are denoted by red and green asterisks (top panel) and are found inside all discernible domains. Gray dashed lines indicate the TAD borders called in the Hi-C study [11]. Cyan squares denote the regions of the map, which are zoomed for the next panel. b) 4C profiles from the *Hoxa5* bait for wild-type and different CTCF-deletion embryonic stem cells (data from Narendra et al. [69], processed and visualized as in Ben Zouari et al. [76]), showing subdomain contact profiles that are not apparent from the Hi-C data, which are altered on deletion of CTCF sites.

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functional architectures are also present at a level below discernible TADs. Does this mean that there are smaller "contact domains," like the "microTADs" recently reported [46], which may also be functionally relevant, or are different architectural principles involved? Computational calling of TADs is heavily dependent on both the Hi-C sequencing depth [6], and the assumptions of the algorithms used [70] (*There may also be other articles within this Special Issue to cite here*), particularly as the contact map patterns are often hierarchical and sometimes partially overlapping (Fig. 4). As sequencing depth of Hi-C experiments has increased, the literature has been filled with vague and confusing terms, such as "sub-TADs" (proposed to be developmentally dynamic sub-parts of stable TADs) [71], "micro-TADs" [46] and "mega-domains" (very large TAD-like structures found on the inactive X chromosome) [72], with a potential hierarchical link between them [44]. Closer analysis suggests that what may be considered "conventional" TADs from functional definitions (e.g., CTCF-mediated borders, cell type conservation, tendency to contain coexpressed genes), are not a special structural level of folding; hierarchical lower- and higher-scale domains are indeed present [73]. These findings raise questions. Which biochemical and physical principles define such chromosome folding: is a combination of loop extrusion and homotypic compartmentalization enough? If hierarchical domains are a ubiquitous feature of chromosome folding, what determines whether they are functional and/or linked to transcriptional control? Despite a large research effort to tackle this question (e.g. Refs. [11,27,40,74]), conflicting findings still arise as to whether transcription is controlled by genome topology or *vice versa*, nor how direct any such links may be.

Conclusions

In summary, better and deeper Hi-C datasets, coupled with elegant genome engineering studies, have greatly expanded our knowledge on chromatin folding principles, in general, but on TADs in particular. General evidence points to chromosomes as folding into genuine physical domains, which are often defined by convergent CTCF sites, consistent with a loop extrusion model, likely performed by cohesins. TAD organization generally correlates very well with underlying chromatin accessibility, replication timing, epigenetic profiles, and transcriptional status, although the mechanistic link between transcription and TAD structure appears complex and possibly indirect.

Despite immense progress in recent years, more questions are raised than answered. Case studies on developmental gene models point to the dangers in making conclusions about the functional consequences of changes in genome topology from simplistic models. An integrative approach of genomics, microscopy, physical modeling, and genome engineering, promises to give the much-needed insight into what remains a fascinating and increasingly tractable question.

Note

Rao et al. report 9448 loop interactions from a "1 kb-resolution" Hi-C map of human lymphoblastoid GM12878 cells [6]. Of these, 3974 (42%) contain a CTCF motif on both anchors of the loop. Of these, 3642 (92%) are in the convergent orientation. A very small number of these interactions span very large distances, larger than known TAD sizes. We have filtered these loops to those within a megabase distance (3412), but this filtering does not change the conclusions.

ENCODE GM12878 CTCF ChIP-seq data gives 43361 bound peaks [78]. Of these, 28002 contain canonical CTCF motifs, as determined by PWM Tools [79]. The ChIP-seq called peaks containing multiple CTCF motifs were only included in the analysis if all motifs had the same orientation, leaving 27766 unambiguous CTCF-bound regions in a single orientation. Taking the genomic locations and orientations of these regions, we counted 6993 regions on the same chromosome and <1 Mb apart containing convergent, bound CTCF motifs with no other bound CTCF regions in between. Thus, 48% of potential CTCF-CTCF loops, based solely on the requirement of CTCF binding and unambiguous convergent orientation, have reported interactions in the Hi-C map. Since "transitive" CTCF loops, skipping CTCF regions whether in convergent or divergent orientations, were frequently observed [6], this estimated number of "potential" loops is likely very conservative, and the proportion of actual loops versus potential loops even smaller.

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