TADs: Dynamic structures to create stable regulatory functions

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Abstract

Mammalian chromosomes are organized at different length scales within the cell nucleus. Topologically Associating Domains (TADs) are structural units of 3D genome organization with functions in gene regulation, DNA replication, recombination and repair. Whereas TADs were initially interpreted as insulated domains, recent studies are revealing that these domains should be interpreted as dynamic collections of actively extruding loops. This process of loop extrusion is subsequently blocked at dedicated TAD boundaries, thereby promoting intra-domain interactions over the surroundings within the Hi-C matrix. In this review, we discuss how mammalian TAD structure can emerge from this dynamic process and we discuss recent evidence that TAD boundaries can have regulatory functions in this process.

Keywords: TADs, Cohesin, CTCF, enhancer-promoter looping, loop extrusion

Introduction

Mammalian interphase chromosomes, together adding up to around 2 meters in length, are subjected to a wide range of biological processes, including transcription, gene regulation, repair and replication. Yet, all this activity takes place in the crowded cell nucleus with a volume of less than 800 μm^3. How the 3D organization of chromosomes permits, or facilitates, these processes, is a topic of intense interest. Whereas initial discoveries on 3D chromosome organization were made using fluorescent imaging of fixed cells, nowadays a wide range of approaches are used. These approaches incorporate genomics, super-resolution and live-cell imaging, the combination of imaging and genomics for spatial and *in-situ* studies and biophysical polymer models of chromatin organization [1,2].

Within the mammalian cell nucleus, 3D organization of chromosomes emerges at different length scales. At the largest scale, as discovered using fluorescence microscopy, individual chromosomes occupy discrete zones called chromosome territories [3]. At a finer scale, the Hi-C assay—a combination of Chromosome Conformation Capture and whole-genome sequencing—has been instrumental to identify additional levels of organization [4]. Hi-C first revealed that chromosomes separate into multi-Megabase (Mb) compartments, so-called A and B compartments ([4], Figure 1a). These compartments are characterized by preferred homotypic interactions between compartments (*i.e.* A-A or B-B) over heterotypic interactions (A-B), creating in a checker-board pattern in a Hi-C interaction matrix. At a sub-Mb scale (typically between 500 kb – 1 Mb), a further level of domain organization appears ([5], Figure 1a). The human and mouse genome are divided into several thousands of Topologically Associating Domains (TADs), which exhibit more frequent intra-domain contacts over their surroundings. Comparison of TADs between cell types and mammalian species revealed a considerable degree of conservation, suggesting that TADs are a more constitutive and structural level of chromosome organization [5-7]. However, insulation between TADs is relatively limited, as intra-domain contact enrichment is only in the order of two-fold [5,8]. Within TADs, further structures can be observed, such as sub-TADs, contact domains and DNA loops between enhancers and promoters [9,10]. In the remainder of this manuscript, we will focus on the structure and function of 3D genome organization in mammalian cells at the level of TADs and within.

Main text

TADs as regulatory neighborhoods

Soon after the discovery of TADs, it was reported that enhancer-promoter pairs mostly localize within the same domain [11]. The insulated nature of TADs may thus create 'regulatory neighborhoods' that restrict inter-TAD loop formation ([8,12], Figure 1a). Indeed, most TAD boundaries are occupied by the CTCF insulator protein, which previously was recognized for its enhancer blocking activity [5]. CTCF binds an asymmetric DNA motif and most TADs are demarcated by convergently oriented motifs, which suggests that binding orientation may play a role in TAD formation ([6,10,13], Figure 1a and below). Since their initial discovery, TADs and other domains restricted by CTCF binding have been identified as units of DNA replication, recombination and repair as well [14-16].

The importance of CTCF binding at TAD boundaries to prevent enhancer hijacking—the inappropriate activation of a gene by an enhancer in a neighboring TAD—has been confirmed at several loci (*e,g.* [17,18]). During embryogenesis, correct hand development requires an intra-TAD interaction between the *EPHA4* gene and its distal enhancer. Various instances of structural variation involving the CTCF binding sites on either side of the *EPHA4* TAD (inversions, deletions, duplications) create chromatin configurations where separation between the *EPHA4* enhancer and genes in the neighboring TADs is lost (Figure 1b). For certain genes, this permits their ectopic activation and ultimately the emergence of hand malformations [17]. In *IDH*-mutant gliomas, the genome undergoes global hypermethylation, which interferes with site-specific CTCF binding. CTCF is therefore absent from an insulator that separates the *PDGFRA* oncogene from an enhancer in a neighboring TAD, thereby allowing enhancer hijacking and subsequent *PDGFRA* activation ([18], Figure 1b).

However, subsequent studies have added further nuance to these observations. Transcriptome analysis in mouse embryonic stem cells where CTCF was rapidly degraded, using the Auxin-inducible degron (AID) system [19], revealed that the nearly complete loss of TAD organization only caused the upregulation of a limited number of genes [20]. Despite their constitutive presence, TADs may thus only prevent a limited number of ectopic promoter-enhancer loops. Explanations for this observation may be that the cell-type specific activity of enhancers limits the potential for ectopic activation in a given cell type or that TADs are required for the establishment of promoter-enhancer loops after mitotic exit but not for their maintenance [21,22]. In parallel, several studies reported that ectopic gene activation and the fusion of neighboring TADs required the removal of multiple CTCF binding sites that were spread out over considerable genomic intervals [23-25]. Conversely, the creation of chromatin configurations where CTCF binding sites were positioned in-between promoters and enhancers revealed that the reduction in gene activity was incomplete [25-28]. Many boundaries between TADs may therefore be composed of sets of CTCF binding sites that are not impermeable and whose impact is restricted to specific cell types.

TADs as collections of actively extruding loops

The discovery of TADs raised the questions how these insulated domains are formed and how CTCF binding can act as a directional boundary. An important hint came from the previously identified colocalization of the Cohesin complex with CTCF [29,30]. The Cohesin complex—well-known for sister chromatid cohesion after DNA replication—is a member of the Structural Maintenance of Chromosomes (SMC) proteins that were proposed to have motor-protein functions [31]. The role of Cohesin in TAD formation was revealed by polymer simulations that could replicate the TAD structure in Hi-C matrices [32,33]. In these models, Cohesin is loaded on the chromosome followed by the bidirectional extrusion of a DNA loop (Figure 2a). Extrusion creates an increasingly large loop that compacts the chromosome until two possible events. Either a roadblock is encountered that halts extrusion on that side, but permits continued extrusion on the other side, or Cohesin dissociates from the chromosome and the loop dissolves (Figure 2a). Whereas CTCF appears to have evolved as a functional roadblock in this process, the encounter with large DNA-associated complexes (*e.g.* RNA polymerase II) may interfere with loop extrusion as well [6,7,34,35]. Consequently, these

polymer simulations revealed that TADs are not insulated domains, but rather a collection of actively extruding loops [32,33].

Numerous studies have contributed to the experimental validation of TAD formation by Cohesin-mediated loop extrusion. Live-cell imaging after biochemical reconstitution of the Cohesin complex has confirmed it possess a motor-protein function for bidirectional extrusion of DNA loops [36,37]. The recent observation of 'jet-like' structures that sprout perpendicularly from the Hi-C diagonal at narrow sites of Cohesin loading further confirmed the bidirectional nature of loop extrusion [38]. Multiple Hi-C studies confirmed that TAD structure was lost in cells where Cohesin was prevented from loading on the chromosome or where it was degraded (RAD21/SCC1-AID cells) [39-41]. The active and energy-consuming aspect of loop extrusion was confirmed in cells with Cohesin ATPase mutations [42]. Finally, single-cell Hi-C and super-resolution imaging studies revealed a large degree of cell-to-cell variation in TAD structure and intermingling. Instead, to obtain defined TAD structure as observed in a Hi-C matrix, data from large numbers of cells had to be averaged [43-46].

Combined with the notion that TADs are collections of extruding loops (see [32,33]), the observed cellular heterogeneity raised the idea that TADs are statistical properties of chromatin ([47,48], Figure 2b). Here, the observation of discrete TADs in population-averaged data is explained by chromosomal configurations where loops can be extruded anywhere in-between two TAD boundaries. In contrast, loops that cross TAD boundaries are rare. Live-cell imaging studies have determined loop extrusion dynamics, by measuring contacts between pairs of nearby CTCF binding sites [49,50]. In population-averaged Hi-C matrices, interactions between such pairs are enriched, creating visible 'corner dots' at the summits of TADs (Figure 2b). Although the distance between the CTCF binding sites was different between these studies (150 kb versus 505 kb), they both detected prolonged periods of contact (in the order of 10-30 minutes). In contrast, the time gap in-between contacts was different and increased with distance [49-51]. Biophysical modeling of looping kinetics indicated that the fully looped state (*i.e.* halting of loop extrusion by both boundaries) was a rare event. Instead, most of the time, the TADs were in a partially extruded state (*i.e.* combinations of loops that are being extruded or halted on one side). These results further confirm that TADs are transient and indicate that intra-TAD loop density is relatively minor [49-51]. The importance of intra-TAD loops was nonetheless confirmed by studies of enhancer-promoter loop formation in cells that lack Cohesin-mediated loop extrusion [52-54]. Whereas local interactions, in the order of 100 kb, were only mildly affected in the absence of loop extrusion, the formation of long-range loops (over 500 kb) was essentially lost. The dynamic nature of TADs is therefore essential to form long-range intra-TAD enhancer-promoter loops, which expands the function of these domains beyond the prevention of inter-TAD enhancer hijacking.

TAD boundaries as regulatory units

The formation of discrete TADs critically depends on the halting of loop extrusion at dedicated boundaries. This raises the question if TAD boundaries can influence loop extrusion, thereby providing means for regulation [8]. Most TADs carry CTCF binding sites in a convergent orientation at their boundaries, suggesting this has an influence on the blocking process [5,6,10,13]. Upon rapid depletion of CTCF (using CTCF-AID cells), TAD structure and pairing of CTCF binding sites was reduced, without interfering with loop extrusion itself [20,41,49,50].

Halting of loop extrusion requires the N-terminus of CTCF, which faces the extruding Cohesin complex when correctly oriented ([55,56], Figure 3). The STAG2 (also known as SA2) and RAD21/SCC1 subunits of the Cohesin complex can subsequently interact with the N-terminus of CTCF. Interestingly, the STAG2 subunit is present only in a variant of Cohesin that is associated with more rapid dissociation and the formation of shorter loops [57,58]. When bound to Cohesin, CTCF competes with the WAPL protein, which is responsible for the dissociation of Cohesin from the chromosome. Indeed, without WAPL, TAD structure was reinforced due to prolonged looping between CTCF binding sites [49,50,59]. For this Cohesin variant we can therefore envision that the CTCF-mediated halting of loop extrusion on one side promotes the formation of longer loops on the other side.

In parallel, the STAG1 (or SA1) containing variant of the Cohesin complex, which is more stably associated with the chromatin, is subject to a different regulation at CTCF binding sites ([41,57,58,60], Figure 3). The STAG1 variant is acetylated by the ESCO1 protein, promoting the association of Cohesin with the PDS5A protein. PDS5A interferes with the ATPase activity of Cohesin, thereby acting as a brake for loop extrusion. Upon halting at a CTCF binding site, this more stable Cohesin variant therefore reduces loop extrusion in the other direction as well. Combined with the regulation of Cohesin variant loading or processivity [61], the balance between loop extrusion promotion (STAG2 Cohesin variant) and reduction (STAG1 variant) may help to fine-tune the diversity of extruding loops, thereby regulating TAD structure and function.

Besides protein-protein interactions, CTCF binding at TAD boundaries itself may also influence the halting of the Cohesin complex. CTCF has an average DNA residence time in the order of 1-2 minutes, which is about ten-fold shorter than the Cohesin complex [62]. Upon CTCF dissociation, Cohesin may thus restart extrusion, resulting in the formation of longer loops. This possibility was confirmed when Cohesin dissociation was perturbed by removing WAPL, which dramatically increased the contacts between neighboring TADs [59]. The permeability of CTCF binding sites is further supported by the biophysical modeling of pairing between nearby CTCF binding sites, which requires the incorporation of a low chance (~12.5%) of loop extrusion blocking [49]. To improve the chance of successful halting of loop extrusion at a boundary, thereby improving the separation between neighboring TADs, multiple CTCF binding sites can be grouped together. Indeed, strong TAD boundaries are characterized by multiple closely-spaced CTCF binding sites [63,64]. Moreover, the complete intermingling between certain neighboring TADs is only achieved upon the removal of multiple CTCF binding sites [23-25]. In summary, the combination of protein-protein interactions, which particularly reduces loop extrusion capacity of the STAG1 variant, with sequential and prolonged halting at multiple DNA-encoded CTCF binding sites may sufficiently tip the balance toward Cohesin dissociation, thereby improving separation between TADs.

Conclusions and future perspectives

The discovery of TADs and their involvement in gene regulation has raised a tremendous interest in their structure-function relationships. Combinations of genomics, imaging and biophysical modeling have identified an active and dynamic mechanism for TAD formation that incorporates the creation of DNA loops through active extrusion by the Cohesin complex,

with halting at dedicated CTCF binding sites [32,33]. Whereas loop extrusion is essential for long-range intra-TAD enhancer-promoter loops [53,54], the sequential halting and regulation of loop extrusion at CTCF binding sites and ultimate the dissociation of Cohesin from the chromosome is sufficient to create separation between neighboring domains [17,18]. Combined, this intricately regulated process explains how dynamic and active loop extrusion can create TADs with stable regulatory functions.

An open question is if loop extrusion can maintain stable regulation over prolonged periods of time, for instance in post-mitotic human neurons where transcriptional programs are maintained for decades. Studies in post-mitotic mouse cells, several days after withdrawal from the cell cycle, confirmed that TAD structure is maintained [20,38,53]. Similarly, a recent Hi-C comparison of young (2 months) versus geriatric (over 28 months) mouse skeletal muscle stem cells confirmed that most TAD boundaries were preserved [65]. Yet, a considerable reduction in insulation at these boundaries was detected as well, suggesting that the efficiency of loop extrusion halting was compromised. It remains to be determined what is the underlying cause for this reduction and if this occurs in other post-mitotic cell types as well.

CRediT author statement

J. A. d. C-N. & D. N. Conceptualization.
J. A. d. C-N. & D. N. Writing- Original draft preparation.
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D.N. Supervision

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that influenced the work reported in this paper.

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Data availability

No data was generated for the research described in the article.

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Figures and legends

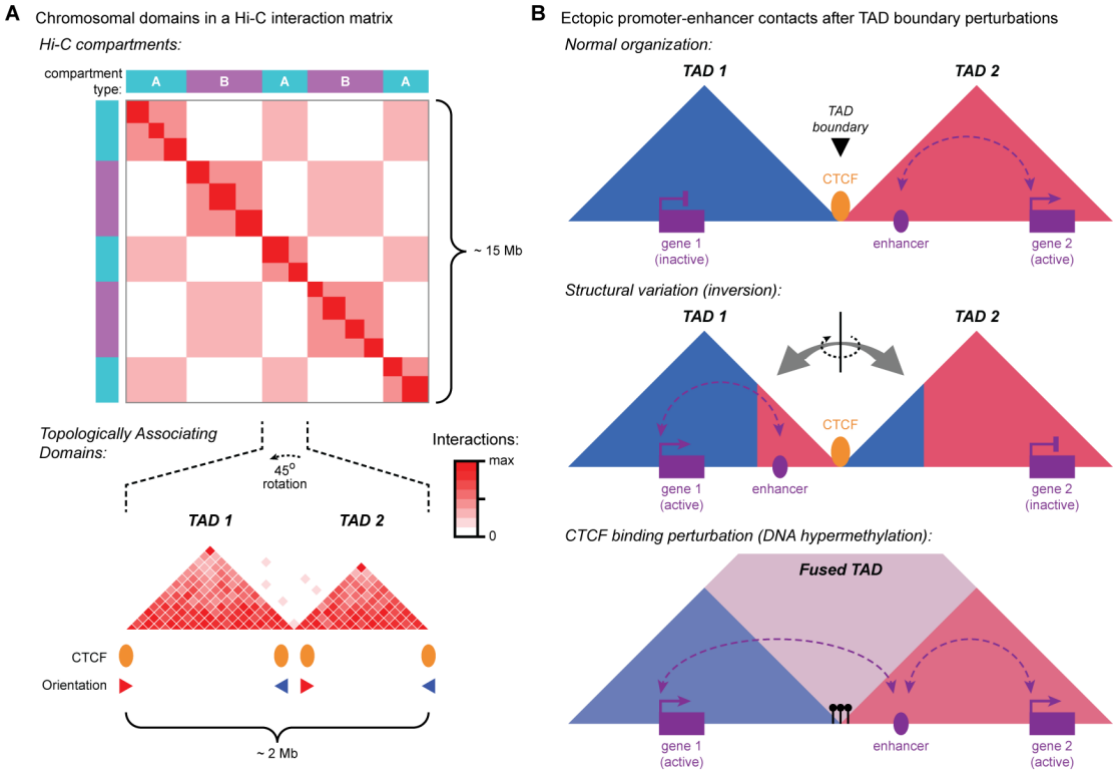

Figure 1: Chromosomal domains and TAD reorganization in disease.

a. Appearance of mammalian chromosomal domains in Hi-C matrices. Top: Hi-C compartments within an ~ 15 Mb chromosomal region. Hi-C compartments engage in local and long-range homotypic interactions, resulting in a checker-board pattern of interactions. Compartment A and B identity is indicated on the top and left. Bottom: Zoom in on TADs in an ~ 2 Mb chromosomal region. TADs are domains with enriched intra-domain interactions and frequent convergent CTCF binding at the boundaries.

b. Examples of enhancer hijacking due to TAD boundary perturbations. Top: normal TAD organization. TAD 1 contains inactive gene 1 and TAD 2 contains active gene 2 that forms a DNA loop with its enhancer. Middle: Example of a chromosomal inversion that brings the enhancer and gene 1 together in the same TAD, thereby allowing the formation of a promoter-enhancer loop and gene activation. Example based on [17]. Bottom: Example of CTCF binding perturbation, caused by DNA hypermethylation (lollipops), that causes the fusion of neighboring TADs. Gene 1 and the enhancer are in the same TAD, thereby allowing the formation of a promoter-enhancer loop and gene activation. Example based on [18].

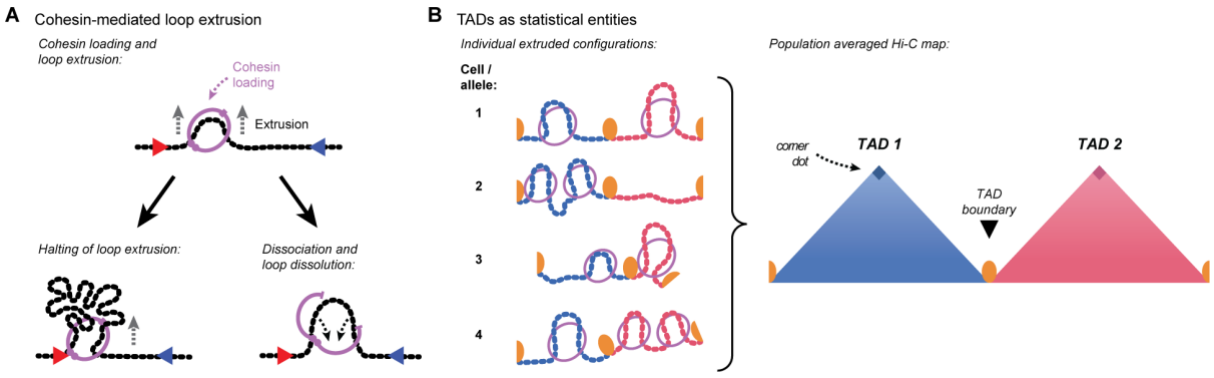

Figure 2: Loop extrusion and TADs as statistical properties of chromatin.

a. Steps of Cohesin-mediated loop extrusion. Top: The Cohesin ring (purple) loads on the DNA, followed by bidirectional loop extrusion (arrows). Bottom left: Loop extrusion is halted on one side when a roadblock is encountered (red arrow: occupied CTCF binding site in the correct orientation). Loop extrusion will continue unidirectionally on the other side, until a roadblock is encountered as well. Bottom right: The Cohesin ring dissociates from the DNA and the extruded loop will dissolve.

b. TADs as statistical properties of chromatin. Left: At a given time, different configurations of loops are extruded in individual cells. The density of loops is relatively minor and loops that cross the boundary are rare. Blue and red lines indicate the different regions covered by two TADs. Orange ovals indicate the boundary. Right: the averaged extruded configurations from large numbers of cells appear as an insulated TAD in a Hi-C matrix.

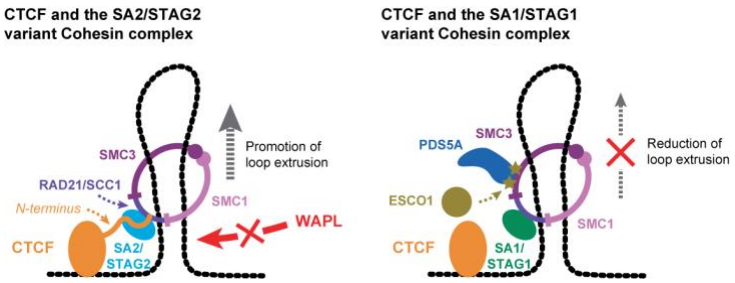

Figure 3: Different regulation of variant Cohesin complexes at CTCF binding sites.

Left: Promotion of loop extrusion of the SA2/STAG2 variant Cohesion complex at CTCF binding sites. Unidirectional loop extrusion is promoted through the direct interaction of the CTCF N-terminal tail with the SA2/STAG2 and RAD21/SCC1 Cohesin complex subunits. This interaction prevents the interaction of the Cohesin complex with WAPL, thereby preventing its dissociation from the chromatin. Figure compiled after [57,58]. Right: Reduction of loop extrusion of the SA1/STAG1 variant Cohesion complex at CTCF binding sites. The SMC3 Cohesin complex subunit is acetylated (stars) by ESCO1, followed by the recruitment of PDS5A and inhibition of Cohesin ATPase activity. Figure compiled after [58,60].