Topologically Associating Domains in Chromosome Architecture and Gene Regulatory Landscapes during Development, Disease, and Evolution

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The packaging of genetic material into chromatin and chromosomes has been recognized for more than a century, thanks to microscopy and biochemical approaches. This was followed by the progressive realization that chromatin organization is critical for genome functions such as transcription and DNA replication and repair. The recent discovery that chromosomes are partitioned at the submegabase scale into topologically associating domains (TADs) has implications for our understanding of gene regulation during developmental processes such as X-chromosome inactivation, as well as for evolution and for the search for disease-associated loci. Here we discuss our current knowledge about this recently recognized level of mammalian chromosome organization, with a special emphasis on the potential role of TADs as a structural basis for the function and evolution of mammalian regulatory landscapes.

The spatial organization of the eukaryotic genome is tightly linked to its functions, including the regulation of gene activity. Microscopy studies have been key for our understanding of how chromosome folding in the nucleus may be related to transcriptional regulation of different parts of the genome but have been limited by their relatively low throughput and resolution. The recent advent of chromosome conformation capture (3C) technologies (Cullen et al. 1993; Dekker et al. 2002; Denker and de Laat 2016) and next-generation sequencing has allowed a degree of molecular resolution that enables us to assess how DNA elements physically interact with each other, opening up exciting new perspectives for our understanding of long-range regulation of gene expression. The first 3C-based analysis at the whole-genome scale (termed Hi-C), using a human cell line, revealed the genome-wide existence of spatially segregated compartments of at least two different types, associated with open or closed chromatin, and spanning very large chromosomal regions (Lieberman-Aiden et al. 2009). Subsequent higher-resolution “C” studies, genome-wide and at the *X-inactivation center* locus, discovered that at the submegabase level, mammalian chromosomes are partitioned into domains of high frequency interactions, which were named topological domains (Dixon et al. 2012) or topologically associating domains (TADs) (Nora et al. 2012). TADs and their boundaries were found to be very well conserved across syntenic regions of mammalian chromosomes, first shown in human and mouse (Dixon et al. 2012) and later in macaque, dog, and rabbit as well (Vietri Rudan et al. 2015). Similar genomic domains were also

identified in the fruit fly—called “physical domains” (Sexton et al. 2012)—and later in different organisms, too; however, these are not necessarily equivalent, both structurally and/or functionally, to mammalian TADs (see Dekker and Heard 2015 for a review on topological domains across different organisms). TADs, as initially defined in mammalian cells, span an average of 900 kb (Dixon et al. 2012) and are often partitioned into smaller sub-TADs of a few hundred kilobases (Phillips-Cremins et al. 2013). The highest resolution “C” maps to date in mammals show the existence of even smaller domains, ~10–100-kb-long “contact domains” (Rao et al. 2014; Bonev et al. 2017). Among these different domains of chromosome folding, the relative stability of TADs across cell types and the conserved locations of TAD boundaries between man and mouse has led to their being proposed as “a structural basis for regulatory landscapes” (Nora et al. 2013). Indeed, in genome-wide association studies (GWASs), TADs can now be taken into account as a means of identifying disease-risk loci, based on potential regulatory variants in candidate single-nucleotide polymorphism (SNP) sets (Way et al. 2017). The concept of TADs, although still debated, has received increasing support from recent functional studies and will be discussed here. We will cover some specific examples, including the *X-inactivation center*, one of the loci where the implication of TAD organization in developmental gene expression was first put forward (Nora et al. 2012), and other complex regulatory loci, including *Shh* and *Hox* loci, where more recent insights have emerged.

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TADs: A FUNCTIONALLY PRIVILEGED SCALE IN THE CHROMOSOME FOLDING HIERARCHY?

The discovery of TADs was based on “C” techniques and supported by microscopy approaches: DNA fluorescence in situ hybridization (FISH) confirmed that sequences in the same TAD showed significantly higher overlap than sequences on either side of a TAD boundary (Dixon et al. 2012; Nora et al. 2012; Sexton et al. 2012). TADs are commonly defined as “domains within which sequences interact preferentially with each other, compared to their interactions with sequences outside.” However, this could be applied to almost all domains identified using C-technologies. Compartments, TADs, subTADs, and contact domains represent preferentially interacting sequences that seem to be insulated from adjacent interacting sequences. So are there any specific characteristics of TADs that would support their definition as distinct entities? Several functional properties have been attributed to TADs, including enrichment of active histone marks (Dixon et al. 2012), CTCF clustering at boundaries (Dixon et al. 2012; Berlivet et al. 2013; Fraser et al. 2015), transcriptional co-regulation (Le Dily et al. 2014; Nora et al. 2012), and enhancer–promoter communication (Shen et al. 2012; Nora et al. 2013). Do TADs correlate with these properties more than other types of domains?

Defining TADs: Function beyond Structure

In most studies so far, the algorithms used to identify TADs take into account changes in the direction, or sum, of the interaction frequencies (as a measure of insulation), but they also impose limitations such as domain length (see Forcato et al. 2017 for a comparison study of different algorithms). It has therefore remained unclear whether TADs are simply an arbitrary scale within a continuum of insulation levels of a nested hierarchy of domains, or whether they represent an intrinsic level of chromosome organization. Recently, this question was addressed by developing an algorithm that identifies and stratifies topological domains from genome-wide interaction frequency maps (Zhan et al. 2017), using a single parameter, reciprocal insulation between adjacent domains, to generate multiple sets of domains from a given map. With this algorithm, compartments, TADs, subTADs, and contact domains emerged at different values of reciprocal insulation, in a continuous spectrum of nested self-interacting domains (Zhan et al. 2017). TADs are therefore not a structurally privileged level within the spectrum of insulated domains. However, the authors found that functional properties previously attributed to TADs but sometimes also enriched at other domains (e.g., transcriptional co-regulation, active histone marks, CTCF clustering at boundaries, enhancer–promoter communication) were maximized at the TAD scale of reciprocal insulation (Zhan et al. 2017).

In summary, TADs cannot be defined solely based on their structural features, as they represent an arbitrary level within a continuum of increasingly/decreasingly insulated

domains. Instead a definition of TADs should take into account their functional significance as well. We thus propose that *TADs represent submegabase domains in which genomic elements interact preferentially with each other, maximizing specific functional properties of the genome.* Importantly the reference TAD “atlas” (Dixon et al. 2012) is actually a good approximation of the scale at which functional features are maximized (Zhan et al. 2017). For the sake of simplicity this is the working definition we use when referring to TADs in this review.

TADs Represent the Combined Interaction Frequencies from an Average Population

Most 3C-based techniques involve analyses of millions of pooled cells. What do TADs represent at the single-cell level? Polymer modeling recapitulating interaction maps within the *X-inactivation center* (*Xic*) region, has predicted highly variable, but nonrandom, conformations within TADs (Giorgetti et al. 2014), suggesting that TADs represent an averaged ensemble of multiple conformations across the cell population. DNA FISH confirmed this and highlighted the cell-to-cell variability regarding the shape, compaction, and spatial separation of *Xic* TADs (Nora et al. 2012; Giorgetti et al. 2014). Recently, single-cell Hi-C experiments confirmed variable cell-to-cell chromosome structures suggested by microscopy studies, with individual contacts at the megabase scale rarely surpassing TAD boundaries (Stevens et al. 2017; Nagano et al. 2013, 2017). Such cell-to-cell variability in TAD organization has important implications for the role of TADs in transcriptional regulation (Nagano et al. 2013; Giorgetti et al. 2014)—if TADs were stable entities in every cell within a population, *cis*-regulatory elements would be confined within a static chromatin configuration and the regulatory input in each cell would be equivalent. If instead TADs reflect an average of the interactions at the single-cell level, enhancer–promoter contacts emerge as probabilistic events in a fluctuating environment (Fudenberg and Mirny 2012; Nora et al. 2013; Giorgetti et al. 2014), providing variable regulatory input across the cell population. This could also explain cell-to-cell transcriptional heterogeneity (Amano et al. 2009; Vera et al. 2016). The relationship between the structural dynamics of TADs and the dynamics of enhancer–promoter interactions within them is still poorly explored and will have to be addressed with live imaging, which remains challenging in mammalian systems (see Fukaya et al. 2016 for a recent example in *Drosophila* embryos). The idea that such interactions are probabilistic as opposed to stable and directed is still debated and is discussed below in the context of recent models for domain and loop formation.

MECHANISMS AND DYNAMICS OF TAD ORGANIZATION

Scanning the topological map of a mammalian chromosome, TADs are as noticeable as the transitions between them. These are generally referred to as “boundaries,” but

they do not always demarcate sharp transitions and can sometimes correspond to rather large regions, with long transitions between TADs (Rocha et al. 2015). It should be noted as well that boundaries correspond to regions across which interactions are markedly reduced but not completely absent. Some TADs seem more insulated than others, which might reflect the strength of their boundaries. One study found that the level of enrichment of architectural proteins at TAD boundaries is correlated with their level of insulation (Van Bortle et al. 2014). It remains unclear, however, what forms a TAD boundary, and whether TAD boundaries represent insulatory elements per se, with specific characteristics such as binding of certain factors (see more below), or whether they arise as a result of adjacent, self-interactions between intra-TAD sequences. Probably both scenarios are possible and may even occur together. Structural elements within TADs have been proposed to help defining boundaries between TADs, by organizing the internal TAD structure and thereby preventing interactions with neighboring TADs (Giorgetti et al. 2014).

CTCF: A Role in Insulation and Loop Formation?

TAD boundaries are enriched in binding of CTCF (Dixon et al. 2012), a zinc-finger nucleic acid-binding protein, which nevertheless binds intra-TAD elements as well. Originally described as a transcription factor and insulator, CTCF was quickly recognized as a candidate for mediating TAD organization and loop formation (for review, see Merkenschlager and Nora 2016). Interestingly, conserved CTCF sites are mostly located at TAD boundaries, whereas species-specific CTCF sites, sometimes derived from retrotransposon expansion (Schmidt et al. 2012), are more often found inside TADs (Gómez-Marín et al. 2015; Vietri Rudan et al. 2015). It is unknown why some CTCF sites seem to participate in the formation of boundaries whereas others do not. Functional studies trying to address CTCF contribution to the topological organization of chromosomes suffer from CTCF being essential for development and cell proliferation, rendering knockout approaches difficult to interpret, whereas knockdown experiments are not efficient enough to completely deplete CTCF (Zuin et al. 2014). A recent study managed to overcome this by using an inducible degron system, which acutely depletes CTCF and can be reversible (Nora et al. 2017). Nora et al. (2017) found that CTCF is absolutely required for insulation of most TADs and loops between CTCF target sites in mouse embryonic stem cells and derived differentiated cells. Interestingly, loss of TADs was not accompanied by loss of active and inactive genomic compartments (Nora et al. 2017), suggesting that genomic organization in compartments does not depend on its folding in TADs, and that different mechanisms underlie their establishment and maintenance.

Molecular Models for the Establishment of TAD Organization

Models of “loop extrusion” (Riggs 1990; Blackwood and Kadonaga 1998; Kimura et al. 1999; Nasmyth 2001;

Alipour and Marko 2012) have been proposed to explain how TADs and chromatin loops arise at the molecular level (Sanborn et al. 2015; Fudenberg et al. 2016; Goloborodko et al. 2016)—an “extruding factor,” able to engulf two DNA chains and move along them, would extrude DNA until it reaches “stalling factors,” that block its progression; a DNA loop would thus be formed and stabilized. CTCF has been suggested as a “stalling factor,” whereas cohesin is proposed as the “extruding factor” (for review, see Merkenschlager and Nora 2016). Cohesin is a protein complex that forms a “ring,” involved in holding sister chromatids together after DNA replication and regulating their separation during cell division. Cohesin-bound sites very often overlap with CTCF-bound sites and several examples show that cohesin also participates in long-range *cis*-interactions (Hadjur et al. 2009; Degner et al. 2011; Guo et al. 2012). Acute depletion of either CTCF or cohesin lead to loss of TADs and loops (Nora et al. 2017; Rao et al. 2017) and mutations in factors involved in the loading or release of cohesin from DNA lead to differences in the length of the loops formed (Busslinger et al. 2017; Haarhuis et al. 2017; Schwarzer et al. 2017; Wutz et al. 2017).

Factors other than CTCF and cohesin might also contribute to the formation of boundaries in mammalian genomes. A proportion of TAD boundaries (<20%) remained unaffected upon acute depletion of CTCF (Nora et al. 2017), indicating that CTCF-independent mechanisms to establish and/or maintain TADs exist. This also implies that there is some heterogeneity among the domains globally classified as TADs. The highest-resolution Hi-C maps available to date show three classes of TAD boundaries: (i) CTCF-bound, (ii) no CTCF and proximity to active promoters, and (iii) no CTCF and no active marks, corresponding to repeat regions (Bonev et al. 2017), consistent with previous studies (Dixon et al. 2012). Cohesin is present in the CTCF-bound boundaries, as well as in those associated with active promoters (Bonev et al. 2017). Transcription seems therefore highly correlated with local chromatin insulation, and cell type-specific TAD boundaries are often associated with the activity of cell type-specific genes (Bonev et al. 2017). Transcription itself can influence the deposition of cohesin along the genome (Busslinger et al. 2017) or could possibly create torsional constraints leading to local insulation (Remeseiro et al. 2016). However, transcriptional activation per se was not sufficient to induce chromatin insulation and create a TAD boundary (Bonev et al. 2017). Considering that loss of boundary elements might lead to dramatic and severe phenotypic consequences (discussed later), the fact that TAD boundaries might be composed of different elements and/or involve different mechanisms suggests that this might represent an evolutionary strategy to buffer the potential effects of mutations at single elements (Lupiáñez et al. 2016).

TAD Dynamics during the Cell Cycle

Exploring how TADs are established (and maintained) at the mechanistic level is particularly relevant in the

context of cell cycle, given that TADs have to be reestablished after each cell division, as revealed by the absence of a compartmentalized organization in mitotic (metaphasic) chromosomes (Naumova et al. 2013). Furthermore, single-cell Hi-C revealed that TADs (as well as compartments, contact insulation, and long-range loops) are dynamic during the cell cycle (Nagano et al. 2017). Specific distributions of short- and long-range contacts characterize each phase of the cell cycle (Nagano et al. 2017), suggesting that the genome is not stably folded at any particular stage. This probably explains at least partially the high cell-to-cell variability in chromosome conformation observed by single-cell Hi-C (Nagano et al. 2013) or polymer modeling and DNA FISH (Giorgetti et al. 2014). Is there a topological memory from the previous cell cycle, or does loop extrusion (or other mechanisms) act completely *de novo* after each cell cycle? The cell cycle itself seems to be important to establish TAD organization. Acute depletion of CTCF in nondividing cells revealed that TAD structure collapsed to the same extent as in dividing cells (Nora et al. 2017), indicating that topological organization can be lost independently of cell division. However, when CTCF was restored, and contrary to dividing cells, structure was not fully recovered in nondividing cells (Nora et al. 2017), implying that the establishment of TADs depends on passage through cell cycle.

TAD Dynamics during Preimplantation Development

Another fundamental question concerns when and how TAD organization emerges during development, given that just after fertilization, the embryonic genome undergoes intensive remodeling in terms of its chromatin, transcription, and organization. Is TAD organization inherited from the gametes or established *de novo* in the developing embryo? The murine sperm genome, wrapped mostly with protamins, is bound by CTCF and cohesin and shows a topological organization similar to that of other cell types, such as mESCs and fibroblasts, but with a higher proportion of long-distance interactions (Battulin et al. 2015; Du et al. 2017; Jung et al. 2017; Ke et al. 2017). However, mature oocytes (arrested in metaphase of meiosis II) lack high-order chromosome structures (such as TADs) (Du et al. 2017; Ke et al. 2017) and their uniform folding configuration resembles that of the mitotic chromosomes (Naumova et al. 2013). Based on single-cell or low-input Hi-C protocols, the topological organization of the early embryo has been recently explored (Du et al. 2017; Flyamer et al. 2017; Ke et al. 2017). Although a full picture is still missing, certain trends emerge from these studies: (1) the organization of the zygotic genome and two-cell embryo is rather unique, with a “diffuse” structure; (2) the organization found in somatic cells (including TADs) seems to be gradually acquired and consolidated from one stage to the other during preimplantation development; and (3) the establishment of this organization is independent of transcription/zygotic genome activation but requires DNA replication. Which TADs appear first and

are they correlated with specific transcriptional programs and/or chromatin states? When a TAD is established at a specific stage, is it maintained in subsequent stages? Are there stage-specific TAD boundaries? Interestingly, cohesin and Wapl (a cohesin release factor) are involved in shaping the topological organization of the zygotic genome, compatible with the “loop extrusion” model (Gassler et al. 2017). Further refinement and functional studies will be necessary to complete our understanding of how TAD organization is set up during development.

TADs: A STRUCTURAL BASIS FOR REGULATORY LANDSCAPES?

The term “regulatory landscape” in the context of gene regulation was first used by the Duboule lab (Monge et al. 2003; Spitz et al. 2003) to refer to large genomic regions containing clusters of enhancers and the promoters within their reach. A reporter gene inserted at hundreds of genomic locations in mice revealed that the activity range of *cis*-regulatory elements extends over large domains, which strongly correlate with TADs (Symmons et al. 2014). Complete regulatory landscapes of many genes are indeed in the range of hundreds kilobases or more, as is the case for the *X-inactivation center*, the full extent of which, however, is still unknown—for an extensive review on the *Xic*, see Augui et al. (2011). Indeed, even the largest *Xic* transgenes tested so far (~460 kb) were found to be unable to function as single-copy ectopic *Xics* to induce X inactivation, and they do not fully recapitulate normal *Xist* expression patterns (Heard et al. 1999). Thus, the discovery that the *Xic* is partitioned into at least two TADs, spanning a total of ~800 kb, suggests that this might be the minimal *Xic* interval (Nora et al. 2012). Despite the largely invariant positioning of TADs across cell types (Dixon et al. 2012; Nora et al. 2012; Smith et al. 2016), variations in the internal conformation of TADs are frequently and reproducibly observed across different cell types (Nora et al. 2012; Dixon et al. 2015; Bonev et al. 2017), suggesting that TADs might represent a structural scaffold within which cell type-specific interactions can occur. In this section, we will discuss the accumulating evidence in support of the role of TADs in shaping and/or reflecting regulatory landscapes, as proposed when they were first discovered (Nora et al. 2013).

Physical and Functional Communication between Enhancers and Promoters

Many known pairs of enhancers and target promoters seem to be found within the same TAD (Shen et al. 2012; Nora et al. 2013), although several exceptions exist (Lower et al. 2009), and developmental genes often have enhancers spread across two neighboring TADs (see later). Several models have been proposed to explain the modes of action of enhancers (Kolovos et al. 2012), the most commonly cited being the “looping” model, whereby physical proximity in the nucleus between enhancer and promoter is required for their function. Numerous studies,

especially using 3C-based technologies support looping by showing that enhancers and promoters establish spatial interactions, with intervening chromatin looping out (Tolhuis et al. 2002; Sanyal et al. 2012; Shen et al. 2012), and at the β -globin locus, forcing looping between the β -major promoter and the locus control region (LCR) induces transcription (Deng et al. 2012). However, specific looping at the time of promoter up-regulation has rarely been shown, and despite the focus on interactions between promoters and enhancers, the most prominently detected interactions in mammalian genomes are those between CTCF bound sites. Whether this is because of the more dynamic or labile nature of the former or because of detection biases with 3C-based technologies that privilege the latter is still not clear. A recent digestion- and ligation-free method for capturing chromatin contacts (GAM, genome architecture mapping) found a particular enrichment for pairwise interactions between enhancer elements and active genes (Beagrie et al. 2017), suggesting that 3C-derived might be less efficient in capturing this type of loops. Interactions between CTCF sites have also been shown to contribute to promoter–enhancer communication in some cases (for review, see Merkenschlager and Odom 2013 and Merkenschlager and Nora 2016). This function of CTCF might be more directly related to its role in shaping TADs than in directly linking promoters and enhancers, as only a few enhancers bind CTCF and many reside far away from CTCF sites (Cuadrado et al. 2015).

Contacts between promoters and their *cis*-regulatory elements may be constitutive, present in all cell types with no association to transcriptional activation (Amano et al. 2009; Montavon et al. 2011; Jin et al. 2013; Ghavi-Helm et al. 2014), or established *de novo* in a cell type–specific manner, accompanying cell type–specific transcriptional activation (Tolhuis et al. 2002; Simonis et al. 2006; Bonev et al. 2017). The latter—the “instructive model” (de Laat and Duboule 2013)—implies that looping events depend on factors present only in specific cell types (Spilianakis and Flavell 2004; Vakoc et al. 2005; Deng et al. 2012; Bonev et al. 2017) and/or that cell type–specific epigenetic modifications influence whether or not a DNA sequence can be bound by TFs or architectural proteins. Constitutive, preformed interactions—the “permissive model” (de Laat and Duboule 2013)—can nevertheless be of functional relevance, as they can be associated with paused RNA polymerase (Ghavi-Helm et al. 2014), probably in a transcriptional poised state because of the lack of a specific set of transcription factors. This could be a rapid way to render cells permissive for transcriptional activation at specific stages or in specific tissues once those transcription factors become present (Amano et al. 2009). Another possible role for these preformed contacts between promoters and enhancers might be to prevent them from establishing interactions with other elements, as proposed in (Lonfat and Duboule 2015)—indeed, ectopic interactions might lead to dramatic phenotypic consequences, as discussed in the last section. The instructive and permissive models are not mutually exclusive: in some contexts, *de novo* contacts can accompany transcriptional activation within a preformed interacting domain

(Montavon et al. 2011). Here, the preformed contacts might contribute to the stabilization of the overall structure—the TAD structural scaffold—to allow the new interactions to be properly established and/or maintained.

In summary, based on current findings, it is likely that correct spatiotemporal gene expression is most often achieved via a combination of insulation from ectopic interactions and permissiveness for appropriate interactions, which usually but not always occurs within the limits of a TAD. A major part of what drives specificity of promoter–enhancer interactions within a TAD is likely to be factors binding to specific DNA sequences; however, cooperativity between transcription factors, presence of RNAs, and preexisting or induced chromatin states may all play a role in enabling the finely tuned usage of *cis*-regulatory elements for appropriate gene expression.

TADs Facilitate Enhancer–Promoter Communication

TADs seem to help the action of remote enhancers by reducing the effects of genomic distances: a recent study investigating a series of chromosomal rearrangements within the ~1-Mb-size TAD that contains the *Shh* gene (Symmons et al. 2016), revealed that reduced or increased intra-TAD distances had no impact on *Shh* expression nor on correct *Shh*-dependent limb development. However, if the TAD was disrupted as a consequence of genomic inversions that place a TAD boundary between *Shh* promoter and its limb-specific enhancer, the contact frequencies between enhancer and promoter—and their transcriptional output—became distance-dependent, leading to a spectrum of phenotypical alterations (Symmons et al. 2016). In this case it would appear that TAD organization indeed promotes distance-independent interactions between distant elements, which would otherwise interact only very sporadically, failing to trigger appropriate gene expression (Symmons et al. 2016). Consistent with a model whereby stochastic fluctuations within a TAD bring regulatory elements and target promoters into closer proximity, favoring transcriptional activation, we have reported fluctuations in TAD conformation coupled to fluctuations in transcription at the *X-inactivation center* locus (Giorgetti et al. 2014). Using sequential RNA–DNA FISH, we found that a more compact TAD correlated with higher expression levels of one of its genes, suggesting a scenario in which enhancers and promoter are in closer proximity (Giorgetti et al. 2014).

TADs and Transcriptional Co-Regulation

The description of TADs at the *Xic* was accompanied by the observation that, during differentiation of mouse embryonic stem cells, expression of genes within the same TAD showed coordinated dynamics (up- or down-regulation) (Nora et al. 2012). This correlation (median correlation coefficient [mcc] of 0.40) was significantly higher than for genes in different TADs (mcc of 0.03) or randomly selected (mcc of 0.09). This was later confirmed

beyond the *Xic*, genome-wide (Zhan et al. 2017), and also reported upon hormone stimulation, with up to 20% of the TADs showing coordinated up-regulation or down-regulation of the majority of the genes therein (Le Dily et al. 2014). TADs might be able to constrain diffusion of factors required for transcriptional activity and therefore may respond to transcriptional stimuli as a whole, as proposed in Nora et al. (2013) and Remeseiro et al. (2016). Physical clustering within TADs could therefore be used to coordinate gene expression programs during development. As mentioned previously, in the hierarchical folding of chromosomes, it is at the scale of TADs that the likelihood of genes within a domain being co-regulated during differentiation is maximized (Zhan et al. 2017).

Another curious example of an intimate link between TADs and transcription is that of the inactive X chromosome in mammals. This almost silent chromosome is mostly devoid of TAD structures (Minajigi et al. 2015; Giorgetti et al. 2016), except at the limited number of loci that retain transcriptional activity (Giorgetti et al. 2016). This raises the question of whether transcription at these loci drives their topological architecture, or whether these loci are transcribed because they retain their three-dimensional organization. Whichever may be cause or consequence, addressing such questions will provide additional insights into understanding the tight association between TADs and transcription.

TADs and Chromatin States

Differentially marked chromatin domains demarcate active and silent regions of the genome and the boundaries of such domains are often demarcated by TAD boundaries (Nora et al. 2012). Do such chromatin domains underlie TAD formation? In mouse embryonic stem cells (mESCs) deleted for the modifiers of H3K9me2 or H3K27me3, TAD organization at the *Xic*—which harbors a large domain of H3K9me2/H3K27me3—was unaffected (Nora et al. 2012), suggesting that TADs form independently of chromatin domains. Could TADs instead define such chromatin domains, by serving as modular units for the action of chromatin modifiers and limiting their spread beyond TAD boundaries (Nora et al. 2013; Ciabrelli and Cavalli 2015)? A recent study using a degron system to deplete the CTCF protein, which abolished most TADs in ES cells, revealed that H3K27me3 domains remained largely unchanged in this context (Nora et al. 2017). Furthermore, deleting a boundary CTCF element in the mouse *HoxA* locus did not lead to spread of H3K27me3, despite a shift in the interaction border (Narendra et al. 2015). However, the authors observed spreading of the active mark, H3K4me3, concomitant with the aberrant activation of previously repressed genes, affected by the boundary shift (Narendra et al. 2015). H3K4me3 spread could simply be due to ectopic gene expression, or it might reflect mechanisms regulating local spread of this chromatin mark (Narendra et al. 2015). Whether transitions between chromatin states can be directly dictated by TADs remains to be

disentangled from indirect effects of rewiring transcriptional activity, and the role of TADs and chromatin states in each other's formation or maintenance needs to be further explored.

TADs AND REGULATORY LANDSCAPES DURING DEVELOPMENT AND EVOLUTION

TAD organization may represent an evolutionary strategy to regulate developmental genes (for review, see Lonfat and Duboule 2015). Mammalian developmental genes often have multiple functions that are cell type- or stage-specific. It is therefore not surprising that they are frequently accompanied by complex regulatory landscapes. Examples of developmentally regulated loci are discussed below, although complex regulatory landscapes can also be found for broadly transcribed genes, including *Myc*, which has a different set of enhancers in ES versus B cells (Ruf et al. 2011; Kieffer-Kwon et al. 2013).

Developmental Genes Often Lie in Regions with Bipartite TAD Organization

We note that many developmentally regulated loci share a remarkably similar topological organization, with the gene promoter(s) lying close to or at the boundary between two TADs that harbor important *cis*-regulatory elements for their regulation (Fig. 1). Such locus architecture is found at the *Hox* clusters (Andrey et al. 2013; Lonfat et al. 2014), the *Xic* (Nora et al. 2012; Giorgetti et al. 2014), the *Six* genes (Gómez-Marín et al. 2015), and the *Tfap2c/Bmp7* locus (Tsujimura et al. 2015). The bipartite structure found at these developmental loci seems to be conserved across evolution, at least in certain animal lineages. The organization of the *Six* cluster is very similar in mouse, zebrafish, and sea urchin (Gómez-Marín et al. 2015), whereas *Hox* clusters are also found partitioned into two domains from mammals to fish (Woltering et al. 2014) and the *Xic/XIC* shows a boundary at the *Xist/Tsix* unit in both human and mouse (Dixon et al. 2012; Nora et al. 2012). This suggests that there are evolutionary constraints to maintain this highly conserved and particular organization in two adjacent TADs. For the *Hox* clusters, these constraints seem easier to understand—located at the boundary between two TADs, the locus needs to switch interactions at specific developmental stages to ensure proper segmentation of the developing limb (see Fig. 1 legend for more details). In the other cases (the *Six* clusters; *Tfap2c* and *Bmp7*; the *Xic*), however, the bipartite organization apparently serves to segregate distinct regulatory elements in two different TADs, either oppositely regulated or with different tissue specificities. Why then keep these domains adjacent to each other across millions of years of evolution if they represent different regulatory landscapes? To us, this suggests that cross-talk regulatory mechanisms probably exist between the two TADs at those loci, imposing evolutionary constraints and favoring the conservation of two adjacent TADs. Spitz and colleagues have reported that at the *Tfap2c/Bmp7* locus

Figure 1. Bipartite TAD organization of developmental genes. (A) Regulation of limb development by the *HoxD* cluster occurs in two phases, which depend on the usage of two different regulatory landscapes, one on each side of the cluster. Accordingly, the locus shows a bipartite TAD structure and lies precisely on the boundary between them (Andrey et al. 2013; Lonfat and Duboule 2015). Although genes in either extremity of the cluster interact preferentially with the TAD they are closer to, central *Hox* genes undergo a topological switch from one TAD to the other at specific developmental stages in specific regions of the developing limb (Andrey et al. 2013). This switch is accompanied by specific gene expression patterns, critical for the patterning of vertebrate limbs (Andrey et al. 2013; Woltering et al. 2014). This type of regulation seems to be present at the *HoxA* cluster as well, which shows similar expression patterns during limb development and a similar TAD organization (Lonfat et al. 2014; Woltering et al. 2014). (B) The *Xic*, the master regulatory locus of X-chromosome inactivation, is also partitioned into two TADs in the mouse (Nora et al. 2012), with an antisense transcription unit at the boundary, composed of the noncoding *Xist* locus and its negative *cis*-regulator *Tsix*. Although the *Xist* promoter is within a TAD with some of its known positive *cis*-regulators, the *Tsix* promoter seems to lie under the influence of the adjacent TAD. Like for the *HoxA* and *HoxD* clusters, each TAD seems important at a specific stage: Whereas genes within the *Tsix*-TAD are coordinately down-regulated during embryonic stem cell differentiation, the genes within the *Xist*-TAD are up-regulated (Nora et al. 2012). (C) *Tfap2c* and *Bmp7* lie close to each other and are active in multiple tissues during embryogenesis, independently regulated by a distinct set of enhancers (Tsujimura et al. 2015). Each enhancer set lies in a different TAD, controlling different tissue-specificities, with a “transition zone” (corresponding to a TAD boundary) in between the *Tfap2c* and *Bmp7* genes, which allows for enhancer competition in specific tissues (Tsujimura et al. 2015). (D) The *Six* homeobox gene clusters are also organized in two TADs, lying close or at the border between them, and the expression patterns of genes on each side of the boundary are markedly different (Gómez-Marín et al. 2015). This organization seems to be conserved in fish and in sea urchin (Gómez-Marín et al. 2015).

the insulation between the two TADs is not absolute—it probably never is between any given TADs—and that the position of *Bmp7* influences the expression of *Tfap2c* in *cis*, despite being located in different, adjacent TADs (Tsujimura et al. 2015). Cross-TAD communication might thus represent the evolutionary constraint that explains this highly conserved bipartite TAD organization of developmental genes.

TADs as Modular Units during Mammalian Genome Evolution

The presence of *cis*-regulatory elements can impose evolutionary constraints, disfavoring the break of synteny with their targets, even if they lie far away from each other (Ahituv et al. 2005; Kikuta et al. 2007). Considering that TADs might host regulatory landscapes, they would not only provide a structural basis for their function but also for their evolution (Nora et al. 2013; Acemel et al. 2017). The position of TADs is robustly conserved, at least in mammals (Dixon et al. 2012; Vietri Rudan et al. 2015), and current available data from evolutionary distant species suggests that synteny breaks found within TADs are rather uncommon (Ahituv et al. 2005; Dixon et al. 2012; Nora et al. 2013; Vietri Rudan et al. 2015; Acemel et al. 2017). Hadjur and colleagues uncovered a number of complex rearrangements between the genomes of mouse and dog, involving duplications, insertions, and inversions, and in each case, the rearrangement never occurred within a TAD but always at the border between two TADs (Vietri Rudan et al. 2015). This suggests that disrupting TADs and *cis*-regulatory landscapes has been negatively selected during evolution. Accordingly, most—if not all—examples in the literature that report disruptions of TADs are associated with deleterious effects (Groschel et al. 2014; Northcott et al. 2014; Lupiáñez et al. 2015; Flavahan et al. 2016; Franke et al. 2016; Hnisz et al. 2016; Vicente-García et al. 2017). Nevertheless, evolutionary changes can also happen within TADs, provided that gain or loss of regulatory elements do not strongly affect TAD structural organization (Acemel et al. 2017)—in support of this, interaction domains in mouse and zebrafish seem to have conserved boundaries but quite different TAD lengths (Woltering et al. 2014). The emerging scenario is that TADs can indeed be reorganized during evolution as intact modules, as proposed in Nora et al. (2013), and also that TAD boundaries can constitute important hotspots for genomic rearrangements during evolution, as noted elsewhere (Acemel et al. 2017).

Genome organization in TADs might also provide new possible mechanisms for the evolution of gene regulation. A recent study has shown that large-scale duplications can lead to the formation of new TADs, bringing together previously insulated regions and leading to aberrant gene expression and limb malformations (Franke et al. 2016). From an evolutionary perspective, as suggested by Gómez-Skarmeta and colleagues, this indicates that processes such as gene duplication and neofunctionalization (the process by which a gene acquires a new function

upon a gene duplication event), classically thought to occur in a stepwise manner, can actually occur simultaneously with the formation of neo-TADs (Acemel et al. 2017). Other chromosome mutations or rearrangements (such as deletions, inversion, and translocations) that split, fuse, or alter TADs in some way can also easily lead to gene expression changes (Groschel et al. 2014; Northcott et al. 2014; Lupiáñez et al. 2015; Flavahan et al. 2016; Franke et al. 2016; Hnisz et al. 2016; Vicente-García et al. 2017). It has also been shown that alterations in a single CTCF site, affecting its orientation or binding of the protein, might be enough to reshape the organization of the TAD and the loops between *cis*-regulatory elements and their targets (de Wit et al. 2015; Guo et al. 2015; Sanborn et al. 2015; Tang et al. 2015). TAD reorganization has thus a considerable evolutionary potential, if even a single mutational event could generate expression patterns in substantially different temporal or spatial contexts (Acemel et al. 2017).

TADs AND REGULATORY LANDSCAPES DURING DISEASE

Chromosomal rearrangements involving disruption or displacement of TAD boundaries, or fusion or fission of TADs, can result in gene expression alterations that underlie specific pathologies. A recent study illustrated this very elegantly by exploring in mouse models the changes in TAD structure and gene expression induced by genomic rearrangements characteristic of human patients with limb malformations (Lupiáñez et al. 2015). These rearrangements—either deletions or inversions—included boundary elements flanking an ~2-Mb TAD harboring a single gene, *Epha4*, which is expressed in the developing limb. Knockout of *Epha4*, however, does not lead to limb skeletal defects (Helmacher et al. 2000), implying that the rearrangements underlying the human pathologies do not involve *Epha4* loss of function. Mundlos and colleagues showed that these rearrangements allowed a set of enhancers within the same TAD as *Epha4*, probably responsible for the limb-specific expression pattern of *Epha4*, to establish new contacts with genes in the neighboring TADs. These genes (*Wnt6*, *Pax3*, *Ihh*) are normally not expressed in the developing limb at the same stage as *Epha4*, but the new ectopic interactions were accompanied by limb-specific activation of these genes, which likely underlies the limb malformations observed in mutant mice at birth. This study highlights the importance of TADs and their boundaries to restrict the range of action of *cis*-regulatory elements, but also how structural variants affecting TAD organization can lead to aberrant gene expression and morphological alterations in vivo. Other examples also illustrate this (Montavon et al. 2012; Franke et al. 2016).

Evidence for disruption of TADs in cancer contexts has also been reported, as a result of either chromosomal rearrangements or compromised CTCF binding. An excess of somatic mutations at CTCF sites is found in essentially all types of cancers, especially at sites involved in higher-

order chromatin structures, such as TAD boundaries (Katainen et al. 2015; Hnisz et al. 2016; Kaiser et al. 2016). CTCF sites can also be affected by DNA hypermethylation, as observed in a subset of gliomas, where this is associated with impaired CTCF binding, loss of insulation between TADs and aberrant oncogene activation (Flavahan et al. 2016). Chromosomal rearrangements, a hallmark of cancers, can also lead to activation of oncogenes by exposing them to the activity of new enhancer elements, a phenomenon known as “enhancer adoption” or “enhancer hijacking” (Groschel et al. 2014; Northcott et al. 2014) and a likely result of TAD reorganization. In addition, chromosomal rearrangements can result in loss of interactions, leading to functional haploinsufficiency (Groschel et al. 2014), a known cause of some cancer syndromes. Recently, microdeletions that eliminate TAD boundaries were recurrently found in T-cell acute lymphoblastic leukemia genomes (Hnisz et al. 2016). Young and colleagues have further showed that in nonmalignant cells, perturbing boundaries of TADs containing proto-oncogenes was sufficient to activate them (Hnisz et al. 2016). Together, these observations suggest that, at least in some types of cancer, disruption of TADs might actually represent a driver phenomenon of tumorigenesis (Kaiser and Semple 2017).

CONCLUSION

The discovery that mammalian genomes are partitioned in TADs (Dixon et al. 2012; Nora et al. 2012) has influenced our understanding of long-range gene regulation, shedding further light on the mechanisms that govern the communication between enhancers and promoters, as well as on the evolution of the regulatory landscapes they underlie. Several open questions remain, however. Are regulatory landscapes restricted by TADs or can they span several TAD boundaries? How deterministic are fluctuations in TAD structure in regulating enhancer–promoter communication? How dynamic is the establishment of TAD structure during preimplantation development in mammals? What is the functional significance of topological domains beyond mammals, how equivalent are they, and which mechanisms underlie the different domains? Further investigations will allow the disentanglement of context-specific mechanisms and pave the way to establish general rules orchestrating the dynamic establishment and maintenance of TADs during development, disease, and evolution.

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