The Role of Chromosome Domains in Shaping the Functional Genome

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The genome must be highly compacted to fit within eukaryotic nuclei but must be accessible to the transcriptional machinery to allow appropriate expression of genes in different cell types and throughout developmental pathways. A growing body of work has shown that the genome, analogously to proteins, forms an ordered, hierarchical structure that closely correlates and may even be causally linked with regulation of functions such as transcription. This review describes our current understanding of how these functional genomic "secondary and tertiary structures" form a blueprint for global nuclear architecture and the potential they hold for understanding and manipulating genomic regulation.

Eukaryotic genomes must be tightly folded and packaged to be contained within cell nuclei. Since initial observations of heterochromatin by Emil Heitz in the 1930s, it has become more and more appreciated that this packaging is highly organized and may be closely linked to transcriptional control. Over the last two decades, many studies have assessed the spatial proximity and nuclear organization of specific genomic loci, using microscopic techniques, such as fluorescent in situ hybridization (FISH), or molecular biology techniques, such as chromosome conformation capture (3C). Collectively, these studies demonstrated a correlation between chromatin topology and underlying gene activity, without resolving whether chromosome folding is a cause or consequence of genomic functions (Cavalli and Misteli, 2013; de Laat and Duboule, 2013).

Topology and activity appear linked at different scales within the nucleus. At the kilobase-to-megabase scale, distal regulatory elements such as enhancers were found to come into direct contact with their target genes via chromatin loops (Palstra et al., 2003). At the megabase scale, genes were observed to significantly co-occupy functional sites within the nucleus, such as foci of Polycomb proteins (Bantignies et al., 2011) or of active RNA polymerase (Schoenfelder et al., 2010), specifically in cells where the genes have the same activity. At the scale of the whole nucleus, chromosomes occupy discrete territories, which are non-randomly organized to place gene-poor chromosomes in the predominantly heterochromatic periphery and gene-rich regions in the euchromatic interior. The transcriptional activity of specific genes has been correlated with their nuclear positioning relative to the periphery, and more specifically the repressive nuclear lamina (Peric-Hupkes et al., 2010), as well as to their position relative to the bulk of the chromosome territory (Chaumeil et al., 2006). Intriguing recent work has even decoupled chromatin decondensation from transcriptional activation, showing that opening chromatin without concomitant gene activation is sufficient for relocalization of genes to the nuclear interior (Therizols et al., 2014). Overall, these case studies support a hierarchical, multi-scale model where expression of a gene may influence or be influenced by its local chromatin interactions, its associations with other potentially coordinately controlled genes and the regulatory environment provided by its nuclear location.

Average conformations of chromatin have been more systematically characterized by coupling 3C to high-throughput sequencing (Hi-C) to derive large catalogs of pairwise chromatin interactions within populations of nuclei (Lieberman-Aiden et al., 2009). Initial, lower-resolution Hi-C studies demonstrated that active chromatin predominantly associates with other active regions, and repressed chromatin associates with other silent regions with little inter-mixing of the two types (Lieberman-Aiden et al., 2009). More recently, high-resolution chromatin interaction maps revealed that metazoan genomes fold into distinct modules called physical domains or topologically associated domains (TADs), whereby genomic interactions are strong within a domain but are sharply depleted on crossing the boundary between two TADs (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). The presence of TADs is less clear for non-animal species. Although Hi-C is unable to give any information on TAD dynamics or cell-to-cell variability, the domains identified correlate well with many markers of chromatin activity, such as histone modifications and replication timing (Dixon et al., 2012; Sexton et al., 2012). TADs can also contain coordinately regulated genes (Le Dily et al., 2014; Nora et al., 2012). The described organization of the genome into functional domains containing different types of chromatin (Ernst et al., 2011; Ho et al., 2014) thus reflects the average folded state of the chromosome.

TADs appear to form the modular basis for higher-order chromosomal structures (Sexton et al., 2012), which in themselves may be built up from key stabilizing interactions between regulatory elements (Giorgetti et al., 2014). Such an arrangement is reminiscent of protein folding, whereby hierarchical stabilization

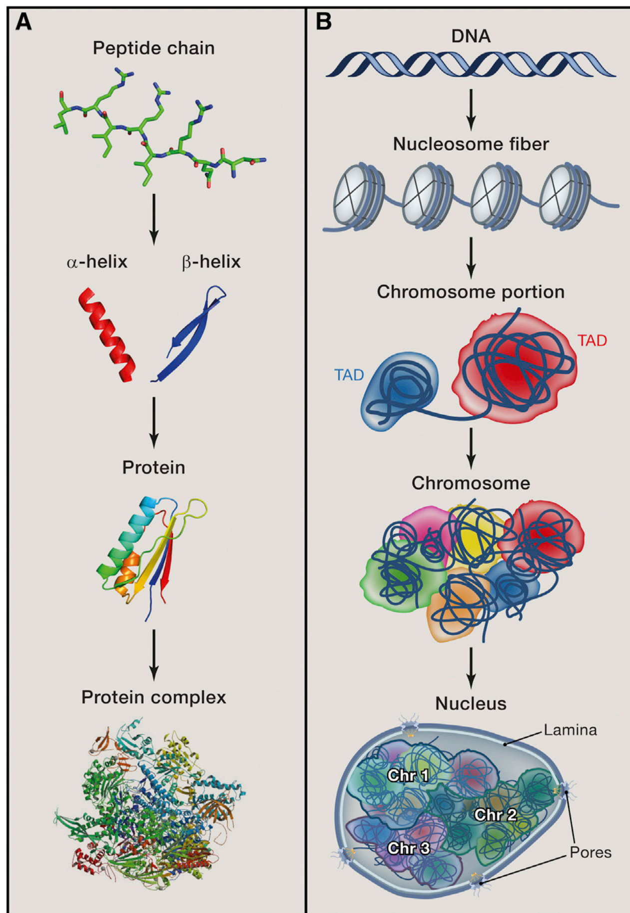

Figure 1. Analogous Hierarchical Organization of Protein and Genome Structure

(A and B) Primary structures comprising the amino acid or nucleotide sequence (packaged into a nucleosomal fiber in eukaryotic chromatin) on a single polymeric chain form locally stabilized interactions to fold into secondary structures, such as polypeptide alpha-helices or beta-sheets, or chromatin TADs. These domains in turn hierarchically co-associate to form a tertiary structure of a protein or chromosome. The co-associations of multiple, separately encoded subunits forms the final quaternary structure of a protein complex or entire genome. Protein structures taken or derived from the RCSB database (PDB 2KVQ, or 4BBR for quaternary structure).

of secondary structures such as alpha-helices leads to the final tertiary structure, whose conformation is crucial to protein function (Figure 1). Genome folding is not as rigidly or thermodynamically defined as protein structure—single-cell experiments reveal a high variability of adopted genomic configurations (Nagano et al., 2013; Noordermeer et al., 2011a). Further, it has not been shown that a specific chromosome structure is *essential* for genomic functions. However, considering chromosome topology as a principle of folding, and TADs as chromosomal secondary structures, is a useful starting analogy. Here, we discuss the relationship between DNA sequence (primary structure), genomic sub-structures such as TADs (secondary structure), overall chromosome folding (tertiary structure), and genome function, positing that TADs and other localized structures form a blueprint for coordinated genome control.

Chromatin Loops in Gene Regulation

Seminal studies of the beta-globin locus showed that the globin gene promoter more frequently interacted with distal enhancers than intervening sequence, specifically in erythroid tissue where the gene was transcribed (Palstra et al., 2003). Such results were confirmed for other enhancer-promoter combinations (Kieffer-Kwon et al., 2013; Li et al., 2012; Sanyal et al., 2012) and suggest that chromatin looping brings genes and their regulatory elements in close proximity. For simplicity, we will also refer to these phenomena as loops, although in many cases they are more likely to represent a statistical ensemble of transient contacts than true stable structures (Giorgetti et al., 2014). Many enhancer-promoter combinations share binding of common transcription factors, and enhancers are also frequently transcribed, especially when involved in interactions with target genes (Sanyal et al., 2012). Such chromatin loops are thus proposed to set up an "active chromatin hub," providing a chromatin environment more permissive to transcription than factors bound directly to the promoter alone (Mousavi et al., 2013; Palstra et al., 2003). In support of this model, enhancer-promoter interactions within the human *OCT4* locus, a gene encoding a key pluripotency transcription factor, distinguish induced pluripotent stem cells from non-reprogrammed cells (Zhang et al., 2013). The non-reprogrammed cells had equivalent binding of the inducing factors at the promoter and enhancer but no *OCT4* expression. However, it remains an open question whether chromatin looping is a cause or consequence of transcriptional activation. Recent elegant experiments have engineered chromatin loops within the mouse beta-globin locus by exogenously targeting the dimerization domain of the transcription factor Ldb1, which is naturally present at the enhancers of the globin locus control region (Deng et al., 2012; Deng et al., 2014). These induced chromatin loops could partially rescue adult beta-globin expression in mutants for erythroid transcription factors (Deng et al., 2012) or stimulate fetal globin expression out of its normal developmental context (Deng et al., 2014). Chromatin topology can thus be causally linked to transcriptional regulation. As the globin genes are very highly expressed in erythroid tissues, it will be interesting to see the functional consequences of induced chromatin loops in less transcriptionally permissive genomic and cell-type contexts.

The beta-globin active chromatin hub is progressively formed during hematopoiesis (Palstra et al., 2003) and involves binding sites for erythroid-specific transcription factors (Drissen et al., 2004 for example), so enhancer-promoter contacts were proposed to occur exclusively in cells where the target gene is being transcribed. Although many cell-type-specific chromatin loops have been characterized from more systematic approaches (Heidari et al., 2014; Sanyal et al., 2012), evidence is also emerging that chromatin topology and transcriptional regulation can be temporally uncoupled. A recent analysis of the interaction profiles of a hundred *Drosophila* mesodermal enhancers found that more than 90% of the interactions were detectable before mesoderm specification and were commonly linked to genes with paused RNA polymerase (Ghavi-Helm et al., 2014). This result suggests that chromatin loops may commonly poise a gene for expression but that another signal is required for complete transcriptional firing. In support of this model, induced looping within the beta-globin locus rescued transcription

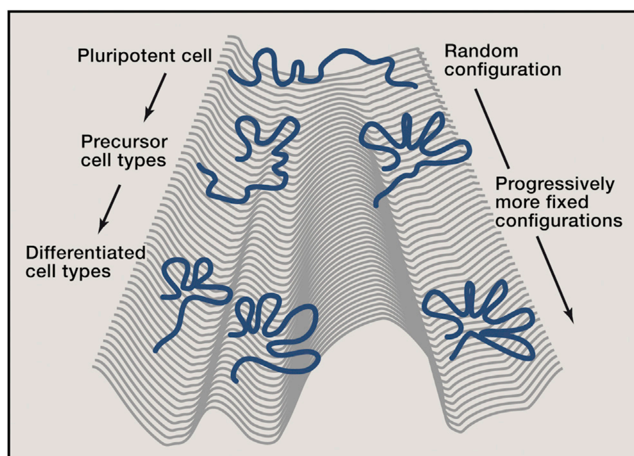

Figure 2. Waddington Landscape of Chromatin Loop Configurations throughout Development

Pluripotent cells able to form any lineage (top) have largely unstructured local chromatin topologies. Progressive lineage restriction throughout development, tracing paths through the landscape from top to bottom, may be accompanied by progressive constraint of the specific chromatin loop topologies as only a limited repertoire of enhancer-promoter contacts are permitted and fixed.

initiation, but not efficient elongation when the essential transcription factor GATA-1 was lacking (Deng et al., 2012). Furthermore, Hi-C analysis of a human fibroblast cell line showed conservation of enhancer-promoter interactions around responsive genes before and after treatment with the cytokine TNF-α (Jin et al., 2013).

These seemingly opposing views of enhancer-promoter chromatin loop dynamics may be reconciled by a Waddington landscape model of chromatin architecture (Figure 2). Non-expressed genes form more promiscuous contacts in pluripotent cells than in differentiated cells (de Wit et al., 2013; Splinter et al., 2011). Repertoires of tissue-specific interactions may then be set up in precursor cells as their differentiation potential is restricted, effectively limiting the sets of genes with a permissive chromatin environment for further induction. Fully differentiated cells may then benefit from their pre-formed active chromatin hubs for rapid transcriptional responses to appropriate signals. Although this model has yet to be formally assessed, chromatin states themselves exhibit a similar progressive developmental restriction (Zhu et al., 2013). Furthermore, there is more tissue-type variation in the chromatin states of enhancers than of promoters (Ernst et al., 2011). Finally, a recent analysis has suggested that enhancer-promoter interactions are variable in different cell types (He et al., 2014). Together, these data suggest that enhancers carry a large regulatory potential, and although the mechanistic details of *when* and *how* they stimulate transcription are not yet clarified, chromatin loops appear a ubiquitous means of relaying enhancer-promoter communication.

Architectural Chromatin Loops—Building up the Secondary Structures

In addition to specific transcription factors, ubiquitous proteins have also been linked to chromatin loops, in particular the insulator protein CTCF (Splinter et al., 2006), the cohesin complex (Hadjur et al., 2009), and the general co-activating Mediator complex (Kagey et al., 2010). Mediator is predominantly found at loops between promoters and enhancers and between promoters, in agreement with its general activation role (Conaway and Conaway, 2011). Consistently, Mediator-linked interactions are more cell-type-specific (Phillips-Cremins et al., 2013). In contrast, CTCF tends not to be present at enhancer-promoter loops. It is more commonly associated with constitutive, longer-range chromatin interactions (Phillips-Cremins et al., 2013; Sanyal et al., 2012), although some cell-type-specific CTCF-mediated interactions have been reported (Hou et al., 2010). CTCF is enriched at TAD borders (Dixon et al., 2012; Hou et al., 2012; Sexton et al., 2012), and CTCF-mediated loops are implicated in maintenance of TAD structure (Giorgetti et al., 2014) and are thus believed to play a more fundamental architectural role in chromosome folding. Various case studies have implicated CTCF-mediated loops in insulator function, preventing communication between distal regulatory elements (Kurukuti et al., 2006 for example). However, many CTCF sites have recently been shown not to be a barrier to enhancer-promoter interactions (Sanyal et al., 2012). The functional consequences of these more developmentally stable chromatin architectures are thus likely to be complex and context-dependent. Similarly, CTCF binding alone cannot account for TAD border function (discussed in more detail in later sections). Cohesins are associated with both cell-type-specific enhancer-based loops and constitutive, CTCF-mediated loops, although both types of loops can also be cohesin-independent (DeMare et al., 2013; Phillips-Cremins et al., 2013). In agreement, cohesin has been shown to interact with CTCF (Rubio et al., 2008) and forms direct complexes with Mediator (Kagey et al., 2010) and certain transcription factors (Wei et al., 2013). The cohesin complex comprises a ring structure that physically maintains sister chromatid attachment after DNA replication (Nasmyth and Haering, 2009). Though yet to be demonstrated, a similar structure could be envisioned to stabilize chromatin loops on cohesin recruitment. Abrogation of cohesin causes perturbation of chromatin loops with subsequent effects on transcriptional control (Hadjur et al., 2009; Seitan et al., 2013; Sofueva et al., 2013; Zuin et al., 2014). Overall, chromatin loops appear important for the possibly inter-linked functions of transcriptional regulation and maintenance of higher-order chromosome folding. A full proteomic appraisal of the factors present at chromatin loops may help us better understand how they are recruited to their specific sites in a developmental context and how and when they are able to effect looping.

Chromosomal Secondary Structures—"Facultative" and "Constitutive" TADs

The three-dimensional organization of many metazoan genomes into discretely folded kilobase-to-megabase sized TADs is particularly striking due to their agreement with many linear (or one-dimensional) measurements of chromatin activity; for example, histone modifications (Dixon et al., 2012; Sexton et al., 2012), coordinated gene expression (Le Dily et al., 2014; Nora et al., 2012), lamina association (Dixon et al., 2012), and DNA replication timing (Dixon et al., 2012; Pope et al., 2014).

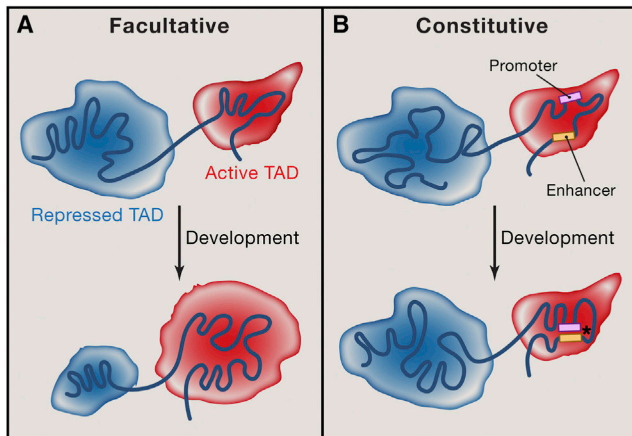

Figure 3. Facultative and Constitutive TAD Models of Regulated Developmental Gene Expression Programs
(A) Active (red) and repressed (blue) chromatin domains form separate facultative TADs which spatially segregate their regulatory environments. During development, some genes are activated and leave the repressive TAD to enter the growing facultative active TAD by a shift in the boundary between TADs.
(B) Boundary positions do not change in constitutive TADs. Gene expression changes are effected via altered intra-TAD chromatin interactions; for example by developmental stage-specific presence of enhancer-promoter chromatin loops (asterisk; positions of sequences participating in this loop in both cell types are highlighted in yellow and pink).

TADs thus appear to be chromosomal secondary structures that reflect a tendency to divide the genome into distinct, autonomously regulated regions. This model is supported by the finding that TADs determine the scope of most enhancers' activities (Ghavi-Helm et al., 2014; Shen et al., 2012; Symmons et al., 2014). The mechanisms of TAD establishment and maintenance are largely unknown. In particular, a critical issue to be resolved is whether TADs constitute a structural blueprint that defines chromosome architecture within which gene regulatory changes are overlaid, or are themselves dynamically built by transcriptional silencing or activation machineries. A case in point for TAD organization by transcription arises from studies aimed at understanding the spatial and temporal collinearity of mouse Hox gene expression. These genes are sequentially activated during development, and according to anterior-posterior body position, in order along the chromosomal fiber. The active genes are marked by trimethylation of lysine-4 of histone H3 (H3K4me3) and the silent regions are coated with trimethylation of lysine-27 of histone H3 (H3K27me3). Hox gene activation is accompanied by a transition in the chromatin modification (Soshnikova and Duboule, 2009). Strikingly, the Hox gene loci form distinct topological domains which mirror these chromatin domains precisely, with the active domain expanding and the silent domain shrinking according to collinear gene activation (Noordermeer et al., 2011b). Such a dynamic model of chromosome topology implies that "facultative TADs" spatially confine co-regulated genomic regions but may actually be defined by the underlying transcriptional activity and/or chromatin state (Figure 3A). However, ablation of H3K27me3 in mouse ES cells by knockout of the Polycomb group gene *Eed* had no effect on TAD structures around the X-inactivation locus (Nora et al., 2012). Further, genome-wide comparisons of TADs in disparate mouse and human cell lines and tissues revealed that most TADs seem invariant with cell type (Dixon et al., 2012). Although many TADs at gene deserts or clusters of ubiquitously expressed housekeeping genes would not necessarily be expected to change in these different cell types, the large number of "constitutive TADs" suggests that many are genuine chromosomal secondary structures. These may thus represent a ground state spatial configuration on which subsequent regulatory features are overlaid (Figure 3B). In support of this view, entire TADs containing coordinately responsive genes to progesterone treatment can be structurally re-modeled while their borders remain unchanged (Le Dily et al., 2014). In between these extreme views of chromosome topology, high-resolution analysis of a handful of TADs during ES cell differentiation identified them to be predominantly stable but noted developmental dynamics of smaller "sub-TADs" within them (Phillips-Cremins et al., 2013). As the resolution of genome-wide chromatin interaction maps improves, so will our appreciation of the interplay between developmentally stable and dynamic chromosomal secondary structures and of the cause-effect relationships between TADs and genome function.

Establishing, Maintaining, and Re-Building Chromosomal Secondary Structures

Despite (or perhaps because of) their many correlations with different epigenomic features, unravelling the causal factors in TAD establishment and maintenance remains a challenge. TAD borders in *Drosophila* are very significantly associated with binding of various insulator proteins (Hou et al., 2012; Sexton et al., 2012); CTCF is the only one of these factors conserved in mammals and is also enriched at constitutive TAD borders (Dixon et al., 2012). However, the full link between insulators and chromosome topology remains unclear—in one genome-wide study around a quarter of TAD borders did not contain CTCF and only 15% of CTCF binding sites were present at TAD boundaries (Dixon et al., 2012). Further, knockdown of CTCF in a human cell line caused an increase in the chromatin interactions spanning TAD borders but did not completely disrupt TAD organization (Zuin et al., 2014). This result is consistent with the persistent demarcation of H3K27me3 domains in *Drosophila* on CTCF knockdown (Van Bortle et al., 2012). In mammals, but not *Drosophila*, cohesin is also significantly found at TAD borders, although again the majority of binding sites are not at borders (Nora et al., 2012; Phillips-Cremins et al., 2013). Furthermore, cohesin abrogation in post-mitotic cells has no (Seitan et al., 2013; Zuin et al., 2014) or weak (Sofueva et al., 2013) effects on TAD border function. Although the effects of persisting levels of functional CTCF or cohesin cannot be ruled out in these studies, collectively it appears that these so-called "architectural proteins" contribute to the functional organization of the genome but that chromosomal secondary structures are largely epistatic to them.

TAD borders are also highly enriched in transcriptionally active genes (Dixon et al., 2012; Hou et al., 2012; Sexton et al., 2012), although the presence of borders at silent domains and the majority of transcribed genes residing inside domains mean that transcription alone cannot account for TAD organization. However, the known effects of RNA polymerase binding and

elongation on local DNA topology (Lavelle, 2014) suggest that gene expression programs and chromatin organization could have a profound effect on higher-order chromosome folding. In active chromatin, not only do enhancers contact promoters, but the promoters of expressed genes also contact each other (Li et al., 2012; Sanyal et al., 2012), and these interactions could favor TAD formation. Furthermore, active yeast genes form loops between their start and end sites to coordinate initiation and termination events, and this phenomenon appears to be conserved for at least some mammalian genes (Grzechnik et al., 2014). Transcription units could conceivably form a type of facultative mini-TAD. In support of this, active topological domains are smaller and more structurally complex than silent domains (Hou et al., 2012; Sexton et al., 2012; Sofueva et al., 2013). TAD borders are also enriched in housekeeping genes (Dixon et al., 2012). Evidence is mounting that housekeeping or widely expressed genes have fundamentally different regulatory sequences and chromatin states than developmentally regulated genes (Rach et al., 2011; Schauer et al., 2013; Zabidi et al., 2014). It will be interesting to see if these features, rather than maintained transcription per se, could contribute to TAD organization.

The tendency of chromatin domains of the same type to establish strong interactions is not limited to active chromatin domains. Polycomb domains are formed by clusters of Polycomb-bound sites that form preferential interactions, both intra-TAD (Lanzuolo et al., 2007; Schuettengruber et al., 2014) and inter-TAD (Bantignies et al., 2011; Sexton et al., 2012). Likewise, HP1-bound heterochromatin is involved in specific interactions (Csink and Henikoff, 1996; Sexton et al., 2012). Recent polymer physics-based modeling showed that the simple assumption of the existence of homotypic interactions between domains formed of these chromatin types is sufficient to generate polymer structures mimicking those shown in Hi-C contact maps (Jost et al., 2014). This result suggests that chromatin components of each type of chromatin domain may contribute to establish TADs. The role of boundary factors such as CTCF could thus be to strengthen the stability of the boundaries between domains of different chromatin types or to sharpen their localization.

One experimental test that has appreciably disrupted topological domain structure was the deletion of a 58-kb region spanning a TAD border within the X-inactivation locus. This perturbation resulted in complete loss of border function and the establishment of a new TAD border approximately 50 kb downstream of the deletion site (Nora et al., 2012). Interestingly, the de novo creation of a TAD boundary near to the deleted one was predicted from physical models and suggests that the chromosomes of many genomes have an intrinsic tendency to fold into topological domains (Giorgetti et al., 2014). Thus, at least some topological domain boundaries have a genetic component. Although it has yet to be demonstrated experimentally, disease phenotype association studies have also suggested that around one tenth of human pathologies caused by genomic deletions could involve perturbed topological domain function (Ibn-Salem et al., 2014). Finer dissection of the *cis*-sequence requirements of TAD borders and testing their function outside of their usual genomic contexts, should be fruitful in explaining the mechanistic basis of chromosome organization and in enabling chromosome domain engineering.

Global chromosome structure is regulated throughout the cell cycle. Hi-C experiments have further shown that, whereas TAD organization is largely conserved throughout interphase, the domains are lost during mitosis (Naumova et al., 2013). The robust detection of conserved TADs in early G1 cells suggests that they can be efficiently re-built. Characterization of the proteins and chromatin marks that persist on mitotic chromosomes, the so-called "bookmarking" factors, is an area of current intense study, which may yield some clues as to how TADs can be established at each cell cycle (Zaret, 2014). For example, it has been shown in *Drosophila* that the Polycomb group protein PSC persists on only a subset of binding sites during mitosis and that these are predominantly interphase TAD boundaries (Follmer et al., 2012). However, it is unclear how this bookmarking is regulated, if or how it controls TAD organization, or how the many TADs that are not mitotically bound by PSC are regulated. DNA damage and the chromatin remodeling accompanying its repair are also likely to affect the organization of the associated TADs. Although previous results have shown that heterochromatin domains have different induced mobility and/or repair mechanisms in response to double-stranded breaks (Chiolo et al., 2011; Lemaître et al., 2014), it is still unknown how TADs are maintained or restored in different nuclear environments. Overall, genetic elements, transcription, and the binding of architectural proteins have all been correlated with TAD borders. Future research should tease out whether they are causes or consequences of TAD folding, how these factors interplay in such organization, and their roles in re-building TADs after mitosis.

Chromosomal Secondary Structures in Genome Evolution

TAD organization appears to be a conserved, but not universal phenomenon (Table 1); TADs are readily observed in *Drosophila* (Hou et al., 2012; Sexton et al., 2012) and mammalian (Dixon et al., 2012; Nora et al., 2012) genomes but are less clearly defined in *Arabidopsis* (Feng et al., 2014; Grob et al., 2014), *Plasmodium falciparum* (Ay et al., 2014), and yeasts (Duan et al., 2010; Tanizawa et al., 2010). Although more systematic chromatin interaction maps of different organisms are required to make further conclusions, it is interesting that species with clear TAD genomic organization match those with conservation of the insulator protein CTCF (Heger et al., 2012), further supporting its role as a genomic architectural protein. However, closer analysis of chromatin interaction maps of non-metazoan species reveals some topological domain-like organizations, such as the very large "structural domains" in *Arabidopsis* (Grob et al., 2014), or the tens of kilobase-sized "globules" in *Schizosaccharomyces pombe*, which correlate with the organization of convergent genes and cohesin binding sites (Mizuguchi et al., 2014). More strikingly, the chromosome of the bacterium *Caulobacter crescentus* also adopts TAD-like domains, which are highly sensitive to transcriptional activity and negative supercoiling (Le et al., 2013). Thus, genomic folding into potentially self-organized modules appears to be a common strategy for very diverse types of chromatin, perhaps reflecting an intrinsic ability for chromatin

Table 1. Overview of the Absence or Presence of Chromosome Topological Domains, as Well as Their Observed Sizes, Based on Current Studies

Organism	Evidence for TADs or Similar Domains	Domain Size	Methods Used	References
C. crescentus	Yes	30–420 kb	Hi-C and a sub-genome-wide derivative (5C)	Le et al., 2013
S. cerevisiae	No	NA	A genome-wide 4C derivative	Duan et al., 2010
S. pombe	Yes	50–100 kb	Hi-C	Mizuguchi et al., 2014
P. fulciparum	Only around a specific group of genes	10–50 kb	Hi-C	Ay et al., 2014
A. thaliana	Controversial	> 1 Mb in one study; no TADs in another	Hi-C	Feng et al., 2014; Grob et al., 2014
D. melanogaster	Yes	10–980 kb	Hi-C	Sexton et al., 2012
M. musculus	Yes	100 kb–5 Mb	Hi-C, 5C	Dixon et al., 2012; Nora et al., 2012
H. sapiens	Yes	100 kb–5 Mb in one study, 40 kb–3 Mb in another	Hi-C	Dixon et al., 2012; Rao et al., 2014

to be compacted in a way that can be easily opened and recondensed without entangling of chromosome fibers (Lieberman-Aiden et al., 2009). Until very recently, the TAD size of an organism appeared to scale with the average gene or chromosome length (Table 1). However, Hi-C coupled to extremely deep sequencing has identified human domains at a similar scale to that observed in *Drosophila* (Rao et al., 2014). Caution with respect to the resolutions afforded by different studies is thus required when trying to make cross-species comparisons of chromosome folding.

Comparison of mouse and human chromatin interaction maps revealed a high degree of TAD organization conservation around syntenic regions (Dixon et al., 2012). If these domains truly represent autonomously functional units of the genome, then rearrangements of whole TADs may be favored over ones that split TADs apart. Although such selection has not been formally proven, random P element insertions are highly enriched at TAD boundaries (Hou et al., 2012), suggesting that they may be genetic loci particularly susceptible or permissive to rearrangement events. It is also curious that distal human sequences which are syntenic in the mouse genome retain long-range chromatin interactions, tens of millions of years after the synteny break (Véron et al., 2011). This is not an isolated observation as Polycomb-dependent long-range contacts between Hox loci are conserved among fly species that diverged around 40 million years ago (Bantignies et al., 2011). Genome evolution could thus potentially be driven by re-arranging their secondary structures, analogous to the evolution of proteins by shuffling domain-coding exons (Liu and Grigoriev, 2004). Conversely, the spatial organization of TADs may also influence the sequence divergence within them. A recent comparative genomics study in Drosophilid species found that the dual transcription factor/Polycomb recruiter protein PHO bound only to consensus motif sequences outside of a Polycomb context but was able to bind far weaker motifs within TADs marked by H3K27me3 (a hallmark of Polycomb-mediated repression) (Schuettengruber et al., 2014). Of note, these Polycomb-linked PHO sites participated in stronger chromatin interactions, consistent with known looped interactions between Polycomb group response elements (Lanzuolo et al., 2007). Such co-operative interactions within specific TADs were proposed to stabilize PHO binding, allowing a greater tolerance of motif sequence divergence (Schuettengruber et al., 2014). Thus DNA sequence appears to influence chromosome folding, and 3D chromosome structure in turn may influence sequence evolution (Figures 4A and 4B). These data call for more work in order to understand whether this principle may apply to the binding of a wide variety of transcription factors in eukaryotes.

Toward Tertiary Chromosomal Structures

At current sequencing depths, Hi-C experiments are able to give fairly detailed views of TAD organization, but the resolution of longer-range (and interchromosomal) contacts is more limited. Although there is evidence to suggest that TADs hierarchically co-associate to build up larger chromosomal structures (Sexton et al., 2012), the precise nature of such spatial configurations remains mysterious. FISH studies of long-range gene co-associations in mouse erythroid cells or *Drosophila* embryos detected specific long-range interactions in only a few percent of cells, despite their robust detection by 4C (a 3C variant detecting all interactions with a specific bait sequence), suggesting that many chromosomal configurations are present within a population of cells (Bantignies et al., 2011; Noordermeer et al., 2011a; Schoenfelder et al., 2010). Despite this apparent diversity in global chromosome structure, several groups have attempted to model the average conformation (or conformations), which best globally fit the underlying interaction maps (for example Duan et al., 2010; Nagano et al., 2013; Figure 4C), whereas others have used more precise physical models to try and explain either the general features of Hi-C maps (Barbieri et al., 2012; Jost et al., 2014; Lieberman-Aiden et al., 2009) or obtain higher-resolution views of smaller genomic regions (Giorgetti et al., 2014; Le Dily et al., 2014). More and higher-resolution interaction maps will allow the validity of these models to be tested, but already they have been able to provide testable hypotheses as to which genomic regions are the most crucial for structural integrity (Giorgetti et al., 2014).

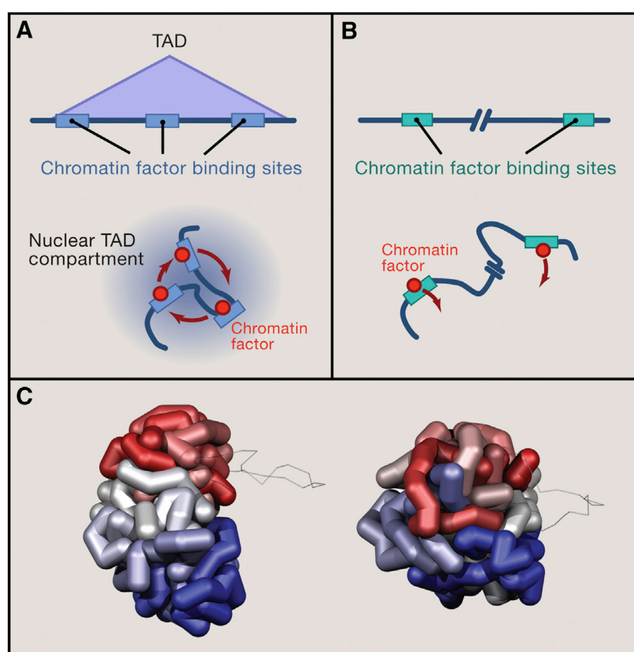

Figure 4. TAD-Dependent Enhancement of Chromatin Factor Targeting and Chromosome Conformation Heterogeneity

(A) Top: A hypothetical TAD that contains three binding sites (in blue) for a chromatin factor is represented. Bottom: Intra-TAD contacts bring the chromatin binding sites in close proximity and form a 3D compartment where the chromatin factor is concentrated via formation of either homodimers or of self-interacting chromatin complexes. This architecture favors the maintenance of factor binding since, once the factor dissociates from a target site, the high relative concentration of other binding sites present in the same TAD favors rebinding.

(B) A genomic region with isolated binding sites for a chromatin factor (green) is shown. In the isolated context, the factor is rapidly lost in the nucleoplasm after dissociation from its target and therefore its replenishment from nucleoplasmic regions with lower relative concentration is less efficient. In this model, proposed by (Schuettengruber et al., 2014), 3D association of factor binding sites via intra-TAD contacts can favor the maintenance of robust chromatin targeting compared to non-TAD isolated factor binding sites.

(C) The tertiary structures of two mouse male T_H1 cell X chromosomes, inferred from two separate single-cell Hi-C experiments, showing that single cells of a population can have diverse chromosome structures (Nagano et al., 2013). The chromosomal position of the fiber is shown as a color scale, going from red (centromeric end) to blue (telomeric end). The gray line represents regions with low constraints due to low mappability in the Hi-C experiment. Image provided by Csilla Varnai and Peter Fraser.

Comparisons of the chromatin interaction maps derived from multiple single-cell Hi-C experiments consistently revealed a high diversity in long-range contact repertoires but found that TADs were surprisingly persistent, suggesting that they are genuinely more stable sub-structures of the chromosome (Nagano et al., 2013; Figure 4C). What is currently unclear is how much of the structural heterogeneity is due to stable alternative genomic configurations and how much can be explained by chromosomal dynamics. Tagging mammalian DNA loci with multiple copies of binding sites for fluorescently labeled *lac* or *tet* repressors has revealed that chromatin is highly mobile but constrained within a restricted subnuclear volume (Lucas et al., 2014; Masui et al., 2011). This constrained diffusion is affected by developmental stage and attachment to nuclear landmarks such as the periphery or nucleoli. On a larger scale, photobleaching studies of fluorescently labeled histones revealed that arrays of chromatin domains can undergo coordinated long-range movements (Cheutin and Cavalli, 2012). It is interesting to speculate that these domains could correlate with TADs (or groups of adjacent TADs), which have also been proposed to form the physical limit for the observed rapid sub-diffusion of chromatin (Lucas et al., 2014). Therefore, TADs may constitute the physical microenvironment in which neighboring functional elements interact, while occasional movements of strings of adjacent TADs may allow for large-scale rearrangement of chromosome structure and for the formation of new contacts among distant chromatin loci. A fascinating research area is to investigate whether these long-range movements might be specifically induced and regulated.

Moreover, very little is known about the conservation of chromosome structures across cell cycles; initial photobleaching experiments gave conflicting results for global chromosome positioning after mitosis (Gerlich et al., 2003; Walter et al., 2003). However, an elegant recent study suggests that at least some chromosome configurations can be remodeled during cell division. Lamina-associated chromatin was tagged during a short time period, and then its nuclear location(s) were traced through subsequent cell cycles (Kind et al., 2013). Only around one third of the lamina-associated chromatin called from population-average studies contacted the lamina at any given point in a single cell and, more strikingly, these regions were reshuffled during mitosis. Recent advances allow fluorescent DNA tagging without the insertion of large exogenous sequences (Chen et al., 2013; Miyanari et al., 2013; Saad et al., 2014). Their systematic application is likely to shed more light on the dynamics underpinning enhancer-promoter contacts, TAD stability and long-range interactions, and ultimately address whether they can be inherited across interphase and through subsequent cell cycles. Overall, whereas chromosomes are organized arrangements of seemingly stable secondary structures, they may adopt many different "tertiary structures" within a population, with as yet unclear dynamics of how these variants may interchange.

Long-Range Interactions—Non-Opposites Attract

Focused 3C variants and FISH studies have uncovered a plethora of co-associations between genes separated by megabases, or occupying different chromosomes, usually occurring at frequencies that are low but much higher than expected by chance. Such long-range interactions are commonly between genes sharing regulation by a common factor, such as Polycomb-mediated repression (Bantignies et al., 2011; Denholtz et al., 2013), or activation by tissue-specific (Papantonis et al., 2012; Schoenfelder et al., 2010), or pluripotency-linked transcription factors (Apostolou et al., 2013; de Wit et al., 2013; Denholtz et al., 2013; Wei et al., 2013), occurring specifically in cell types where the regulation is mediated. Many groups have proposed the existence of functional spatial gene networks, whereby the clustering of genes at nuclear foci enriched in their regulatory factors facilitates their coordinate expression (Bantignies et al., 2011; Papantonis et al., 2012; Schoenfelder et al., 2010). Support for this model has come from detailed analysis of the acute co-association of three human TNF-alpha

stimulated genes: an induced double-stranded DNA break in one gene completely abolishes its transcription but also severely impairs expression of the other target genes, concomitant with loss of co-association (Fanucchi et al., 2013). Most strikingly, this network is hierarchical, as break formation in the gene *SAMD4A* perturbs expression of both the genes *TNFAIP2* and *SLC6A5*, but *SAMD4A* is unaffected by breaks in either of the other genes. Similarly, a break in *TNFAIP2* perturbs *SLC6A5* expression but not vice versa. These examples of spatial co-regulated gene networks are very evocative; however in general, many combinations of genes sharing modes of regulation are not uncovered as interacting partners in 4C experiments. Furthermore, some gene co-associations linked to embryonic stem cell differentiation and formation of induced pluripotent cells precede the transcriptional changes by several days (Apostolou et al., 2013; Wei et al., 2013). It is also noteworthy that the observed spatial association of co-regulated genes in *S. cerevisiae* (Duan et al., 2010) was completely recapitulated when chromosomal structures were modeled from a few basic physical principles (Tjong et al., 2012). Thus, seemingly regulated spatial gene networks may actually be an indirect effect of chromosome folding mechanics, although the principles behind any potential direct regulation are even less clear than those determining enhancer-promoter communication or TAD organization at this stage.

Over multiple scales of chromosome organization, a recurring theme is the prevalence of homotypic or ‘‘like-with-like’’ interactions, whether this is the dimerization of proteins within chromatin loops (Deng et al., 2012), potential spatial networks of co-regulated genes (Schoenfelder et al., 2010) or a tendency for active and repressed chromatin to segregate (Lieberman-Aiden et al., 2009). Such configurations are the expected outcomes of self-organizing systems: a chance encounter between two loci bound by common regulatory factors increases the factors’ local concentrations, so that when a factor dissociates it is more likely to be re-trapped by the cluster of binding sites within its locale than to diffuse away to another location (Kang et al., 2011; Rajapakse et al., 2009). As association of the majority of DNA-bound factors with their cognate sites is transient (Phair and Misteli, 2000), self-organized spatial clustering of related genetic loci may be important for their efficient regulation. This model is consistent with the maintenance of active chromatin hubs at expressed genes (Palstra et al., 2003), the formation of Polycomb repressive domains (Lanzuolo et al., 2007), and perhaps their evolutionary robustness to motif mutations (Schuettengruber et al., 2014), and heterochromatic clustering (Taddei et al., 2009). As TAD organization mirrors underlying functional chromatin domains so well, we posit that TADs may be similarly self-organized structures that increase the local concentrations of diffusible regulatory factors around their sites of activity (Figures 4A and 4B). TADs may thus not only be an effective manner of preventing aberrant communication between genetic loci, but they may also allow for genes to be more efficiently bound by their effectors for stronger or more rapid transcriptional responses. Furthermore, the surprising finding that large-scale chromosome structures are actually more compact on perturbation of intra-TAD loops also suggests that TADs may be important for global chromosome structure maintenance (Tark-Dame et al., 2014).

Perspectives

Mounting evidence shows that the genome is a dynamic yet highly organized hierarchical structure, built up from progressive stabilization of homotypic, potentially functional contacts between genes and regulatory elements. TADs present some conceptual analogy to secondary structures of proteins. These structures clearly have dynamics and cell-to-cell variability but also show a surprising developmental and evolutionary robustness, suggesting that they may be chromosome building blocks required for appropriate genome function. However, hypotheses about how TADs are organized and their functions are difficult to directly assess for two main reasons. First, up till now they have only been detected by population-average studies in fixed cells; TADs have yet to be visualized in single cells or followed in real time. Clearly, the way in which TADs may impinge on gene expression depends on whether they are genuinely stable structures or more a reflection of a ground state of inherent chromatin dynamics. Second, TADs appear robust to the initial perturbation studies that have been tried (for example, CTCF or cohesin abrogation), so it has been difficult to pinpoint any ‘‘causative’’ factor. Major advances in the future will tackle these two issues with live imaging of chromatin interactions in single cells (and following such interaction dynamics over the cell cycle), proteomic studies of which factors (if any) distinguish interacting loci from non-interacting ones and finer genetic dissection of the elements contributing to TAD borders or key architectural loops.

Returning to the protein folding analogy, genomes appear to be built up from the stabilization of progressively higher-order conformations, from TAD secondary structures to chromosomal tertiary structures, to the organized arrangement of chromosome territories into a final quaternary structure. With few exceptions, the structure of a protein cannot be predicted solely from its amino acid sequence. However, once the structure is resolved, the key residues contributing to the protein’s function can be readily identified and engineered. As our knowledge of TADs and specific chromatin loops increases, we posit that similar structure-informed reverse genetic engineering will allow us to manipulate the genome, with myriad applications. For example, de novo creation of autonomously regulated TADs would reduce any side effects of linked transgenes, and the engineering of switchable chromatin loops may allow for fine manipulation of gene expression. In summary, we are entering an exciting time in the field of nuclear organization. Mechanistic links are beginning to be assigned to what were previously only correlations between chromatin conformations and transcriptional regulation. Combined with the revolution in genome engineering tools such as CRISPR, we are in an unprecedented position to not only model, but also modulate, genome structure.

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