CellPress

Molecular Cell
Review

Regulatory Landscaping: How Enhancer-Promoter Communication Is Sculpted in 3D

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https://doi.org/10.1016/j.molcel.2019.05.032

During embryogenesis, precise gene transcription in space and time requires that distal enhancers and promoters communicate by physical proximity within gene regulatory landscapes. To achieve this, regulatory landscapes fold in nuclear space, creating complex 3D structures that influence enhancer-promoter communication and gene expression and that, when disrupted, can cause disease. Here, we provide an overview of how enhancers and promoters construct regulatory landscapes and how multiple scales of 3D chromatin structure sculpt their communication. We focus on emerging views of what enhancer-promoter contacts and chromatin domains physically represent and how two antagonistic fundamental forces—loop extrusion and homotypic attraction—likely form them. We also examine how these same forces spatially separate regulatory landscapes by functional state, thereby creating higher-order compartments that reconfigure during development to enable proper enhancer-promoter communication.

Overview

During development, intricate changes to gene expression transition single-celled embryos to complex organisms with hundreds of cell types. Robustly regulated transcription in time and space is essential for such precision and is thus critical for embryogenesis. However, in metazoans, many core promoters proximal to transcription start sites (TSSs) do not drive such robust and precise gene expression alone. Rather, regulatory information is distributed throughout a promoter's genomic surroundings in non-coding elements with diverse spatiotemporal activities, termed enhancers. In this way, promoters and enhancers together create gene regulatory landscapes that drive the complex and flexible patterns of transcriptional activity necessary for metazoan life.

Remarkably, enhancers can communicate their defined activities across large genomic distances by physically contacting distal promoters via chromatin folding. To achieve this, regulatory landscapes are highly organized in 3D nuclear space at a number of scales, each of which differently influences enhancer-promoter communication (Figure 1). Interactions within regulatory landscapes create enhancer-promoter contacts that support and modulate cell-type-specific gene expression (Andrey et al., 2017; Javierre et al., 2016). Higher-order chromatin folding constrains these contacts within self-interacting topological-associated domains (TADs) separated by insulating boundaries, thereby partitioning the genome into discrete functional blocks (Dixon et al., 2012; Nora et al., 2012). At a chromosomal scale, multi-megabase interactions between TADs with similar epigenetic states further spatially segregate chromatin according to activity, creating the structurally and functionally distinct active A and inactive B compartments (Lieberman-Aiden et al., 2009). Subsequent positioning of A compartments near nuclear speckles and B compartments at the nuclear envelope/nucleolus localizes regulatory landscapes at sites conducive or intolerant to transcription, respectively (Chen et al., 2018b; Kim et al., 2019; Rao et al., 2014). Together, these organizational scales sculpt regulatory landscapes. Enhancers define their information content, promoter contacts transmit that information, TADs determine their limits, and compartments reflect their functional state. (For a complete glossary, see Box 1.)

Although such details are increasingly clear, precisely what mechanisms drive these organizational scales or enable them to influence enhancer-promoter communication have remained elusive. Here, we discuss recent advances addressing these questions, driven by developments in genome engineering, protein-depletion technologies, and single-locus structural mapping. We describe how enhancers combinatorially coordinate promoter transcription and how invariant and tissue-specific chromatin interactions influence their spatial proximity. We evaluate how TADs define regulatory landscapes, what they physically represent at single loci over time, and which mechanisms drive their formation. Finally, we will examine how regulatory landscapes physically transition between active and inactive compartments, thereby providing a unified model of enhancer-promoter communication throughout all organizational scales.

Enhancers Define the Information Content of Gene Regulatory Landscapes

In metazoans, regulatory information is uncoupled from the proximity of TSSs and transmitted to promoters from distal enhancers (Figure 2). Though enhancers are defined as blocks of non-coding sequences that induce spatiotemporally precise transcription in even distal promoters, how they achieve this is still fundamentally unknown (Furlong and Levine, 2018). What is clear is that enhancer activities stem from their recruitment of distinct combinations of sequence-specific transcription factors (TFs). Once bound, these TFs recruit coactivator proteins that promote RNA polymerase II (Pol II) recruitment and

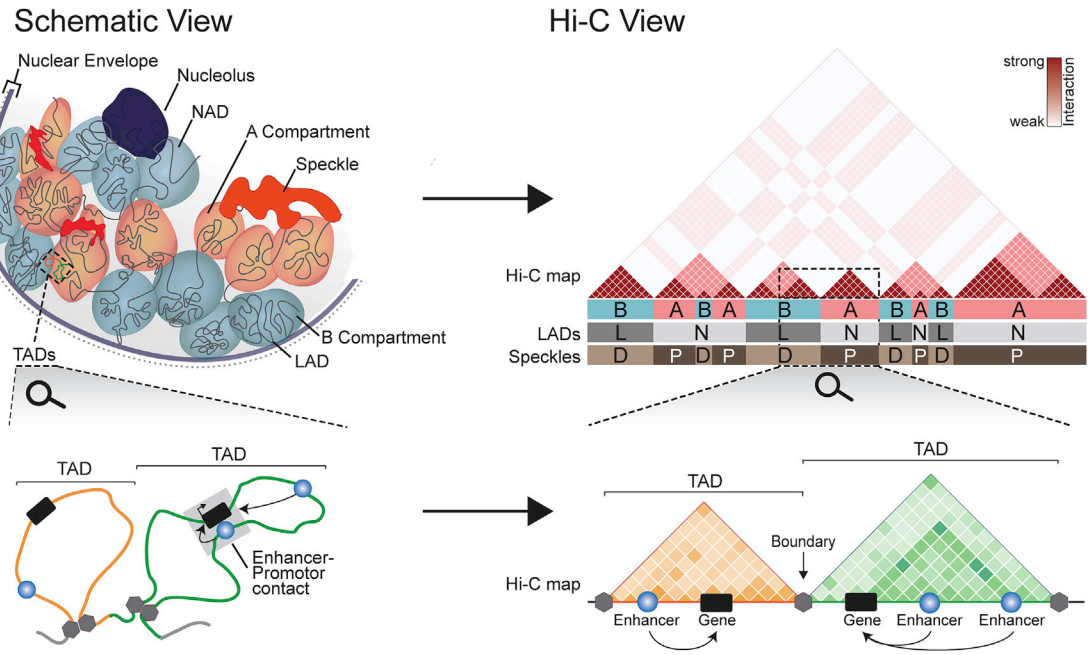


Figure 1. The Hierachical 3D Organization of the Genome
Schematic (left) and Hi-C (right) views of genome organization. Upper panel: at higher-order scales, chromatin with a transcriptionally active or repressive signature separates into A (red) and B (blue) compartments, respectively. B compartments frequently overlap with nucleolar-associated domains (NADs) and LADs (L) but are distant from speckles (D). A compartments are coincident with non-LADs (N) and are speckle-proximal (P). Lower panel: at smaller scales, enhancers transmit regulatory information to genes by physical proximity within, but not between, TADs. Separated by insulating boundaries, TADs preferentially internally self-associate to create discrete functional and structural blocks.

processivity at target genes (Haberle and Stark, 2018; Long et al., 2016). Nevertheless, not all enhancers can activate all promoters. Thus, different enhancers likely utilize diverse mechanisms to stimulate transcription of only compatible promoters, thereby refining the potential targets they can regulate (Haberle and Stark, 2018).

Though candidate enhancers can be identified from a distinct signature of accessible chromatin, H3K4me1, H3K27ac, and transcribing Pol II, these features do not guarantee an ability to function as one (Catarino and Stark, 2018). Consequently, putative elements must be experimentally validated through, for example, enhancer-reporter assays performed on single elements *in vivo* or many elements in parallel *in vitro* (Figures 2A and 2B) (for review, see Catarino and Stark, 2018). Through these approaches, thousands of putative enhancer activities have been mapped in space and time *in vivo* across different cell types of developing embryos (Manning et al., 2012; Pennacchio et al., 2006). As such, enhancers are significant contributors to the diversity of gene expression patterns.

In metazoans, many promoters are controlled by multiple enhancers with differing spatiotemporal activities, each of which regulates a distinct subset of a gene's overall pattern of activity (Figure 2A) (Andrey and Mundlos, 2017). For instance, the composite activities of at least 11 enhancers drive *Sonic hedgehog* (*Shh*) in multiple tissues, including central nervous system, epithelial linings, and limbs, during mouse embryogenesis (Anderson et al., 2014). Consequently, loss of a single element is thought to exclusively eliminate only its corresponding portion of a gene's expression pattern (Figure 2A). Supporting this, removal of the limb-specific *ZRS* enhancer specifically eliminates *Shh* expression only in that tissue, thereby disrupting limb outgrowth (Anderson et al., 2014). However, such situations are rare. More commonly, complex expression patterns are generated by multiple redundant enhancers with overlapping activities that resist genetic variation. Indeed, the overlapping activities of multiple ultra-conserved enhancers at the *Gli3* and *Shox2* genes require that several elements are deleted before expression is pathogenically disrupted in developing mouse limbs (Osterwalder et al., 2018). Nevertheless, such redundant enhancers are frequently not completely interchangeable (Will et al., 2017). Thus, regulatory landscapes assemble combinatorially complex and genetically resistant expression patterns through multiple enhancers, each of which can contain both distinct and redundant overlapping activities (Figure 2B). Further, by allowing mutations in enhancers to accumulate without total gene loss of function, such redundancy likely also provides a rich template from which to generate new regulatory activities during evolution (Long et al., 2016).

Beyond complexity and genetic resistance, overlapping activities of enhancers also provide transcriptional robustness under sub-optimal conditions. In *Drosophila melanogaster*, redundant *shavenbaby* enhancers can be deleted without regulatory or phenotypic effects when the embryos develop at 25°C. However, when developing at 17°C or 32°C, *shavenbaby* gene expression is disrupted in individual enhancer deletions, ultimately perturbing trichome formation (Frankel et al., 2010).

Box 1. Glossary

- Topologically associated domains (TADs): chromatin domains with high self-association that are insulated from the wider genome by boundary elements.
- Structural Variant (SV): large chromosomal rearrangements, including duplications, deletions, and translocations, that can restructure the genome and TADs.
- Lamina-associated domains (LADs): chromatin domains detected by DamID to interact with the nuclear envelope.
- Nucleolar-associated domains (NADs): chromatin domains that interact with the nucleolus.
- Replication-timing domain: chromatin domains that engage in DNA replication at different times in S phase.
- A/B compartments: higher-order structures formed by preferential inter-TAD interactions between chromatin of the same A or B type.
- Loop extrusion: postulated mechanism of TAD formation whereby loops are produced by progressive extrusion of chromatin by a loop extrusion factor.
- Homotypic attraction: postulated force driving chromatin with similar epigenetic properties or DNA binding proteins to preferentially self-associate, for example, into compartments.
- Liquid-liquid phase separation (LLPS): process in which molecules separate in the absence of membranes into discrete liquid condensates with distinct compositions.
- Intrinsically disordered region (IDR): portion of a protein that lacks a fixed or ordered structure and can induce LLPS.

Such redundant enhancers may mediate this robustness by maintaining target expression in excess of minimal requirements within each cell, thereby buffering against adverse conditions. Indeed, deletion of individual redundant enhancers at the *Gli3* or *Shox2* loci normally yields no phenotype. However, when *Gli3* or *Shox2* baseline expression is reduced by 50%, loss of single enhancers lowers their expression beyond a critical level and disrupts limb development (Osterwalder et al., 2018). Alternatively, redundancy may overcome the inherent probability that each enhancer fails to activate at least one copy of its target gene per cell, thereby achieving consistent transcription across cell populations (Perry et al., 2011). Regardless, additive cooperativity between redundant enhancers ensures robust and consistent expression, both within single cells and across cell populations.

Taken together, these examples demonstrate how complex and robust expression patterns are assembled from ensembles of enhancers and promoters within modular regulatory landscapes. However, with regulatory landscapes spanning hundreds to thousands of kilobases, the question remains: how does chromatin's spatial organization influence the transmission of enhancer activities to target genes?

Fixed and Tissue-Specific Contacts Support Transmission of Enhancer Activities

Enhancers utilize chromatin folding to bypass up to megabase (Mb) genomic distances and transmit their regulatory outputs to promoters by physical proximity. Supporting this, artificial enhancer-promoter contacts are sufficient to induce robust gene transcription (Deng et al., 2014). Similarly, live-cell imaging of labeled enhancers, promoters, and nascent RNA products has unequivocally visualized that their direct proximity corresponds with bursts of target gene transcription in *Drosophila* (Chen et al., 2018a; Lim et al., 2018). Within endogenous regulatory landscapes, many naturally occurring enhancer-promoter contacts have been identified through derivatives of chromosome conformation capture (see Box 2) and other structural mapping approaches (for review, see Bonev and Cavalli, 2016). However, the relative strengths of these contacts vary significantly. Indeed, numerous enhancer-promoter contacts were only observed when averaged together in recent ultra-high-resolution Hi-C maps or when selectively enriched through Promoter-CaptureC or H3K27ac-HiChIP (Andrey et al., 2017; Bonev et al., 2017; Mumbach et al., 2016). Thus, enhancer-promoter contacts appear to represent genuine, albeit variable, structural features of regulatory landscapes.

Interestingly, systematic analysis of different developmental time points and cell types by high-resolution Promoter-CaptureC have demonstrated both preformed (invariant) and facultative (activity-dependent) enhancer-promoter contacts, each of which influences gene regulation differently (Andrey and Mundlos, 2017). At invariant contacts, multiple proteins maintain enhancers and target promoters in close proximity independently of their activity. In this way, pre-established proximities can poise enhancer-promoter communication, allowing enhancers to immediately activate target genes. Indeed, during development, the PRC1 and PRC2 complexes generate Polycomb-repressed enhancer-promoter contacts, maintaining them in the close proximity necessary for robust gene induction during subsequent differentiation (Cruz-Molina et al., 2017; Ogiyama et al., 2018). Similarly, the zinc-finger protein CTCF and cohesin DNA-bridging complex generate a 1-Mb-spanning contact between the *Shh* promoter and *ZRS* enhancer (Paliou et al., 2019). This contact constrains *Shh* and *ZRS* within an average distance of ~400 nm, irrespective of their activation or repression in different parts of the developing limb (Williamson et al., 2016) (Figure 3A). However, 80% of *ZRS* and *Shh* loci further move to within 200 nm when activated in posterior limb cells. As such, their pre-established proximity appears to support the generation of a closer activity-dependent contact that is necessary for *Shh* transcriptional activation (Williamson et al., 2016). Moreover, similar analogous progressions from a protein-driven contact to an activating proximity have also now been observed directly by live-cell imaging in *Drosophila* (Chen et al., 2018a). Thus, proteins such as CTCF and cohesin or PRC1 and PRC2 can constrain inactive enhancers and promoters in close proximity and enable subsequent activation-associated contacts to robustly form and induce transcription.

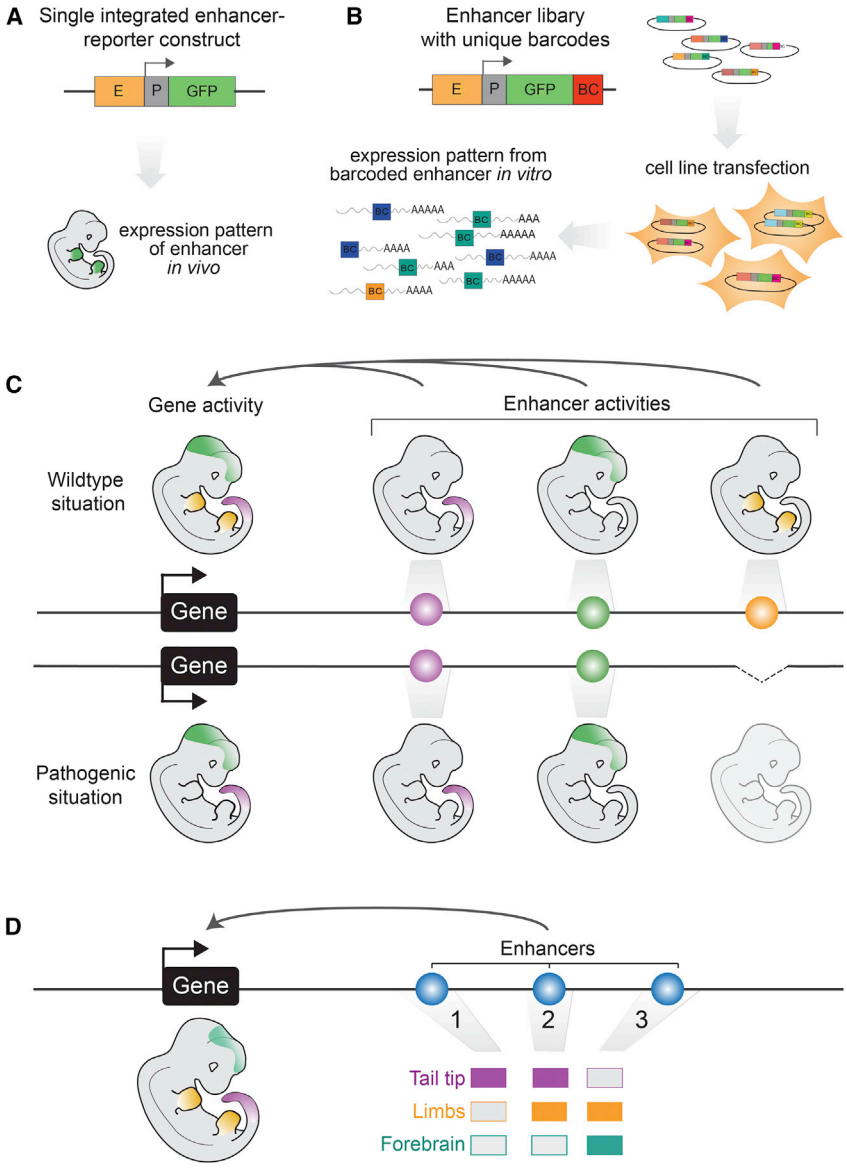


Figure 2. Enhancers Combinatorially Drive Complex Gene Expression in Regulatory Landscapes

(A). *In vivo* enhancer-reporter assay. Integration of putative enhancers (E) together with a minimal promoter (P) and reporter gene (e.g., GFP) at ectopic sites enables mapping of spatiotemporal activities in embryos.

(B) Massively parallel reporter assay (MPRA). Cloning thousands of putative enhancers into barcoded libraries enables their activities to be assayed in parallel within selected cell types *in vitro*.

(C) Three enhancers generate a composite gene expression pattern in the brain (green), tail tip (purple), and limbs (yellow). Loss of the limb enhancer selectively removes that expression domain.

(D) Gene expression in the tail tip (purple), limbs (yellow), and forebrain (green) is driven by three enhancers (1, 2, 3), each possessing distinct as well as overlapping and/or redundant spatiotemporal activities. Only loss of enhancer 3 will result in a loss of expression in the forebrain.

By contrast, facultative interactions form and disassemble dynamically during development, coinciding with transcription and active chromatin modifications at contacting enhancers and promoters (Andrey et al., 2017; Bonev et al., 2017; Javierre et al., 2016). This link to transcription suggests facultative contacts act to physically transmit enhancer activities. However, their dynamic nature also allows facultative interactions to guide enhancer activity in different ways. For instance, though active in both embryonic hind- and forelimbs, the *Pen* enhancer drives *Pitx1* expression only in the hindlimb through a dynamic topological switch. Specifically, a hindlimb-specific locus configuration reduces the large distance normally separating *Pen* and *Pitx1*, thereby bringing them into close spatial proximity to drive *Pitx1* transcription uniquely in that tissue (Figure 3B) (Kragesteen et al., 2018). Similarly, locus control region (LCR) enhancers switch contacts between embryonic and adult *β-globin* genes during erythroid differentiation, thereby sequentially activating them at the correct developmental stages (Figure 3C) (Deng et al., 2014; Palstra et al., 2003). Hence, unlike invariant interactions, facultative contacts can flexibly refine enhancer activities toward selected promoters to regulate transcription.

Multiple proteins are implicated in directing facultative contacts. For example, *LCR-β-globin* interactions are mediated by the self-associating nuclear factor LDB1 that is recruited to sites of contact in a protein complex with zinc-finger transcription factors (Deng et al., 2012). Similarly, other tissue-specific contacts derive from site-specific developmental alterations in PRC1/PRC2, CTCF, or YY1 binding (Andrey et al., 2017; Bonev et al., 2017; Weintraub et al., 2017). However, many facultative contacts seemingly depend on the mediator complex, a large multi-subunit component of the transcriptional apparatus that interfaces between enhancer- and promoter-bound TFs and Pol II (for review, see Carlsten et al., 2013). In doing so, mediator is thought to generate facultative contacts that it further stabilizes by recruiting cohesin to aid transcriptional activation (Kagey et al., 2010; Phillips-Cremins et al., 2013). As such, multiple proteins contribute to facultative contacts and, in many cases, link their formation to the assembly of the transcriptional machinery itself.

In summary, preformed and facultative contacts coexist within regulatory landscapes to support the varied and different demands of gene functions. However, much remains unclear. For example, though frequently corresponding with mediator binding, how enhancer proximity induces promoter transcription or if this even requires direct physical contact remains fundamentally unclear (Furlong and Levine, 2018). Indeed, observations of enhancers altering the frequency, but not magnitude, with which

Box 2. Mapping Chromatin Structure by Chromosome Conformation Capture (3C)

C-technologies employ digestion of crosslinked chromatin to generate complexes of DNA fragments and their bound proteins. Proximity between loci (contact frequency) is then determined by the incidence that fragments are ligated together. Many 3C derivatives exist (for review, see Bonev and Cavalli, 2016). Hi-C enables genome-wide identification of contact frequencies, though resolution is often limited by cost. However, selected interactions can be enriched through immunoprecipitation or complimentary oligonucleotide probes. This enables interactions from selected proteins (HiChIP), specific viewpoints (CaptureC), or entire genomic regions (Capture-Hi-C) to be affordably analyzed at high resolution.

genes engage in bursts of transcription suggests that contacts deliver a binary instruction for core promoters to activate (Haberle and Stark, 2018). Similarly, many active enhancers do not display significantly higher contact frequencies with target promoters than with surrounding chromatin, even when analyzed using high-resolution C technologies (see Box 2) (Andrey et al., 2017; Bonev et al., 2017; Despang et al., 2019). Though perhaps due to current technical sensitivity limits, this may indicate that many enhancers require only transient promoter proximity to induce transcription and so communicate without specific higher-intensity contacts. Finally, given that enhancers can act over great genomic distances, the open question remains: how is their great regulatory potential constrained to only their target genes?

Chromatin Folding into TADs Defines the Operational Limits of Regulatory Landscapes

Enhancers transmit their activities to promoters across often large genomic distances, the extent of which can be mapped by integration of "regulatory sensors" (Figure 4A). Consisting of only a minimal promoter and a reporter gene, integrated sensor activities solely reflect that of available enhancers (Symmons et al., 2014). Such experiments have demonstrated promoters can widely and non-specifically sample large regions for regulatory information, revealing that the landscapes of many genes extend for hundreds of kilobases (Kragesteen et al., 2018; Marinić et al., 2013). However, this long-distance communication is not unlimited. Rather, the extent to which regulatory information is sensed by promoters appears delimited by self-associating structural domains separated by insulating boundaries, termed TADs (Figure 4) (Dixon et al., 2012; Nora et al., 2012; Symmons et al., 2014). Thus, while enhancers encode the information content of regulatory landscapes, TADs seemingly physically define their operational limits by isolating enhancers and promoters within the same structural unit (Figure 4B).

Unfortunately, TAD identification varies significantly with Hi-C data resolution and detection algorithms, largely due to chromatin structure appearing as a series of nested hierarchies in Hi-C maps (Bonev and Cavalli, 2016). Indeed, TADs themselves associate into higher-order metaTADs while simultaneously encompassing smaller, less insulated domains, variously termed sub-TADs and loops (Fraser et al., 2015; Phillips-Cremins et al., 2013; Rao et al., 2014). Consequently, determining exactly which of these Hi-C structural features define the fundamental functional units of the genome remains challenging. Nevertheless, unlike their frequently reconfiguring sub-domains, TADs and their boundaries are largely conserved between cell types and species (Dixon et al., 2012; Fraser et al., 2015; Harmston et al., 2017). Similarly, TADs display functional properties not found in their subdomains. For example, genes within the same TAD frequently display coordinated expression across different cell and tissue types, a property that is significantly weaker at other organizational scales (Zhan et al., 2017). Moreover, TADs closely overlap with other independently identified genomic features, including replication timing domains and lamina-associated domains (LADs), arguing that each represents an orthogonal measure of the same fundamental feature (Pope et al., 2014; Rao et al., 2014). Together, these observations support TADs being a functionally privileged scale of organization that defines units of gene regulation within spatially insulated domains.

Importantly, it has now been confirmed experimentally that TADs are insulated gene regulatory blocks through pathogenic structural variant (SV) mutations (for review, see Spielmann et al., 2018). These large duplication, inversion, deletion, and translocation SVs reassemble TADs in new configurations, causing promoters to acquire new enhancer inputs in a process termed enhancer adoption (Figure 4). For example, duplications that include enhancers, promoters, and the TAD boundary normally separating them combine these elements together in so called *neo-TADs* while leaving the original domains unaffected (Franke et al., 2016; Weischenfeldt et al., 2017). By contrast, boundary-covering inversions and deletions induce *TAD reshuffling* and *TAD fusion*, respectively, thereby disrupting existing landscapes to create novel TAD structures (Lupiáñez et al., 2015). In this way, SVs generate new composite regulatory landscapes with novel enhancer-promoter combinations, thereby driving their pathogenic misexpression in disorders from cancer (Weischenfeldt et al., 2017) to congenital malformations (Spielmann et al., 2018). However, not all SVs are detrimental. TAD restructuring can also be a major source of morphological and physiological novelty during evolution, as genes acquire new function through their altered spatiotemporal expression (for review, see Acemel et al., 2017).

Together, these data demonstrate that TADs behave as discrete insulating blocks of gene regulation that constrain enhancer activity. Nevertheless, this view is likely too simplistic for several reasons. First, there is a significant fraction of the genome wherein TADs cannot be clearly detected, such as near *Wnt6*, which is located in a gene-dense region composed of weakly defined, less insulated structures (Dixon et al., 2012; Kraft et al., 2019). Why some critical developmental genes require strong insulation within large and highly structured TADs to maintain precise expression while others do not has not yet been explained (Dixon et al., 2012). In these latter situations, dynamic facultative contacts or the specificity of enhancers for certain promoters may play a greater role in

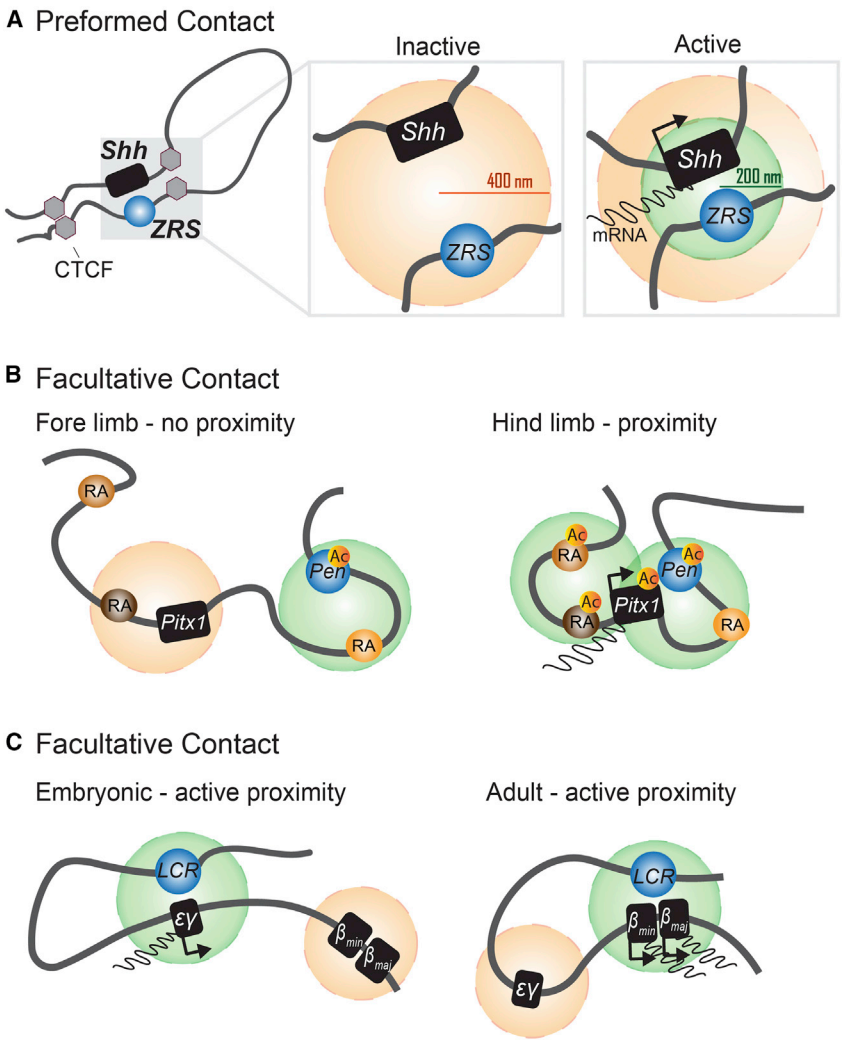


Figure 3. Invariant and Facultative Contacts Support Enhancer-Promoter Communication

(A) A preformed CTCF-dependent contact maintains *Shh* and the *ZRS* enhancer on average within 400 nm and likely supports a closer activation-dependent proximity of 200 nm (Paliou et al., 2019; Williamson et al., 2016).

(B) In the embryonic forelimb, physical separation prevents the active *Pen* enhancer from inducing *Pitx1*. By contrast, in the hindlimb, a conformational change to an active configuration associated with H3K27ac (Ac) of regulatory anchors (RA) drives *Pen-Pitx1* proximity and *Pitx1* transcription (Kragesteen et al., 2018).

(C) LCR enhancers switch contacts between embryonic ($\varepsilon\gamma$) and adult (βmin and βmaj) globin genes during early and late stages of development, respectively (Palstra et al., 2003).

restricting *cis*-regulatory activities. Second, while TAD fusion has been observed following boundary elimination, this is not always sufficient to disrupt TAD insulation and cause enhancer adoption (Despang et al., 2019; Franke et al., 2016). Hence, other features, such as the substructures of TADs themselves, could also redundantly contribute to a TAD's insulating capacity. Finally, the degree of promoter responsiveness to enhancers can vary based on position within a TAD, indicating that more parameters beyond inclusion in the same domain influence their communication (Marinić et al., 2013). Thus, though providing a conceptual framework for the partitioning of regulatory information, a greater mechanistic understanding is required of how TADs and their enhancer-promoter contacts are formed and operate.

Mechanisms of TAD Formation and Enhancer-Promoter Contact

TADs Are Probabilistic and Dynamic Assemblies

Enhancer-promoter contacts regulate transcription within insulated TADs. However, determining what mechanisms drive these contacts and TADs remains challenging due to a fundamental problem: as TADs are identified from bulk analyses of millions of cells, it has been unclear what they physically represent at individual loci over time. *In silico* polymer modeling and conventional FISH microscopy have previously suggested TADs are superpositions of loci occupying many distinct configurations that, on average, maintain contacts within TAD boundaries (Giorgetti et al., 2014). Recent data now strongly support this. Indeed, TADs are not observed in reproducible conformations at individual loci in single-cell Hi-C experiments but can be "reassembled" when individual maps are combined in bulk (Flyamer et al., 2017). Similarly, TADs observed directly by super-resolution FISH microscopy appear largely as discrete physical structures, though the exact position along the chromatin polymer that separates neighboring domains varies (Figure 5B) (Bintu et al., 2018; Szabo et al., 2018). Thus, while interactions adopted by a single locus *can* violate domain borders, across cell populations they are *most frequently* constrained within a discrete physical unit delimited by TAD boundaries. In this way, dynamic enhancer-promoter contacts seemingly drive transcription within an ensemble of fluctuating TAD structures that achieve insulation over time by preferential self-association. Nevertheless, these observations raise a further question: what factors and mechanisms could generate such highly dynamic TAD structures and the enhancer-promoter contacts they contain?

TAD Formation by Loop Extrusion

The formation of many TADs is dependent on cohesin and CTCF, both of which are highly enriched at TAD boundaries (Nora et al., 2017; Rao et al., 2017; Schwarzer et al., 2017). The most current model—loop extrusion—accounts for this dependence as well as for the self-associative, insulating, and dynamic properties of TADs (Fudenberg et al., 2016; Sanborn et al., 2015). In this model, cohesin progressively extrudes chromatin loops through its tripartite ring structure until reaching "roadblocks," like CTCF boundaries or constitutive transcription, that stall its activity (Figure 5A). Through this, TAD self-association and insulation

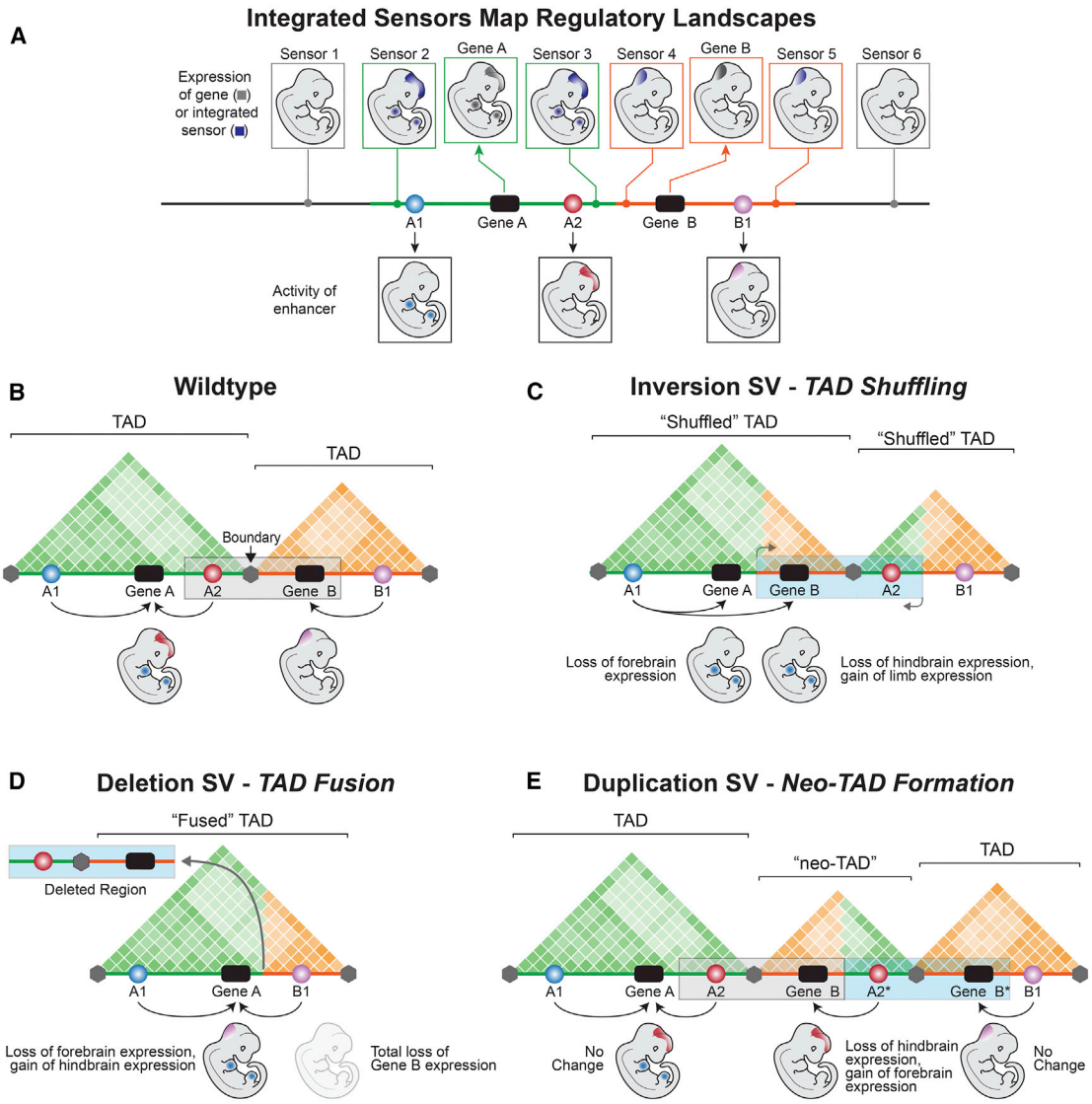


Figure 4. Structural Variants Create Composite Regulatory Landscapes by Altering TADs

(A) Regulatory landscapes (green and orange) can be mapped by contrasting the expression of genes (A and B) with that of integrated "regulatory sensors" whose activity reflects that of available enhancers (A1, A2, and/or B1). Genes and sensors respond to enhancers within the associated but not neighboring landscape.

(B) Regulatory landscapes of genes A and B and corresponding enhancers A1, A2, and B1 are separated in two TADs. Different SVs (gray box) involving the same region (blue box) cause drastically different effects.

(C) Inversion SV *shuffles* landscapes to drive gene A and B misexpression.

(D) Deletion SV *fuses* landscapes to drive gene A misexpression by eliminating the boundary while also removing gene B.

(E) Duplication SV creates a composite landscape in a *neo-TAD* that drives gene B misexpression while leaving gene A and the gene B* copy unaffected.

arise from loop extrusion by forcing loci to continuously sample all points between—but not beyond—their domain's roadblocking boundaries.

Excitingly, the loop extrusion model explains multiple observations. For example, many TADs are formed by CTCF sites in convergent (inward-pointing) orientations, a detail readily explained by CTCF blocking cohesin extrusion in an orientation-dependent manner (Fudenberg et al., 2016; Rao et al., 2014). Similarly, boundaries without strong corresponding partners generate one-sided "stripes" of interaction rather than TADs, as expected from boundary-stalled cohesin extruding in only the unobstructed direction (Kraft et al., 2019). However, most compellingly, recent advances have now experimentally confirmed predictions made by loop extrusion. For instance, TADs found in bulk Hi-C are predicted to be continuously changing but reproducible sets of loops found in populations of cells. Accordingly, a majority of TADs are erased from population-averaged Hi-C maps following elimination of cohesin and its presumptive extrusion activity (Rao et al., 2017; Schwarzer et al., 2017). However, this is not due to a complete loss of structure, as similar self-associating domains are still observed by super-resolution microscopy within individual cohesin-depleted cells (Bintu et al.,

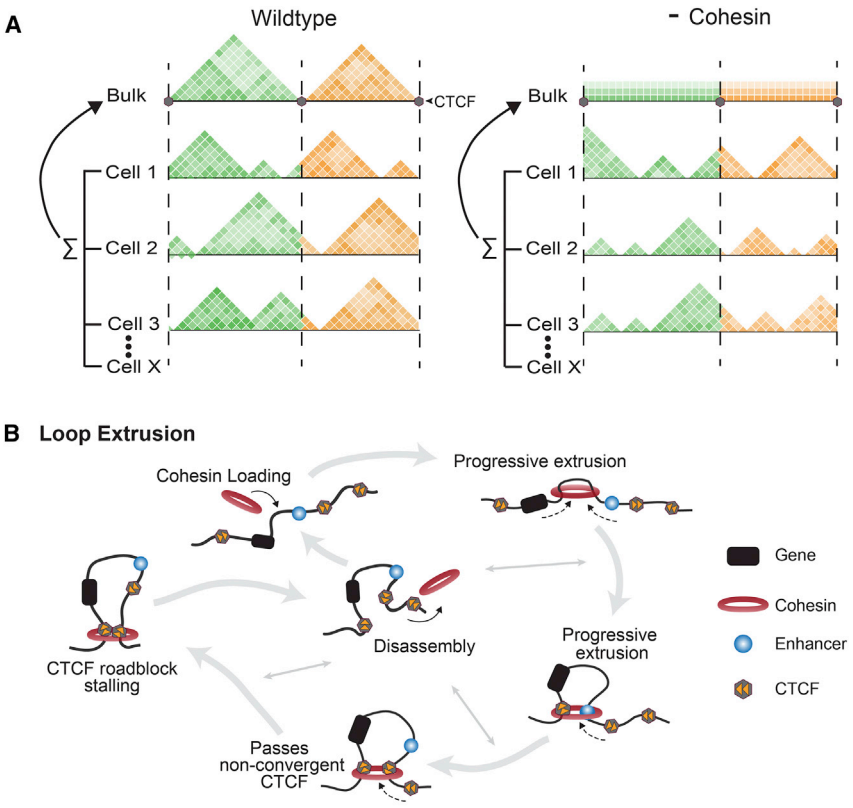


Figure 5. Mechanisms of TAD Formation

(A) Contact frequency-like maps inferred from single cells by super-resolution FISH microscopy. Wild-type cells: TADs in bulk Hi-C emerge from ensembles of self-associating domains that are most frequently separated at CTCF boundary sites. Without cohesin: self-associating domains are still observed but with boundary positions that are random and independent of CTCF sites, preventing TADs from emerging in bulk (Bintu et al., 2018).

(B) Loop extrusion model. Cohesin extrudes chromatin through its ring-shaped protein complex to create loops that progressively grow in size. Extrusion stalls at roadblocks, e.g., at convergently oriented CTCF sites, that block cohesin activity. Cohesin disengagement and loop disassembly can potentially occur at any time (Fudenberg et al., 2016; Sanborn et al., 2015).

(C) Homotypic attraction model. Regions with compatible protein compositions (orange or blue balls) interact via molecular bridges formed by bound complexes or through phase-separated condensates.

2018). Rather, TADs appear absent because these residual domains are considerably more randomly distributed along the chromatin fiber, thereby preventing reproducible structures emerging in bulk analyses (Figure 5B). Similar bulk erasures of TADs are also observed when boundaries are weakened by CTCF depletion, as would be expected if extruding loops are no longer reproducibly constrained within TAD borders (Nora et al., 2017). Thus, loop extrusion driven by cohesin but delimited by CTCF seemingly generates TADs by enforcing continuous but reproducible sampling within defined boundaries. As a result, TAD insulation derives from a careful balance of boundary strength and extrusion processivity, the respective weakening or increase of which causes TAD borders to be violated (Haarhuis et al., 2017; Hanssen et al., 2017; Nora et al., 2017).

Despite these fundamental insights into TAD formation, exactly how specific dynamics of loop extrusion impact enhancer-promoter communication is unclear. For example, two varieties of cohesin complexes with distinct subunit compositions seemingly mediate different aspects of TAD structure. While SA1-containing complexes largely drive overall TAD formation, intra-TAD enhancer-promoter contacts are occupied almost exclusively by SA2-containg complexes (Kojic et al., 2018). This enhancer/promoter-specific accumulation of cohesin may represent SA2-specific extrusion complexes stalling at such intra-TAD contacts, thereby stabilizing them. Alternatively, cohesin accumulation could derive from its preferential recruitment to these elements by the cohesin loading factor NIPBL that is also enriched at active enhancers/promoters (Kagey et al., 2010). In this way, rather than stabilizing their contact, cohesin may selectively enable active enhancers and promoters to more rapidly scan for and find one another by extrusion. Regardless, determining precisely how cohesin and loop extrusion alters enhancer-promoter proximities will be critical to determine.

Chromatin Structure through Homotypic Attraction

Though compelling, loop extrusion does not explain all features of 3D genome organization or enhancer-promoter communication. Indeed, self-associating domains with more random positions persist following cohesion depletion, as do higher-order A and B compartments (see below) (Bintu et al., 2018; Rao et al., 2017; Schwarzer et al., 2017). Similarly, multiple enhancer-promoter contacts are cohesin independent, and only relatively mild disruptions to gene expression are observed following cohesin depletion (Andrey et al., 2017; Monahan et al., 2019; Rao et al., 2017; Schwarzer et al., 2017). Thus, additional forces must structure chromatin and mediate enhancer-promoter communication.

One such force appears to be the inherent homotypic attraction that exists between loci with compatible chromatin states and that drives multiple enhancer-promoter interactions and higher-order compartmentalization. Such chromatin states appear to be defined by a region's collective epigenetic

modifications, protein composition, and transcriptional activity (Andrey et al., 2017; Javierre et al., 2016; Rao et al., 2014). Consequently, several studies have proposed homotypic attraction structures chromatin through dynamic "molecular bridges" formed at sites of contact by multivalent interactions between bound complexes (Figure 5C) (Pombo and Dillon, 2015). Supporting this, known architectural proteins, including LDB1, YY1, and PRC1, are proposed to function in a bridging manner (Deng et al., 2014; Isono et al., 2013; Weintraub et al., 2017).

Interestingly, a flurry of recent studies suggest that homotypic attraction may be driven by a mechanism of liquid-liquid phase separation (LLPS) rather than classical stoichiometric protein interactions (Figure 5C). Here, the intrinsically disordered regions (IDRs) of associating proteins engage in many weak interactions, thereby driving their assembly into phase-separated condensates with specific compositions (for review, see Banani et al., 2017). As such, loci would be physically recruited to selected condensates through their compliment of bound IDR-containing proteins. Supporting this, targeting artificial condensates to specific loci is sufficient to pull them together physically, thereby demonstrating that LLPS can exert a mechanical force on chromatin (Shin et al., 2018). Moreover, as would be expected if active and repressive contacts were directed by LLPS, a number of their facilitators, including TFs, Pol II, mediator, and heterochromatin protein 1 (HP1), have now been observed forming phase-separated condensates (Boija et al., 2018; Cho et al., 2018; Chong et al., 2018; Sabari et al., 2018; Strom et al., 2017). Thus, similar to the mechanisms structuring a number of membraneless nuclear bodies such as nucleoli and speckles, significant aspects of chromatin structure may be directed by LLPS (Banani et al., 2017).

Excitingly, LLPS may link contact formation to the mechanism of enhancer activation of promoters (Hnisz et al., 2017). Specifically, enhancers would function as binding surfaces that concentrate the transcriptional apparatus in "transcriptional condensates," allowing them to induce gene expression when promoters come into close, but not absolute, proximity. While still speculative, TFs, mediator, and Pol II all have been directly observed in dynamic condensates, up to 300–400 nm in size, associated with actively transcribing chromatin (Boija et al., 2018; Cho et al., 2018; Chong et al., 2018; Sabari et al., 2018). As a result, transmission of enhancer activities by LLPS may explain several confusing observations, e.g., how individual enhancers can activate multiple promoters simultaneously (Lim et al., 2018) or why the transcriptional bursting from others occurs without direct enhancer-promoter physical overlap (Alexander et al., 2018). Similarly, apparent enhancer-promoter specificities may derive from biochemical differences between transcription factor IDRs that prevent their joint incorporation into the same condensate (Chong et al., 2018).

Homotypic Attraction and Loop Extrusion Coexist to Structure Regulatory Landscapes

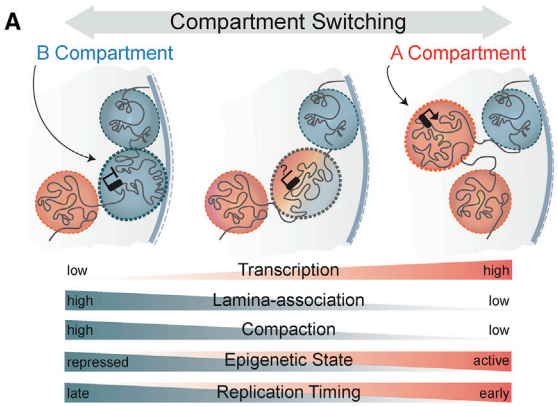
Thus, current evidence indicates that loop extrusion and homotypic attraction coexist and together structure regulatory landscapes and direct enhancer-promoter contacts. Supporting this, a recent computational model incorporating loop extrusion and molecular bridging, as well as differences in active and inactive chromatin compaction, effectively simulated the structure of the *Pax6* locus from epigenetic data alone (Buckle et al., 2018). Thus, a tentative generalized model is emerging. Loop extrusion forms TADs that drive loci to continuously sample every position within their boundaries, thereby controlling how frequently enhancers and promoters are physically close enough for homotypic contacts to form.

Nevertheless, recent data suggest competition between loop extrusion and homotypic attraction, both within TADs and at compartment scales (Nuebler et al., 2018). For example, upon cohesin depletion, finer-scale homotypic interactions emerge within mammalian TADs that closely reflect their diverse underlying chromatin state (Rao et al., 2017; Schwarzer et al., 2017). Thus, the activity of cohesin seemingly breaks homotypic interactions by forcing all chromatin within a TAD to self-associate, regardless of its varied epigenetic states. As a result, it is tempting to speculate that an antagonistic equilibrium between these forces may be necessary to enable proper enhancer-promoter communication within TADs. Specifically, loop extrusion may break inappropriate homotypic contacts while simultaneously initiating functional interactions through the extensive sampling that cohesin drives. Indeed, cohesin disruption impairs the initiation of new regulatory programs in neurons acquiring responses to novel stimuli and in differentiating myeloid cells, thereby suggesting loop extrusion is necessary for transitions between expression states (Cuartero et al., 2018; Yamada et al., 2019). Consequently, determining precisely how these forces relate to each other, particularly at the level of enhancer-promoter contacts, will be of critical importance to understand transcriptional regulation.

Regulatory Landscapes Are Organized in Compartments that Reconfigure during Development

The balance between loop extrusion and homotypic attraction likely structures enhancers and promoters within TADs. Nevertheless, at genome scales, homotypic contacts between TADs in similar chromatin states dominate and assemble regulatory landscapes into A or B compartments (Figures 1 and 6) (Lieberman-Aiden et al., 2009; Nuebler et al., 2018). Indeed, while A compartment domains are enriched in an active chromatin signature, their B equivalents display features of a repressed state and lower transcription (Lieberman-Aiden et al., 2009; Rao et al., 2014). In Hi-C maps, this higher-order spatial segregation manifests as an alternating "checkerboard" or "plaid" contact pattern in which A or B domains preferentially interact with other A or B regions, respectively (Figure 1A). However, the physical separation of A and B compartments can also be observed directly by super-resolution microscopy and *in silico* modeling of single-cell Hi-C data (Bintu et al., 2018; Nir et al., 2018; Stevens et al., 2017; Wang et al., 2016). Thus, whereas TADs internally self-associate to partition regulatory information, compartment domains homotypically associate among themselves to spatially organize chromatin by functional state.

However, beyond spatial association, overlaps between compartmentalization and many other genomic features indicate that regulatory landscapes in different compartments are in profoundly distinct states (Figure 6). Indeed, while A compartments are positioned proximal to transcription-associated nuclear bodies termed speckles, B compartments intersect



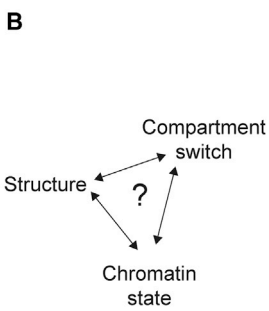


Figure 6. Compartment Switching Transitions Regulatory Landscapes Between Profoundly Different States

(A) Regulatory landscapes undergo concomitant structural and functional changes as they switch between B and A compartments during differentiation.

(B) Due to their close relationship, it remains unclear if or which of these changes drive the others.

with Polycomb-repressed domains, nucleolar-associated domains (NADs), and nuclear envelope-attached LADs (Chen et al., 2018b; Quinodoz et al., 2018; Rao et al., 2014; Robson et al., 2017). Such localized compartments appear to generate distinct functional environments that influence nuclear functions. For example, while promoters integrated into LADs or recruited to the nuclear envelope are frequently repressed, those contacting nuclear speckles display significantly elevated expression (Kim et al., 2019; Leemans et al., 2019; Reddy et al., 2008; Robson et al., 2016). Correspondingly, nuclear envelope-associated B compartments display reduced mobility, higher chromatin compaction, and later DNA replication than A compartments positioned more internally (Chubb et al., 2002; Ou et al., 2017; Pope et al., 2014). Nevertheless, how such distinct environments are established remains unclear. Intriguingly, heterochromatin domains have recently been reported to form through phase-separated HP1 condensates that selectively enrich heterochromatin factors while excluding components of euchromatin (Strom et al., 2017). Thus, though speculative, homotypic attraction mediated by LLPS may link the coalescence and structures of compartments to the generation of their distinct capacities to support or inhibit transcription.

Considering these profoundly distinct states, it is unsurprising that many regulatory landscapes transition between repressed configurations devoid of activity and those supporting active enhancer-promoter communication. Accordingly, during development many regulatory landscapes concomitantly alter, among other features, transcription, compartment identity, and nuclear envelope attachment (Figure 6) (Bonev et al., 2017; Dixon et al., 2015; Fraser et al., 2015; Peric-Hupkes et al., 2010; Pope et al., 2014). For example, during T cell differentiation, activation of *BCL11B* corresponds with its ~2-Mb-sized domain concomitantly adopting an active structural conformation, releasing from the nuclear envelope, and undergoing a B-to-A compartment switch (Isoda et al., 2017). However, as these changes seemingly occur simultaneously, it remains unclear which—if any—individually drives the switch between active and inactive states. Nonetheless, multiple observations suggest initial chromatin state changes may ignite a series of further self-reinforcing alterations to structure and localization that are necessary for full activity. Indeed, initial transcription of an enhancer/lncRNA induces subsequent activation and contact of additional enhancers with BCL11B, both of which are required for its activation (Isoda et al., 2017). Thus, rather than snapping immediately between states, regulatory landscapes may instead progressively remodel between active and inactive configurations. Consequently, by requiring that loci are actively restructured, it is tempting to suggest that compartment transitions and the processes driving them constitute an additional layer of gene regulation.

Perspectives

Recent advances have uncovered a vast array of mechanisms that construct regulatory landscapes in 3D and control the transcriptional instructions received by promoters. Loop extrusion and homotypic attraction drive promoters to dynamically sample enhancer activities from regulatory landscapes occupying large but reproducible ensembles of different configurations delimited by boundaries. Such observed variability elegantly matches the stochastic bursting dynamics of transcription that are seemingly induced by enhancer proximity (Chen et al., 2018a; Lim et al., 2018). This in turn enables promoters to integrate many distinct and overlapping enhancer activities to achieve precise, complex, and robust spatiotemporal patterns of expression. Nevertheless, many questions remain. How do enhancers induce transcription? Do all operate by a proximity mechanism? How does the antagonism between loop extrusion and homotypic attraction influence enhancer-promoter communication? What generates the transcriptional environments of compartments and drives regulatory landscapes to transition between them? We believe a combination of rapidly advancing computational models, high-resolution single locus observations, and functional interventions hold great promise in addressing such questions.

Nevertheless, despite these exciting possibilities, predicting which features are critical to controlling any single locus is likely to remain immensely challenging. For instance, many features so far found to sculpt regulatory landscapes rarely operate to the same extents as at other loci. Hence, rather than a one-fits-all system, individual landscapes each utilize combinations of any number of mechanisms acting at different strengths to achieve a gene's regulatory requirements. Moreover, many of these mechanisms act redundantly to support desired expression patterns, thereby creating landscapes that are highly resistant to perturbations that disrupt other loci. Thus, individual organizational features optimize but are frequently dispensable for enhancer-promoter communication within such seemingly "over-engineered" regulatory landscapes. Consequently, this diversity and redundancy of landscape construction should be

carefully considered before extrapolating generalized trends observed genome-wide to individual loci. Indeed, despite the many mechanisms described here, predicting the consequences of SVs in both evolution and human disease remains a significant problem, but one that must be overcome.

ACKNOWLEDGMENTS

We apologize to colleagues whose work was not discussed or cited owing to space constraints. We thank the Mundlos group, Ruth Shah, and Alexandra Mattei for helpful discussions. M.I.R. was supported by the EMBO LTF (ALTF-1554-2016) and Henry Wellcome Postdoctoral Fellowship (206475/Z/17/Z). Work in S.M.'s laboratory is funded by the Deutsche Forschungsgemeinschaft, the Berlin Institute for Health, and the Max Planck Society.

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