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Review

Decoding the organization, dynamics, and function of the 4D genome

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SUMMARY

Understanding how complex cell-fate decisions emerge at the molecular level is a key challenge in developmental biology. Despite remarkable progress in decoding the contribution of the linear epigenome, how spatial genome architecture functionally informs changes in gene expression remains unclear. In this review, we discuss recent insights in elucidating the molecular landscape of genome folding, emphasizing the multilayered nature of the 3D genome, its importance for gene regulation, and its spatiotemporal dynamics. Finally, we discuss how these new concepts and emergent technologies will enable us to address some of the outstanding questions in development and disease.

INTRODUCTION

The acquisition of distinct cellular identities during development of multicellular organisms requires the coordinated change in gene expression in a spatially and temporally controlled manner. Significant progress has been made in generating high-resolution maps of the *cis*-regulatory landscape of the genome in a variety of contexts across development (Cusanovich et al., 2018; Gorkin et al., 2020; Grubert et al., 2020), but the functional intersection of molecular mechanisms that contribute to the regulation of gene expression is incompletely understood. In addition to the more widely studied epigenetic regulatory layers such as DNA methylation, chromatin accessibility, and histone modifications, the 3D organization of the genome has also been proposed to functionally contribute to gene regulation. Acquisition of cellular identity is associated with a reorganization of the 3D genome, but whether these changes are the cause or consequences of changes in transcription remains unclear (Bonev et al., 2017; Dixon et al., 2015; Rubin et al., 2017; Stadhouders et al., 2018). Emerging evidence describing how genome folding occurs has provided new mechanistic insights into the molecular logic of gene expression and has established an important framework for understanding the complexities of metazoan development.

Chromatin is non-randomly arranged in the 3D space of the nucleus and such patterns can be measured using a variety of high-throughput chromosome conformation capture (3C) or imaging techniques. In short, 3C-based methods measure the frequency of interaction between genomic regions within crosslinking distance (Dekker et al., 2002). Observation of the 3D nuclear architecture has provided a view of the multiple interconnected layers of chromatin organization (Bonev and Cavalli, 2016; Rowley and Corces, 2018; Mirny et al., 2019; Schoenfelder and Fraser, 2019). At the megabase to submegabase scale, chromosomes are organized into distinct compartments and topologically associating domains (TADs). At the local scale, DNA is packaged around nucleosomes to form chromatin, which is marked by a variety of biochemical modifications and can be bound by trans-acting proteins such as transcription factors (TFs). Communication between regulatory elements, such as promoters and enhancers, is thought to require close physical proximity of the chromatin regions, although alternative models have been proposed (Schoenfelder and Fraser, 2019). Contact maps constructed from different developmental times, cell types, and genetic mutants have contributed significantly to our growing understanding of the mechanisms and functions of genome folding and the relationship with gene expression.

Recent improvements in single-cell technologies have uncovered remarkable heterogeneity in the transcriptome, chromatin accessibility, and DNA methylation patterns during development (Carter and Zhao, 2021). Consistent with these findings, the 3D genome architecture has also been shown to vary substantially from cell to cell, raising important questions about the basis and functional role of genome folding heterogeneity (Flyamer et al., 2017; Ma et al., 2018; Nagano et al., 2017; Tan et al., 2018). In contrast to 3C methods that are limited to a static, steady-state view of pairwise genomic contacts, visualization of DNA interactions in single cells or at single loci has highlighted the dynamic nature of chromatin interactions (Boettiger and Murphy, 2020). Together, these data-rich approaches are uncovering new mechanistic insights into the emergence and maintenance of nuclear organization and into how 3D genome architecture can inform spatiotemporal regulation of gene expression. Here, we review recent findings in the molecular features that play roles in establishing genomic contacts at the different levels of 3D genome organization and the dynamic nature of chromatin folding. We discuss the core molecular machinery that facilitates the higher-order structuring of the genome and conclude with a perspective on the use of tools that measure genome contacts to contribute to our understanding of development and disease processes.

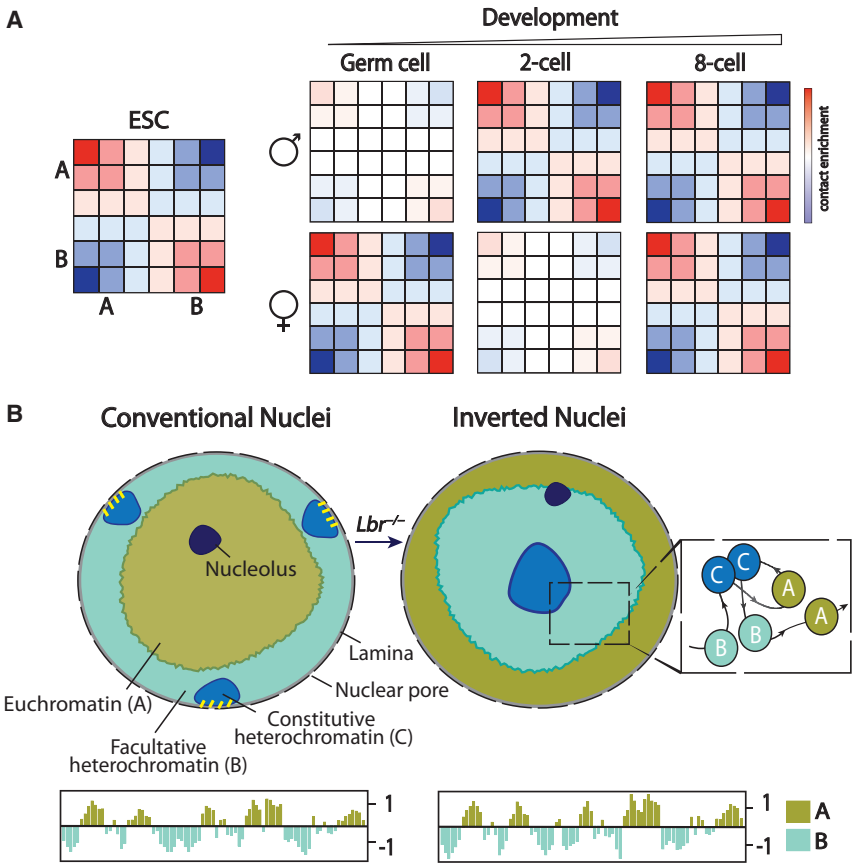

Figure 1. Large-scale topological reorganization of the 3D genome in development

(A) Saddle plot schematic showing genome-wide changes in compartmentalization during early developmental cell divisions, compared to an ESC. Data summarized from (Collombet et al., 2020; Du et al., 2017, 2020).

(B) Distinct radial organization of chromatin in conventional and inverted nuclei is independent of compartmentalization but associated with tethering to the nuclear lamina. Compartmentalization is largely driven by the attraction of homotypic interactions between constitutive heterochromatin (C-C) and facultative heterochromatin (B-B), but not euchromatin (A-A) interactions or heterotypic interactions. The schematic is based on (Falk et al., 2019).

Large-scale topological reorganization of the genome during development

Quantification of DNA contacts using Hi-C has been used to partition the genome into TADs and compartments. Although different and somewhat interchangeable definitions exist in the field (please see Beagan and Phillips-Cremins, 2020; Rowley and Corces, 2018 for a review), here we use the term TADs to denote genomic regions that exhibit an increase in interactions between loci within the domain compared with adjacent regions (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012), and we refer to compartments to describe the set of interactions between domains with a similar epigenetic nature. Compartments are typically subdivided into "A" and "B," which correlates with transcriptionally active euchromatin or inactive heterochromatin, respectively (Dixon et al., 2012; Lieberman-Aiden et al., 2009; Sexton et al., 2012). Significant progress in elucidating the mechanisms driving compartmentalization shows that heterochromatin contacts play a primary role (reviewed in Penagos-Puig and Furlan-Magaril, 2020). In comparison, the mechanisms of TAD formation are distinct from that of compartments, suggesting that these features are formed independently (Nora et al., 2017; Rao et al., 2017; Schwarzer et al., 2017).

Studies of genome organization dynamics during development have provided an intriguing context to interrogate the mechanisms and heritability of genome folding, as well as to ask when and how these large-scale topological features emerge? Observation of chromatin folding during early cell divisions in mouse gametes shows that compartments are weakly formed by the late 2-cell stage and exhibit a discernible parent-of-origin pattern until the 8-cell stage (Collombet et al., 2020; Du et al., 2017, 2020) (Figure 1A). Compartment strength increases concurrently with a gain in the facultative heterochromatin mark, H3K27 trimethylation (H3K27me3), and the establishment of strong B compartments requires the H3K27 methyltransferase, *Eed* (Collombet et al., 2020; Du et al., 2020; Zheng et al., 2016). Similarly, *C. elegans* embryos lacking the constitutive heterochromatin mark, H3K9me3, show significantly reduced compartment strength (Bian et al., 2020). Ectopic recruitment of the H3K9 methyltransferase, SUV39H1 but not the H3K27 methyltransferase, EZH2, is sufficient to drive the repositioning of genomic regions from the A to the B compartment but both result in transcriptional repression, indicating that transcriptional silencing alone may not be sufficient to cause immediate relocalization or change in compartment identity (Wijchers et al., 2016).

Though Hi-C is agnostic about the radial positioning of chromatin in the nucleus, imaging studies have shown that compartmentalization is readily observable (Misteli, 2007). In most cells, euchromatin localizes to the nuclear interior and heterochromatin is sequestered to the periphery to form lamina-associated domains (LADs) (reviewed in Crosetto and Bienko, 2020). LADs appear not to be derived from direct parental inheritance in the zygote and LAD formation precedes the emergence of compartments, suggesting that the sequestration of heterochromatin may prime the establishment of B compartments (Borsos et al., 2019). During the maturation of rod photoreceptors in nocturnal animals, these cells adopt a specific "inverted" morphology with heterochromatin positioning to the nuclear interior and euchromatin to the nuclear membrane (Solovei et al., 2009, 2013) (Figure 1B). Heterochromatin inversion is both necessary and sufficient to increase the contrast sensitivity of rods, demonstrating a functional role for chromatin organization in cellular functions (Subramanian et al., 2019). Despite the dramatically altered spatial arrangement, the degree and magnitude of A/B compartmentalization are retained in inverted cells, and polymer modeling supports the role of heterochromatin interactions in driving

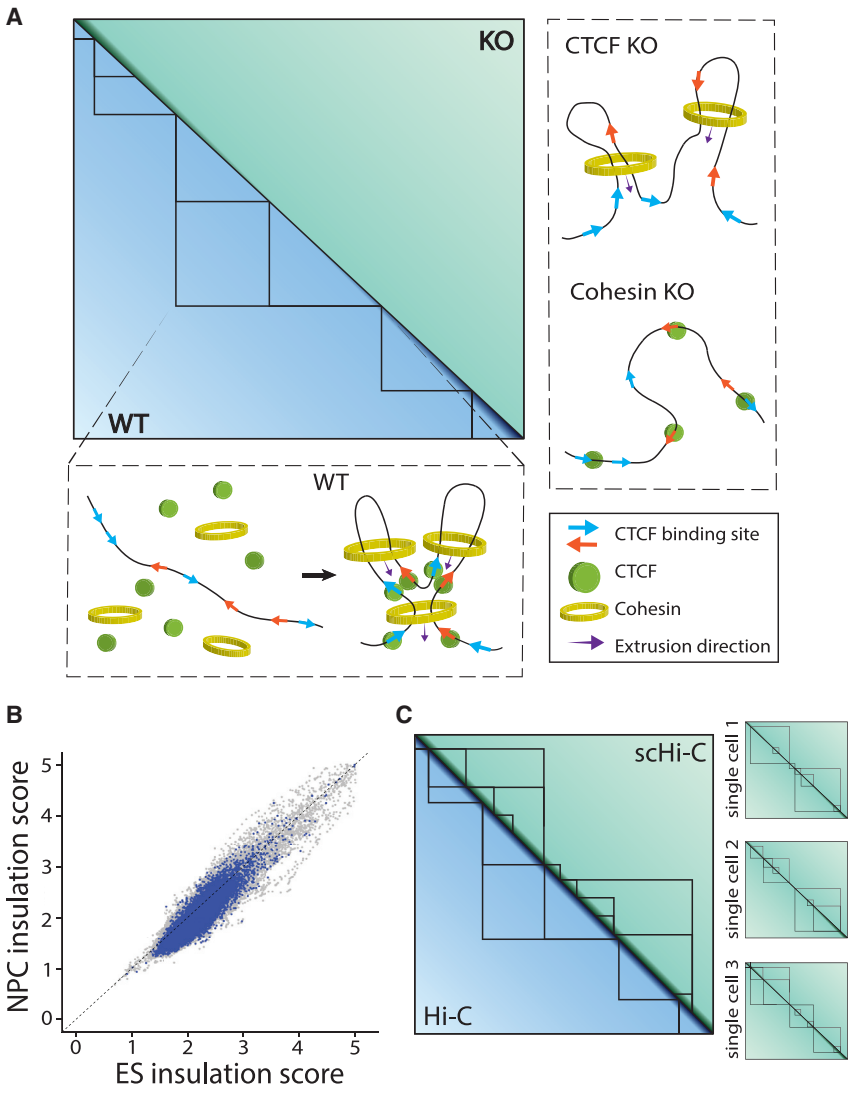

Figure 2. Dynamic loop extrusion mediated by CTCF and cohesin underlies chromatin insulation

(A) Schematic representing the loss of chromatin insulation in CTCF or cohesin-depleted cells (KO) compared with wild type (WT). Cohesin extrudes DNA until it encounters a CTCF-bound site in a particular orientation and depletion of CTCF or cohesin leads to loss of loops.

(B) Scatter plot comparing ESCs and neural progenitor cells (NPCs) in 10-kb genomic regions shows that insulation scores are a continuum, with bound CTCF sites shown in blue. Data reanalyzed from (Bonev et al., 2017).

(C) Schematic representation comparing bulk and single-cell Hi-C experiments. Single-cell Hi-C aggregate contact maps show similar average contact probability, but maps from individual cells show the stochastic nature of boundaries.

compartmentalization (Falk et al., 2019) (Figure 1B). Changes in heterochromatin domains, such as spreading of H3K9me3-marked chromatin and disruption of LADs, are observed as cells undergo senescence, raising the prospect that changes in genome organization may play a role in cell growth arrest and aging (Evans et al., 2019). Compartment switching and formation of large CCCTC-binding factor (CTCF) clusters correlate with gene expression changes in replicative senescence (Criscione et al., 2016; Zirkel et al., 2018). Furthermore, increased frequency of long-range interactions between heterochromatin regions in oncogene-induced senescence has been proposed to lead to the formation of senescence-associated heterochromatin loci that correlate with changes in gene expression of cancer-related genes (Sati et al., 2020). These results highlight the context-dependent nature of 3D genome folding changes in response to developmental cues.

In contrast to the establishment of compartments, TADs do not become apparent in the fertilized embryo until the 8-cell stage, when strong A/B compartments are already well established (Collombet et al., 2020; Du et al., 2017, 2020; Ke et al., 2017). Observation of 3D genome organization in CTCF or cohesin-depleted cells supports the critical role of loop extrusion to establish TADs (Nora et al., 2017; Rao et al., 2017; Schwarzer et al., 2017) (Figure 2A). In short, a putative extrusion complex (most likely cohesin) associates to chromatin and progressively extrudes DNA in opposite directions until it reaches a boundary element such as a CTCF-bound site in a particular orientation (Banigan et al., 2020; Fudenberg et al., 2016; Kim et al., 2019c). Maternal knock out of cohesin shows that mouse zygotes fail to acquire TADs, whereas compartments are strengthened (Gassler et al., 2017), consistent with previous observations that these chromatin features form independently and may function antagonistically (Rao et al., 2017; Schwarzer et al., 2017).

Organisms that lack known CTCF homologs, such as plants, nevertheless exhibit TAD-like structures with actively transcribing genes enriched at TAD boundaries (Dong et al., 2017). Similarly, boundaries in mammals are enriched in housekeeping genes, RNA polymerase II (RNAPII), and active chromatin marks, suggesting that transcription may contribute to the formation of TADs (Bonev et al., 2017; Dixon et al., 2012; Sexton et al., 2012). Several insights into the relationship between transcription and 3D genome organization have come from developmental studies. Although the timing of zygotic genome activation (ZGA) differs between organisms, it is similarly characterized by recruitment of RNAPII and an increase in DNA accessibility (Xu and Xie, 2018). Prior to ZGA in *Drosophila*, TAD-like structures are visible but unstructured and reform after ZGA, raising the prospect that the establishment of chromatin organization requires zygotic transcription (Hug et al., 2017). However, inhibition of Pol II transcription in *Drosophila* or early mouse embryos does not prevent the formation of TADs (Du et al., 2017; Hug et al., 2017; Ke et al., 2017).

Understanding the dynamics of chromatin folding requires us to have robust definitions of TADs. Identification of TAD

boundaries frequently relies on the quantification of chromatin insulation via a "insulation score"—the inverse probability of interactions to cross boundary regions (Crane et al., 2015; Olivares-Chauvet et al., 2016). Though many prediction algorithms have been proposed to improve the identification of TADs (Norton et al., 2018; Zufferey et al., 2018), we argue that these methods face a major limitation—trying to classify a quantitative phenomenon such as chromatin insulation as binary. This has led to the emergence of many different "flavors" of topological domains (TADs, subTADs, insulation neighborhoods, etc.), which likely represent different levels of chromatin insulation. Reanalysis of one of the highest resolution Hi-C datasets (Bonev et al., 2017) shows that insulation levels across the genome represent a continuum that does not fall into readily identifiable groups (Figure 2B). Emerging evidence about the mechanisms that lead to the formation of TADs may suggest more stringent and quantifiable criteria upon which to define these structures (Beagan and Phillips-Cremins, 2020). In contrast to biochemical measurements taken from cell populations, single-cell Hi-C and high-resolution chromatin tracing show that, although TAD-like structures can be identified, their boundaries are highly variable between individual cells (Bintu et al., 2018; Finn et al., 2019; Flyamer et al., 2017; Szabo et al., 2020) (Figure 2C). The heterogeneous chromatin contacts observed in single cells indicate that the positioning of TADs measured at the population level represents the statistical preference of certain sites to act as boundaries. At the same time, the ability of boundary regions to form new domains in a different chromatin context (Zhang et al., 2020) and the functional importance of TAD boundaries for correct cell type specification in development and disease has been shown (Franke et al., 2016; Lupiáñez et al., 2015). Furthermore, TADs have also been independently confirmed by orthologous approaches such as native 3C/4C and DamC (Brant et al., 2016; Redolfi et al., 2019) and may represent a functionally rather than structurally privileged scale in 3D genome folding (Zhan et al., 2017). These findings raise the important question of whether and how cell-to-cell variability in boundary location can influence gene expression and stochastically affect cell-fate decisions in development.

Regulatory interactions as a distinct molecular layer in gene-regulatory networks

The formation of long-range interactions between genomic regulatory elements and their target genes is a critical determinant of the spatiotemporal control of transcription (Schoenfelder and Fraser, 2019). Enhancer pairing is differentially utilized to coordinate transcriptional responses, as is seen during cellular differentiation (Furlong and Levine, 2018). However, deciphering if and how genome folding informs the specificity of enhancer-promoter (E-P) interactions and the mechanisms by which these contacts regulate transcriptional activation remain a key questions. Remarkably, dramatic rearrangement of the *Drosophila* genome using balancer chromosomes causes differential expression of only a few hundred genes despite significant reshuffling of TADs (Ghavi-Helm et al., 2019). It remains unclear whether analogous experiments in mammalian cells or acute TAD reshuffling instead of stable transgenic lines will lead to the same conclusions. Similarly, though depletion of either CTCF or cohesin results in global disruption of TADs, changes in gene expression are limited to only a few hundred to ~1,000 genes (Nora et al., 2017; Rao et al., 2017; Schwarzer et al., 2017). Furthermore, CTCF appears to be dispensable for lineage reprogramming, despite the strong effect on chromatin insulation and TAD boundaries (Stik et al., 2020). These observations suggest that additional, CTCF-independent mechanisms contribute to gene regulation and 3D regulatory interactions (Rao et al., 2017; Rowley et al., 2017). Intriguingly, genes upregulated upon cohesin ablation fall into regions of chromatin that lost insulation, supporting the correlation between chromatin insulation and adjacent contact frequencies (Rao et al., 2017; Thiecke et al., 2020). Consistent with this idea, enhancer interactions are largely limited to genes within the same TAD, and genes found within a TAD flanked by CTCF are often coregulated in cellular processes such as differentiation (Bonev et al., 2017; Ramírez et al., 2018; Zhan et al., 2017).

Are developmental changes in the 3D genome a simple consequence of transcriptional activation? Several recent studies have investigated the requirement for RNAPII and active transcription in 3D genome folding. Inhibition of RNAPII-mediated transcription in fly and mouse embryos shows that transcription is dispensable for the formation of TADs but is required for both proper localization of boundaries and for fully establishing appropriate insulation strength (Du et al., 2017; Hug et al., 2017). However, CRISPR-mediated recruitment of RNAPII was not sufficient to drive the formation of insulated boundaries, nor increase the insulation level at the target loci, suggesting that additional factors may be required to mediate local chromatin contact changes (Bonev et al., 2017). Consistent with these findings, prolonged depletion of RNAPI, RNAPII, or RNAPIII causes a modest reduction of local chromatin interactions, which is likely an indirect effect of reduced chromatin accessibility and cohesin binding (Jiang et al., 2020b). Mediator, a large complex that is involved in RNAPII activation, has been proposed to act as a bridge that tethers E-P contacts (Malik and Roeder, 2016). However, acute degradation of Mediator results in few detectable changes in E-P contacts, whereas prolonged degradation, similar to extended RNAPII depletion, leads to a reduction in chromatin contacts (El Khattabi et al., 2019). These studies support an indirect model for the transcription machinery in maintaining local chromatin contacts and insulation.

Strikingly, E-P contacts were reported to be remarkably similar across time and different tissues in *Drosophila* (Ghavi-Helm et al., 2014), suggesting that, at least in this model organism, E-P interactions largely exist as preformed topologies. In mammals, dynamic contacts have been observed in several independent studies, such as those formed *de novo* in response to stimuli, during differentiation or in circadian gene regulation (Javierre et al., 2016; Joshi et al., 2015; Kim et al., 2018; Mermet et al., 2018). These contrasting observations raise numerous questions about the underlying mechanisms that drive the establishment and maintenance of E-P contacts and how regulatory interactions contribute to the control of gene expression (Figure 3). Specifically, what mechanisms and molecular factors contribute to pre-established and dynamic contacts, and what are the contributions and consequences of these different classes of interactions to transcriptional output?

One model for how preformed E-P contacts instruct gene expression is by promoting the physical association of genes

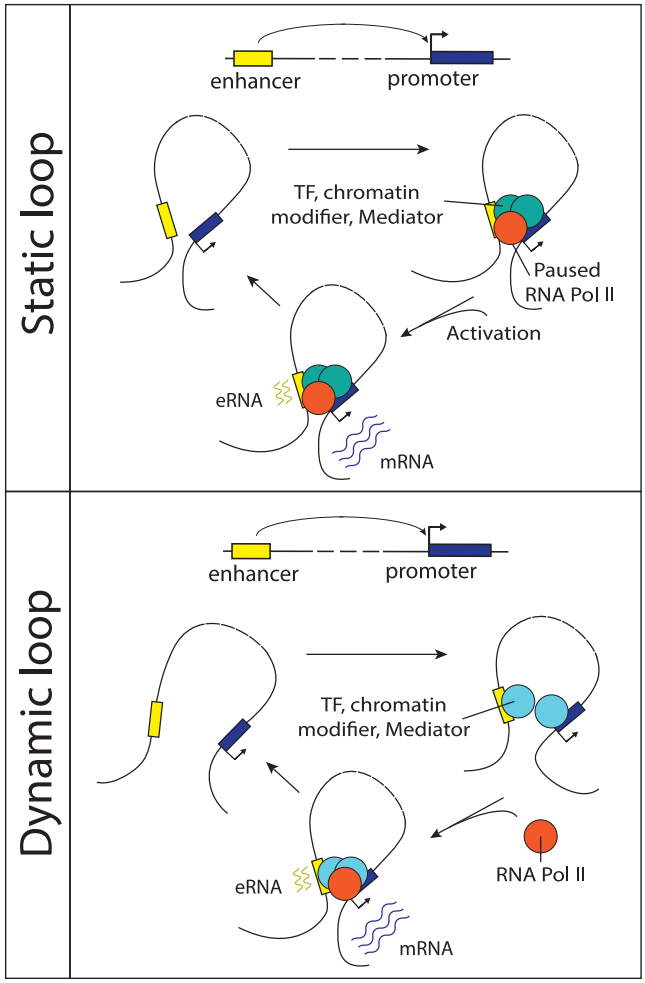

Figure 3. Functional importance of regulatory loops for gene expression and lineage specification

(A and B) Schematic representation of the two proposed models of regulatory interactions. Enhancers can be brought into proximity with target promoters by TFs, chromatin modifiers, local phase transitions, and/or the Mediator complex. In some cases, the E-P interaction is stably maintained in the presence of these factors (A), while other E-P pairs are dynamically established and reset during development (B).

that are coregulated, enabling robust and timely gene expression (Osborne et al., 2004). Enrichment for paused RNAPII at preformed E-Ps supports the model that transcription is primed at these loci, but E-P proximity is not sufficient to activate expression and likely requires recruitment of additional factors or signals to induce transcription (Ghavi-Helm et al., 2014). Contact between the developmentally regulated *Shh* gene and its enhancer, *ZRS,* are stable in embryonic stem cells (ESCs), midbrain, and limb bud tissue but gene activity is only observed in the latter tissues (Paliou et al., 2019). In comparison, *Pitx1,* which is crucial for hindlimb development, interacts with its enhancer, Pen, only in the hindlimbs but not in the forelimbs, although the enhancer is active in both (Kragesteen et al., 2018). These results suggest that regulatory interactions represent an additional regulatory layer in development and cannot simply be inferred from enhancer activity or transcription.

Changes in genomic architecture in mouse and human differentiation, both *in vitro* and *in vivo*, show that the majority of E-P contacts are highly dynamic and cell-type-specific and that these interactions strongly correlate with gene expression (Bonev et al., 2017; Freire-Pritchett et al., 2017). Loops that are stable across differentiation are frequently bound by CTCF, whereas interactions that change between tissues or across development correlate with alteration of the underlying chromatin landscape (Andrey et al., 2017). Promoter Capture Hi-C of primary human blood cells similarly shows that while promoter-promoter (P-P) interactions occur between only ~10% of genes, nearly all promoters engage in cell-type-specific interactions, and 60% of promoters establish interactions that are invariant across cell types (Javierre et al., 2016). Furthermore, changes in transcription initiation rather than a release from pausing, have recently been observed in mammalian cells at developmentally regulated E-P contacts at the α- and β-globin loci (Larke et al., 2021). Together, these observations suggest that a complex arrangement of promoter-interacting regions is required to achieve proper gene expression and the evidence supports that both stable and dynamic chromatin structures can contribute to gene expression.

Our ability to robustly determine the developmental dynamics of regulatory 3D interactions is limited due to the inherent challenges in detecting these interactions with standard 3C or imaging methods. Such interactions may be transient, present in only a subset of cells in a heterogeneous population, and may occur in close proximity along the linear genome. With sufficient sequencing depth and background normalization, Hi-C can robustly detect regulatory interactions (Bonev et al., 2017) but it is still conceptually limited to restriction-enzyme-based fragments. The introduction of Micro-C, which utilizes micrococcal nuclease fragmentation, has led to increased resolution (~100–200 bp) and a higher signal-to-noise ratio, thus improving the detection of transient loops (Hsieh et al., 2015, 2016). Application of Micro-C in mammalian cells has identified more than 10,000 novel chromatin loops and has improved signal at loops that are also identified by Hi-C (87% common) (Hsieh et al., 2020; Krietenstein et al., 2020). Although better suited for the identification of regulatory loops, Micro-C has some limitations, such as the difficulty in identifying compartments and very-long-range interactions. Crosslinking assisted proximity capture (CAP-C) has recently been reported, which enables the purification of smaller DNA fragments without DNA-bound proteins using ultraviolet irradiation instead of chemical crosslinking (You et al., 2021). CAP-C can be performed on native chromatin (nCAP-C), although fewer loops are detected, possibly due to the more dynamic nature of unfixed chromatin. Continuing technological development and integration of 3D proximity information with other regulatory layers, such as transcription or DNA methylation (Lee et al., 2019; Li et al., 2019), is critical to further our understanding of the importance of the 3D genome in gene regulation.

Functional importance of 4D regulatory interactions

As noted above, genes in TADs are often coregulated, however, there can also be both active and silent genes within a domain and expressed genes can have vastly different expression levels, indicating that spatial genome folding represents only one layer of regulation. The physical properties of enhancers likely drive at

least a portion of this heterogeneous transcriptional output (Jindal and Farley, 2021). Assays to measure whether a DNA sequence contains enhancer activity and the strength of that activity have the potential to greatly expand our knowledge of the *cis*-regulatory code (Arnold et al., 2013; Gordon et al., 2020). The prevailing model of how enhancer interactions inform transcriptional output is through changes in spatial proximity to the target gene promoter, though there are important exceptions (Furlong and Levine, 2018). Forced chromatin looping has shown that E-P pairing is not only sufficient to drive transcriptional activation but also causes changes in local chromatin architecture and binding of TFs (Bartman et al., 2016; Deng et al., 2012; Morgan et al., 2017). Disentangling the cause and consequence of dynamic 3D contacts from changes in the linear epigenome and their role in modulating precise timing and levels of gene activity will likely be a key challenge in our understanding of the regulatory code.

Temporal analysis of the dynamics and kinetics using live-cell imaging is emerging as a powerful tool to begin addressing these critical questions. Unlike proximity ligation experiments, microscopy-based tools enable visualization of the changes in proximity between targets of interest in tandem with transcriptional output in real time. Surprisingly, 3D fluorescent *in situ* hybridization in mESCs undergoing differentiation showed that transcriptional activation of *Shh* is coupled with an increase in the E-P distance (Benabdallah et al., 2019). Simultaneous measurement of E-P proximity and transcription at the *Sox2* locus did not show a positive correlation, suggesting that proximity between E-P contacts does not directly determine transcription (Alexander et al., 2019). These studies indicate that there are likely additional steps involved in the uncoupling of proximity and transcription kinetics, for example, binding of additional factors or formation of chromatin condensates. Alternatively, 3D proximity of regulatory elements may only be required for the initial activation (or repression) at developmentally regulated genes and not for maintenance of the current state. Contrary to the imaging studies discussed above, measurement of gene activity in *Drosophila* embryos using multi-color live imagining has suggested that E-P pairing is necessary for continued transcription, and that transcription reinforces local compaction (Chen et al., 2018). E-P proximity has also been shown to correlate with increased burst frequency, and enhancer strength increases the frequency but not the duration of bursts (Bartman et al., 2016; Fukaya et al., 2016). Expanding these studies to additional loci is critically needed to determine what the molecular logic of enhancer activation is and what the contribution of spatial proximity to gene activation is.

In addition to pairwise interactions, several studies have revealed that contacts between three, and likely more, chromatin loci are highly prevalent (Beagrie et al., 2017; Bintu et al., 2018; Quinodoz et al., 2018). Actively transcribed genes are coordinated in 3D space by association with transcription factories and splicing foci, which likely influences chromatin compaction (Papantonis and Cook, 2013; Razin et al., 2011). Such transcriptional hubs have been proposed to generate microenvironments that enrich for factors that mediate efficient and specific gene expression (Tsai et al., 2017). For example, deletion of a Ubx-responsive enhancer from the *svb* locus in *Drosophila* embryos is sufficient to decrease the local concentration of the Ubx TF, leading to misexpression of the *svb* TF and trichome formation defect (Tsai et al., 2019). Some enhancers can create clusters called super-enhancers (SEs) that are characterized by a high density of associated factors (TFs, chromatin regulators, and RNAPII) and the production of enhancer RNA (eRNA) (Arnold et al., 2019). The assembly of such features has been suggested to drive phase separation, a model that aims to explain both condensations of E-P contacts and the increased pairing distances caused by the assembly of multi-molecular components at these loci (Hnisz et al., 2017). Nevertheless, considerable controversy exists around whether the formation of condensates is the cause or consequence of transcriptional activation (Leslie, 2021).

Identification of the proteome of paired *cis*-elements will also greatly aid in the development of new hypotheses about transcriptionally relevant contacts. Probing the long-term protein occupancy at a CRISPR-induced E-P contact showed that the recruitment of RNA helicases was necessary to maintain looping, suggesting resolution of DNA-RNA hybrids or proper splicing could play an important role in stabilizing loops (Giraud et al., 2018). However, whether RNA helicases are a ubiquitously utilized mechanism remains an open question and the context may be particularly important, for example, do loci with no introns require RNA helicases? Some E-P pairs are also preferentially established between sites bound by the same TF (Bonev et al., 2017; Di Giammartino et al., 2019; Stadhouders et al., 2018). A large mutagenesis screen coupled to the measurement of genome folding demonstrated that a combination of TFs is sufficient to mediate pairing at an inducible contact (Kim et al., 2019b). One possible mechanism for TF-mediated contacts is exemplified by YY1 and LDB1, which form homodimers and are both necessary and sufficient to increase looping frequency (Deng et al., 2012; Weintraub et al., 2017). Whether multimerization is the prevalent way of how TFs can facilitate chromatin looping or other mechanisms such as recruitment of cofactors and formation of condensates remain to be discovered. Many exciting questions remain to not only understand how E-P pairs occur but how these features are coordinated in 3D space and what mechanisms contribute to proper spatiotemporal gene expression.

Additional molecular features inform spatial genome architecture

The establishment of genome contacts in *cis* occurs in the context of reversible covalent modifications that occur on both histone proteins and DNA. The genome is marked by distinct patterns of chromatin marks and DNA methylation that can be used to annotate and predict functions of the genome region. For example, chromatin marks such as H3K27ac and H3K4me1 correlate strongly with enhancer activation states and are commonly used to classify enhancers (Kleftogiannis et al., 2016). The Polycomb group (PcG) proteins are classified into two complexes: Polycomb Repressive Complex 1 (PRC1) and PRC2. Polycomb binding is associated with both local chromatin compaction and silencing as well as with the formation of long-range contacts between distal PcG loci (Kundu et al., 2017; Schoenfelder et al., 2015). Such long-range contacts are relatively strong in ESCs but become disrupted during differentiation (Bonev et al., 2017; Schoenfelder et al., 2015). The presence of PcG-mediated contacts demonstrates that not all chromatin

loops are associated with transcriptional activation but instead can be utilized to create targeted gene silencing and to maintain a permissive regulatory state for future activation (Cruz-Molina et al., 2017).

Functional testing of PcG domains on higher-order chromatin structure and the corresponding effect on transcription suggests that PcG proteins play an essential role in the prevention of ectopic gene expression. In contrast to the essential role of PRC for normal development, loss of PRC does not result in global changes in topological genome organization but does result in decompaction of PcG domains (Denholtz et al., 2013; Joshi et al., 2015; Kundu et al., 2017). Similarly, deletion of a Polycomb-bound element in *Drosophila* is sufficient to reduce chromatin looping and cause gene derepression (Ogiyama et al., 2018). Polycomb-dependent interactions are maintained and modestly increased in the absence of cohesin, which likely acts as an antagonist to PcG domain formation (Rhodes et al., 2020). Genes located in PcG domains were further downregulated upon cohesin depletion, suggesting that cohesin can counteract PcG associated repression. PcG domains are also present in oocytes, where cohesin is also not required for their organization (Du et al., 2020). Intriguingly, these studies raise the prospect that the mechanisms that promote high-order genome folding can be uncoupled from one another, specifically that PcG domains act largely independent of loop extrusion.

DNA methylation also plays a critical role in embryonic development. Covering 70%–80% of CpG residues throughout the mammalian genome, DNA methylation occurs in a tissue-specific manner and is associated with both transcriptionally silent and active DNA (Greenberg and Bourc'his, 2019). Genome-wide mapping shows that DNA methylation is largely depleted at TF-bound loci, though some TFs are insensitive to DNA methylation and other DNA-binding proteins are specific for methylated DNA (Héberlé and Bardet, 2019). Indeed, TF binding specificity can be affected by many means, such as cooperative and competitive binding with other proteins, chromatin environment, and DNA shape. Modulation of the binding properties of *cis*-acting proteins is one mechanism through which DNA methylation can alter genome folding, although the *in vivo* role of DNA methylation can be difficult to predict and likely depends extensively on the local context of the genome region. For example, CTCF occupancy is strongly abrogated by DNA methylation both *in vitro* and *in vivo*. However, upon genome-wide depletion of DNA methylation, CTCF occupancy was largely unaffected (Maurano et al., 2015). Of the sites that exhibited methylation sensitivity, 3,237 sites became strongly bound by CTCF when DNA methylation was reduced. It is important to note that being unmethylated is not sufficient to recruit CTCF, suggesting that additional factors are involved in recruitment. Furthermore, active demethylation, non-CpG methylation, and additional DNA marks such as hydroxymethylation can likely contribute to the dynamic mechanisms by which these modifications influence 3D genome architecture. Future studies that incorporate the dynamics of DNA marks and their association with altered genome contacts in a tissue- or developmental-dependent context are of significant interest.

DNA methylation can also influence transcription output by affecting RNAPII elongation rate, mRNA splicing, and transcription initiation. For example, recruitment of methyl-binding-domain proteins, such as MeCP2, to methylated DNA was shown to be required to repress intragenic transcription by slowing RNAPII initiation (Boxer et al., 2020). MeCP2 has also been shown to repress enhancer activity and downregulate target genes, showing that DNA marks are associated with both chromatin folding and transcription (Clemens et al., 2020). DNA methylation likely plays important instructive roles in mediating genome regulation through epigenetic crosstalk with high-order chromatin organization. Advances in technologies that enable simultaneous detection of genome features and spatial proximity in single cells have contributed significantly to understanding how DNA methylation and chromatin architecture are coordinated. For example, using a combined Hi-C and DNA methylation approach, it has recently been shown that DNA methylation is coordinated between spatially proximal regions (Li et al., 2019). Using a similar approach, single-nucleus methyl-3C sequencing (sn-m3C-seq), revealed cell-type-specific DNA methylation and contact profiles defining 14 distinct cell types from human prefrontal cortical tissue (Lee et al., 2019). These tools provide a new basis for studying the heterogeneity in genome profiles in cells from complex tissues, allowing testing new hypotheses about the mechanisms and functions of these differences.

Perturbation of the 3D genome in disease and diagnostics

DNA sequence variation has been shown to affect phenotypic variation and disease susceptibility in the human population and more recently has been shown to result in 3D genome conformation alterations. Haploinsufficiency or mutation of the CTCF gene is linked to a variety of cancers, likely through disruption of chromatin domains (Akhtar et al., 2020; Ciriello et al., 2013; Kemp et al., 2014; Mei et al., 2019; Pinoli et al., 2020; Yang et al., 2019), but more recently it was uncovered that sequence variation in CTCF binding sites can directly affect loop strength (Gorkin et al., 2019). Mutation of CTCF/cohesin-binding sequences is prevalent in multiple cancer types (Katainen et al., 2015), although it is challenging to correlate other types of SNPs with 3D conformational changes. Chromatin contact changes were found to occur within the regulatory regions of putative cancer-driver mutations, suggesting that therapeutic targeting of these features could have positive outcomes (Gorkin et al., 2019; Zhu et al., 2020).

Mutations can also indirectly affect 3D genome conformation by altering other underlying epigenetic features, such as DNA methylation, therefore future studies will be important to uncover the mechanisms of the direct and indirect effect driving altered cellular function and disease states. It remains of great interest to determine how, and if alterations in the epigenome contribute to neoplastic changes (Danieli and Papantonis, 2020). Hypermethylation observed in gastrointestinal tumors and gliomas results in disruption of CTCF binding that promotes activation of known oncogenes (Flavahan et al., 2016, 2019). Multiomic analysis of colon cancers compared with healthy tissue showed that compartments but not TADs were largely reorganized. Furthermore, DNA hypo-methylation, which occurs frequently in these cancers, was correlated with large-scale compartment reorganization, suggesting that such regions interact preferentially and lead to the formation of a third, intermediate compartment

(Johnstone et al., 2020). These studies highlight how analyzing 3D genome dynamics can shed significant light on the contribution of these changes to disease and aging.

A recent analysis of breast cancer genomes identified susceptibility loci in cell-specific enhancers, suggesting changes in enhancer activity influence disease risk (Zhang et al., 2020). Pathogenic rewiring of enhancer-promoter interactions can also be associated with tissue-specific developmental phenotypes. One prominent example of this is congenital limb abnormalities, where alterations of E-P interactions can lead to phenotypes such as polydactyly (additional digits) and syndactyly (fusions of digits). One such condition results from misregulation of the *Pitx* gene by aberrant activation of its hindlimb-specific enhancer, *Pen*, in the forelimbs (Kragesteen et al., 2018), and in another case, disruption of CTCF boundaries leads to ectopic chromatin interactions of limb enhancers (Lupiáñez et al., 2015). These results highlight the direct role that 3D genome dynamics can play in the spatiotemporal control of gene expression.

Large-scale chromosomal rearrangements are also highly prevalent in diseases and can result in changes to 3D genome folding (Akdemir et al., 2020). Detection of structural variations in the genome has been a nontrivial problem but has important applications in diagnostics. Long-read sequencing technologies can be used to map structural variation (Jiang et al., 2020a), however, this approach does not contain information about genome folding. Hi-C data have recently been used to identify copy-number variations and translocations breakpoints at single-base-pair resolution (Wang et al., 2020). The ability to analyze a variety of aspects of 3D genome architecture constitutes an important new diagnostic tool for analyzing cells from a variety of diseases, for example, comparison of prostate and breast cancer cells shows that spatial re-patterning of genes exhibit tissue-of-origin and gene-specific repositioning in a predictable and reproducible pattern (Meaburn, 2016). In another example, rearrangement of the *HLXB9* locus in leukemia is associated with transcriptional changes (Federico et al., 2019). The specificity of genomic repositioning of particular genes raises important questions about the functional relevance of these features to disease etiology, the mechanisms that lead to this misregulation and the potential therapeutic relevance of targeting such perturbations. Understanding how the 3D genome is misregulated during disease will also further inform us about its role in development. Furthermore, developing tools to manipulate and correct 3D genome abnormalities, such as light-directed forced chromatin looping (Kim et al., 2019a) or reversing the methylation state of a TAD boundary (Flavahan et al., 2016, 2019) can also be used to facilitate lineage reprogramming or reinforce certain lineage decisions in development.

Concluding remarks and future directions

Despite tremendous progress, fundamental questions remain regarding the functional relevance of the 4D genome and the molecular mechanisms mediating the establishment and maintenance of chromatin domains and contacts. Recent research, especially through developmental studies, has advanced our understanding of mechanisms of chromatin architecture formation and how the high-order folding informs gene expression. Although segregation of the genome into compartments and TADs appears to arise largely independent of each other, the different layers of genome folding may be at least partially redundant, perhaps helping to maintain robust gene expression patterns. One critical question in the field is how regulatory loops are established and remodeled during development and how to disentangle the functional importance of physical proximity from changes in the linear epigenome. The observation that 3D genome organization is highly heterogeneous across single cells suggests that the relationship between genomic contacts and molecular events like transcription is highly dynamic and to some extent stochastic. Loops are highly context dependent, relying on *cis*-acting elements and the local chromatin environment to coordinate transcription in time and space. High-resolution and multi-way contact maps have suggested the existence of multiple, simultaneous interactions between several loci and it is intriguing to speculate that such contacts could be used to stabilize chromatin states and/or coordinate transcriptional events. Furthermore, interaction strength between regulatory elements likely represents a continuum and not binary loop/no-loop events, analogous to chromatin insulation scores. Such observations in population-based methods likely reflect the frequency of interactions in individual cells and reinforce the importance of dissecting the temporal sequence of folding events in individual cells. Future studies will greatly benefit from the integration of multiple molecular-omic layers, ideally at the single-cell level, and together with advancing technologies such as long-read sequencing and live-cell imaging will likely be the key to decode the functional importance of the 4D genome organization for gene regulation in development and disease.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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