The role of 3D genome organization in development and cell differentiation

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Abstract | In eukaryotes, the genome does not exist as a linear molecule but instead is hierarchically packaged inside the nucleus. This complex genome organization includes multiscale structural units of chromosome territories, compartments, topologically associating domains, which are often demarcated by architectural proteins such as CTCF and cohesin, and chromatin loops. The 3D organization of chromatin modulates biological processes such as transcription, DNA replication, cell division and meiosis, which are crucial for cell differentiation and animal development. In this Review, we discuss recent progress in our understanding of the general principles of chromatin folding, its regulation and its functions in mammalian development. Specifically, we discuss the dynamics of 3D chromatin and genome organization during gametogenesis, embryonic development, lineage commitment and stem cell differentiation, and focus on the functions of chromatin architecture in transcription regulation. Finally, we discuss the role of 3D genome alterations in the aetiology of developmental disorders and human diseases.

Gametogenesis
The process in which diploid gamete-precursor cells undergo meiotic division and differentiation to form mature sperm and oocytes.

Primordial germ cells
(PGCs). The germline ancestor cells of both sperm and oocytes. PGCs are diploid and are first found in the primary ectoderm of the epiblast.

Protamines
Small, arginine-rich nuclear proteins that are specifically found in the haploid phase of mature sperm and that largely replace histones as the DNA-packaging proteins.

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In mammalian cells, the DNA inside the nucleus is hierarchically packaged to form chromatin fibres, and the 3D structure of the genome is proven to function in many biological processes[1–6]. For example, higher-order chromatin organizations are frequently linked to long-distance gene regulation that in turn controls development and cell fate commitment[2]. Furthermore, chromatin condensation and decondensation are crucial for proper chromosome segregation during mitosis and meiosis[7], and defects in higher-order chromatin organization can lead to development abnormalities and human diseases[8].

The conformation of the 3D genome is highly dynamic during animal development, including in gametogenesis, early embryonic development and lineage commitment. In mammals, primordial germ cells (PGCs) undergo a series of events during meiosis I and meiosis II to generate mature haploid gametes[9]. In males, spermatogonia rapidly pass through a mitotic period and two rounds of meiotic cell division to give rise to haploid spermatids[10]. The generation of spermatids is followed by global chromatin condensation and genome-wide transcription silencing during spermiogenesis to form mature sperm[10], when protamines replace most of the histones to serve as the DNA-packaging proteins[11–13]. In females, PGCs enter meiosis I and become arrested at the diplotene stage for a long period — months in mice and up to decades in humans[14]. On stimulation by hormones such as luteinizing hormone and follicle-stimulating hormone, a small number of oocytes exit diplotene arrest to resume meiosis I, before being arrested again in metaphase of meiosis II before fertilization[15]. After fertilization, the two parental nuclei fuse to form a totipotent zygote, a process associated with extensive epigenetic reprogramming[16,17]. The genome remains silenced at this stage until it is reactivated during zygotic genome activation (ZGA)[18]. ZGA occurs at the two-cell stage in mice and in humans begins between the 4-cell stage and the 8-cell stage[19]. After several cycles of embryo cleavage, lineage specification occurs as early as the morula stage in mammals before the formation of the blastocyst. During gastrulation, three germ layers, namely the ectoderm, mesoderm and endoderm, emerge in postimplantation embryos; these layers further develop into virtually all types of tissue in the body[20].

Unravelling the molecular basis of the chromatin architecture underlying these crucial developmental events has been historically challenging due to the limited availability of tools to study the 3D genome. However, remarkable progress has been made recently in the development of cutting-edge technology to probe chromatin organization. For example, fluorescence in situ hybridization (FISH), which is widely used to detect the location of particular genomic regions as well as to calculate the distance between them[21,22], can be combined with super-resolution imaging to investigate chromatin organization at unprecedented resolution[23]. Microscopy combined with CRISPR–Cas-based genome editing tools allowed the flexible spatiotemporal visualization of genomic regions in live cells[24]. Furthermore, chromosome conformation capture and its derivative technologies (such as circularized chromosome

Diplotene
A stage of prophase in meiosis I. During diplotene, the synaptonemal complex degrades and two homologous chromosomes separate from each other and uncoil.

Totipotent
A totipotent cell has the capacity to divide and produce all the differentiated cells of both embryonic and extraembryonic tissues.

Zygotic genome activation
(ZGA). The activation of gene transcription from the zygote genome after fertilization.

conformation capture (4C), chromosome conformation capture carbon copy (5C) and Hi-C) have been instrumental in detecting 3D organizations of chromatin at the DNA level[25–30]. In these experiments, cells are first treated with formaldehyde to fix the chromatin structure. The crosslinked DNA is then digested with restriction enzymes, and DNA ends within spatial proximity can be ligated. The frequencies of such ligation events can be measured by PCR or by use of high-throughput DNA sequencing. These new technologies have dramatically expanded the toolset of 3D genome research and elevated our understanding of higher-order chromatin organization.

In this Review we first discuss recent discoveries regarding the basic principles of higher-order chromatin organization before summarizing 3D genome dynamics in gametogenesis, early embryonic development, lineage commitment and cell differentiation. In our discussion of development and cell differentiation, we focus on the relationship between the reorganization of chromatin structure and transcription regulation. Finally, we discuss the latest findings on the role of 3D genome alterations in developmental disorders and human diseases. It is worth noting that the discussions here mainly focus on findings in mice and humans, with some data from other species also included for comparison.

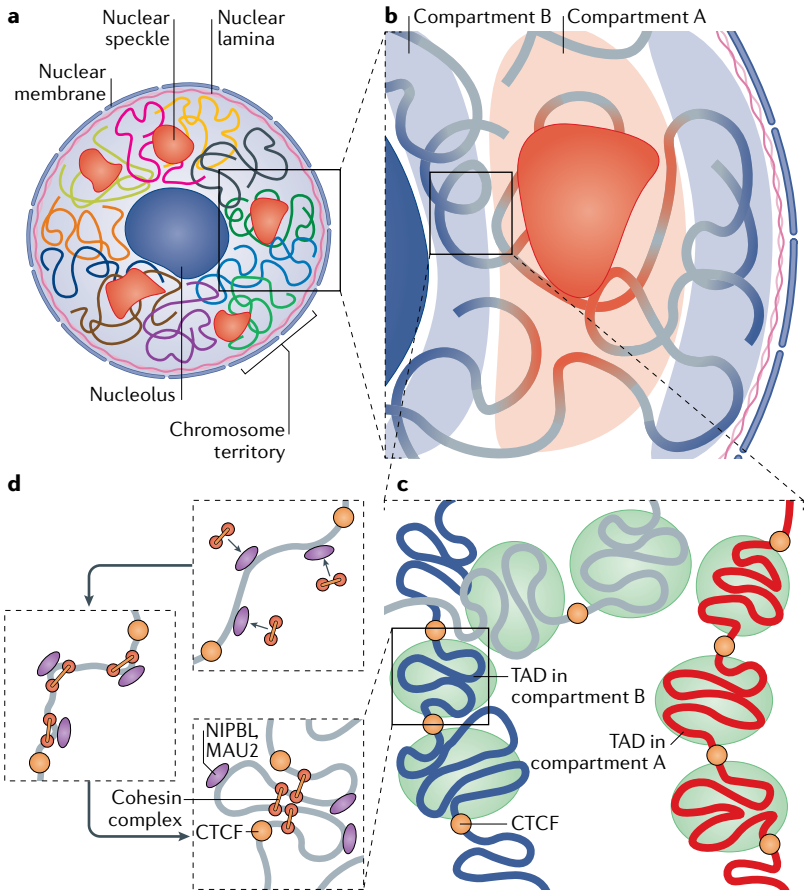


Fig. 1 | **The hierarchical organization of the 3D genome. a** | Inside the interphase nucleus, individual chromosomes (represented by different colours) occupy separate territories. **b** | At a smaller scale, transcriptionally active regions, which preferentially reside in the interior nuclear space, tend to interact with other active regions, forming compartment A. Compartment A regions from different chromosomes tend to spatially cluster near nuclear speckles. Inactive regions, which are preferentially associated with the nuclear lamina and nucleolus, tend to interact with other inactive regions to form compartment B. **c** | Locally, genomic domains show strong self-interactions and are insulated from nearby regions, forming topologically associating domains (TADs). In mammals, CCCTC-binding factor (CTCF)-binding sites on chromatin are preferentially found at TAD boundaries. **d** | Inside each TAD, cohesin-mediated loop domains facilitate chromatin folding. Once cohesin is loaded on to chromatin by a loading complex containing nipped-B-like protein (NIPBL) and MAU2, it moves along chromatin and presumably extrudes chromatin loops. Cohesin sliding can be blocked by CTCF, where loop boundaries form. Parts **a** and **b** adapted with permission from REF.[53], Elsevier.

Hierarchical 3D genome organization

Chromosomes are hierarchically folded at different levels in the nucleus; the basic principles of this 3D chromatin organization are also well reviewed elsewhere[2,4,31–33].

Chromosome territories and compartments

Individual interphase chromosomes preferentially reside in separated chromosome territories[34] (FIG. 1a), as revealed by both microscopy-based approaches such as chromosome painting[35] and various 'C technologies'[26,36]. Inside each chromosome territory, the positioning of genomic regions is non-random and is correlated with transcriptional activity[37,38]. Gene-rich regions with the potential to be transcribed tend to reside near the borders of chromosome territories[39–42], although this is not a universal rule and exceptions do exist[43,44]. For example, the *Hoxd* gene is activated in the limb bud of mouse embryonic day 9.5 embryos without detectable movement to the boundary of the chromosome territory[43]. Different chromosome territories can intermingle, particularly at their boundaries[45]. At a smaller, megabase scale, genomic regions with similar chromatin characteristics tend to interact with each other[26]. For example, transcriptionally active regions frequently interact with other active regions. These regions tend to have higher levels of gene density, chromatin accessibility and active histone modifications. By contrast, inactive regions, which typically consist of gene deserts and heterochromatin, tend to interact with other inactive regions[26,46,47]. These compartments, termed 'compartment A' ('active') and 'compartment B' ('inactive'), respectively, were identified through Hi-C, a genome-wide approach for measuring interaction intensities between any pair of regions in the genome[26]. The spatial segregation of compartments A and B was also confirmed by microscopy-based methods[48].

The spatial segregation of chromatin is often associated with various nuclear structures[49]. For example, compartment A is frequently found in the interior nuclear space, whereas compartment B is preferentially associated with either the nuclear lamina — forming lamina-associated domains[50] — or the nucleolus[38] (FIG. 1b). In human fibroblasts, about 40% of the genome is associated with lamin proteins[51]. During differentiation of mouse embryonic stem cells (ESCs) to neural progenitors and further to astrocytes, the interaction pattern between the genome and the nuclear lamina is gradually altered at hundreds of sites[52]. The spatial segregation of chromatin is not limited to intrachromosomal compartments but also applies to compartments from different chromosomes. Indeed, a recent study identified two

Gastrulation
A process of early embryonic development during which the single-layer blastula is reorganized to form a multilayer structure known as the gastrula.

Heterochromatin
A condensed form of chromatin in which gene activity is usually repressed.

Nuclear lamina
A mesh structure just inside the nuclear membrane that is composed of lamins and lamin-associated proteins.

Nuclear speckles
Also known as splicing speckles, these are nuclear domains enriched in pre-mRNA splicing factors and located in interchromatin regions of the nucleoplasm.

classes of hub for interchromosomal interactions[53]. The inactive interchromosomal hubs were found to be organized around the nucleolus and to contain centromeres and regions of ribosomal DNA; this finding is consistent with previous observations that identified nucleolus-associated chromosomal domains[54–56]. By contrast, the active hubs are organized around nuclear speckles. Mechanistically, the positioning of gene-poor compartment B regions near the nuclear periphery depends on lamin B receptor, lamin A and lamin C, as the absence of all three proteins results in a relocalization of heterochromatin to the nuclear interior[57]. Chromatin compartments were also proposed to be formed through liquid–liquid phase separation[33]. For example, the formation of heterochromatin is driven by heterochromatin protein 1 (HP1)-mediated phase separation in NIH3T3 cells[58]. In *Drosophila melanogaster*, HP1a proteins aggregate as phase-separated puncta in nuclei when heterochromatin domains begin to emerge in early embryos[59]. Nevertheless, direct evidence showing that the formation of compartment A and compartment B is driven by phase separation is still lacking.

TADs and loop domains

Another major type of chromatin organization is the self-interacting domain termed the topologically associating domain (TAD) or contact domain[60–62] (FIG. 1c). TADs were initially identified through Hi-C and 5C data of populated cells as interacting squares along the diagonal, which represent local contacts, in 2D chromatin interaction maps[60,61]. It has been proposed that one major function of TADs is to restrict promoter–enhancer interactions[63,64]. The TAD boundaries preferentially remain stable across cell types, with a small subset of boundaries showing cell-type specificity[60,65]. In mammals, TAD boundaries are usually demarcated by the chromatin architectural proteins CCCTC-binding factor (CTCF) and cohesin[60,61,66–68]. Other boundary features include histone modifications associated with active-gene regions, such as trimethylation of histone H3 Lys4 (H3K4me3) and H3K36me3, and the presence of housekeeping genes[60,67].

The cohesin complex forms a ring-like structure and can translocate on chromatin. Cohesins are recruited to chromatin by their loading factors nipped-B-like protein (NIPBL, also known as SCC2) and MAU2 sister chromatid cohesion factor homologue (also known as SCC4) and are released from chromatin through interaction with wings apart-like protein homologue (WAPL)[69–73]. The translocation of cohesins on chromatin requires ATP, as inhibition of global ATPase or mutation in the ATPase domain of the cohesin-complex component structural maintenance of chromosomes protein 3 (SMC3) can inhibit such activities[74]. Transcription may also help move cohesin along chromatin to shape its structure[75,76]. Mechanistically, CTCF and cohesin were proposed to promote 'loop extrusion', which contributes to TAD formation[77,78]. In this model, cohesin extrudes chromatin outwards until it meets chromatin barriers that are often created by CTCF[32,77–81]. In this way, loop-like structures that promote interactions within TADs, but insulate regions across TAD boundaries, are formed (FIG. 1d). In Hi-C contact matrices, these chromatin loops appear as 'peaks' of high contact frequency[67]. Notably, pairs of CTCF-binding sites at loop anchors occur predominantly in a convergent orientation, with the asymmetrical motifs 'facing' each other[67,82], and changes in the orientation of CTCF motifs can disrupt chromatin loops and TADs[78,82]. These findings strongly support the notion that pairs of CTCFs help to form chromatin loops. Furthermore, depletion of the proposed loop-extruding factors, cohesin or its loading factor NIPBL results in the global loss or reduction of chromatin domains[83–85]. Nevertheless, it is worth noting that direct evidence that cohesin can extrude chromatin loops, similar to the evidence shown for condensin[81], is currently lacking. In addition, one recent study reported that the knockout of one CTCF-associated TAD boundary does not affect local chromatin interaction patterns, as measured by 4C analysis (4C-seq)[86]. Although a more comprehensive assessment of the chromatin organization (for example, assessment using 5C or Hi-C) in this mutant has not yet been reported, it is possible that other factors besides CTCF and cohesin may contribute to the formation of TADs.

Although TADs are widely observed in many species[60–62,66,87–91], single-cell Hi-C analyses indicate that they may not exist as constant structures in individual cells but may instead represent an ensemble of chromatin conformation enrichment in a cell population[91,92]. For example, a single-cell Hi-C analysis in mice showed that contact domains are indeed found in individual cells but show cell-to-cell variation[93]. Recent research using super-resolution imaging revealed TAD-like structures in *Drosophila*[94] and in single human cells[48,95]. In human cells, the domain boundaries differ from cell to cell but are preferentially located at CTCF-binding sites and cohesin-binding sites[95]. Intriguingly, TAD-like structures can still be seen at the single-cell level after cohesin depletion, although the boundary preference for CTCF-binding sites and cohesin-binding sites is lost in these cells. It remains unclear whether the cohesin-independent TAD-like structures and cohesin-dependent TADs are fundamentally different. For example, one possibility is that cohesin-independent TAD-like domains might reflect a chromatin-intrinsic organization based on the polymer characteristics of chromatin, as polymer modelling of DNA results in globular structures that form stochastically through random polymer interactions[91]. Overall, the nature of TADs at the single-cell level still awaits further investigation.

Regulation of compartments and TADs

Chromatin compartments and TADs are likely regulated by distinct mechanisms[33,85]. Growing evidence indicates that they may actually antagonize each other[33,73,80,84,85,90]. Depletion of the cohesin-complex component RAD21 in human cancer cells or of NIPBL in mice results in the disappearance of loop domains and TADs but in the emergence of enhanced and finer compartmentalization[84,85]. A similar antagonistic relationship was confirmed by simulation analyses[33]. In humans and *D. melanogaster*, higher-resolution Hi-C has identified much smaller compartmental domains or subcompartments than

Superenhancers
Genomic regions comprising multiple enhancers that are collectively bound by multiple transcription factors to drive gene transcription.

Synaptonemal complex
A structure that forms between homologous chromosomes during meiosis and functions in mediating chromosome pairing, synapsis and recombination.

Pachytene
A stage of prophase in meiosis I during which the paired chromosomes shorten and thicken. Homologous recombination occurs during this stage.

Meiotic sex chromosome inactivation
In spermatogenesis, the transcriptional silencing of the X and Y chromosomes during meiotic pachytene.

the conventional compartments identified by lower-resolution Hi-C[67,96]. These fine-scale compartmental domains correlate with transcription activity and chromatin state. In mammalian cells, such fine-scale compartmental domains are nevertheless confounded by CTCF-mediated and cohesin-mediated loop domains, but become significantly enhanced when loop-extruding factors are depleted[84,85,90]. It is possible that compartmentalization can facilitate the general activation and repression of transcription by clustering genes to nuclear territories that are enriched in or depleted of transcription resources. Loop extrusion may facilitate region-specific promoter–enhancer interactions and provide insulation against the global transcription environment. Intriguingly, cohesin loss also causes superenhancers, even those from different chromosomes, to preferentially colocalize spatially to form clusters of transcription factor-binding domains[84]. This observation could perhaps be explained by the enhanced phase separation of highly transcribed units in the absence of restriction from CTCF and cohesin[97]. A recent study showed that the transcriptional coactivators bromodomain-containing protein 4 (BRD4) and mediator complex subunit 1 (MED1), which are both enriched in superenhancers, could form nuclear puncta[98]. Mechanistically, the intrinsically disordered regions of MED1 participate in a liquid–liquid phase separation, and such droplets can incorporate BRD4 and RNA polymerase II in vitro. Other transcription factors, including OCT4 and GCN4, can also be incorporated into phase-separated droplets with the mediator complex[99]. These results offered new insights into the relationship between chromatin organizations and the regulation of gene expression. Whether cell type-specific transcription factors could participate in phase separation and regulate 3D genome architectures during in vivo development awaits further investigations. Taken together, these results indicate that the genome is organized in a hierarchical manner through mechanisms such as loop extrusion and transcription-related compartmentalization. It is expected that additional factors involved in this process will continued to be unveiled in the future.

3D genome dynamics in gametogenesis

A life cycle starts with the fusion of an oocyte and a sperm, which are highly specialized cells called gametes[9]. These haploid cells are generated through several elegantly orchestrated processes, starting with the specification of PGCs. PGCs undergo extensive epigenetic reprogramming before entering meiosis, when homologous chromosomes are first paired and then separated into different daughter cells[9]. This event is followed by a second round of cell division to produce haploid gametes. Besides these shared features, male and female gametes undergo sex-specific development to serve their distinct functions[100]. For example, at later stages of development, oocytes undergo rapid growth and size expansion to accommodate abundant RNAs and proteins for future embryonic development[101]. Sperm, by contrast, adopt a highly compact chromatin and cell configuration during the final stage of maturation. Developmental processes that are common to, and distinct between, oocytes and sperm are accompanied by the drastic reconfiguration of chromatin organization[91,102–108], as described and discussed below.

Genome organization in spermatogenesis

In mammals, the sperm genome is packaged mainly by protamine, with only ~1–15% of DNA wrapped by histones[11–13]. Sperm DNA is reported to be coiled into large and condensed protamine toroids[109]. Consistently, Hi-C analysis revealed that sperm show more long-range chromatin interactions than ESCs and fibroblasts, which likely reflects the condensation of sperm chromatin[102,103,105]. Surprisingly, despite the chromosomes being packaged by different sets of proteins, the position of TADs and the separation of compartment A and compartment B in mouse sperm and that in ESCs and somatic cells are similar[102–105] (FIG. 2a). CTCF and cohesins bind similar loci in sperm, round spermatids and ESCs[104,110]. It is possible that Hi-C may lack the resolution to identify fine-scale differences between chromatin packaged by histone and protamine. Alternatively, such fine-scale differences may differ between individual cells and become lost in cell population data.

Chromatin organization undergoes extensive reprogramming during spermatogenesis[9,111,112]. Multiple events involving chromatin organization occur during the male meiotic cell cycles, including homologous chromosome pairing, the formation of a synaptonemal complex, meiotic recombination, desynapsis and so on[113–115]. Recent studies have examined 3D genome organization during spermatogenesis in mice and the rhesus monkey[106–108]. Interestingly, in both mice and the rhesus monkey, TADs are strongly depleted at the pachytene stage of prophase in meiosis I, during which homologous chromosomes pair to form the synaptonemal complex. As pachytene chromatin is actively transcribed[115], these results show that transcription can be largely independent of TADs at certain developmental stages. Notably, this idea is in line with the notion that CTCF is dispensable for the maintenance of synaptonemal complexes and homologous recombination[116]. Conventional compartmentalization is also weakened (but still exists) at the pachytene stage[106,107]. Compartment A, in particular, is enriched in meiotic double-strand break hotspots and crossover sites[108]. In addition, in pachytene spermatocytes from the rhesus monkey, a new type of local compartment appears and is termed the 'refined A/B compartment', which alternates between transcribing and non-transcribing regions. As its name suggests, the refined A/B compartment is more refined in resolution than conventional A and B compartments[106] (FIG. 2a). Refined A/B compartments can also be observed in mouse pachytene spermatocytes, although at a lower level than in the rhesus monkey[106]. In mouse pachytene spermatocytes, certain regions of active transcription are spatially clustered to form 'hubs' or 'point interactions'[107,108], which resemble the interaction found between refined A regions in pachytene spermatocytes from the rhesus monkey[106]. Refined A/B compartments and transcription-linked 'hubs' are absent from X chromosomes in sperm, which are inactivated during meiotic sex chromosome inactivation[106–108]. Notably,

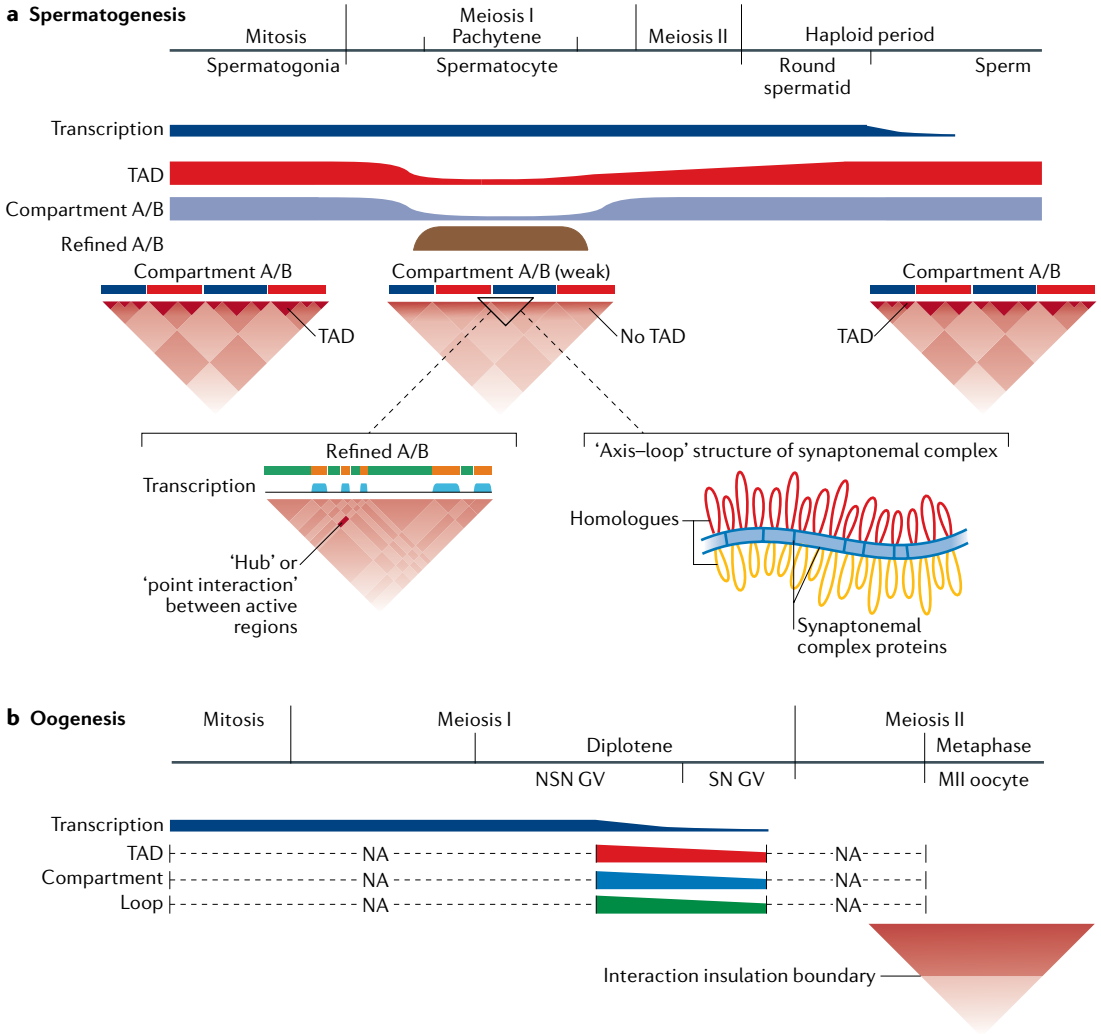


Fig. 2 | **Chromatin architecture dynamics during mammalian gamete development. a** | Reprogramming of chromatin organization during mammalian spermatogenesis, based on data from the mouse and the rhesus monkey. The stages of meiosis and spermatogenesis are indicated at the top. The strengths of topologically associating domains (TADs), compartment A and compartment B (compartment A/B) during meiosis and spermatogenesis are indicated by the widths of the bars. Both TAD and conventional compartment A/B are depleted at the pachytene stage, when a highly refined local compartment (refined A/B) emerges. Such a refined A/B compartmentalization is not found at other stages of spermatogenesis. The alternation between refined A and refined B regions correlates with alternations between transcribing and non-transcribing regions, respectively. Certain transcribing regions show strong interactions termed 'hubs' or 'point interactions'. Also at the pachytene stage, the synaptonemal complex forms; in this complex homologous chromosomes (indicated by red and orange loops) align in parallel and attach to a protein axis, forming an 'axis–loop' structure. Transcription is active throughout spermatogenesis, apart from in mature sperm. **b** | Chromatin organization dynamics in mouse germinal vesicle (GV) oocytes and oocytes arrested in metaphase of meiosis II (MII oocytes). The stages of meiosis and oogenesis are indicated at the top. The strengths of TADs, compartments and loops are indicated by bar widths. During the transition from non-surrounded nucleolus (NSN) GV oocytes to surrounded nucleolus (SN) GV oocytes, conventional chromatin organizations such as TADs, compartments and loops are present with decreasing strength. Interphase chromatin structures are lost in MII oocytes. Dashed black lines indicate stages of oogenesis for which information on chromatin organization is not available (NA). Transcription is active until the later stages of oocyte growth (that is, in SN GV oocytes and in MII oocytes). Chromatin interactions in MII oocytes drop markedly beyond a certain distance (as indicated in the interaction heatmap, termed 'interaction insulation boundary'), which likely reflects the interaction limits for linearly compressed loop arrays as observed for mitotic chromatin.

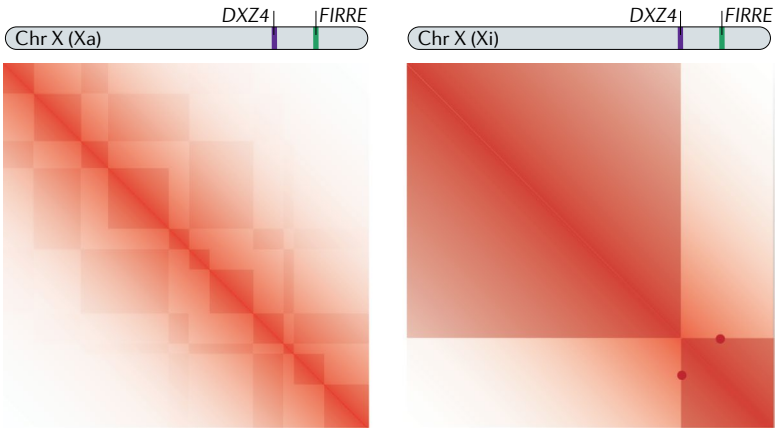
the structure of inactive X chromosomes in this case is distinct from that of the randomly inactivated X chromosome in female somatic cells, which comprises two megadomains[117] (BOX 1). By contrast, the silent male X chromosome in meiosis has no megadomains but only weakened TADs and conventional compartments.

In mouse mutant spermatocytes deficient in synaptonemal complex protein 2 (*Sycp2*) (a structural component of the scaffold of synaptonemal complex) or in type 2 DNA topoisomerase 6 subunit B-like gene (*Top6bl*) (a DNA topoisomerase that regulates double-strand breaks), the refined A/B structure is weakened and

Box 1 | Chromatin organization of the inactive X chromosome

In most mammalian species, one of the female X chromosomes is inactivated to achieve dosage compensation between male and female cells[210,211]. The random X chromosome inactivation is initiated by upregulation of the non-coding RNA X inactive specific transcript (*XIST*) on the X chromosome that is about to be inactivated[212]. Recruitment of the X chromosome to the nuclear lamina, which is induced by *XIST*, is thought to be important for its inactivation[213–215], although this recruitment alone may not be sufficient to induce gene silencing[216]. Although Hi-C analyses show that in the activated X chromosome (see the figure, left) both topologically associated domains (depicted by squares along the diagonal) and compartments (depicted by a checkerboard pattern) are observed, topologically associated domains are largely undetectable or attenuated on the inactivated X chromosome, except in regions where genes escape X chromosome inactivation[61,213,217–219]. Instead, two superdomains are formed on the inactivated X chromosome (see the figure, right), which are separated by the microsatellite repeat element *DXZ4*, which contains multiple CCCTC-binding factor (CTCF)-binding sites[48,67,220–222]. The induction of *XIST* expression can initiate the formation of superdomain boundaries at *DXZ4* (REF.[213]). In addition, megabase-long superloops were identified between the *DXZ4* locus and functional intergenic repeating RNA element (*FIRRE*; see the figure, right, indicated by the red dots) and a few other loci (not shown in the figure) in humans and mice[67,221,223,224]. Knocking out *DXZ4* from inactivated X chromosomes results in loss of both superdomains and superloops but does not have a major effect on the transcription of the inactivated X chromosome[213,221].

Several recent studies have shed light on how the inactive X chromosome is folded in a stepwise manner[219,225,226]. During the spreading of *XIST* along the X chromosome, conventional compartments are reorganized and fused into 'S1' and 'S2' compartments. Then the non-canonical cohesin protein structural maintenance of chromosomes flexible hinge domain-containing protein 1 (SMCHD1) binds to the inactivated X chromosome and further consolidates the formation of a compartmentless chromatin architecture. Loss of SMCHD1 causes defects in *XIST* spreading and heterochromatin-mediated gene silencing[219]. How such a unique chromatin structure contributes to the inactivation of the X chromosome requires further investigation.



Chr, chromosome; Xa, active X; Xi, inactive X.

absence of TADs may help promote refined chromatin compartmentalization, like refined A/B, hubs and point interactions[83–85]. It is, however, worth noting that such transcription-correlated refined compartmentalization in pachytene spermatocytes is even stronger than that found in cohesin-depleted cells[106].

Besides TADs and compartments, it has long been observed that the synaptonemal complex has an axis–loop chromatin structure, with homologous chromosomes aligned in parallel and symmetrical loops extruding from the axis[114] (FIG. 2a). Such loops are, however, not immediately evident from Hi-C data of mouse and monkey pachytene spermatocytes[106–108]; thus, it remains unclear whether refined A/B structures correspond to such loops. These results contrast with those obtained from Hi-C analyses of *Saccharomyces cerevisiae*, which showed strong looping interactions between cohesin-binding sites across the genome during pachynema[118,119]. It was suggested that in mammals the positions of such loops may differ from cell to cell, therefore becoming lost in average population profiles[108]. Nevertheless, by comparing the average interaction-to-distance patterns in zygotene spermatocytes with those in pachytene spermatocytes, one study suggested that the sizes of chromatin loops may be dynamic and increase from early to late prophase[108]. The analysis of interchromosomal interactions between homologous chromosomes appears to confirm that synapsed homologues are aligned, with loops from each homologue interdigitated[108]. Overall, these data provide an early overview of meiotic chromatin dynamics during gametogenesis.

Genome organization in oogenesis

Unlike the genome of mature sperm, the genome of mouse MII oocytes, which are arrested in metaphase of meiosis II until fertilization, lacks both TADs and compartments[103,105] (FIG. 2b). Hi-C analysis showed that, at the population level, mouse MII oocytes have a locus-independent chromatin interaction pattern that largely mimics that of cells in metaphase[36,88,120]. The lack of interphase chromatin structures in MII oocytes indicates that the maternal genome organization from the previous stage of oogenesis is extensively disassembled before fertilization[103,105]. A very similar process occurs during mitosis when interphase chromatin organization is globally disassembled and reassembled to mitotic chromatin that is composed of linearly compressed loop arrays[88,120]. However, it remains to be determined whether any architectural proteins or epigenetic marks continue to associate with chromatin in MII oocytes and, if so, whether they carry parental memory during the maternal-to-zygotic transition. A single-nucleus Hi-C study examined chromatin conformation in mouse full-grown germinal vesicle oocytes (which are at the late stage of size growth during a meiosis arrest period at the diplotene stage, after pachytene of meiosis I)[91]. There are two types of fully grown germinal vesicle oocyte: those that have a 'ring-like' chromatin rim surrounding the nucleolus (known as the 'surrounded nucleolus' conformation or SN oocytes) and those that lack this structure (known as the 'non-surrounded nucleolus' or NSN oocytes)[121]. The transition of NSN oocytes to SN oocytes is accompanied

the conventional compartments and TADs is partially restored[106]. Both mutations result in defects in the formation of synaptonemal complex and in cell cycle arrest before pachytene is entered. An intact synaptonemal complex is likely to be important for the pachytene-specific features observed when Hi-C is used.

Mechanistically, it remains unknown why pachytene spermatocytes show a depletion of TADs and the emergence of refined A/B. Synaptonemal complexes may restrict chromatin from TAD-related free extrusion and therefore eliminate TADs[106], although direct evidence for this is lacking. Alternatively, the activity of cohesin could be repurposed for the assembly of a stable chromatin loop array[108]. In both cases, the

Maternal-to-zygotic transition
The stage in early embryonic development when the zygotic genome takes control of development from the maternal genome. This transition requires zygotic genome activation and the degradation of maternal RNA and proteins.

Germinal vesicle oocytes
Growing or grown oocytes arrested in prophase of meiosis I before ovulation. The germinal vesicle refers to their nucleus, which is clearly visible under the microscope.

Pronuclei
The paternal and maternal nuclei just after fertilization, when they are still physically separated in the zygote.

by genome-wide transcription silencing and extensive chromatin compaction[122]. Conventional chromatin organizations such as loops, TADs and compartments are present in germinal vesicle oocytes, with variations between individual cells[91]. Consistent with their more compact chromatin, SN oocytes show more long-range chromatin interactions than NSN oocytes and notable decreases in loop, TAD and compartment strength[123,124] (FIG. 2b). Taken together, these data show that chromatin undergoes dynamic transitions throughout oogenesis.

3D genome dynamics in early development

Dramatic reprogramming of 3D chromatin architecture also occurs after fertilization. A study using electron spectroscopic imaging showed that chromatin is dispersed in 1-cell mouse embryos[125]. However, substantial chromatin compaction occurs during the 1-cell to 2-cell transition, resulting in the formation of large chromatin blocks that accumulate near the nuclear envelope. Recent studies using low-input Hi-C approaches have probed the reprogramming of higher-order chromatin organizations in mouse preimplantation embryos[91,103,105]. Consistent with the electron spectroscopic data[125], these studies revealed that early-stage mouse embryos contain chromatin with a relaxed structure because conventional chromatin organizations, such as TADs and compartments A/B, are unusually weak (FIG. 3). Chromatin is also relaxed in *D. melanogaster* embryos before ZGA[126]. However, one study was able to identify loops and TADs in mouse zygotes as early as in the G1 phase of the cell cycle using genomic-distance-normalized contact frequency in aggregate averaging analysis[127]. The conditional knockout of *Rad21* from oocytes results in almost complete absence of TADs and loops in both pronuclei in zygotes[127]. Conversely, TADs and loops in zygotes were strengthened when the residence time of cohesin on chromatin was increased by deletion of the cohesin release factor WAPL[127]. These results suggest that cohesin contributes to TAD regulation in early embryos. Nevertheless, the consensus is that TADs are much weaker at early stages of development than at later stages of development[103,105,127]. During preimplantation development before the 8-cell stage, the re-establishment of higher-order chromatin structure occurs at a slower rate[103,105,127] than the re-establishment of chromatin structures seen in other cell types once they are released from mitotic metaphase[36,88,120]. During early development, the 3D genome architecture of both parental genomes also undergoes allele-specific reprogramming. In 1-cell mouse embryos, chromatin compartmentalization on the maternal genome appears to be much weaker than chromatin compartmentalization on the paternal

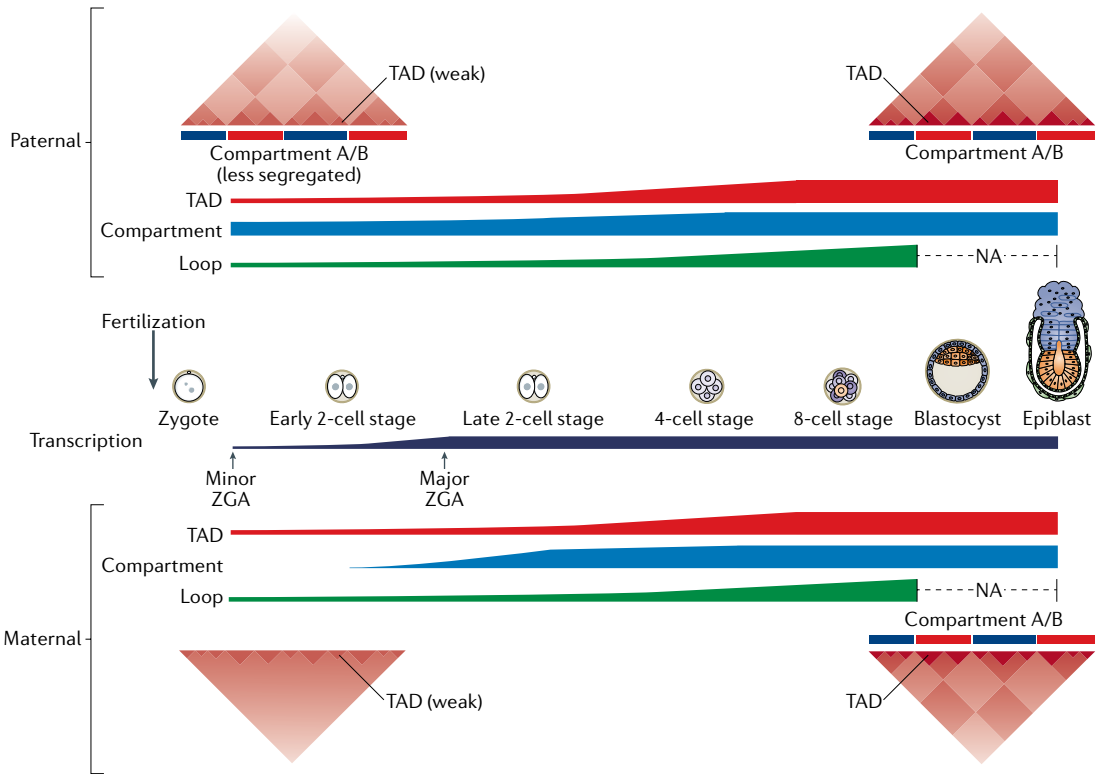


Fig. 3 | **Chromatin architecture dynamics during early development in mammals.** Reprogramming of chromatin organization during mouse early embryogenesis. The strength of topologically associating domains (TADs), compartmentalization and loops is indicated by the width of the bars. Minor zygotic genome activation (ZGA) starts at the one-cell stage of development and major ZGA starts at the middle to late two-cell stage. Weak TADs, loops and compartments start to appear in zygotes and gradually become stronger at later stages of development. Compartments appear in the paternal genome as early as the one-cell stage (although they are less segregated than those observed later in development) but are very weak or largely absent in the maternal genome in zygotes. Loop information is not available (NA) for the blastocyst and epiblast stages of early development in mammals.

genome[91,103,105] (FIG. 3). This variation may reflect the differential starting states of the gametes as sperm, but not oocytes, have highly compartmentalized chromatin[91]. Allelic differences in chromatin structure can be found as late as the 8-cell stage[103], and analysis of interchromosomal interactions revealed that the two parental genomes remain partially segregated as late as the 8-cell stage[103]. Notably, a recent study showed that two bipolar spindles exist in mouse zygotes, allowing the parental genomes to remain apart during the first cleavage[128].

Intriguingly, the reprogramming of chromatin compartmentalization in early embryos is accompanied by the reprogramming of epigenetic modifications. For example, DNA in compartment A regions is more rapidly demethylated in mouse preimplantation embryos, and more efficiently remethylated in extraembryonic tissues, than DNA in compartment B regions[105,129]. The difference in DNA methylation dynamics could be because compartment A regions are more accessible to methyltransferases and demethylases than compartment B regions, although this model awaits testing.

It remains unclear why chromatin domains are weaker in the early stages of embryo development than in later stages of development and in somatic cells. Notably, the addition of mRNA encoding the H3K9me3 demethylase lysine-specific demethylase 4D (KDM4D), KDM4B, or the histone deacetylase inhibitor trichostatin A promoted the development of mouse and monkey embryos generated by somatic cell nuclear transfer[130–133]. H3K9me3 is involved in the formation of heterochromatin[134], whereas histone acetylation promotes chromatin accessibility[135]. Therefore, adding these two factors may help promote chromatin relaxation and the appropriate reprogramming in early development. It is possible that a relaxed 3D chromatin organization may similarly promote the efficient resetting of chromatin organization from parents to early embryos. Further investigations are needed to test these hypotheses.

One striking finding from the Hi-C analysis of early mouse embryos is that ZGA occurs in 2-cell embryos before TADs are well established[103,105]. These findings appear to uncouple TADs and transcription at this embryonic stage and raise the question of how enhancers and promoters function properly without TAD restriction; additional mechanisms may exist to ensure the fidelity of transcription in these unique developmental windows. Notably, acute depletion of cohesin in the human colorectal carcinoma cell line HCT-116 (REF.[84]) or of CTCF in mouse ESCs[80] results in loss of loop domains and TADs, but has only moderate immediate impact on gene expression. By contrast, cohesin appears to be crucial for the proper activation of nearly 40% of lipopolysaccharide-induced genes in mouse primary macrophages[136]. It remains unclear whether cohesin is involved in ZGA in mouse early embryos and whether promoter–enhancer interactions are widely present in mouse ZGA. Furthermore, the consolidation of TADs can still partially proceed when ZGA is blocked in mouse embryos[103,105] and in fly embryos[126]. These findings were echoed by a recent report showing that, in mature B cells, the establishment of cohesin-mediated loop domains does not require transcription[74]. Conversely, artificially activating endogenous genes using nuclease-deactivated Cas9 showed that transcription is correlated with but not sufficient for local chromatin insulation at TAD boundaries[137]. Notably, although initial weak TADs can be found in G1 zygotes before DNA replication[91,127], the gradual consolidation of TADs in mouse preimplantation embryos was reported to require DNA replication[105]. However, this does not seem to be the case for TAD consolidation in HCT-116 cells[74]. It is worth noting that these two studies used different inhibitors of DNA replication: mouse preimplantation embryos were treated with aphidicolin[105] and HCT-116 cells were treated with thymidine[74]. In summary, the relationship between TADs, gene expression and DNA replication may differ at individual loci and may depend on cell type.

Differentiation and lineage commitment

During lineage specification and cell differentiation, the proper organization of chromatin architecture is crucial for many key molecular events, including the spatiotemporal regulation of the transcription of cell type-specific genes. In addition, chromatin is extensively reorganized during X chromosome inactivation[117] (BOX 1). Here, we briefly review how different levels of chromatin architecture can contribute to gene regulation during cell differentiation.

Chromatin compartmentalization

During cell differentiation, chromatin compartments undergo dynamic switches as cells transition between cell types. For example, during the differentiation of human ESCs, 36% of the genome undergoes compartment switching in at least one of the four derived lineages[65]. Notably, these changes correlate only moderately with changes in gene expression; a subset of genes is upregulated when their loci switch from compartment B to compartment A, and vice versa. However, most genes remain unaffected[65]. This observation is perhaps not surprising given the large sizes of compartments and the relatively small sizes of genes. Rather, genomic compartmentalization was found to be better explained by the density of transcription within a genomic neighbourhood rather than by the transcription activities of individual genes[53]. A more in-depth understanding of chromatin compartments is needed to decipher the weak correlation between compartment switching and changes in gene expression during differentiation of ESCs.

Cell differentiation also involves global compartment dynamics. For example, differentiation of mouse ESCs into cortical neurons is accompanied by an increase in compartment size, an increase in the interactions between B compartments and a decrease in the interactions within A compartments in neurons compared with mouse ESCs[137]. These changes may be related to the expansion and segregation of heterochromatin during cell differentiation[57,65,138,139].

TAD dynamics

TADs appear to be relatively more stable than compartments on cell differentiation. For example, a large portion of TAD boundaries are conserved between different cell types[60,65,140,141], although changes in chromatin

Somatic cell nuclear transfer
A technique for creating a viable embryo by transferring a donor nucleus of a somatic cell to an enucleated oocyte.

Naive pluripotency
In preimplantation embryos, pluripotent stem cells in the epiblast are in a 'naive' state. They become 'primed' during postimplantation development.

structure can occur within TADs during cell differentiation. Indeed, compared with human ESCs, differentiated cell types contain hundreds of TADs with increased and decreased intradomain interactions in regions with active epigenetic modifications (and CTCF binding) and repressive chromatin modifications, respectively[65]. It was shown that the topologies of 'metaTAD trees', which represent large-scale contacts between nearby TADs, undergo a degree (~20%) of reorganization during the differentiation of mouse ESCs to neural progenitor cells[141]. Such chromatin rearrangements are linked to changes in gene expression. The strengths of CTCF-anchored loop domains are enhanced when mouse ESCs exit naive pluripotency[142]. In addition, a study of chromatin organization in 21 types of human tissue and cells revealed genomic regions with unusually high levels of local chromatin interactions termed 'frequently interacting regions'[140]. Compared with TADs, frequently interacting regions are smaller, show stronger tissue specificity in local interaction frequencies and are enriched in active enhancers and superenhancers. These results indicate that, although the positions of TADs are often stable during development, the dynamics of chromatin interactions in local regions may correlate with transcriptional regulation.

Interactions between regulatory elements

Compared with TADs, chromatin structures are perhaps more extensively reorganized locally. This reorganization is exemplified by the cell type-specific interactions that occur between genes and *cis*-regulatory elements such as enhancers[2,143]. However, aside from using ultra-deep Hi-C datasets that contain billions of contact reads, it remains challenging for Hi-C to detect promoter–enhancer interactions due to its relatively low resolution[67,137]. Alternative genome-wide assays with higher resolution than Hi-C include protein-centric or region-centric chromatin interaction analyses such as chromatin interaction analysis by paired-end tag sequencing (ChIA–PET)[144], capture Hi-C[145], HiChIP[146] and proximity ligation-assisted chromatin immunoprecipitation and sequencing (PLAC–seq)[147]. A study using promoter capture Hi-C provided high-resolution interaction information on *cis*-regulatory elements in 17 human haematopoietic cell types[148]. Such rich resources can also be used to identify novel *cis* elements and link non-coding disease variants to their target genes.

Promoter–enhancer looping interactions. How promoters and enhancers interact has been extensively investigated. It has long been proposed that enhancers activate promoters through 'looping'[2,149]. It is worth noting that the 'loops' between enhancers and promoters typically refer to local interactions and are different from the long-range chromatin loops that are mediated by CTCF. Here, we refer to promoter–enhancer loops as 'interaction loops', which reflects increased interaction between these elements. CTCF-mediated loops, however, can facilitate enhancer–promoter interactions, either by bringing enhancers and promoters together or by separating active and silent chromatin regions[150–152]. As a result, most promoter–enhancer interactions between regulatory elements are restricted within TADs[153,154]. This restriction is in line with the model that these interactions are facilitated by cohesin-mediated loop extrusion, which cannot proceed beyond chromatin barriers that are often created by CTCF[77–80]. Besides interaction loops, chromatin architectural 'stripes' (also termed 'flames' or 'lines') were recently characterized and refer to the observation that one anchor region interacts with entire domains at high frequency[74,77,83]. This interaction results in a stripe on one side of TADs or contact domains. Stripes are often found at superenhancers, which are bound by a large amount of NIPBL. In B cells, one stripe near the immunoglobulin heavy chain gene promotes class switch recombination in this region and also facilitates translocation-mediated oncogene deregulation[74].

Although CTCF contributes to insulation domains that restrict enhancer functions, it does not directly mediate promoter–enhancer interactions. Instead, such functions are believed to be carried out by mediators and transcription factors such as yin and yang 1 (YY1)[155,156]. The roles of cohesin appear to differ between different promoter–enhancer pairs (see later). Chromatin immunoprecipitation and sequencing revealed that cohesin and NIPBL preferentially bind to chromatin at *cis*-regulatory elements that are occupied also by key transcription factors[66,76,157]. However, other promoter–enhancer interactions can be mediated by transcription factors independently of cohesin[158] (see below), suggesting that transcription factors can function with or without architectural proteins such as CTCF and cohesin to regulate promoter–enhancer interactions.

Promoter–enhancer loops and the regulation of transcription. One critical question is what is the causal relationship between promoter–enhancer loops and transcription? Developmental stage-specific and cell type-specific promoter–enhancer interactions are widely observed in the genome[2,137,159]. For example, during the differentiation of mouse ESCs to neural progenitors and further to cortical neurons, promoter–enhancer contacts are often established concomitantly with changes in gene expression[137]. Such cell type-specific interactions can even be observed for some broadly transcribed genes, which can also associate with different sets of enhancers in different cell types[160,161] (FIG. 4a). Perhaps one of the best examples of how enhancer–promoter looping can trigger transcription comes from studies of the β-globin locus. This locus contains several β-like globin genes, a large upstream regulatory element termed the 'locus control region' and multiple additional regulatory elements[162]. In humans, γ-globin and β-globin genes are specifically expressed in the fetus and the adult, respectively, and these genes undergo developmental stage-specific contacts with the locus control region when they are activated (FIG. 4b). Artificially tethering the repressed γ-globin genes to the locus control region in adult human erythroid cells substantially activates their expression[163]. Similar observations were made in mice when *Ldb1* was targeted to a silenced embryonic β-like globin (βh1) gene promoter in adult erythroblasts[163,164]. These results indicate that forced chromatin looping can

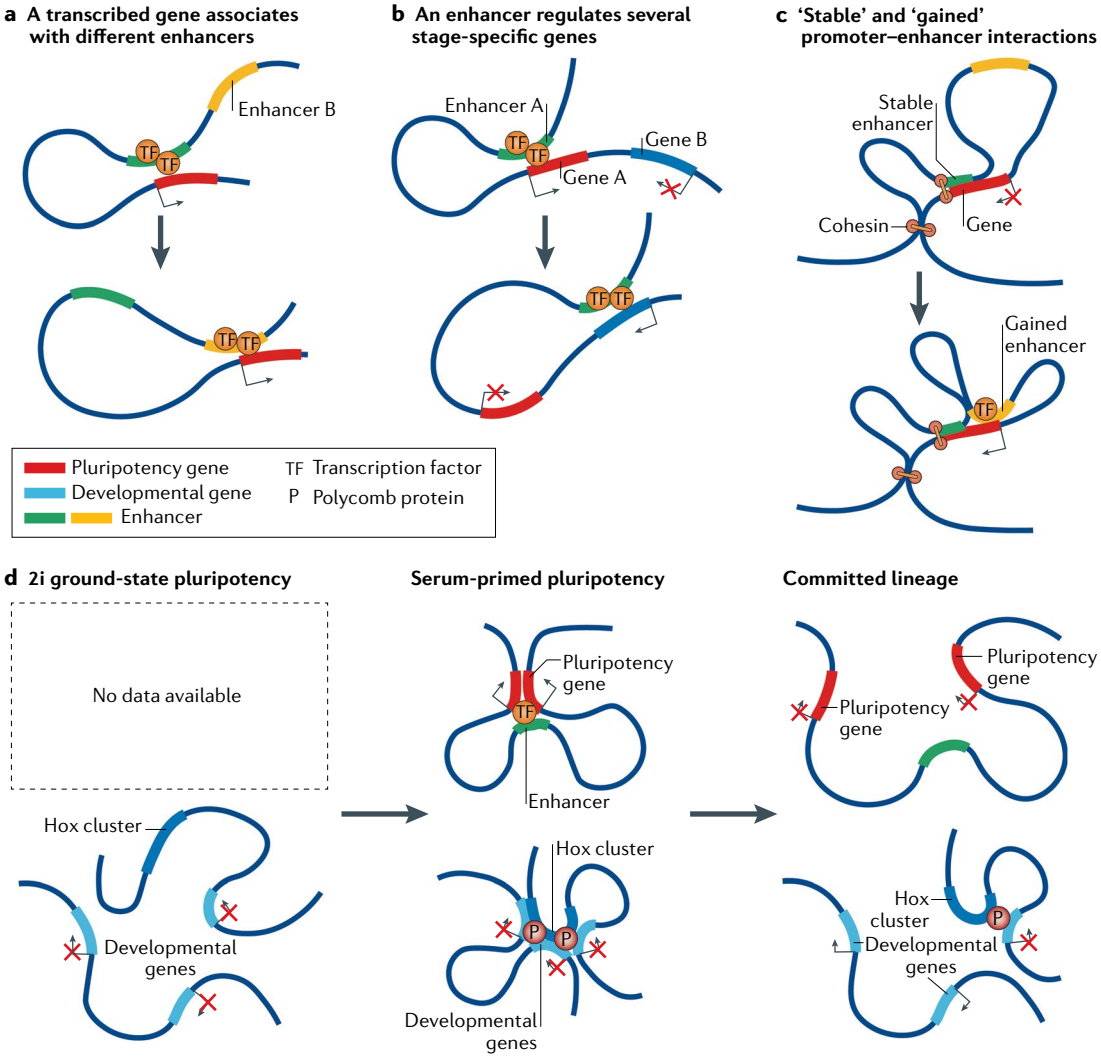


Fig. 4 | **Different modes of interactions among cis-regulatory elements in development. a** | Transcribed genes can associate with different sets of enhancers in different cell types. **b** | Different stage-specific genes (such as the β-globin gene locus) can be activated by the same enhancers through stage-specific promoter–enhancer interactions. **c** | Two types of promoter–enhancer interaction exist in stage-specific gene activation: 'stable' promoter–enhancer interactions that pre-exist before the induction of target genes are preferentially mediated by cohesin, whereas promoter–enhancer interactions that are gained on the activation of target genes are typically independent of cohesin. **d** | The dynamics of two interaction clusters during development from ground-state pluripotency to committed lineages. Pluripotency genes and enhancers are found to spatially cluster in serum-cultured primed embryonic stem cells (ESCs; top), with these interactions being lost when cells are committed to a lineage. No information is available on the spatial clustering of pluripotency genes and enhancers for 2i cultured ground-state pluripotency cells, as indicated by the empty box (top panels). In serum-cultured primed ESCs, Polycomb target genes are spatially clustered to form repression clusters, with Hox genes serving as hubs. Such interactions are not found in 2i cultured ground-state pluripotency ESCs, and a large portion of these interactions are lost when cells are committed to a lineage (bottom panels).

override the endogenous gene expression programme, providing a possible therapeutic approach to treat diseases such as sickle cell anaemia, which is caused by mutations in the β-globin gene.

Interactions between *cis*-regulatory elements, however, do not necessarily always trigger transcription. For example, enhancer–promoter interactions can also pre-exist before gene activation[165] and might be associated with paused transcription. Many enhancers that respond to signalling cues interact with their target genes before activation, which presumably allows rapid transcription activation[87]. Similarly, during fly embryogenesis, most of the promoter–enhancer interactions appear to be established before gene activation[165]. Studies of the Hox genes (a subset of homeobox genes) in mouse development indicate that pre-existing chromatin contacts may help recruit the appropriate transcription factors to establish tissue-specific promoter–enhancer interactions[166,167]. The mechanisms underlying how promoter–enhancer interactions can be either dynamic or stable during gene activation were recently elucidated. Using an epidermal differentiation system, one study showed that enhancers that stably interact with promoters before and after differentiation, but not enhancers that only interact with

Insulators
cis-regulatory elements that can block the function of enhancers or the spreading of gene silencing. The word can also refer to the protein complexes that bind to these elements, such as CTCF.

2i ground-state pluripotent mouse ESCs
Mouse embryonic stem cells cultured in the presence of MEK and glycogen synthase kinase 3 inhibitors and thought to be in a naive ground state.

Primed-like pluripotent state
Mouse embryonic stem cells cultured in serum that are thought to be epigenetically more restricted and developmentally primed than 2i ground-state pluripotent mouse embryonic stem cells and are similar to cells in the postimplantation epiblast.

promoters upon differentiation, are preferentially associated with cohesin[158]. The preloaded cohesin and interactions in progenitor cells are however not present in human ESCs. These data suggest that certain promoter–enhancer interactions may be pre-established in progenitor cells through cohesin loading (FIG. 4c), perhaps to provide a regulatory infrastructure before terminal differentiation.

It is worth noting that the conventional model of relatively stable promoter–enhancer looping has been challenged at some loci[168–170]. In *D. melanogaster* embryos, live imaging revealed that enhancers control a dynamic burst of transcription[169]. The frequency of these bursts can be reduced by introducing insulators. These results indicate that communication between promoters and enhancers is vibrant. Another study analysed the relationship between the morphogenesis gene sonic hedgehog (*Shh*) and its brain-specific enhancers during the differentiation of ESCs to neural progenitor cells[168]. Strikingly, during *Shh* activation, *Shh* and its enhancers show increased spatial distance and do not form stable chromatin loops. Finally, *cis*-regulatory elements such as promoters and enhancers within the same TAD can show higher mobility when activated[170]; the increased mobility provides additional opportunities for these elements to meet and interact. These results suggest a 'collision' model that allows more dynamic promoter–enhancer communications within a decompacted domain. Nevertheless, as this model has been shown for only a few regions, how widely it holds true remains unclear. Furthermore, what determines the choices between collision and looping for enhancers awaits further investigation.

Other types of regulatory interaction. In addition to interacting with enhancers, promoters, especially those in an active state, can interact with each other on multiple genomic scales[137]. Genes with interacting promoters tend to be coexpressed in a tissue-specific manner[171,172]. Furthermore, active promoters can function as enhancers as well. A study of the 2-Mb-long *OCT4* locus in human ESCs revealed 17 annotated promoters that can function as enhancers by interacting with and activating the *OCT4* promoter[173]. In mouse ESCs, promoters of key pluripotency genes such as the homeobox gene *Nanog* are connected by a pluripotency-specific interactome that includes many other pluripotency-related genes and binding sites of pluripotency key regulators[174–176] (FIG. 4d). Key transcription factors in ESCs, such as NANOG and OCT4, are important for the formation of this interactome[176]. The interactions are lost following differentiation and can be gradually re-established during the reprogramming of induced pluripotent stem cells[174,177]. Other promoter-anchored interactions include those between inactive genes and elements marked by repressive chromatin features, which may act as long-range silencers[145,175].

Contact network of Polycomb targets. Polycomb group proteins and the histone modifications that they introduce repress key developmental genes[178]. Emerging evidence shows that Polycomb complexes, including Polycomb repressive complex 1 and Polycomb repressive complex 2, contribute to both local and long-range chromatin architectures. Polycomb group proteins help compact local chromatin both in vitro and in vivo[179,180]. Indeed, 4C and 5C analyses of Polycomb-occupied regions reveal that these loci tend to form isolated self-interacting domains, which are smaller than TADs[181,182]. In addition, these loci interact with each other in both *D. melanogaster* and mammalian cells[177,183–186] and may contribute to the corepression of developmental genes[187] (FIG. 4d). Notably, these Polycomb-associated promoter interactions are depleted in 2i ground-state pluripotent mouse ESCs but are established when cells transit to a primed-like pluripotent state[185] (FIG. 4d). This observation indicates that such interactions may poise developmental genes for transcriptional activity in cell differentiation. In mouse ESCs, four Hox gene clusters and other genes encoding early developmental transcription factors appear to be at the centre of these repression clusters[188]. On differentiation from ESCs to neural cells, the interaction network between Polycomb target genes is progressively disrupted, while de novo interactions are established between neural transcription factors[137]. The interaction dynamics of Polycomb target genes appear to correlate with the binding affinity of the Polycomb repressive complex 1 component RING1B for these sites rather than with the presence of H3K27me3, which is a histone modification catalysed by Polycomb repressive complex 2 (REF.[137]). Polycomb proteins are necessary, but not sufficient, for the establishment of the interactions of the Polycomb network. Loss of Polycomb results in the depletion of such interactions between Polycomb target genes without alteration of the overall organization of the genome[177,185] or promoter–enhancer interactions within the Polycomb network[188]. Collectively, these data suggest that Polycomb-mediated interactions may contribute to the repression of developmental genes.

3D genome alteration in human diseases

As the proper folding of chromatin is crucial for gene regulation, there is growing attention on the relationship between alterations in chromatin structure and diseases. Mutations in the genes that encode CTCF and cohesin have frequently been linked to human diseases and developmental abnormalities[189–191]. In mice, cardiac-specific depletion of CTCF can lead to heart failure, owing to aberrant chromatin organization and the misregulation of disease-causing genes[191]. The disruption of local chromatin domains can also cause developmental disorders[8,192]. In many cases, altered boundary elements may lead to abnormal promoter–enhancer interactions known as 'enhancer adoption' or 'enhancer hijacking'. One of the best-studied cases is limb malformation caused by alterations in chromatin structure at the *EPHA4* locus[193]. In humans, limb syndromes, including brachydactyly, F syndrome and polysyndactyly, are caused by deletions, inversions or duplications near the boundaries of one TAD spanning *EPHA4* (REF.[193]) (FIG. 5a). Using CRISPR–Cas9 genome editing, researchers generated mouse models with chromosomal rearrangements corresponding to those seen in humans with these limb

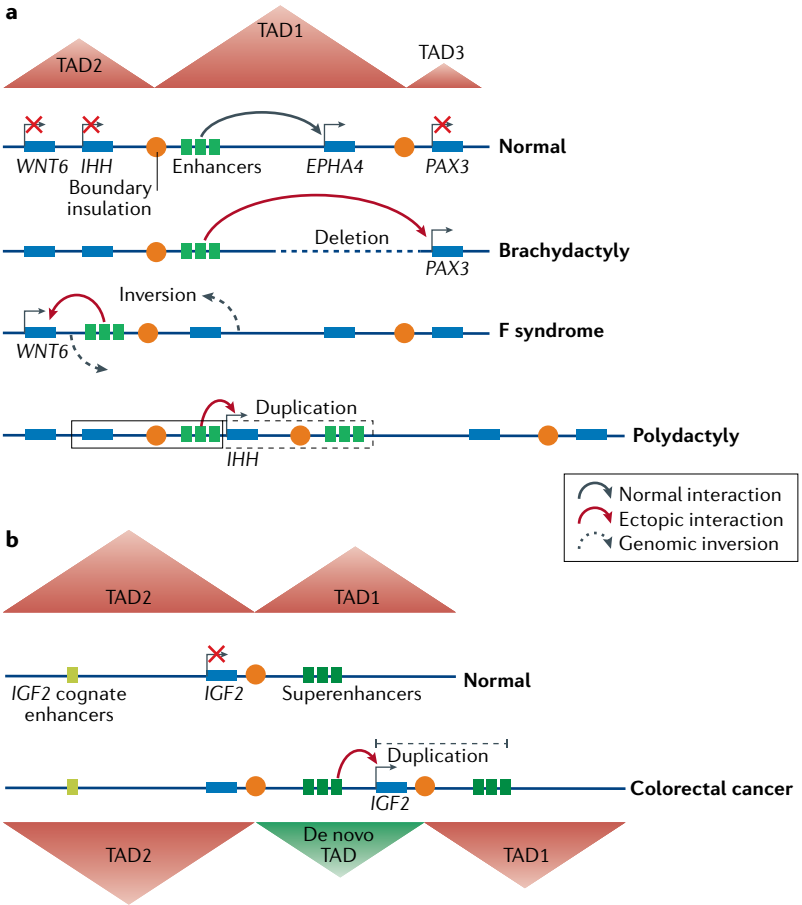


Fig. 5 | **Misregulation of the 3D genome in human diseases. a** | Three types of limb malformation that are caused by alterations in chromatin structure at the *EPHA4* locus. Normally, the interactions of enhancers in the topologically associating domain (TAD) marked 'TAD1' are restricted to *EPHA4* inside TAD1 (black arrow); the other three neighbouring genes are repressed. On deletion of the TAD1–TAD3 boundary, the enhancers aberrantly interact with *PAX3* (red arrow), resulting in the ectopic activation of *PAX3* and brachydactyly. In other cases, a genomic inversion can occur that moves these enhancers out of TAD1 and repositions them near *WNT6*. This repositioning results in the ectopic activation of *WNT6* but prevents the expression of *EPHA4*, causing F syndrome. Finally, following a genomic duplication, the enhancers are placed next to the duplicated Indian hedgehog gene (*IHH*), resulting in the formation of an ectopic interaction with and the aberrant expression of *IHH* and polydactyly. **b** | How a genomic duplication causes the formation of a new TAD and thus the overexpression of *IGF2* in colorectal cancer. The superenhancers inside TAD1 are normally insulated from *IGF2* in TAD2. The duplication results in the formation of a de novo TAD (green triangle), which contains the superenhancer and a duplicated *IGF2* gene, leading to the interaction between the superenhancer and *IGF2* (red arrow) and the ectopic activation of *IGF2*.

conditions. In mice, 4C analyses revealed that in the presence of these rearrangements a cluster of limb-specific enhancers that are normally associated with *Epha4* were spatially misplaced and aberrantly activated neighbouring genes, including *Pax3*, *Wnt6* and *Ihh*[193]. Disruption of TAD boundaries that leads to ectopic interactions between three enhancers and the promoter of *LMNB1* (encoding lamin B1) was also identified as a path to autosomal dominant adult-onset demyelinating leukodystrophy[194]. In another case, duplication of a region upstream of the gene encoding the transcription factor SOX9 in humans (termed the 'RevSex region') causes female-to-male sex reversal[195]. A larger structural variant with duplications that include the RevSex region was found to extend over the boundary of the *Sox9* TAD, resulting in the formation of new chromatin domains (neo-TADs), the ectopic activation of a nearby gene (*Kcnj2*) and limb malformation[196]. In addition, balanced chromosomal abnormalities can disrupt TADs. For example, breakpoints of balanced chromosomal abnormalities in eight individuals resulted in the repression of *MEF2C*, the deregulation of which is closely linked to 5q14.3 microdeletion syndrome. These breakpoints could disrupt a single TAD that contains *MEF2C*[197]. Up to 11.8% of the disease-related deletions recorded in the Database of Genomic Variation and Phenotype in Humans Using Ensembl Resources (DECIPHER) could involve enhancer adoption[198]. The growing chromosome conformation data provide an excellent resource that can be used to revisit the causes of many diseases[8].

Besides developmental disorders, variations in chromatin structure can also lead to tumorigenesis[192,199]; these variations are often caused through enhancer adoption or enhancer hijacking by oncogenes[200–202]. Genomic mutations are frequently found at CTCF-binding sites and cohesin-binding sites[203,204] and, importantly, the perturbation of certain domain boundary sites in non-malignant cells was sufficient to upregulate proto-oncogenes[204]. For example, a study of 7,416 cancer genomes across 26 tumour types found that deletions of one specific TAD boundary are associated with the dysregulation of *IRS4* in sarcoma and squamous cancers[205]. Also in this study it was observed that genomic duplications caused the formation of a new chromatin domain and the overexpression of *IGF2* in colorectal cancer (FIG. 5b). Another study showed that a single rearrangement in the enhancer of the transcription factor gene *GATA2* can ectopically activate *EVI1* (also known as *MECOM* or *PRDM3*) and simultaneously confer *GATA2* functional haploinsufficiency, both of which contribute to leukaemia[200].

Chromatin domains can also be affected through epigenetic mechanisms that do not disrupt the underlying CTCF motifs. For example, gain-of-function mutations in *IDH1* and *IDH2* initiate several classes of glioma[206]. Mutant isocitrate dehydrogenase proteins disrupt the proper function of ten–eleven translocation methylcytosine dioxygenase (TET) proteins and thus cause hypermethylation at CTCF-binding sites. The resulting reduction in CTCF binding is associated with loss of insulation between TADs and the aberrant upregulation of a prominent glioma oncogene, which encodes platelet-derived growth factor receptor A[206].

Future perspectives

The eukaryotic genome has long been known to be hierarchically packaged inside the nucleus. During the past few decades, key principles of chromatin folding and its functions have been progressively unveiled. Nevertheless, as in many rapidly developing fields, these studies have raised more questions than they have answered.

First, to reveal fine-scale chromatin structure, current Hi-C analyses require extremely deep sequencing. Such data depth is practically challenging when working with limited numbers of cells and/or a limited laboratory

budget. In many circumstances, loop interactions between enhancers and promoters are difficult to detect. Compounding the problem of low-resolution data is the fact that most of the detected chromatin interactions may come from constitutive chromatin interactions, rendering those that are related to spatiotemporal gene regulation rare in these datasets. Thus, how the chromatin architecture can be probed in greater detail at high spatiotemporal resolution is a major challenge in the field. Other genome-wide methods, such as capture Hi-C, ChIA–PET, HiChIP and PLAC–seq, can reach high resolution with reasonable sequencing depths although, at present, these assays usually require large amounts of cells. Advanced computational methods are also needed to help filter out background arising from constitutive chromatin interactions to better reveal regulatory interactions.

Second, despite the great successes of C technologies, observations made using them should be complemented by results from orthogonal assays. These independent approaches are crucial not only in validating results obtained by C technologies but also in directly observing chromatin organization inside the nucleus. Recently, encouraging progress was made in the development of FISH technologies[23]. DNA FISH analysis using probes derived from array-synthesized oligo libraries (Oligopaint FISH) allows imaging of regions of up to megabases in size[207]. Often combined with super-resolution microscopy, FISH has validated chromatin structures, such as TADs and compartments, that were initially detected in Hi-C maps[48,94,95]. A recent study using RNA FISH that interrogated introns from nascent transcripts showed that active genes tend to localize on the surface of chromosome territories, and that their nuclear organization is highly variable between individual cells[39]. With the advancement of CRISPR–Cas technology, robust imaging of repetitive elements and non-repetitive genomic regions can now be achieved in living cells[208], with multiple chromosomal loci being simultaneously monitored[209]. Nevertheless, independent assays with even higher resolution and greater coverage of the genome are needed to better dissect chromatin architecture.

Third, although remarkable progress has been made towards understanding the mechanisms underlining how chromatin architectures form, their functions are far from clear. Although CTCF and cohesin-dependent TADs and loop domains have crucial roles in the expression of individual, inducible genes, depletion of cohesin or CTCF does not appear to have an immediate major impact on steady-state transcription[80,84,85]. Finally, at certain developmental stages (such as in early embryos and in pachytene spermatocytes), transcription occurs in the absence of strong TADs and CTCF-mediated chromatin loops[103,105,106,126]. It would be intriguing to decipher how transcription is regulated at these unique developmental stages. Unravelling these mysteries may rely on the continuing invention of new technologies. We believe that this is just the beginning of an exciting era to understand the nature of biological processes in three dimensions.

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