

Three-dimensional genome architecture: players and mechanisms

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Abstract | The different cell types of an organism share the same DNA, but during cell differentiation their genomes undergo diverse structural and organizational changes that affect gene expression and other cellular functions. These can range from large-scale folding of whole chromosomes or of smaller genomic regions, to the re-organization of local interactions between enhancers and promoters, mediated by the binding of transcription factors and chromatin looping. The higher-order organization of chromatin is also influenced by the specificity of the contacts that it makes with nuclear structures such as the lamina. Sophisticated methods for mapping chromatin contacts are generating genome-wide data that provide deep insights into the formation of chromatin interactions, and into their roles in the organization and function of the eukaryotic cell nucleus.

Chromatin immunoprecipitation

A method in which chromatin bound by a protein is immunoprecipitated with an antibody against that protein, to allow the extraction and analysis of the bound DNA by quantitative PCR or genome-wide sequencing.

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 doi:10.1038/nrm3965

Published online
 11 March 2015;
 corrected online
 5 August 2015

The 2-metre length of DNA in a mammalian cell is organized into chromosomes, which are packaged and folded through various mechanisms and occupy discrete positions in the nucleus. The multiple levels of DNA folding generate extensive contacts between different genomic regions. These contacts are influenced by the proximity of DNA sequences to one another, by the folding architecture of local and long-range chromatin contacts and by proteins that associate directly or indirectly with the DNA. Packing of chromosomes in the nucleus also brings them into contact with one another and with nuclear compartments, such as the nucleolus and the nuclear envelope. As cells progress through the cell cycle and as they differentiate into specialized cell types, their chromosomes undergo structural re-organizations that influence gene expression and cell behaviour and function, which in turn modulate the organization of contacts between chromosomal regions. Techniques such as chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) have yielded large amounts of data on transcription factor binding and post-translational histone modifications in a range of cell types. Increasingly sophisticated methods of chromatin conformation capture (3C) are also being used to map genome-wide chromatin contacts. These approaches are enabling the study of how different cellular functions are influenced by the constraints of chromatin and chromosomal organization.

Contacts between chromatin regions are increasingly thought to have important roles in gene regulation mechanisms. In particular, since the discovery of enhancers

more than 30 years ago, it has become clear that this type of genetic element can regulate gene transcription over large distances. Looping out of the DNA that separates promoters and distally located enhancers was proposed as a mechanism by which factors that are bound to enhancers can directly contact their target promoters and influence the composition of transcription initiation complexes (FIG. 1a). Evidence supporting looping models was initially obtained by fluorescence *in situ* hybridization (FISH) and by measuring the effects of genomic distance on enhancer function^{1,2}.

In this Review, we discuss how various cellular factors are thought to contribute to the formation of higher-order chromatin structures such as active chromatin hubs (ACHs) and lamin-associated domains (LADs). We also describe the organization of the nucleus into distinct compartments and chromosome territories. We then discuss evidence that genome architecture contributes to the regulation of cellular functions such as the cell cycle, differentiation and senescence.

The organization of chromosomes

The development of the 3C³ technique and its subsequent maturation into chromosome conformation capture on chip (4C)⁴, 3C combined with high-throughput sequencing (4C-seq)⁵, multiplexed 3C sequencing (3C-seq)⁶, carbon-copy chromosome conformation capture (5C)⁷, capture-C⁸, genome conformation capture (GCC)⁹, Hi-C¹⁰, tethered conformation capture (TCC)¹¹ and targeted chromatin capture (T2C)¹² (BOX 1) have been critical for the rapid progress in the study of

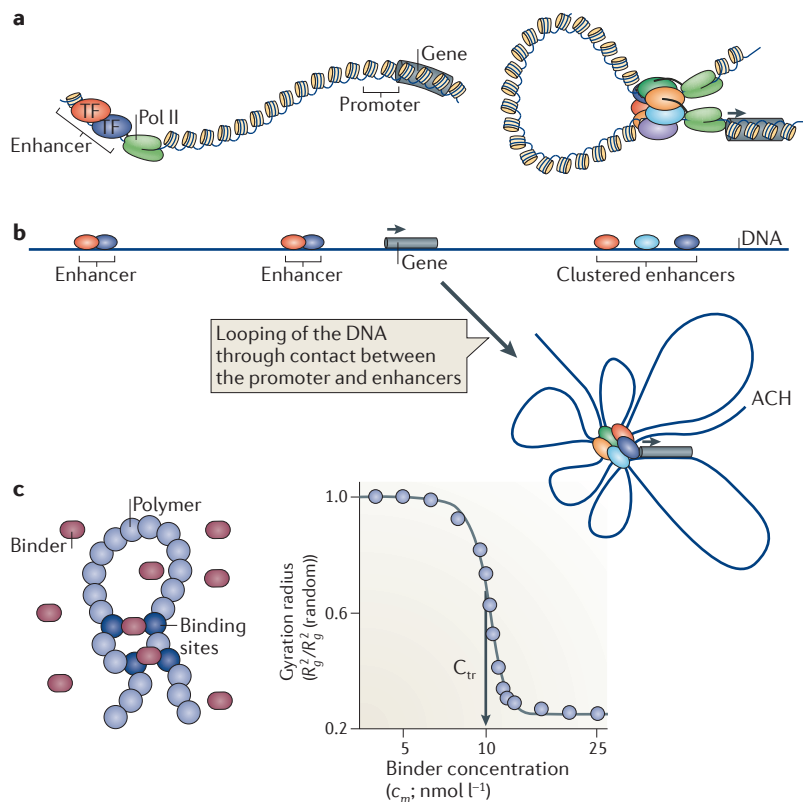


Figure 1 | Chromosome organization. **a** | Direct contact between enhancers and promoters occurs through looping out of intervening genomic sequences. RNA polymerase II (Pol II) has been shown to bind to enhancers in conjunction with transcription factors (TFs) before activation of transcription. **b** | Proposed structure of an active chromatin hub (ACH). The ACH structure is formed through direct contacts between multiple enhancers and the promoter of their target gene through looping out of intervening sequences. Coloured ovals represent factors bound to enhancers. **c** | The strings and binders switch (SBS) model considers a simple scenario in which chromatin conformations arise by interactions of specific binding sites across the polymer with randomly diffusing binding factors. SBS modelling shows that polymer loop formation displays a ‘switch-like’ response to changes in the concentration of binding factors. Gyration radius is a measure of the average polymer compaction. c_m , concentration of binding factors; C_{tr} , concentration of binding factors at threshold between open and closed polymer states. Figure, part c, is modified from REF. 113, PNAS.

chromatin contacts, allowing high-throughput mapping of putative contacts across different genomic distances and between chromosomes, with varying levels of resolution. The 3C-based methods involve crosslinking and ligation of interacting regions, after cell fragmentation by restriction enzyme digestion, followed by quantification of ligated products, which are interpreted as chromatin contacts. They have been used to measure chromatin contact frequencies that correlate well with functional studies of regulatory elements in several loci and support looping models^{13–16}. However, it should be noted that important caveats have been raised about the nature of the contacts that are detected by ligation-based methods^{9–12} (BOX 2), which should be kept in mind when interpreting results obtained with these techniques.

The formation of active chromatin hubs. Early 3C-based analyses indicated that the erythroid-specific mouse β -globin gene and its locus control region (LCR), which

is located 50 kb away, are brought into close contact in fetal liver cells, where the β -globin gene is expressed, but not in fetal brain cells, where it is not. The interaction sites in the active mouse β -globin locus are clustered and are thought to form an ACH¹³, where multiple contacts occur simultaneously (FIG. 1b). ACH-type contacts have also been described in the human *RUNX1* (also known as *AML1*)¹⁷ and *CFTR* loci¹⁸ and in the mouse *Cd8* locus¹⁹.

Formation of the hub structure in the mouse β -globin locus precedes activation of the β -globin genes in erythroid progenitor cells. The core ACH includes the DNase I hypersensitive sites of the LCR and additional hypersensitive sites at the 5' and 3' ends of the locus. The embryonic and adult β -globin genes are recruited to the ACH as they become sequentially activated during mouse development²⁰. Formation of the complete ACH requires the presence of the erythroid-specific transcription factors GATA1 and Krüppel-like factor 1 (KLF1; also known as EKLF). The cofactor LIM domain-binding 1 (LDB1) binds to both the β -globin LCR and the β -globin promoter in erythroid cells and promotes contact between them. LDB1 is part of a complex containing the transcription factors GATA1 and T cell acute lymphocytic leukaemia 1 (TAL1) together with another cofactor, LIM domain-only 2 (LMO2; also known as rhombotin 2)²¹. Tethering LDB1 to the β -globin promoter using artificial zinc fingers enhanced the formation of a chromatin loop between the LCR and the promoter in GATA1-deficient cells, with an accompanying increase in transcription²². The same effect was achieved by tethering the LDB1 self-association domain, which promotes the multimerization of the LDB1 complex²².

The ACH hypothesis has considerable implications for our understanding of how chromosomal regions that contain active genes are organized in the interphase nucleus (FIG. 1b). ChIP-seq data for histone modifications and transcription factors associated with enhancer function indicate that enhancers are present at variable distances around highly expressed genes in many cell types²³. Clustering of enhancers into ACHs would be expected to greatly affect the folding of these chromatin regions, through the formation of large constrained loops. ACHs would be established initially through the action of pioneering factors that can access condensed chromatin, or facilitated by opening of the chromatin during the S phase of the cell cycle.

The formation of ACHs seems to be a property of LCRs, which are functionally defined as transcription-activating sequences that are dominant over chromatin position effects when randomly inserted into different parts of the genome, and often contain multiple elements that would be expected to adopt an ACH-type configuration^{24–26}. This suggests that ACH formation could, at least in some cases, create folding structures that are dominant over other, more generic types of chromatin folding. Arranging single elements from LCRs into tandem repeats in transgenic mice was shown to recreate the dominant LCR transcriptional effect^{25,27}, suggesting that contacts driven by multiple transcription factors,

Box 1 | Summary of currently used chromosome conformation capture-based techniques

Chromosome conformation capture (3C)-based techniques involve the preparation of chromatin after mild formaldehyde crosslinking, followed by sonication and/or digestion with restriction enzymes, and ligation of digested DNA fragments attached to chromatin remnants. The ligation products are captured by a variety of approaches and amplified by PCR or sequenced using unbiased next-generation sequencing methods. More extensive technical reviews of 3C-based methods can be found in REFS 105–107.

Technique	Detection of chromatin contacts	Refs
3C	PCR amplification using primers of two known ('biased') genomic regions of interest	3
Chromosome conformation capture on chip (4C)	PCR amplification of genome-wide ligation products using one known (biased) genomic region of interest, followed by microarray analysis	4
Chromosome conformation capture combined with high-throughput sequencing (4C-seq)	Bait-specific library amplification from circularized 3C products using adapter-containing primers, followed by high-throughput sequencing	108
Multiplexed 3C sequencing (3C-seq)	Bait-specific library amplification from circularized 3C products using adapter-containing primers; uses restriction enzymes that recognize 4-bp sequences for finer detection of chromatin contacts	6
Carbon-copy chromosome conformation capture (5C)	Ligation and PCR similar to 3C; a collection of PCR primers covering a whole genomic region are annealed to 3C products and ligated to produce a 'carbon copy' of a section of the 3C library; amplification and sequencing of the ligated primers generates a high-coverage map of interactions in a single region	7
Capture C and targeted chromatin capture (T2C)	After ligation, DNA fragments are ligated to sequencing primers; genomic regions of interest are captured by bead or array technologies and subjected to paired-end sequencing	8,12
Genome conformation capture (GCC)	The first genome-wide unbiased 3C-based approach: global sequencing of the 3C material simultaneously measures the frequency of ligation events and the presence or absence of each genomic fragment; achieving good contact frequency signals in larger genomes is expensive, but the technique allows normalization of contact frequencies relative to the detectability and copy number of each DNA fragment	9
Hi-C	Ligation products are tagged with biotinylated nucleotides; after crosslink reversal, biotinylated fragments are pulled down to enrich for ligation products, prior to paired-end genome-wide sequencing	10
Tethered conformation capture (TCC)	<ul style="list-style-type: none"> • Biotinylation of chromatin proteins is performed before restriction enzyme digestion • Immobilization of the biotinylated chromatin on streptavidin beads removes non-crosslinked DNA • DNA overhangs are filled with an exonuclease-resistant nucleotide analogue containing α-thio-triphosphate and with a biotinylated nucleotide analogue to generate blunt ends • After blunt-end ligation, crosslinking is reversed and DNA is purified • Non-ligated DNA ends are digested with <i>Escherichia coli</i> exonuclease III, DNA is sheared and biotinylated ligation products are purified as for Hi-C and subjected to high-throughput sequencing 	11

irrespective of the factors involved, can impose alterations in chromatin organization by establishing ACH-type loops. Results obtained by polymer modelling support the idea that chromatin looping promoted by multivalent binders can have a switch-like behaviour ('on' or 'off') that is able to translate a range of transcription factor concentrations or affinities into a single, robust chromatin folding behaviour, which can be open or interacting (FIG. 1c). A discussion of approaches for characterizing the functional effects of chromatin contacts can be found in [Supplementary information S1](#) (Box).

There is still much to be learned about contacts between enhancers, promoters and silencers. Contacts can be highly flexible and diverse rather than following a single pattern of groups of enhancers

clustering around a promoter. For example, in the *KIT* locus, distally located sequences contact the promoter early in erythropoiesis and enhance transcription¹⁶. At later stages of differentiation, the promoter forms contacts with downstream intronic sequences, leading to repression. In the *MYB* locus, contact between upstream enhancers and an intronic element seems to promote transcriptional elongation¹⁵.

A high proportion of the genes in vertebrate genomes are located in large gene-dense regions. It is possible that genes located in these regions might form different contacts from the ACH structure that is observed in loci such as the β -globin locus, which are relatively distant from other active genes in the cells in which they are expressed. To address this question, the 4C technique

Box 2 | Interpreting 3C data and using them to model chromatin folding

The precise nature of the chromatin contacts detected by chromosome conformation capture (3C)-based techniques is unclear: they were initially thought to quantify contacts between distant DNA sequences brought into close proximity by sequence-specific protein bridges. However, recent studies suggest that many of the putative contacts could reflect varying degrees of proximity within larger nuclear structures (reviewed in REF. 109). A significant fraction of interactions detected by 3C-based techniques may result from ligation events that occur in the insoluble nuclear remnant of the chromatin preparation¹¹⁰. Electron microscopy of this remnant identifies nuclei that retain their typical morphology and have heterochromatin located in the periphery. This suggests that many of the ligation events captured by 3C-based methods could be happening in the context of the higher-order organization of intact nuclei rather than isolated sonicated fragments that would reflect specific contacts¹¹⁰. These issues introduce a level of imprecision about exactly what is being measured by 3C-based techniques, which undermines, for example, the unbiased identification from genome-wide datasets of regulatory regions that are thought to interact specifically with individual promoters. Ligation events generated between sequences located close to specific contacts and baseline ligation levels arising from random contacts between chromatin¹⁰⁵ will add to the background noise. Ligation-based methods are also affected by inherent biases in restriction enzyme cutting efficiency, ligation frequency and the fact that the contacts detected represent the average of the chromatin states of the individual cells in a population at the moment of crosslinking¹¹¹.

The development of effective mathematical models that use 3C data to predict the folding behaviours of single loci or whole chromosomes is likely to provide an important complement to the experimental approaches, and should provide insights into the question of which chromatin contacts are most important for folding, for gene regulation and for chromosome architecture. Pioneering efforts to mathematically model chromosome organization began by modelling chromatin folding using approaches that reflect broad-scale organization of chromosomes, but have only recently started to take advantage of the more detailed information gained from chromatin contacts measurements by 3C-based approaches (reviewed in REF. 112). Further developments in the modelling of chromatin contacts will be particularly important for capturing and shedding light on the mechanisms of folding that underpin the variability in chromatin folding observed at the single cell level as well as in different chromatin states, at different stages of the cell cycle, and in different cell types and organisms^{11,113–115}.

was used to measure contacts between *Rad23a*, a housekeeping gene located in a gene-dense region on mouse chromosome 8, and other genomic regions and to compare these with contacts made by HS2 of the mouse β -globin LCR⁴. The analysis showed that *Rad23a* preferentially interacts with many other gene-dense chromosomal regions, both locally and through *trans* interactions with other chromosomes, and that the interactions are largely conserved between the erythroid cells in the fetal liver and the fetal brain. By contrast, the β -globin gene interacts with other active erythroid-specific genes in fetal liver cells, but with silent genes in the fetal brain.

The roles of CTCF and cohesin in chromatin folding. As discussed above, current evidence supports a mechanism for action at a distance based on direct contacts between enhancers and promoters. These contacts bring together factors and co-factors that are bound to the contacting elements, thereby facilitating the formation and correct function of the transcription pre-initiation complex. In addition, another class of proteins has been identified that promotes interactions between distally located sequences, the best-characterized being cohesin and CCCTC-binding factor (CTCF).

Cohesin has a crucial role in establishing sister chromatid cohesion during S phase and maintaining it through G2 and mitosis. The core cohesin complex is made up of a heterodimer of the proteins SMC1 (structural maintenance of chromosomes 1) and SMC3, in combination with SCC1 and SCC3. The complex forms a ring structure and is loaded onto DNA early in G1, through a process that depends on the cohesin-loading complex NIPBL–MAU2 (reviewed in REF. 28).

In addition to its cell cycle functions, cohesin is involved in regulating gene expression through the stabilization of long-range contacts between *cis*-acting regulatory elements. Cornelia de Lange syndrome, which is caused by mutations in NIPBL and the cohesin subunits SMC1A and SMC3, is associated with dysregulation of gene expression in many tissues²⁸. A large number of cohesin-binding sites, mapped globally in the mouse genome, colocalize with binding of CTCF²⁹. Cohesin-binding sites were shown to be important for the organization and transcriptional regulation of the mouse interferon- γ locus³⁰ and for correct rearrangement of the T cell receptor- α locus³¹. The mechanisms that allow cohesin to stabilize long-range interactions have not yet been elucidated but could involve the same ring structure that maintains sister chromatid cohesion.

CTCF was originally characterized as a factor that binds to the promoter of the chicken *Myc* gene³² and functions as an activator or a repressor of transcription depending on the context. A major reason for the interest in CTCF has been its association with insulators, which are sequences that block the action of enhancers on promoters (reviewed in REF. 33). The canonical example is the insulin growth factor 2 (*Igf2*)–*H19* locus, in which DNA methylation-sensitive binding of CTCF is involved in regulating an insulator that controls the epigenetic imprinting of the locus. However, long-range interactions are unimpeded by the presence of CTCF-binding sites in 79% of loci where such sites are present between enhancers and promoters³⁴. This suggests that the relationship between CTCF and insulator effects is more complex than was originally thought.

In addition to its well-established functions in enhancer blocking, CTCF is involved in promoting long-range interactions that stimulate transcription. These include the interaction between the promoter of the insulin gene and synaptotagmin 8 (*SYT8*), which enhances *SYT8* transcription in pancreatic islets³⁵, and the enhancer–promoter interactions that generate multiple protocadherin isoforms on the surface of neurons^{36,37}. Overall, the evidence to date suggests that CTCF is multi-functional and can act as a gene activator or a silencer as well as a promoter of looping between distant regions. These different roles are likely to depend on the association of CTCF with different binding partners. It is also possible that the common feature of all three functions is the ability of CTCF to promote interactions between distal sequences.

Topologically associating domains. Global analyses of chromatin contacts revealed genome-wide patterns that suggest that chromatin contacts operate at different

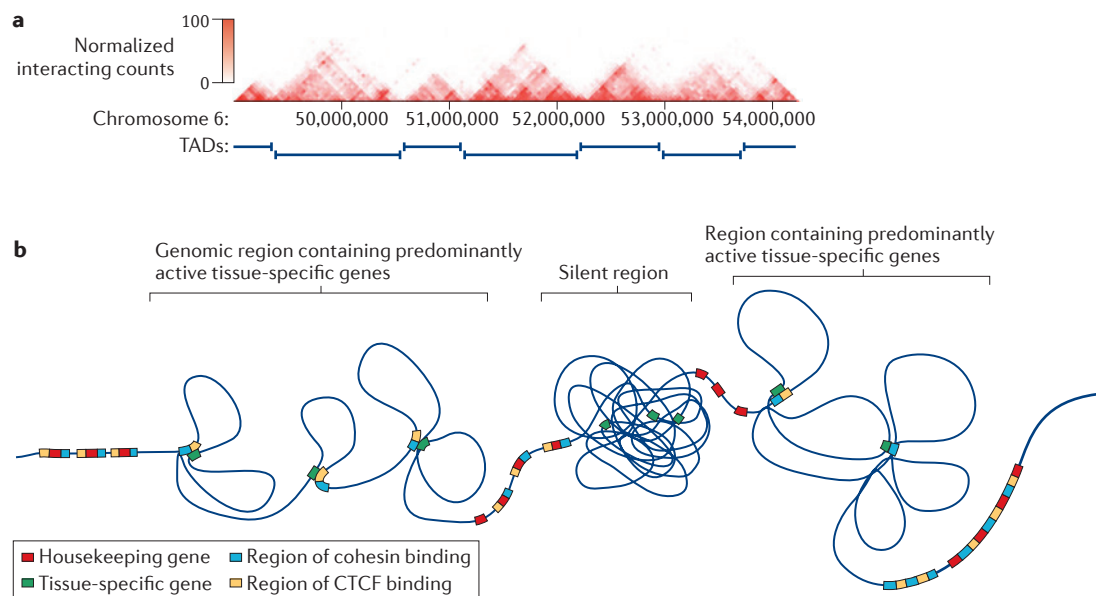


Figure 2 | Topologically associating domains. **a** | Hi-C profiles reveal that the mammalian genome is organized into topologically associating domains (TADs): regions that show high levels of interaction within the region and little or no interaction with neighbouring regions. The heat map represents normalized Hi-C interaction frequencies. **b** | Schematic of putative TAD structures. The central regions of TADs show high levels of chromatin interaction and coincide with the presence of tissue-specific genes and their associated enhancers, the interactions of which with their cognate promoters are facilitated by the presence of cohesin and CCCTC-binding factor (CTCF). The border regions between TADs are enriched for housekeeping genes, which are often clustered together and generally lack the widely dispersed distal enhancers that are found around tissue-specific genes. The border regions show high levels of CTCF and cohesin binding, although only CTCF seems to prevent interactions between TADs. Figure, part **a**, is reprinted from REF. 38, Nature Publishing Group.

levels^{38,39}. Hi-C analyses of mouse and human embryonic stem cells (ESCs) and human fibroblasts³⁸, and of the chromosomal region around the mouse X inactivation centre³⁹, revealed that the genome contains large regions that are defined by high levels of chromatin interactions occurring within the region or domain, interspersed with genomic regions with fewer interactions (FIG. 2a). These regions are generally referred to as topologically associating domains (TADs). A genome-wide Hi-C analysis identified 2,200 TADs occupying 91% of the mouse genome, with an average size of 880 kb³⁸. Interestingly, the boundaries between TADs are enriched for housekeeping genes (FIG. 2b). Histone marks associated with enhancers, such as histone 3 Lys4 monomethylation (H3K4me1), and with transcription repression, such as histone 3 Lys9 trimethylation (H3K9me3), were not found to be enriched at TAD boundaries. The structural organization of the genome into TADs is maintained across different cell types and is partially conserved between the human and mouse genomes³⁸. TADs have also been mapped by Hi-C in the *Drosophila melanogaster* genome and have been found to have many of the characteristics of vertebrate TADs^{40–42}. *D. melanogaster* TADs are smaller than their vertebrate counterparts, perhaps reflecting the smaller size of the *D. melanogaster* genome and the closer packing of genes within it. To date, TADs have not been identified in *Saccharomyces cerevisiae*.

The relatively large size of TADs^{38,39} means that they mostly contain a large number of genes with a variety of

cell-type specificities. This makes it unlikely that they represent a general partitioning of the genome into functional domains. However, there is evidence that boundaries between TADs can demarcate functionally distinct regions of the genome in some cases. For example, the *Hoxa* locus is separated into an active and a repressed compartment by an insulator that binds CTCF⁴³ and was found to correspond to a TAD boundary in differentiated cells³⁸.

Several recent studies have looked at the roles of cohesin and CTCF in TAD organization^{44,45}. In all of these studies, knockout or depletion of cohesin reduced interactions within TADs but the TADs retained their basic shape. Two studies reported that reducing cohesin levels did not lead to increased interaction between domains^{44,45}, whereas a third reported significant increases in inter-domain interactions⁴⁶. Depletion of CTCF had the same effect, but it also increased the levels of interactions between different TADs^{44,45}. The different conclusions from these studies could relate to differences between the cell types used. The study reporting increased inter-domain interactions⁴⁶ analysed interactions in differentiated astrocytes, which could have a different chromatin organization from the non-cycling thymocytes⁴⁴ or cycling embryonic kidney cells⁴⁵ used in the other two studies. These results suggest that cohesin and CTCF are primarily involved in promoting promoter–enhancer interactions but could also have some involvement in delineating the boundaries between TADs.

Box 3 | The organization of chromatin into functional compartments

Different factors can cluster together in the nucleus to form functional nuclear compartments. Well-characterized examples of this include the clustering of active DNA and RNA polymerases, as well as chromatin regulators such as the Polycomb group (PcG) repressor complexes. The clustering of DNA polymerases during replication in discrete domains, termed replication factories, is observed in yeast, plants and mammals^{116,117,72}. Strikingly, in mammalian cells, clustered origins of replication that initiate replication simultaneously can remain associated during several rounds of cell divisions⁷².

RNA polymerases are also found in discrete domains called transcription factories (see the figure). RNA polymerase I (Pol I) factories transcribe the 45S ribosomal RNA genes, which are clustered in the genome into groups of many tens of copies. Pol I factories are found within nucleoli (see the figure, left), which in HeLa cells contain on average of 500 active enzymes and about four 45S rRNA genes, each of which is transcribed simultaneously by approximately 125 enzymes^{118,119}. Individual nucleoli bring together 45S rRNA genes from clusters present in different chromosomes, constituting one of the first known examples of gene expression coordinated within a single structure (reviewed in REF. 120). By contrast, Pol II and Pol III factories are more abundant but contain far fewer polymerases (see the figure, right). In HeLa cells there are about 8,000 Pol II and about 2,000 Pol III factories, each containing approximately 6 to 8 active enzymes^{118,121,122}. The number of factories per nucleus seems to scale with genome size, but their spatial density and size remain broadly constant. For example, diploid mouse embryonic stem cells (ESCs) have 4,000 transcription factories. By contrast, the salamander, the genome of which is 11 times larger than that of diploid mouse ESCs, has 33,000 transcription factories¹²³. The transcription factory model is further supported by observations that active genes associate with one, or a few, active RNA polymerases¹²⁴, and that single genes can be found co-associated at shared Pol II sites^{125,126}.

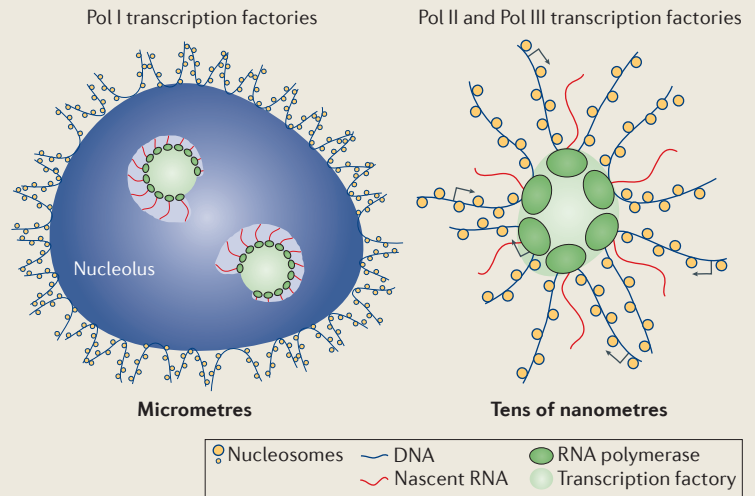
Recent evidence suggests that Pol II transcription factories are functionally specialized according to the activation state of the polymerase complexes that they contain. Transcription factories in a 'poised' state contain the initiating form of Pol II (in which Ser5, but not Ser2, is phosphorylated in the carboxy-terminal domain repeats)⁶³ and are associated with genes repressed by PcG complexes^{63,127}. In HepG2 cells, *UPA* (urokinase-type plasminogen activator) is not expressed but is found associated with poised factories⁶³. Following transcriptional activation, *UPA* associates with active factories that contain Pol II phosphorylated on both Ser5 and Ser2. Developmental genes repressed by PcG in ESCs, such as *Nkx2-2*, *Msx1* and *HoxA7*, are also found associated with poised factories¹²⁷.

PcG bodies are another type of functional nuclear compartment, present in the nucleoplasm of *Drosophila melanogaster* and mammalian cells. In *D. melanogaster*, Polycomb response elements (PREs) colocalize with PcG bodies in anterior nuclei of the syncytial embryo¹²⁸. In the posterior region of the embryo, inactive PREs such as *bxd* are also associated with PcG bodies, whereas active PREs (such as *Fab7*) lose contact with these domains, supporting the idea that the association with PcG bodies is important for gene silencing. The pairing of two *Fab7* elements from different genomic regions, and the co-association of three PREs (*Fab7*, *Mcp* and *bxd*) within the BX-C *Hox* gene locus, also occur within shared PcG bodies^{128,129}. Indeed, analyses of chromatin immunoprecipitation combined with chromosome conformation capture of a major Polycomb protein, enhancer of zeste 2 (*Ezh2*), demonstrated its presence at intra- and inter-chromosomal hubs that are associated with gene silencing¹³⁰.

Overall, the data suggest that TADs are complex entities, with structures that are governed by several different parameters and that are potentially generated by heterogeneous mechanisms. ACH formation through enhancer–promoter contacts is likely to contribute strongly to the contacts that are observed within TADs. The enrichment of housekeeping genes and the depletion of the H3K4me1 enhancer mark at TAD boundaries suggest that some aspects of TAD organization might be the product of differential distribution of cell-type-specific and housekeeping genes along chromosomes.

Interaction of chromosomes in the nucleus

The specificity and frequency of chromatin contacts are affected by the organization of the nucleus into structural and functional compartments, such as the nuclear lamina and various proteinaceous bodies (BOX 3). The behaviour of whole chromosomes also depends on their size, the number and state of activity of genes that they contain, and the distribution of heterochromatin. Chromatin contacts and their functions are also likely to be affected by the biophysical environment in which chromosomes exist. Important parameters include the nuclear shape, mechanical stress generated by cellular



Nuclear lamina

A protein meshwork made of intermediate filaments (such as lamins) and membrane-associated proteins (such as emerin) that covers the inner nuclear membrane and is responsible for maintaining nuclear shape, organization and function.

Heterochromatin

Highly condensed chromatin that shows dark staining. Constitutive heterochromatin remains in this state throughout the cell cycle. Facultative heterochromatin is cell-type-specific condensed chromatin that is often a feature of terminally differentiated cells.

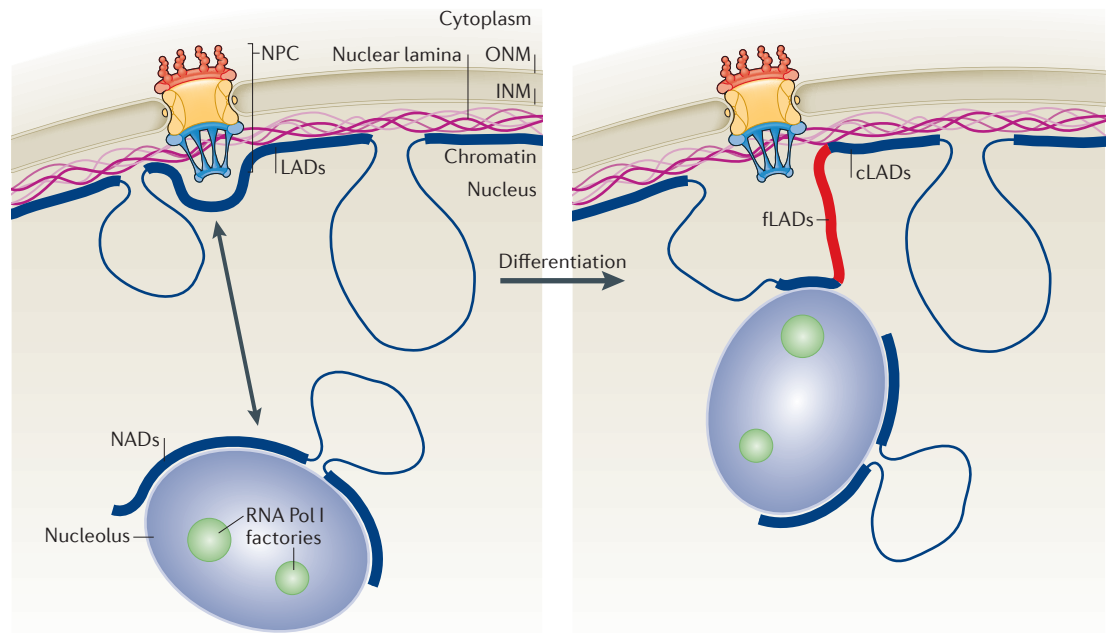


Figure 3 | The nuclear envelope affects genome organization and function. Lamina-associated domains (LADs) are regions of condensed chromatin that are bound by the nuclear lamina. LADs are also enriched for marks such as histone 3 Lys9 dimethylation (H3K9me2), which is a mark of heterochromatin and silent genes. Regions of open chromatin, in which genes are actively transcribed, loop out into the interior of the nucleus. As cells differentiate, constitutive LADs (cLADs) remain associated with the lamina, whereas facultative LADs (fLADs) become detached as the genes that they contain become active. After mitosis, some LADs relocate to the periphery of the nucleolus in the following G1 phase of the cell cycle, although the mechanism underlying this remains unclear. Sequences preferentially located at the nucleolar periphery (nucleolus-associated chromatin domains; NADs) have been identified independently by high-throughput sequencing of DNA associated with biochemically isolated nucleoli. NADs are enriched for pericentric satellite repeats, A- and T-rich sequences and gene-poor regions. INM, inner nuclear membrane; NPC, nuclear pore complexes; ONM, outer nuclear membrane.

movements and the cell cycle. Below, we discuss some of these features of nuclear organization.

Nuclear and nucleolar peripheries organize chromosomes. The nuclear envelope is a membrane bilayer, made up of the inner and outer nuclear membranes (FIG. 3). Embedded in the envelope are the nuclear pores, which are large and highly organized structures that span the two membranes and mediate transport in both directions between the cytoplasm and the nucleus. The inner side of the nuclear envelope is coated with lamins and many other proteins. Together, they form the nuclear lamina, which is thought to have key roles in the spatial organization of chromosomes, as it is a large surface that establishes contacts with chromatin while remaining spatially constrained by its location on the inner wall of the nuclear envelope.

Nuclear lamins are members of the intermediate filament protein family. They are found in all multicellular animals but are absent from plants, fungi and protozoa. They are divided into two types, lamin B and lamins A and C. Type B lamins are present in all cell types; type A and C lamins are absent from ESCs, and their expression increases as cells differentiate, although they are expressed at low levels in terminally differentiated neurons⁴⁷. A key function of the nuclear lamins is to maintain the shape and mechanical properties of nuclei;

indeed, cells that are depleted of lamins or that express mutant lamins often have misshapen nuclei⁴⁸. Lamins also have important roles in many nuclear processes, including transcription, DNA replication, cell cycle control and DNA repair (reviewed in REF. 49).

In most cell types, the nuclear periphery is associated with heterochromatin, whereas euchromatin is more found centrally located. A remarkable exception to this general feature is the central position of heterochromatin and the peripheral position of euchromatin that is observed in the rod cells of the eye in nocturnal animals, which is thought to be important for the light transmission properties of rod cells (see below). Chromatin attachments to the lamina have been mapped at high resolution in *D. melanogaster*, mouse and human cells, using DNA adenine methyltransferase identification (DamID) or ChIP-seq (reviewed in REF. 50). Mapping of lamin B-chromatin interactions revealed that large genomic regions, termed LADs⁵⁰, are bound to the nuclear lamina (FIG. 3). LADs vary in size (0.1–10 Mb) and are strongly enriched for H3K27me3 and, to a lesser degree, for H3K9me2 (REF. 51), which are indicative of Polycomb-repressed and heterochromatic sequences, respectively.

Two types of LADs have been identified: constitutive LADs (cLADs) and facultative LADs (fLADs; FIG. 3). The genomic distribution of fLADs varies in different cell types, whereas cLADs, which are A-T rich, are

DNA adenine methyltransferase identification

A method based on expression of fusion proteins with bacterial Dam methylase, and detection of methylated DNA as a measure of its contact with the fusion protein.

maintained across a wide range of cell types and show a high degree of cross-species conservation (91% between mouse and human, compared with 67% for fLADs)⁵². It has been suggested that constitutive interaction of LADs with the lamina could act as a structural backbone for the organization of interphase chromosomes⁵². The mechanisms that direct the interaction of cLADs and fLADs with the lamina are likely to involve interactions with DNA-binding factors, although these factors have yet to be identified.

LADs have a low gene density, but owing to their overall genomic coverage, they still contain thousands of genes, most of which are not expressed. This provides some support for the longstanding hypothesis that peripheral nuclear localization promotes gene silencing. Several studies have tested this hypothesis by expressing a lamina protein tagged to *lacI* and artificially inducing its ectopic binding to *lacO* sites inserted at different chromosomal locations. Particularly illuminating results were obtained by repositioning a chromosome to a more peripheral location in the nucleus, by tethering it to a protein of the inner nuclear membrane⁵³. Microarray analysis showed that several genes on that chromosome were silenced, but also that expression of other genes was not reduced, which suggests that localization to the nuclear periphery is not incompatible with gene expression. These conclusions are supported by the observation that transgenes are expressed even when integrated into heterochromatin⁵⁴, indicating that heterochromatin is not always restrictive for transcription.

In vivo analyses of the dynamics of Dam-methylated chromatin show that the association of LADs with the nuclear periphery is not fully re-established after each cell cycle; a proportion of the LADs that appear in the nuclear periphery during one cell cycle can be found at the periphery of the nucleolus in the following cell cycle⁵⁵ (FIG. 3). Such a dynamic organization suggests that both the lamina and the nucleolar periphery have the potential to organize silent chromatin, although it is not yet clear whether similar gene silencing mechanisms operate in both compartments. The mechanisms underlying this dynamic organization are not known. Inactive ribosomal RNA gene arrays are found at the periphery of the nucleolus, forming a layer that has many of the characteristics of heterochromatin (reviewed in REF. 56). High-throughput sequencing has been used to characterize the DNA sequences bound to the nucleolus (nucleolus-associated chromatin domains; NADs), and these have been found to be enriched for pericentric satellite repeats, A- and T-rich sequences and gene-poor regions^{57,58}. cLADs are also enriched for A- and T-rich sequences and gene-poor regions, suggesting that cLAD and NAD sequences share a mechanism of attaching to the nuclear or nucleolar periphery. Identifying this mechanism is likely to provide important insights into the systems that govern chromosomal organization in interphase cells.

Chromosome territories in the nucleus. Above, we discuss the evidence that distinct genomic regions, TADs, are potential units of chromosomal organization. At the next level of organization, whole chromosomes

occupy discrete chromosome territories in the interphase nucleus, with preferred positioning that depends on the cell type and is conserved between humans and other primates^{59,60}. Striking changes in chromosome positioning are rare, but have been reported to occur within minutes⁶¹. The positioning of many genes changes in response to environmental stimuli, during cell commitment and in disease^{62–65}.

Many models of chromosome organization have been put forward to describe genomic features that are specific for cell type or species. In mammalian cells, the existence of chromosome territories coupled with the observation that some genes are repositioned outside these territories following transcriptional activation, led to the formulation of a model in which chromosome territories are separated by an inter-chromosomal compartment that allows the formation of rare long-range interchromosomal interactions⁶⁶. Higher-resolution analysis of chromosome territory organization was subsequently carried out using cryo-FISH, a high-resolution method that combines FISH with ultrathin sectioning of cryoprotected cells⁶⁷. This analysis showed that chromosomes intermingle extensively in primary human lymphocytes, with an estimated 20% of the nuclear volume containing sequences from intermingled pairs of chromosomes⁶⁷; levels are likely to be even higher in resting lymphocytes⁶⁸. An important functional consequence of chromosome intermingling is that it seems to promote preferential rearrangements between specific chromosomes, depending on their physical proximity^{59,67}. Comparisons of the extent of intermingling in human lymphocytes with the likelihood of chromosomal translocations following ionizing radiation revealed a strong linear correlation with the frequency of chromosomal translocations. This suggests that sequences at regions of intermingling are more likely to recombine than those at the interior of chromosome territories following DNA double-strand break formation⁶⁷. A direct correlation between translocation frequencies (measured using high-throughput genome-wide translocation sequencing (HTGTS)) and inter-chromosomal contacts (quantified by Hi-C) was also observed in G1-arrested mouse pro-B cells⁶⁹. Analyses of specific loci, such as *Igh* and *Myc*, also show a direct relationship between chromatin contacts (measured by 4C-seq) and sporadic translocation events (measured by translocation capture sequencing (TC-seq))⁷⁰.

Quantitative analyses of chromosome territories by cryo-FISH revealed additional unexpected features of chromosome organization in human lymphocytes⁶⁷. First, the volumes of chromosome territories are not simply proportional to their DNA content but depend strongly on the linear density of active genes on each chromosome. The observation that chromosomes with higher transcriptional activity occupy larger nuclear volumes may help to explain the positive relationship observed between higher RNA polymerase II (Pol II) occupancy, active histone marks and expression, and increased translocation frequency^{69,70}. This is particularly apparent where these parameters are associated with increased chromatin mobility that favours chromosomal translocation events⁷¹.

Cryoprotected cells
Cells that have been treated with a cryoprotectant to prevent structural damage during freezing.

Perhaps surprisingly, regions of chromosome intermingling are not enriched for open chromatin or for active genes. This raises the possibility that both active and repressed gene regions intermingle, and that positioning at the periphery of chromosome territories is not generally necessary for gene expression^{63,67}. This suggests that the chromosome specificity, but not the overall level of chromosome intermingling, depends on transcription. In addition, nuclear volume expansion during lymphocyte activation is accompanied by chromosome-specific changes in chromosome territory decompaction during gene activation, intermingling and radial positioning⁶⁸. These observations confirm the view that gene expression and genome architecture are closely linked, and form the basis for the ‘inter-chromosomal network’ model⁶⁷. This model proposes that chromatin contacts within (and, to a lesser extent, between) chromosomes and with nuclear compartments such as the nuclear lamina or nucleoli, determine the way in which the genome is folded in a dynamic and physiological manner. The effect of locally determined chromatin contacts would be to promote preferential positioning of chromosomes, and of the active and repressed genes within them in the nucleus.

Nuclear organization and cell function

Chromatin organization and chromosome positioning undergo extensive changes during progression through the cell cycle, during cell differentiation and during senescence. In this section, we consider how these processes challenge the maintenance of gene expression patterns and cell identity. We also examine the concept that they constitute an opportunity for chromatin remodeling, and for changes to gene expression patterns and nuclear organization.

Cell cycle. Progression through the cell cycle is probably the most disruptive challenge to maintaining nuclear organization. Replication occurs within defined nuclear domains, called factories, which can be visualized as replication foci and contain stable chromatin associations that are maintained through as many as 15 cell divisions⁵¹. Replication factories are clusters of synchronously replicated replicons that extend over regions of about 1 Mb of DNA^{72,73}.

DNA replication poses a challenge to the maintenance of gene expression patterns because of the disruption of transcription factor binding and chromatin loop formation during the passage of the replication fork. As discussed above, current evidence suggests that most genes transcribed by Pol II are regulated by a combination of promoters and enhancers, with the latter often distributed over large genomic regions. It is possible that the passage of the replication fork disrupts only one of these elements at a time, allowing contact with other regulatory elements to act as a form of cellular memory, which would promote transcription factor binding and reconstitution of the enhancer.

Mitosis is likely to present the cell with an even greater challenge to maintaining epigenetic and regulatory information. During mitosis, there is widespread

displacement of sequence-specific and basal transcription factors from chromatin, some of which is triggered by protein phosphorylation⁷⁴. Chromatin is condensed by up to 50-fold, which results in the formation of metaphase chromosomes⁷⁵. The details of this process have not been fully elucidated but it seems likely to have a highly disruptive effect on chromatin domain structures, which can potentially break up loops that bring enhancers and gene promoters together. A recent study using 5C and Hi-C analyses of chromatin contacts showed that the TAD organization of interphase nuclei largely disappears in metaphase⁷⁶. Unlike the disruption of loops that occurs during the passage of replication forks, chromatin condensation in metaphase is not a brief event and affects the entire chromosome simultaneously. This creates specific challenges for the maintenance of epigenetic information. The disruptive effects of mitosis are further emphasized by the observation that some LADs do not re-establish contact with the nuclear lamina following exit from mitosis, but instead are repositioned to the periphery of the nucleoli⁵⁵ (FIG. 3).

Genomic organization through mitosis is likely to involve epigenetic marks of active genes and open chromatin, as well as marking by transcription factors, cofactors and histone modification readers that remain associated with regulatory elements during metaphase. Immunofluorescence combined with FISH (immunofluorescence-FISH) and ChIP analyses have shown that H3K4 methylation, H3 and H4 acetylation and the histone variants H3.3 and H2A.Z are maintained at active genes during mitosis^{77–79}, even when the genes are integrated into pericentric heterochromatin⁷⁷.

Transcription factors and cofactors, such as FOXA1 (REF. 80), GATA1 (REF. 81), RUNX2 (REF. 82), mixed-lineage leukaemia protein 1 (MLL1)⁸³ and bromodomain-containing protein 4 (BRD4)⁸⁴, have also been reported to remain associated with mitotic chromosomes. These factors could act in conjunction with histone modification marks to promote loop formation and facilitate the re-establishment of transcriptionally active and inactive chromatin structures as cells exit mitosis. In support of this idea, mitosis-specific depletion of GATA1 resulted in delayed expression of GATA1-binding genes following exit from mitosis into the G1 phase (REF. 81).

Quiescence and differentiation. Quiescent cells are non-dividing cells that can still be stimulated to divide and proliferate by appropriate signals. Terminally differentiated cells exit the cell cycle permanently and cannot, in general, be induced to divide. Quiescent and terminally differentiated cells have significantly different chromosomal organization and epigenetic marks compared with dividing cells: for example, naive, quiescent lymphocytes have small nuclei with a significantly higher fraction of facultative heterochromatin compared with dividing lymphocytes.

Chromatin condensation in naive, quiescent T cells is mediated, at least in part, by the activity of condensin 2, which is also involved in condensing mitotic chromosomes⁸⁵. Facultative heterochromatin in resting B cells is marked by a combination of the

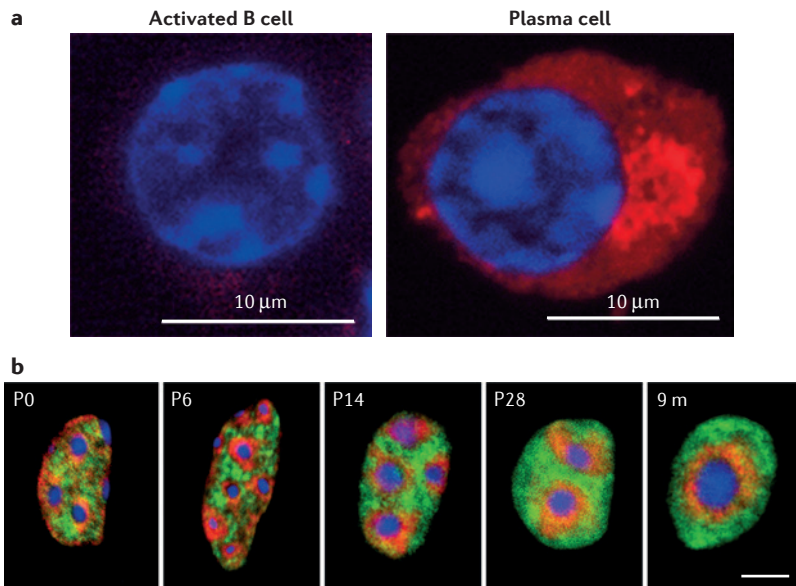


Figure 4 | Chromatin structure of post-mitotic cells. **a** | During differentiation of an activated B cell (left panel) into a plasma cell (right panel), the dense DAPI (4',6-diamidino-2-phenylindole; blue) staining of the heterochromatin forms a characteristic 'cartwheel' structure as the nucleus shrinks to accommodate the expanded endoplasmic reticulum, which is required for high-level production of immunoglobulins (red; indirect immunodetection of immunoglobulins). **b** | Rod photoreceptor cells from adult mice show an unusual nuclear organization in which the heterochromatin (red) is located in the centre of the nucleus and the euchromatin (green) at the periphery. Blue staining shows major satellite repeats-containing chromocentres, detected by fluorescence *in situ* hybridization (FISH) with a major satellite-specific probe. Heterochromatin was detected with probes for long interspersed elements (LINE-1) and euchromatin with probes for B1 short interspersed nuclear elements. The images show rod photoreceptor cells at birth (postnatal day 0 (P0)), P6, P14 and P28 and for the fully adult animal aged 9 months (9m). The nuclei have a conventional organization at birth, which is inverted into a centrally localized heterochromatin arrangement in the adult. Images in the figure, part **a**, courtesy of P. Sabbattini, MRC Clinical Science Centre, London, UK. The figure, part **b**, is reprinted with permission from REF. 91, Elsevier.

double H3K9me3–H3S10ph (H3S10 phosphorylation) and H3K27me3–H3S28ph modifications, which are not present in activated or terminally differentiated B cells, and are instead reminiscent of the epigenetic profile of metaphase chromosomes⁸⁶. Quiescent resting lymphocytes have greatly reduced levels of transcription compared with activated lymphocytes⁸⁷, and it is tempting to link this feature with the increase in chromatin condensation found in the resting cells. However, it is important to note that resting lymphocytes maintain gene expression programmes and are among the most mobile cells in the body, using amoeboid movement to migrate and seek out antigens that will stimulate their activation and proliferation. Chromatin condensation could have a role in reducing global transcription levels, but its main function may be to reduce the size of the nucleus, thereby reducing the overall size of the cell.

Terminally differentiated, post-mitotic cells differ from quiescent cells in that their exit from the cell cycle is irreversible. The differentiated post-mitotic cells that have been studied so far have a variety of chromosomal arrangements that may relate to the different functions of the cells. The end stage of B cell differentiation is the

post-mitotic antibody-producing plasma cell, which has a small nucleus with a substantial amount of facultative heterochromatin that is organized into a characteristic cartwheel structure, with radial spokes extending to the periphery from a central region of heterochromatin (FIG. 4a). Plasma cells are highly specialized for the production and secretion of large amounts of immunoglobulin and thus have a highly developed endoplasmic reticulum to cope with this role. Conversion of their genome into mainly facultative heterochromatin may function to reduce nucleus size and free space for the enlarged endoplasmic reticulum. Unlike constitutive heterochromatin in dividing cells, plasma cell heterochromatin does not contain heterochromatin protein 1 (HP1) and is marked by the double H3K9me3–H3S10ph modification⁸⁸. The presence Ser10 phosphorylation has been shown to prevent HP1 from binding to the adjacent H3K9me3-marked pericentric heterochromatin during mitosis⁸⁹, thereby altering the properties of heterochromatin and ultimately affecting transcription factor access and chromatin condensation⁸⁹.

Rod photoreceptor cells in the eyes of nocturnal mammals (see above) are another cell type in which the organization of heterochromatin has been studied in detail, revealing a fascinating link between chromatin organization and cellular function. Mouse rod cells have an unusual, inverted chromatin organization whereby heterochromatin is concentrated in a dense chromocentre in the centre of the nucleus and is absent from the nuclear periphery (FIG. 4b). This inversion is caused by developmentally controlled silencing of the expression of lamins A and C, and of the lamin B receptor⁹⁰. In contrast to the genomic distribution observed in most cell types, in rod cells both active and inactive genes are found mainly at the nuclear periphery⁹¹, which also has higher levels of transcription factors⁹² and nascent transcripts⁹¹. Analysis of 16 species (nine nocturnal and seven diurnal) revealed a strong correlation between the presence of such an inverted nuclear arrangement and a nocturnal lifestyle. The main functional effect of the inverse organization seems to be changes in the optical properties of the cell, which have been hypothesized to contribute to the much higher sensitivity of the rod cells of nocturnal mammals to low light levels⁹¹.

Senescence. In response to exogenous and endogenous stress, cells can undergo permanent cell cycle arrest through a process termed cellular senescence. Among the stimuli that can induce senescence are replicative stress and overexpression of oncogenes. The latter induces hyper-proliferation and activation of the DNA damage response in S phase, which helps to trigger the senescent phenotype. Senescent cells, which are enlarged and generally secrete substantial amounts of protein, often have partially replicated DNA and cytoplasmic DNA fragments⁹³.

Human fibroblasts that undergo oncogene-induced senescence have an unusual chromatin organization, which is characterized by the presence of senescence-associated heterochromatin foci (SAHFs). Each SAHF is formed by the condensation of a single chromosome,

with telomeric and centromeric chromatin located mainly at the periphery of the SAHF⁹⁴. The formation of SAHFs depends on the HIRA histone chaperone complex, which translocates to PML nuclear bodies soon after senescence has been induced and can initiate SAHF formation⁹⁵. A detailed analysis of the composition of SAHFs revealed that they are organized in concentric non-overlapping layers of chromatin that carry the H3K9me3 and H3K27me3 marks. Interestingly, SAHFs were not observed in mouse fibroblasts, which instead exhibit a more diffuse distribution of heterochromatin, suggesting that there are species-specific differences in the nuclear organization of senescent cells. Lamin B1 is also depleted in senescent fibroblasts^{96,97}. Knockdown of lamin B1 results in heterochromatin reorganization and facilitates the formation of SAHFs⁹⁸, emphasizing its role in tethering heterochromatin to the nuclear envelope. Moreover, the binding of lamin B1 to regions of chromatin enriched for H3K27me3 is increased in senescent cells, suggesting that redistribution of lamin B1 binding could contribute to gene silencing in these cells^{98,99}.

The notion that the cell cycle generally has a disruptive effect on genome organization implies that non-cycling cells should have a stable genome and nuclear organization that can last for months or years. The reality is likely to be more complex. For example, quiescent lymphocytes move rapidly around the secondary lymphoid organs and through the bloodstream and lymphatic ducts in search of antigens. Migration alters the shape of the nucleus¹⁰⁰ and could affect nuclear organization. Moreover, a significant proportion of haematopoietic stem cells (HSCs) is maintained in a quiescent state during the lifetime of the organism, which is thought to protect them from DNA damage. However, when damage does occur in these quiescent cells, it is repaired by error-prone, non-homologous end joining, instead of by homologous recombination, which is used in the fraction of HSCs that are proliferating^{5,101}. This exposes quiescent HSCs to an increased risk of mutagenesis, which may contribute to HSC ageing. Other examples are adult mouse neurons and hepatocytes, which display substantial levels of polyploidy and aneuploidy^{102,103}; the polyploidy can be reversed by hepatocyte cell divisions¹⁰³. Differentiated, post-mitotic cells seem to block

re-entry to the cell cycle by epigenetic mechanisms, which could be mediated in part by changes in chromosome organization and the conversion of the genome into mainly heterochromatin. Our understanding of these processes is still limited, but it is clear that the cell cycle not only poses a challenge for maintaining nuclear organization but also presents opportunities for renewing nuclear structures and maintaining cell plasticity during differentiation. Understanding the role of these processes and the consequences of their absence in different types of quiescent and differentiated cells is likely to be an expanding area of research in the future.

Summary and perspectives

3C-based techniques have markedly influenced our understanding of chromatin contacts in the nucleus and will continue to do so with further methodological developments. However, it is necessary to be aware of their limitations and to integrate the results that they provide with information obtained using different approaches. It will be important to link studies of chromatin contacts and chromosomal organization with information about the functional characteristics of the sequences that control transcription, and DNA replication and repair. Genome-wide analysis of regulatory sequences presents significant challenges, but ingenious approaches are being devised that should allow the effects of gene position on transcription to be studied at a global level (see Supplementary information S1 (Box)). Efficient generation of large-scale deletions and other chromosomal rearrangements through homologous recombination is now possible using genome editing tools such as the CRISPR-Cas9 system¹⁰⁴. In the future, our understanding of the role of large-scale chromatin organization in controlling a wide range of cellular functions should be enhanced by combining chromatin interaction mapping and genomic deletion data with experimental manipulation of key cellular factors such as cohesins, CTCF and transcription factors that bind to enhancers and promoters. These developments will be particularly important for understanding cancer and congenital diseases, in which disruption of genomic organization by chromosomal rearrangements markedly affects gene expression.

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Acknowledgements

The authors thank P. Sabbattini for providing the images shown in FIG. 4a. Work in N.D.'s laboratory is supported by the Medical Research Council, UK. A.P. thanks the Helmholtz Foundation for support.

Competing interests statement

The authors declare no competing interests.

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