Dynamic genome architecture in the nuclear space: regulation of gene expression in three dimensions

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Abstract | The regulation of gene expression is mediated by interactions between chromatin and protein complexes. The importance of where and when these interactions take place in the nucleus is currently a subject of intense investigation. Increasing evidence indicates that gene activation or silencing is often associated with repositioning of the locus relative to nuclear compartments and other genomic loci. At the same time, however, structural constraints impose limits on chromatin mobility. Understanding how the dynamic nature of the positioning of genetic material in the nuclear space and the higher-order architecture of the nucleus are integrated is therefore essential to our overall understanding of gene regulation.

PML bodies
Nuclear structures that are enriched for the promyeolocytic leukaemia (PML) RING-finger protein and are implicated in transcriptional regulation, viral pathogenicity, tumour suppression, apoptosis and DNA repair.

Cajal bodies
Spherical structures that are involved in the processing of nuclear RNA. Marker proteins include p80 coilin and the survival of motor neuron (SMN) protein.

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A striking feature of nuclear architecture is the existence of distinct structural and functional compartments (FIG. 1). Well characterized nuclear substructures include the nuclear lamina, nucleoli, PML and Cajal bodies, and nuclear speckles[1–4]. Also, a growing number of components of the machinery that is required for transcription or its repression are known to have a non-homogeneous distribution in the nucleoplasm[5–7]. At the level of the genome itself, the genetic material is folded and packaged in the nucleus into higher-order structures that are likely to contribute to the regulation of gene expression[8–12].

A major goal of ongoing studies to understand the potential influence of nuclear architecture on nuclear functions is to identify the principles that govern the spatial organization of the genome. One classical example of organization within the nucleus is the distinction between decondensed, transcriptionally active euchromatin and more condensed, generally inactive heterochromatin[13]. It is now also known that individual chromosomes occupy distinct positions in the nucleus, referred to as chromosome territories[14,15]. As a result of different compaction levels, different chromosome segments adopt a complex organization and topography within their chromosome territory[16–18]. A polarized intranuclear distribution of gene-rich and gene-poor chromosomal segments has been shown to be an evolutionarily conserved principle of nuclear organization[19,20]. Gene-rich regions tend to be oriented towards the nuclear interior, whereas gene-poor regions tend to be oriented towards the periphery (for review, see REF. 10).

Although chromosomes are organized as distinct territories in the interphase nucleus, evidence obtained from various biological models — using techniques ranging from classical genetics to molecular tools — has shown that chromosomes are dynamic structures and that individual chromosomal regions can be repositioned, with respect to both nuclear structures and other chromosomal regions. There is also increasing evidence that repositioning of genomic regions in nuclear space is important for the regulation of gene expression[21]. Recent technological advances now allow the large-scale identification of interacting loci across the genome, and initial results have highlighted the potentially widespread importance of such interactions for proper gene regulation. As will be discussed later, the possibility that spatial networks of genomic loci exist in the nucleus[11] implies the existence of a previously unexplored level of gene regulation that coordinates expression across the genome.

Here we review the evidence that supports the dynamic nature of gene positioning and its link with gene expression, focusing mainly on mammalian systems and *Drosophila melanogaster*, but referring to yeast data when appropriate. We first describe chromatin movements that have been observed in living cells and briefly discuss the structural constraints that limit

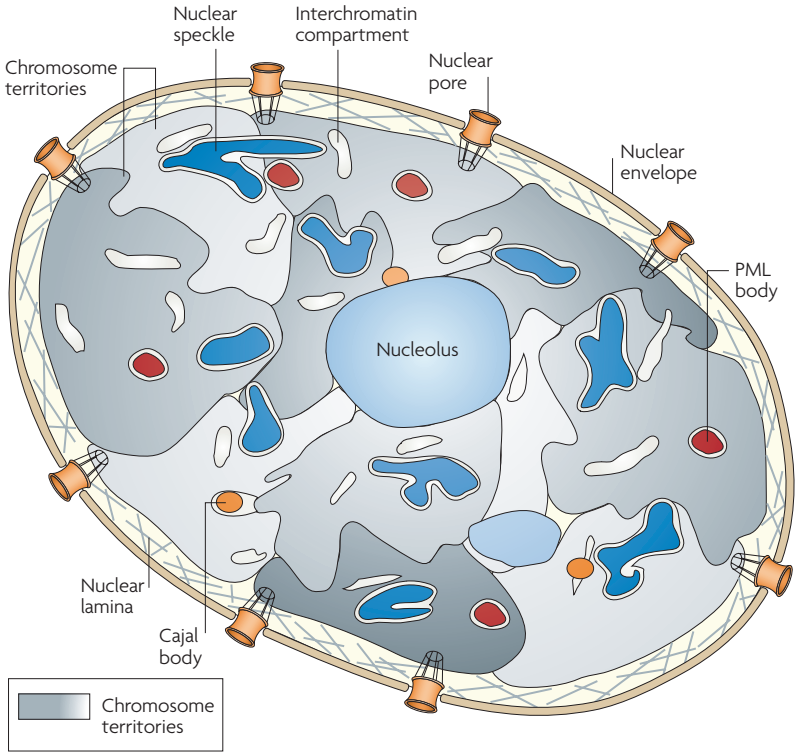

Figure 1 | **Organization of the mammalian cell nucleus.** The nucleus is characterized by a compartmentalized distribution of functional components. The nuclear envelope contains pores and rests on a meshwork of intermediate filaments, the nuclear lamina. Nucleolar organizer regions cluster to form nucleoli. Further topographical details that are shown in this schematic nuclear section are representative for the chromosome territory–interchromatin compartment (CT–IC) model. Chromatin is organized in distinct CTs. Also depicted are nuclear speckles, PML bodies and Cajal bodies located in wider IC lacunas (sections through smaller channels of the contiguous, three-dimensional IC network are not depicted). Nuclear topography remains a subject of debate, expecially with regard to the extent of chromatin loops expanding into the IC and intermingling between neighbouring CTs and chromosomal subdomains. For in-depth discussions of the CT–IC and other models see REFS 15, 32.

Nuclear speckles
Irregular structures that contain high concentrations of splicing factors and small nuclear ribonucleoprotein particles (snRNPs).

Two-photon microscopy
An imaging technique in which a fluorochrome that would normally be excited by a single photon is stimulated quasi-simultaneously by two photons of lower energy. This allows reduced light scattering and less photodamage of the sample.

Sampling volume
The volume or area of a cell or an organelle in which fluorophores are excited or from which emitted photons are collected. Temporal resolution can be increased during imaging by decreasing sampling volume.

chromatin mobility in the interphase nucleus. We then present examples of repositioning of specific loci with respect to nuclear landmarks concomitant with their transcriptional regulation. Finally, we review recent results that have demonstrated the existence of specific interactions between loci, and discuss the significance of such interactions for the regulation of gene expression. Throughout the review, we discuss how the increasing evidence for substantial chromatin mobility can be reconciled with the structural organization of the nucleus.

Chromatin movement: extent and timing

Initial studies in living cells indicated that the positions of labelled chromatin and subchromosomal segments are constrained during interphase within a radius of approximately 0.5–1 μm — that is, less than 1% of the volume of a typical spherical mammalian nucleus that has a diameter of 10 μm[17,22–24]. The exception is during early G1, when long-range movements of >2 μm are frequently observed[24]. At the level of single genomic regions, a study that exploited the specific binding of fluorescently labelled topoisomerase II to a heterochromatic repeat block on *D. melanogaster* chromosome X supported the conclusion that the mobility of individual loci can also best be described as constrained diffusion. However, in this case the radius of confinement (0.9 μm) was about half the radius of the nuclei (2 μm), indicating a much greater relative mobility of chromatin in this experimental model[25].

These initial findings were complemented by several studies that tracked the position of fluorescently labelled, stably integrated arrays of lac operator transgenes in living *D. melanogaster*, *Arabidopsis thaliana* and human cell lines[26–29]. Quantitative time-lapse analysis showed two different types of chromatin motion in *D. melanogaster* spermatocytes: rapid, localized movements (0.3–0.7 μm) and slower long-range movements that were confined to an average radius of 2.6 μm, which is comparable to the dimensions of individual chromosome territories in the large nuclei of this cell type (10–17μm in diameter)[29]. Imaging of fluorescently tagged chromosomal loci in living human cells supported the finding that the average mobility of chromatin is increased during early G1 (REF. 30), as had been observed for chromosome subdomains[24]. Furthermore, the mobility of individual loci depended on their nuclear localization, with peripheral and nucleolar-associated transgenes being significantly less mobile than nucleoplasmic ones[26]. A recent analysis of chromatin dynamics in chinese hamster ovary cells was performed at a much higher spatial (~20 nm) and temporal (~30 ms) resolution using two-photon microscopy and a small sampling volume[31]. Particle trajectories showed periods of constrained diffusion, interrupted approximately every minute by rapid 'jumps' of ~150 nm that were sensitive to depletion of cellular ATP and to temperature, indicating that chromatin might undergo active, possibly directed movements at the level of single loci.

Altogether, these observations in living cells indicate that long-range chromatin repositioning during interphase occurs only during a relatively short time window, which corresponds in most cells to approximately the first third of G1. Once this window has closed, chromatin movements are constrained within small nuclear subdomains. The size of these subdomains relative to the size of the nucleus varies depending on the species, the cell type and, as described below, the cellular differentiation status. Chromosome segments can adopt irregular shapes within these subdomains, with numerous protrusions and invaginations forming as a result of chromatin mobility. Examples of the protrusion of specific chromosomal segments from the core territory have been well documented[32–34]. Currently, there is some controversy as to the extent to which these protruding segments intermingle with chromatin from neighbouring chromosomes[15]. Using three-dimensional fluorescence *in situ* hybridization (FISH) on thin cryosections, Branco and Pombo reported extensive intermingling of chromatin[35], whereas Albiez *et al.* arrived at the opposite conclusion on the basis of experimental manipulations of the nuclear architecture[32].

It is important to determine whether the chromatin movements that have been observed during interphase are correlated with changes in gene expression. This issue is now being addressed by imaging individual

loci in living cells. Because current DNA hybridization techniques cannot be used in living cells, the visualization of specific genomic sequences *in vivo* requires the introduction of heterologous binding sites for fluorescently labelled proteins at or near the locus of interest. Currently, the GFP–lac repressor/lac operator system is the most widely used system of this type. Although the physiological relevance of studying large arrays of tandemly repeated genes has been questioned, this technique has yielded important insights into the relationships between chromatin movement and gene regulation. For instance, chromatin movement was analysed during transcriptional activation in mammalian cells by using a fusion of the acidic activation domain (AAD) of the viral protein VP16 and the DNA-binding domain of the lac repressor in conjunction with an integrated array of lac operators[36]. The targeted locus moved from the periphery towards the nuclear interior after mitosis in both control and VP16-AAD-expressing living cells. However, it returned to the nuclear periphery within 3–4 hours in the absence of VP16 AAD.

In a further refinement, recruitment of VP16 AAD to the lac array was made rapamycin-dependent[37], allowing the timing of transcriptional activation to be controlled. Long-range movements of the locus (with an average distance of 2.6 μm) were observed in 14 out of 43 rapamycin-treated cells, whereas movement was constrained within a small radius in the other 29 cells[38]. In most cases, movement was oriented toward the nuclear interior and was dependent on nuclear actin and myosin, suggesting the involvement of an active process.

Movement with respect to nuclear landmarks

Movement with respect to the periphery or interior. The preferential relocation of a locus to the nuclear interior on transcriptional activation that is described above is consistent with results from a number of previous studies that looked at the positioning of genes in fixed cells. Repositioning of the activated gene towards the nuclear interior has been documented for *IgH* (immunoglobulin heavy chain) in committed B lymphocytes[39], *c-maf* in T cells[40], *Mash1* in neuronal cells[41] and *Cftr* in adenocarcinoma cells[42]. It is generally assumed that the nuclear periphery is an inactive compartment and that repositioning towards the nuclear interior is required to allow efficient transcription. However, there is not always a strict correlation between movement away from the periphery and gene activation. The interferon-γ (*Ifng*) locus, for instance, is detected at the nuclear periphery irrespective of its transcriptional activity[40]. Furthermore, it was recently shown that although the mouse β-globin locus relocates to the nuclear interior during erythroid differentiation, expression of the gene actually precedes movement away from the nuclear periphery[43]. Finally, analysis of a 2-Mb segment encompassing the *Mash1* gene showed that the entire region relocalized to the nuclear interior in *Mash1*-expressing neuronal cells, even though some of the genes in this region are not expressed in this cell type[41]. This is consistent with the idea that transcriptional status can be independent of position relative to the nuclear periphery.

Recent findings in yeast have further complicated our understanding of the relationship between transcriptional activation and gene positioning relative to the nuclear periphery. In *Saccharomyces cerevisiae*, this region contains silencing compartments[44], but also activating ones. Indeed, the nuclear pores are important sites of transcription in this species and genome-wide analysis has shown that nuclear-pore-associated proteins bind preferentially to active genes[45,46]. Transcriptional activation is frequently accompanied by an apparent movement of genes in the opposite direction to that observed in mammalian cells — that is, from the nuclear interior to the periphery[47,48]. Recently, living-cell observations of fluorescently tagged loci in yeast cells have shown that the movement of genes becomes confined alongside the nuclear envelope on transcriptional activation[49–51]. Analysis of yeast mutants indicated that this constrained diffusion of active genes depends on their dynamic association with components of the nuclear pores, a finding that is consistent with the idea that the efficiency of transcription might be increased by tethering active chromatin at the site of mRNA export ('gene gating')[52].

Although it is possible that this model applies to a subset of mammalian genes, it is unlikely that gene gating can account for the regulation of the mammalian transcriptome in general. Indeed, although chromatin is able to probe the whole nuclear volume in yeast due to the small size of the organelle, it is confined to a much smaller volume in the larger nuclei of mammalian cells, which prevents association with the nuclear pores for most genes. This difference highlights the important role of the structural constraints that are imposed on chromatin mobility in the organization of the nucleus. As discussed below, the presence of large amounts of heterochromatin is thought to constitute another form of structural constraint that influences gene expression in mammalian cells.

Movement with respect to heterochromatin. Several studies have reported a correlation between gene silencing and intranuclear positioning close to constitutive heterochromatin in mammalian cells. It has been suggested that this type of transcriptional repression could be explained by competition between heterochromatin proteins and transcriptional activators at the target locus[53], or by chromatin compaction that is mediated by heterochromatin-associated remodelling complexes[54]. Analyses of differentiating cells of the haematopoietic lineage have shown that gene activation is often associated with movement of the locus away from the heterochromatin compartment[40,55] and, conversely, that gene silencing is correlated with repositioning of the locus close to heterochromatin[56].

A similar correlation is observed in a well studied case of variegated expression in *D. melanogaster,* in which a chromosomal inversion brings a heterochromatic block of ~2 Mb into the *brown* locus, which lies near the distal end of chromosome 2. The mutant allele, *bwD*, exerts a dominant repressive effect on the homologous wild-type *brown* allele, resulting in expression that

Fluorescence *in situ* hybridization
A technique in which a fluorescently labelled DNA probe is used to detect a particular chromosome or gene with the help of fluorescence microscopy.

GFP–lac repressor/lac operator system
A technology that allows the imaging of a DNA locus in living cells, which works on the basis of the high-affinity binding of a fluorescent lac repressor to an episomal or stably integrated array of lac operators.

Variegated expression
A mosaic pattern of gene expression that results from epigenetic modifications that are associated with the presence of heterochromatic sequences near euchromatic regions of the genome.

is limited to less than 2% of cells in the eye. Both the mutated and the wild-type alleles are more frequently associated with centromeric heterochromatin in the nuclei of cells that carry the rearrangement than in those of wild-type cells[57,58]. By assessing on a cell-by-cell basis the transcriptional status of three other genes that are subject to variegation effects, Harmon and Sedat found a strong positive correlation between gene silencing and spatial proximity to heterochromatin in the nucleus[59]. The distances between each locus and centromeric heterochromatin were not only smaller on average in non-expressing cells, but also less variable. This reduced variability was interpreted to mean that silenced loci often form stable and persistent interactions with heterochromatin, whereas active loci might be more loosely tethered in the nucleus.

Similar to observations that relate to the repositioning of loci relative to the nuclear periphery, chromosomal context seems to be an important determinant of association between specific loci and heterochromatin. This was demonstrated in a study of the human α-globin and β-globin loci[60]. In lymphocytes, in which both loci are silenced, the β-globin locus but not the α-globin locus localizes close to centromeric heterochromatin. A possible explanation for this observation is that the two loci reside in regions of the genome that differ in important respects: the α-globin locus lies in an early-replicating subtelomeric region that contains genes that are highly and widely expressed, whereas the β-globin cluster is embedded within a late-replicating AT-rich region that contains genes that show tissue-specific expression patterns. It is plausible that the lack of association between the inactive α-globin locus and heterochromatin reflects the need for neighbouring genes to be expressed, in which case these might exert a dominant effect on the positioning of the entire genomic region away from heterochromatin.

Integrating spatial and temporal aspects

Currently, it is not clear at which stage of the differentiation process interactions with heterochromatin are established. An important clue has come from the observation that blocking the development of *D. melanogaster* larvae to preclude terminal differentiation does not prevent the association between the *bwD* locus and heterochromatin[61]. Similarly, the association of other variegating loci with heterochromatin is still detected in G2-arrested eye imaginal disc cells[59]. Interestingly, a previous work had shown that the interaction between *bwD* and heterochromatin is disrupted at the beginning of S phase and must be re-established during G1 (REF. 62). It has therefore been postulated that, in dividing cells that are proceeding towards the terminally differentiated state, the length of G1 rather than the exact differentiation status is a crucial factor in the establishment of interactions between specific loci and heterochromatin. According to this model, the longer the amount of time that a locus spends in G1 exploring nuclear space, the higher the probability that it will encounter centromeric heterochromatin and make stable contacts with this structure[59,61].

After exit from the cell cycle, temporal constraints on chromatin repositioning, such as the length of G1, might be replaced by tighter structural constraints. In *D. melanogaster* imaginal eye discs, chromatin motion was found to be much more constrained in differentiated than in undifferentiated cells[61], indicating that differentiation is accompanied by changes in overall chromatin mobility, possibly as a result of increased tethering to nuclear structures or changes in nuclear volume and chromatin compaction levels.

Advances in imaging living, differentiating cells should prove important in understanding how the spatial and temporal aspects of chromatin repositioning are combined to activate and maintain cell-type specific gene expression programmes. Clearly, mitosis offers a window of opportunity for changes of higher-order chromatin arrangement that might be required for cellular differentiation[63] and, as discussed above, interactions that are established during or shortly after mitosis might become stabilized later in the cell cycle. On the other hand, reports of extensive re-organization of centromeric heterochromatin in post-mitotic cells[64–67] indicate that such cells retain the ability to form new chromatin configurations. Therefore, individual genomic loci have the opportunity to move substantially during both the proliferative phase and the terminal stage of the differentiation process. These movements might favour not only the repositioning of loci with respect to heterochromatin and other nuclear compartments, but also the meeting of specific genomic regions in the nucleus. These specific intra- and interchromosomal associations between loci could contribute to the establishment of cell-type specific gene expression patterns, a topic that we explore below.

Nuclear architecture and gene–gene interactions

The spatial co-localization of gene loci that are located on different chromosomes, and of remote sites within a chromosome, is one of the most striking examples of three-dimensional genome organization (FIG. 2). Because of the low probability that distant loci will co-localize by chance in the nucleus, the observation of such 'gene kissing' in a large fraction of nuclei is considered to be highly significant.

Gene kissing and transcriptional silencing. One well documented case of gene kissing involves the function of Polycomb group (PcG) proteins, transcriptional repressors that are capable of maintaining the memory of silent gene states through cell division[68,69]. Transcriptional repression is achieved through binding of PcG proteins to *cis*-acting PcG response elements (PREs). In *D. melanogaster*, insertion of the 3.6 kb PRE-containing regulatory sequence *Fab7* into chromosome X leads to variegated repression of neighbouring genes, an effect that is more pronounced in homozygous female flies. Because homologous chromosomes are paired in somatic cells of dipterans, this phenomenon is referred to as pairing-sensitive repression.

Polycomb group
A class of proteins, originally described in *Drosophila melanogaster*, that maintain the stable and heritable repression of several genes, including the homeotic genes.

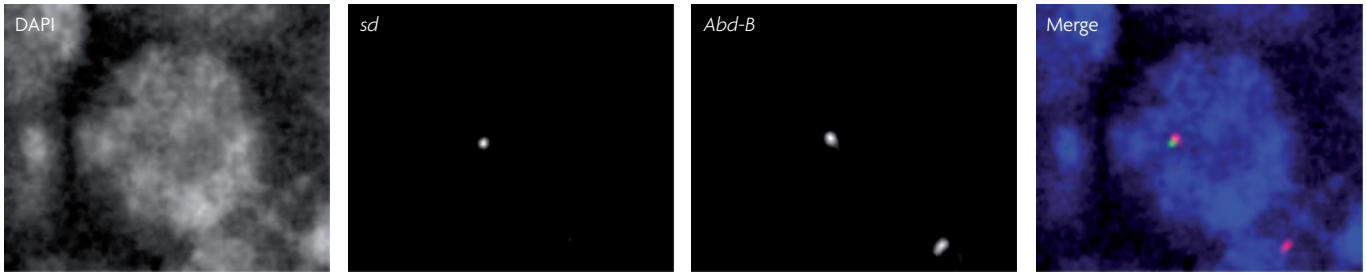

Figure 2 | **Gene kissing.** In this example of gene kissing, copies of the *Drosophila melanogaster Fab7* regulatory element that are present on two different chromosomes co-localize in the cell nucleus. DAPI (4′,6-diamidino-2-phenylindole) is the DNA counterstain, *sd* shows the position of a transgenic *Fab7* copy that is inserted in the X chromosome at the *scalloped* (*sd*) locus. *Abd-B* indicates the locus that is regulated by the endogenous copy of the *Fab7* element. The two loci 'kiss' each other in a significant fraction of the nuclei[70], as seen in the merged panel.

Surprisingly, repression that is mediated by transgenic *Fab7* depends on the presence of the endogenous *Fab7* element in the bithorax complex, a locus that is located on the right arm of chromosome 3 (REF. 70). Three-dimensional FISH showed that these two loci co-localized in 23% and 43% of nuclei in female embryos and female larvae, respectively. The presence of *Fab7* was sufficient to give chromosomal sites the ability to interact in *trans*, as shown by the fact that a *Fab7* element that was inserted on the left arm of chromosome 2 could associate with the endogenous *Fab7* element at the bithorax complex, or with another transgenic *Fab7* element on chromosome X. The spatial association between loci was not detected in a Polycomb null background. Because PcG proteins are known to be compartmentalized in the nucleus[71,72], these data led to the suggestion that "…endogenous PcG target genes may undergo physical associations at nuclear PcG bodies dedicated to their regulation."[70] Another PcG target element, Mcp, was also shown to be able to drive interchromosomal associations in a tissue-specific manner, indicating that this property might be widespread among PcG target elements[73].

The importance of dynamic interchromosomal interactions in transcriptional silencing of large genomic regions is further demonstrated by the transient pairing of X-inactivation centres (XIC) in differentiating mouse female embryonic stem (ES) cells[74,75]. The frequency of XIC–XIC distances smaller than ~1–1.5 μm increased at the time of X-chromosome counting and before the expression of *Xist* RNA, which is the earliest known cytological marker of X inactivation. The association between XIC loci was abolished in cell lines in which X inactivation proceeds abnormally, such as *Tsix*$^{-/-}$ mutants. XIC pairing could not be detected at late stages of differentiation, suggesting that this interchromosomal interaction is transient and is required for the initiation of the inactivation process, but not for the maintenance of the inactive state.

Gene kissing and transcriptional activation. Long-range interactions between genomic regions are also important for gene activation. For instance, interactions between the locus control region (LCR) and downstream regulatory elements at the β-globin locus drive the formation of a 200-kb loop, specifically in expressing cells[76]. Interactions between regulatory elements and target genes that are located on different chromosomes can also occur, as shown by the recent finding that an enhancer element that is present as a single copy in the genome contacts only one of the ~1,300 olfactory receptor (OR) genes in any given sensory neuron in the mouse[77]. The observation that expression of OR occurred specifically from the interacting allele points to the functional importance of this interaction.

As well as long-range interactions between regulatory elements and the genes that are under their control, other insights into the potential functional relevance of gene-kissing events for transcriptional activation have started to emerge. It has recently been suggested that genes must associate with 'transcription factories' — foci that are enriched in RNA polymerase II — in order to be efficiently transcribed and that, owing to the limited number of factories, "…many genes are obliged to seek out and share the same factory."[78] By combining RNA FISH and immunostaining, a set of actively transcribed genes were shown to associate with the same RNA polymerase II focus in mouse erythroid progenitors[78]. Alleles of genes that are transcribed from the subtelomeric region of chromosome 7 (such as *Eraf*, *Uros*, *Igf2*, *Kcnq1ot1*) frequently co-localized at such foci with active β-globin alleles that were located 24–39 Mb away on the same chromosome.

Advances in our ability to analyse three-dimensional interactions between genes have come from the chromosome conformation capture (3C) method, which has revealed further examples of the correlation of gene-kissing events with transcriptional activity. This method measures the formation of crosslinks between chromatin segments after formaldehyde fixation of whole cells or isolated nuclei[79]. A frequency of crosslinking that is above control levels is indicative of spatial juxtaposition, although the need to carry out appropriate controls has recently been stressed[80]. In addition to affording a higher resolution than light microscopy, this biochemical approach averages three-dimensional interactions over a much larger number of cells.

Using this method, the interferon-γ gene, which is located on mouse chromosome 10, was shown to physically interact with parts of the T_H2 cytokine gene locus, which is located on chromosome 11, in naive

X-inactivation centre
A genomic region (of ~35–400 kb in mice) comprising genes and regulatory elements that are responsible for the *cis* inactivation of the entire X chromosome.

X-chromosome counting
A mechanism by which the cell detects the number of X chromosomes for each diploid set of autosomes. This process ensures that a single X chromosome is inactivated.

RNA FISH
A technique in which a fluorescently labelled nucleic-acid probe is used to detect cellular transcripts *in situ*. Probes that are complementary to intronic sequences will hybridize at the site of nuclear transcription.

Box 1 | **Microscopes and microarrays: two complementary assays**

Understanding the orchestration of gene regulation, from individual regulatory sequences to global principles of nuclear organization, has become a feasible goal with the introduction of biochemical and molecular techniques combined with advanced three-dimensional and four-dimensional (space–time) imaging approaches. The chromosome conformation capture (3C) technique has already provided a powerful approach to identify spatial arrangements at a resolution that far surpasses the Abbe resolution limit of conventional light microscopy. The development of a 4C assay that combines 3C with DNA microarrays will further help to uncover the links between nuclear architecture and genome activity.

However, the heterogeneity of cell populations can easily be overlooked in 4C assays, and observations that are made using imaging techniques ranging from electron microscopy to advanced three-dimensional and four-dimensional light microscopy are still indispensable. Furthermore, because gene activity can vary greatly from cell to cell and is frequently detected at only one of the two alleles in a given cell, the ability to study individual cells and distinguish between alleles is crucial to test the hypothesis that gene expression is a function of gene positioning in the nucleus. The use of multicolour DNA and RNA FISH is helping to analyse the cell-to-cell variability of chromatin arrangements, and could reveal cell-type-specific arrangements of certain chromatin domains. It is hoped that techniques for fluorescent labelling of nucleic acids in living cells can be developed further and combined with four-dimensional imaging at a resolution beyond the classical Abbe limit.

Molecular mechanisms that are responsible for the three-dimensional organization of the genome must be validated in the context of the whole cell, and even in the context of tissue organization. It seems then that the problem of deciphering nuclear organization is best tackled by a combination of imaging, cell biology and molecular approaches. This is well illustrated by the work on gene kissing, which usually combines molecular and FISH techniques. In this era of systems biology, we are fortunate to be witnessing a convergence of cellular and molecular interests, which promise to bring about a more complete understanding of nuclear function.

T lymphocytes[81]. This interchromosomal interaction was dependent on the presence of a DNase I hypersensitive site (HS) at the 3′ end of *Rad50* in the T_H2 cytokine gene locus. Deletion of this HS on chromosome 11 affected expression of *Ifng* on chromosome 10, indicating the existence of regulatory cross-talk between kissing loci. Because *Ifng* and genes of the T_H2 locus are rapidly and coordinately expressed at low levels following *in vitro* stimulation of naive T lymphocytes, it was proposed that the interacting loci formed a 'poised chromatin hub' that was responsible for early activation of the genes. The authors suggest that this hub might recruit remodelling complexes or histone acetyltransferases to form a positive environment for the early expression of cytokines.

The 3C technique can be used to validate candidate interactions but, owing to limited throughput and the need to design sequence-specific primers, cannot identify unknown sequences that are crosslinked to a locus of interest. In order to overcome this limitation, the sequence-specific PCR step of the 3C technique can be replaced by recircularization of the template and an inverse PCR step. This novel approach was used to assay potential interactions between specific genomic regions and the rest of the genome. For instance, using this method the spatial environment of the mouse *Hoxb1* gene during ES cell differentiation has been probed with respect to other genomic regions[82]. Sequencing analysis of more than 2,000 PCR fragments showed that that the majority of crosslinking partners were located within ~2 Mb of the *Hoxb1* gene, and that the proportion of interchromosomal interactions increased during differentiation. This is consistent with a previous report that used FISH, which showed that during ES cell differentiation the *Hoxb1* locus is repositioned away from its chromosome territory[83].

A similar method that has been named 4C (BOX 1) has been used to characterize the spatial genomic environment of the mouse β-globin locus[84]. However, instead of sequencing the amplicons that were generated in this study, they were hybridized to microarrays that contained probes for all potential partner sites on seven chromosomes in order to efficiently obtain a complete coverage of crosslinking partners. Most of the loci that interact with the β-globin locus were found in *cis* on chromosome 7, and clusters of 20–50 hits were identified, often tens of megabases away from the β-globin locus. Interestingly, the interacting partners were entirely different in fetal liver cells that express this gene and fetal brain cells that do not. In fetal liver, 80% of the loci that interacted with the active β-globin locus contained one or more active genes, 74% of which were not liver-specific; by contrast, 87% of the interacting loci in brain cells showed no detectable activity. On the basis of these patterns, the authors suggested that chromosome 7 folds into areas of active and inactive chromatin. Gene–gene interactions might therefore be governed mainly by features of the surrounding chromatin (for example, transcriptional activity, histone code and gene content) rather than by a specific gene function being shared by the interacting partners.

The same study also analysed the ubiquitously expressed *Rad23a* gene, which resides in a gene-dense cluster that mainly contains housekeeping genes. For this locus, highly similar interactions were detected in both fetal liver and brain cells. As was observed for the active β-globin locus, most *cis*-interacting regions (~70%) contained at least one actively transcribed gene, which was consistent with gene–gene interactions being determined by chromosomal context rather than specific gene functions. Because FISH analysis showed that the *Rad23a* gene is located at the edge (82% of signals) or outside (14% of signals) its chromosome territory, the possibility that this gene contacts regions on other chromosomes was also investigated, and 68 interacting regions from other chromosomes were identified, two of which were validated by cryo-FISH. Similar to what was found for interactions in *cis*, many of the genomic regions that interacted in *trans* featured a high gene density.

The predominance of *cis* interactions among those identified in the 4C studies is consistent with previous models of nuclear architecture in which the territorial organization of chromosomes represents the main constraint that is imposed on potential interactions between chromatin segments (FIG. 3). In line with these models, the 4C approach also showed that a gene that was exposed at the surface of a chromosome territory (*Rad23a*) contacted genes that were located on other chromosomes, whereas a gene that is known from previous studies to be buried in its territory (mouse β-globin)[60] made few interchromosomal contacts. This suggests that the positioning of a locus within the

DNase I hypersensitive site
A chromosomal region that is highly accessible to cleavage by DNase I. Such sites are associated with open chromatin conformations and transcriptional activity.

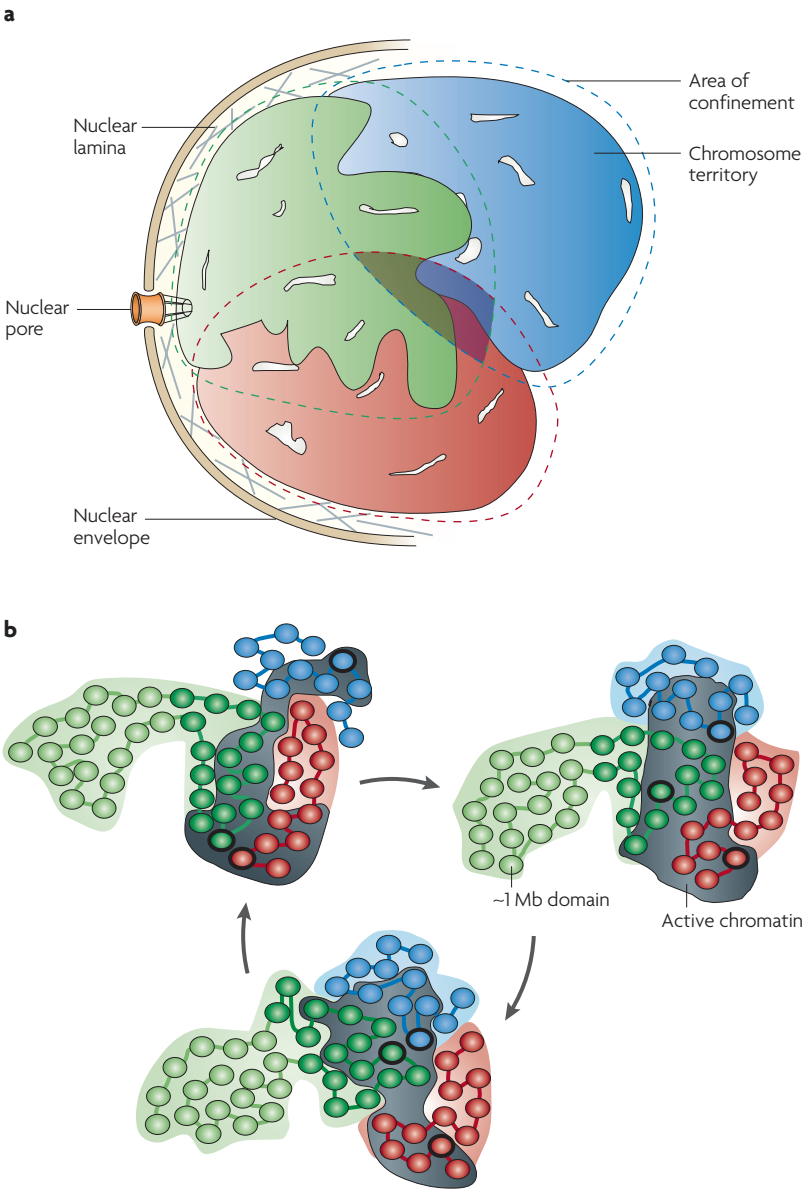

Figure 3 | **A model of structural constraints on chromatin mobility and gene–gene interactions. a** | Three hypothetical chromosome territories — green, blue and red — are shown. **b** | The three panels show how the chromatin that is located where the same three territories are adjacent to each other (shown as a shaded region in panel **a**) might become repositioned over time; the panels indicate three consecutive time points during interphase. Subchromosomal ~1 Mb domains (corresponding to DNA replication units that are labelled during early S phase[32]) are depicted as filled circles. For the green territory, the lighter-green circles indicate gene-poor chromatin; for all territories, dark-coloured circles indicate gene-dense chromatin. Areas that contain transcriptionally active genes are indicated by dark background shading. As the chromatin moves over time, there are concomitant changes in the positioning of genes that are involved in interchromosomal interactions (the positions of three such genes, one from each territory, are indicated by black circles around the ~1 Mb domains in which they are located). Chromatin mobility is constrained in several different ways. As indicated in panel **a**, the territorial organization of chromosomes represents one level of constraint (an area of confinement). The gene-density-related polarity of chromosome territories provides a second level of constraint, as the positioning of the gene-poor chromatin towards the nuclear periphery means that it stands a lower chance of taking part in interchromosomal interactions. Finally, chromatin mobility is constrained by the tendency of transcriptionally active and transcriptionally inactive chromatin regions to cluster in three-dimensional space.

territory is an important determinant of *trans* interactions. Interestingly, the interchromosomal interactions that were detected in the case of *Rad23a* preferentially involved regions of increased gene density. This observation suggests that the clustering of gene-dense chromosomes or chromosomal segments in the nuclear interior[16,20,85] might serve to facilitate interactions between genomic regions that are located on different chromosomes (FIG. 3).

Gene kissing and epigenetic regulatory networks. Recently, the use of 3C and 4C methodologies has indicated the exciting possibility that long-range chromatin interactions are involved in the epigenetic regulation of gene expression, such that epigenetic regulatory mechanisms that affect a given gene can extend to its neighbour in nuclear space. The allele-specific expression of the adjacent *Igf2* and *H19* genes on mouse chromosome 7 is thought to result from differential interactions between an imprinting control region (ICR) that is located upstream of the *H19* gene and sequences near the *Igf2* gene. On the paternal allele, the interaction between the *Igf2* gene and the methylated ICR brings the *Igf2* gene close to enhancer elements and leads to expression of *Igf2* from this allele[86]. On the maternal allele, however, physical contact between two sequences that flank the *Igf2* gene (MAR3 and DMR1) and the unmethylated ICR results in the gene looping out, which prevents its association with enhancer elements and therefore leads to silencing of the *Igf2* gene on this allele[87]. Formation of this tripartite structure on the maternal allele was found to depend on the binding of CTCF, a zinc-finger protein, to both DMR1 and the ICR. Strikingly, mutation of the CTCF binding sites in the maternal ICR, which leads to methylation of this sequence, also abolished the binding of the protein to the maternal DMR1 and led to *de novo* methylation of DMR1. This raises the possibility that epigenetic changes can be coordinated through long-range chromatin interactions.

To determine whether the *H19* ICR could interact with and possibly regulate genes other than *Igf2*, two groups performed 4C using the mouse *H19* ICR sequence as bait[88,89]. Ling *et al.* identified only three interacting sequences in a bone marrow fibroblastic cell line, one of which is located between the *Wsb1* and *Nf1* genes on chromosome 11, whereas Zhao *et al.* identified 114 partners in neonatal liver, distributed on all chromosomes but with a clear overrepresentation of sequences from chromosome 7, which harbours the *H19* ICR. Both groups reported that the interactions primarily involve the maternal unmethylated *H19* ICR and showed, using 3D FISH analysis of selected partners, that intact CTCF binding sites are needed for the interactions to take place. Zhao *et al.* noted that 21 interacting sequences (18%) represent known or candidate imprinted regions. Furthermore, two clones were retrieved within the *Osbp1Ia* gene, which is located 5′ of the imprinted gene *Impact* on chromosome 18. Analysis by 3C revealed that the maternal *H19* ICR interacts with a differentially methylated region of *Impact*. The influence

Box 2 | **The quantitative nature of gene expression**

Microarray data indicate that up to 30% of the genome might be transcribed in one form or another (ribosomal RNA, heteronuclear RNA and processed messenger RNA, guide RNA, transfer RNA, microRNA, non-coding RNA)[95,96]. Whatever their roles might be, the wealth of non-coding RNA forces us to re-evaluate the classical notion of well defined regions of the genome being permanently silenced. Furthermore, the ever-increasing sensitivity of profiling techniques often shows very low levels of widespread expression for genes that were previously thought to be stringently regulated. In this context, gene expression should not be viewed as a qualitative state (that is, on or off), but rather as a quantitative process (that is, relative levels of gene expression). According to this view, nuclear architecture should be interpreted in light of its dynamic nature. In other words, the exact spatial positioning of a genomic segment might not be as important as the length of time that it remains at a given position.

of long-range interactions on gene expression was assessed in both studies. When the interactions were disrupted, either by depleting CTCF[88] or by mutating CTCF binding sites in the ICR[89], gene expression was either reduced by 50% (*Wsb1* or *Nf1* and *Impact*) or increased 2.5-fold (*Osbp1Ia* gene). The existence of regulatory cross-talk between spatially interacting loci opens up a new dimension in the study of gene regulation. Not only does it constitute an additional level of complexity in the search for regulatory elements in the genome, it also implies that chromatin mobility itself, and therefore the ensuing long-range gene–gene interactions, might be a target of regulation. The possibility that gene repositioning represents a means to propagate chromatin states across entire gene networks remains to be explored.

The probabilistic nature and significance of gene–gene interactions. Chromosome territories occupy preferred but not absolute positions in the nucleus[14], and the probabilistic nature of this positioning has been stressed[90]. Initial results suggest that gene kissing also follows probabilistic rules. In the previous examples, co-localization of loci was rarely observed in more than 30–50% of nuclei within a cell population[70,77,88] and most nuclei that were scored as positive in three-dimensional FISH assays had only one pair of co-localizing alleles [78,81]. In one study in which gene kissing was linked to regulation of gene expression, this heterogeneity was observed even when only active genes detected by RNA FISH were included in the analysis[78]. This result indicates that the observed heterogeneity cannot solely be ascribed to the fact that expression does not take place simultaneously across the cell population and on both alleles. Results obtained using the 4C technique, which show that a locus of interest can interact with various genomic regions both in *cis* and in *trans*, further support the idea of probabilistic long-range chromatin interactions[84,88,89]. The dynamic nature of chromatin might be particularly relevant here in view of the fact that a large fraction of the genome now seems to be transcribed and subjected to regulation (BOX 2).

One might ask about the importance of gene kissing in the regulation of transcriptional activity in light of the probabilistic nature of these events. Currently, the answer is that it is not yet clear whether the existence of gene networks in the nucleus constitutes a cause of gene activity, its consequence, or simply reflects structural constraints on the folding of chromosome territories. In support of causality is the finding that silencing mutations in one gene can affect the expression of interacting genes on a different chromosome[81,89]. On the other hand, recent work by Brown *et al.* seems to support the alternative explanation. These authors found that the proportion of active human α-globin and β-globin alleles that kiss each other increases as erythroblasts differentiate, but they interpreted this observation as a consequence of cellular differentiation rather than a prerequisite for increased transcription[91]. Furthermore, they did not observe an association between active globin alleles in mouse cells, and therefore argued that gene kissing is not essential for efficient transcription and that the chromosomal context, which is different for the mouse- and human-globin loci, probably has an important role in determining the frequency of gene kissing. Analysis of other genomic regions should help to uncover the respective influence of the chromosomal context and transcriptional co-regulation in driving gene kissing.

Conclusions and perspectives

Mounting evidence points to an important relationship between the repositioning of the genetic material in the nucleus and the regulation of gene expression. One important set of questions for future investigation relate to the molecular mechanisms that underlie the mobility of chromatin in some situations and its immobilization in others. These are aspects that we have only touched on in this Review, although a brief overview of what is known so far about such mechanisms is given in BOX 3.

Much remains to be understood about the integration of nuclear architecture and the dynamic nature of chromatin with the cell-type specific orchestration of gene expression and silencing. The view that emerges is one of chromatin being partitioned, both at the chromosome and nuclear levels, into regions that are endowed with the ability to move to different extents. A causal link between chromatin mobility and gene expression remains to be firmly established. However, insights have emerged that indicate possible mechanistic links. Gene silencing at the nuclear periphery and in the vicinity of heterochromatin could be the result of decreased chromatin mobility and, conversely, gene activation and sustained transcription might be associated with long-range movement of chromatin, as was shown recently for a transgenic locus[38]. In such a model, regulatory complexes could work by freeing chromatin from tethering, possibly at a given time of the cell cycle or during postmitotic terminal cell differentiation. This might increase chromatin mobility and allow new interactions to form, both between different regions of chromatin and between chromatin and nuclear compartments. Another set of regulatory complexes could then be responsible for stabilizing the subset of interactions that lead to proper gene expression.

Imprinting control region
A genomic region that is subject to monoallelic DNA methylation during gametogenesis. This modification results in the differential activity of the paternal and maternal alleles.

Box 3 | **Mechanisms of chromatin movement and immobilization**

Chromatin mobility

A recent study showed that nuclear translocation of a lac operator array following recruitment of the acidic activation domain of the viral protein VP16 was an active process, on the basis of the partial co-localization of nuclear myosin I with the tagged locus, and the inhibitory effect of 2,3-butanedione monoxime, an inhibitor of actin turnover[38]. So, actin and myosin might have roles in the repositioning of genes in the nucleus, adding to the growing interest in the functions of these proteins in various aspects of nuclear metabolism[97].

Owing to the presence of a large number of ATP-dependent remodelling proteins in the nucleus, chromatin is subjected to a wide range of external forces. Interestingly, transcription could be one of the prime 'movers' of chromatin. Indeed, the RNA-polymerase holoenzymes are some of the most powerful cellular motors, developing far greater forces than do kinesin- or myosin-type motors[98]. These forces, aimed at displacing nucleosomes and unwinding the template, generate significant torsional stress behind the transcription bubble due to topological constraints in the DNA molecule[99]. The necessary dissipation of the accumulated torsional stress might trigger chromatin movement under certain conditions. However, it should be noted that the effects of chromatin remodelling are probably localized. It should also be recognized that in the case of long-range chromatin movements there might actually be no need to invoke force-generating mechanisms as this type of motion could be the result of an energetically-favoured process, that is, chromatin decondensation.

Chromatin immobilization

Whereas the mechanisms of gene movement are still poorly characterized, those leading to gene immobilization are understood in greater detail. The idea of tethered nuclear components has a long history, dating back to the efforts of Berezney, Coffey and Penman to prove the presence of a nucleoskeleton and achieve its biochemical characterization (reviewed in REF. 100).

Although protein complexes undoubtedly have an important role in interactions of chromatin with other nuclear structures, recent results also point to a role for RNA in stabilizing such interactions. In 1999 Berezney and co-workers reported that the maintenance of chromosome-territory structure requires RNA[101]. Recently, Casolari *et al.* found that RNase treatment of *Saccharomyces cerevisiae* cells abolished the association of α-factor induced genes with Mlp1, a component of the nuclear pore complex, suggesting that repositioning of these genes to the nuclear pores following transcriptional activation is achieved through an RNA intermediate[102].

An RNA-dependent mechanism might also account for the clustering of *Fab7* transgenes that contain Polycomb response elements in *Drosophila melanogaster*[93]. In this case, the presence of small RNA species of 21–23 nucleotides, which were derived from the transgene arrays and generated by the RNAi machinery, was found to be correlated with spatial co-localization and silencing of the corresponding genomic regions. Mutations in components of the RNAi machinery led to disruption of long-range *Fab7* interactions in larval nuclei. Interestingly, RNAi mutant backgrounds did not affect interchromosomal interactions at *Fab7* in embryonic nuclei, indicating that RNA intermediates might be required to maintain, but not to initiate, gene clustering. These observations raise the exciting possibility that RNAs might have both structural and regulatory roles in nuclear architecture. This would be in agreement with the growing list of cellular functions that have been attributed to this class of molecules.

The compartmentalized nature of the nucleus raises the question of how compartments are established and maintained. Two non-exclusive models have been proposed to account for the fact that metabolic activities are spatially organized in the nucleus: compartments could correspond to pre-existing structures, or they could be built up as a result of the self-assembly properties of macromolecular complexes that are engaged in gene regulation (FIG. 4). Although the observation of profound changes in nuclear organization after inhibition of transcription and rapidly exchanging pools of proteins within nuclear compartments would seem to support the view of the nucleus as a self-organizing entity in which regulatory processes drive the interactions between functional components[92], the issue remains largely unresolved and should continue to fuel debate in the coming years.

Biochemical and genetic approaches are already at hand to dissect the molecular mechanisms that are involved in gene repositioning and, more generally, in nuclear compartmentalization[93,94] (BOX 1). At the same time, living-cell microscopy should continue to provide insights into chromatin mobility. However, the experimental manipulation of nuclear architecture remains difficult, raising the question of how the functional relevance of changes in nuclear organization can be investigated. Evolutionary conservation has always been considered a strong indication of functional relevance. The observation that radial positioning of chromosomes according to gene density is an evolutionarily conserved feature of the interphase nucleus indicates an important role for this pattern of chromatin organization[19]. Now that a growing number of genome sequences are becoming available, comparative studies of nuclear architecture are needed to distinguish between sequence-dependent and epigenetic determinants of chromatin organization and dynamics. Systematic phylogenetic analyses could also prove useful in determining the influence of reshuffling of syntenic regions and chromosomal context on intranuclear gene positioning. This is best demonstrated by the case of the active α-globin and β-globin loci, which spatially cluster in human but not in mouse erythroblasts[91]. The analysis of the cell nucleus using complementary approaches should lead to an integrated understanding of nuclear structure and function.

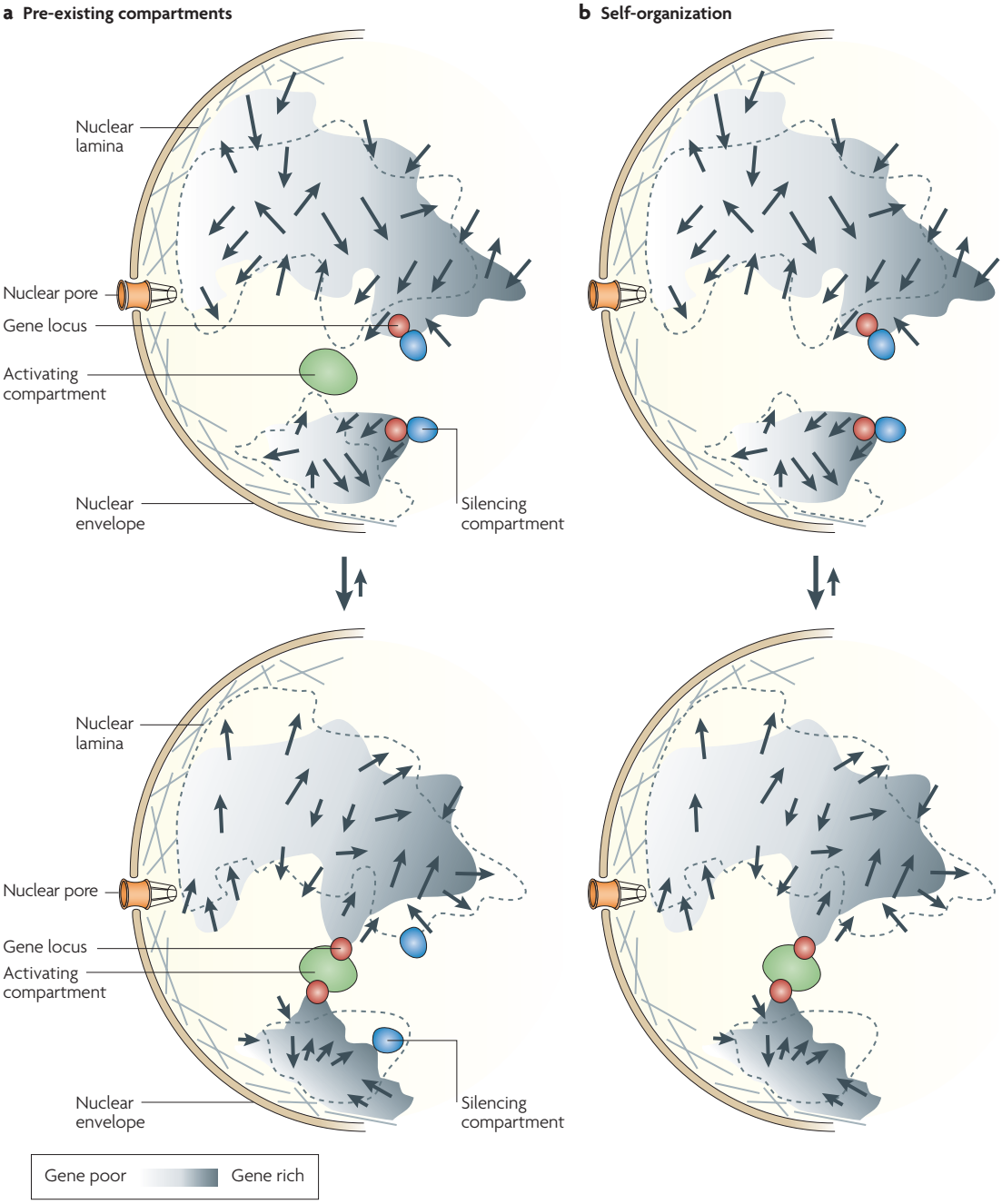

Figure 4 | **Chromatin mobility and principles of nuclear organization.** Chromatin mobility allows dynamic interactions between genomic loci and between loci and other nuclear structures. In both **a** and **b**, two chromosome territories are shown, and within each territory a gene locus is indicated in red. Movement of chromatin is depicted by arrows. Two possible configurations are represented for each territory, with the dotted outline of one superimposed on the other. The transition involves repositioning of the two loci within the three-dimensional space of the nucleus. In this hypothetical example, the bottom configuration in each case is favoured on transcriptional activation of the loci. Note that despite chromatin mobility, the polarized organization of the territory according to gene density is maintained. To account for the compartmentalization of nuclear functions that are involved in gene expression, two alternative views have been proposed. In the model shown in **a**, compartments are pre-existing structures containing molecular machineries that are dedicated to specific nuclear functions. Movement of chromatin from one compartment to another leads to changes in expression of the corresponding genomic regions. Activation is triggered by repositioning of the gene loci to an activating compartment, away from silencing compartments. By contrast, in the model shown in **b**, compartments are transient self-organizing entities. In this case, gene activation leads to dissolution of the silencing compartments, changes in gene positioning and *de novo* assembly of an activating compartment. Once initiated, this state can be maintained by the self-assembly of components that are involved in gene regulation, as well as the clustering of chromatin regions that contain actively expressed genes.

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Competing interests statement
The authors declare no competing financial interests.

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