Nuclear organization of the genome and the potential for gene regulation

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Much work has been published on the *cis*-regulatory elements that affect gene function locally, as well as on the biochemistry of the transcription factors and chromatin- and histone-modifying complexes that influence gene expression. However, surprisingly little information is available about how these components are organized within the three-dimensional space of the nucleus. Technological advances are now helping to identify the spatial relationships and interactions of genes and regulatory elements in the nucleus and are revealing an unexpectedly extensive network of communication within and between chromosomes. A crucial unresolved issue is the extent to which this organization affects gene function, rather than just reflecting it.

What we know about the organization of the genome in the nucleus has been driven to a large extent by two technologies — interphase fluorescent *in situ* hybridization (FISH) and chromosome conformation capture (3C). In FISH, the relative nuclear positions of genes, genomic regions or even whole chromosomes are analysed by the hybridization of probes to nuclei fixed on glass slides. The hybridization signals are then visualized with fluorescence microscopy. In 3C, chromatin fragments in close proximity in the nucleus are captured by fixation, restriction-enzyme digestion and intramolecular ligation. The interaction between two designated genomic loci is then tested by polymerase chain reaction with primers that are specific for the loci under investigation. The limitation with both these approaches is that you only see what you are actively looking for. The advent of 4C — 3C combined with either large-scale sequencing of captured fragments or hybridization to microarrays[1–3] — has facilitated a more unbiased search for regions of the genome that interact with a particular locus, both *in cis* and *in trans*.

In this review, we discuss new data from vertebrates that help to clarify how genes function in the context of the nucleus, and that suggest that the genome's spatial organization is a key contributor to its function. We start by looking at levels of gene organization within the nuclear space occupied by individual chromosomes — the so-called chromosome territory — and ask whether some genes need to escape from this space if they are to be expressed efficiently. We then review long-range (multi-megabase) gene interactions, both *in cis* and *in trans*, and gene interactions with transcription factories. The emphasis is on the potential of such organization for gene regulation and, in particular, epigenetic regulation.

The chromosome territory as a unit of nuclear organization

The largest unit of organization of the eukaryotic genome is the chromosome. The idea that the chromosome territory is a unit of nuclear organization was advanced with the suggestion that chromosomes are discrete nuclear bodies separated by an interchromatin compartment — a more or less continuous space between adjacent chromosomes[4]. Chromosomes have preferred positions with respect to the centre or periphery of the nucleus[5] and with respect to each other[6]. Thus, a chromosome's neighbour in the nucleus is far from random — it varies between cell types, and it has consequences for a chromosome's ability to interact in *trans* with other parts of the genome, as revealed by the frequency of specific chromosome translocations[7,8]. The positional organization of chromosomes within the nucleus could therefore impinge on other aspects of genome function and, in particular, on the regulation of gene expression[9,10].

Studies have probed deep into the substructure of chromosome territories to analyse the position of sub-chromosomal regions and genes. Their results showed that for some genes, the interior, exterior or surface localization relative to their chromosome territory correlates with gene activity or inactivity[11,12]. Despite interest in interactions in the nucleus between loci on different chromosomes, one clear message that has emerged from 4C analyses is that the main sequences captured by a given locus are other regions from the same chromosome[1,3]. Thus the two-dimensional organization of the genome and the three-dimensional organization of the chromosome territory are major determinants of a gene's nuclear environment. However, experiments with 4C provide only an averaged conformation of a locus relative to its nuclear environment, which is gleaned from thousands of cell nuclei. Such a 'consensus snapshot' may not reveal transient dynamic functional states or events, especially since chromosome territories clearly do not have rigid boundaries and have a plastic organization.

Escape or eviction from the chromosome territory

Genes sometimes relocate substantial distances outside of their chromosome territory. This relocation occurs either in domains of constitutively high gene expression[13,14] or, in some instances, when gene expression is induced[15–18]. For example, the *Hoxb* gene cluster is activated at the same time as it relocates away from the chromosome territory[17]. A consequence of this relocation, as assessed by 4C, is that the 'looped out' *Hoxb* locus can now interact more with other chromosomes[1]. This finding, together with the observation of extensive intermingling of DNA from different chromosomes at the boundary of, or just outside of, chromosome territories suggests that chromosome territories might not be as discrete as previously thought[19]. It also raises the issues of why and how genes are leaving the chromosome territories and where they are going, especially since not all active genes are located outside chromosome territories[12].

Relocation outside of chromosome territories and intermingling are reduced when RNA polymerase II (Pol II) is inhibited experimentally[13,19], suggesting that these events are driven partly by the process of transcription itself. The localized chromatin decondensation that occurs

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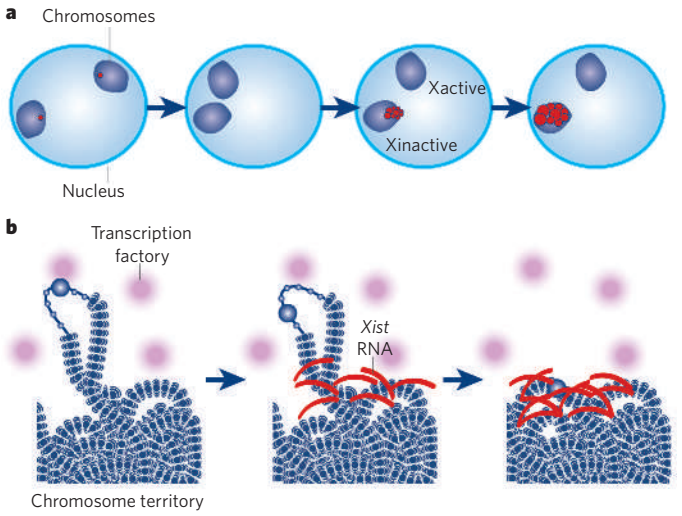

Figure 1 | Events of nuclear reorganization during X-chromosome inactivation. **a**, Soon after female embryonic stem cells start to differentiate, the two X chromosomes (purple) come together in the nucleus, and the X-inactivation centres, which initiate X-chromosome inactivation, interact[9,10]. These events occur concomitantly with the process of X-chromosome counting and choice[38] and lead to upregulation of *Xist* transcription (red) from the future inactive X chromosome (Xinactive). **b**, The coating of the inactive X chromosome by *Xist* RNA molecules excludes Pol II and the transcriptional machinery (pink) from the inactive X-chromosome territory[22]. Genes initially located outside the domain (purple circles) coated by *Xist* RNA are retracted back inside the *Xist* compartment as they become silenced through a mechanism dependent on the A repeats of *Xist* RNA[22].

in conjunction with transcriptional activation might also release constraints on chromatin's mobility and so provide it with the flexibility to facilitate looping out and to establish long-range contacts between genes and their distal regulatory elements *in cis* or *in trans*. An unresolved issue is the extent to which there might be directed chromatin movement that is driven, for example by an actin–myosin system. Compelling evidence for this idea has come from live-cell imaging of a transgene, after a transcriptional activator was targeted to it[20]. The resultant motion was curvilinear, in a direction generally perpendicular to the nuclear periphery, and perturbed by actin/myosin mutants[20]. These properties are inconsistent with the constrained-diffusion mode of chromatin motion usually seen in the mammalian genome[21], and activated endogenous genes will therefore need to be similarly analysed as they move out of their chromosome territories in living cells.

Whether the looping out is a passive response to transcriptional activity or whether it also helps to regulate this process has not been determined. A recent analysis[18] of the *Hoxd* locus in mouse development revealed that its activation along the anterior–posterior embryonic axis is accompanied by a looping out from the chromosome territory, but that activation in the limb bud, at the same stage of development, occurred in the absence of any detectable looping out from the chromosome territory in this zone even though the locus was still decondensed. This finding raises the interesting idea that the relocation of a gene outside of its chromosome territory depends on how it was activated. Evidence in favour of a role for looping out in the regulation of gene expression has come from a study of the early events in inactivation of the mammalian X chromosome. The first event that occurs after *Xist* RNA coats the chromosome to be inactivated is that Pol II is excluded from the compartment that contains the bulk of the X-chromosome territory[22]. Chromosome territories are not usually barriers to the transcriptional machinery[23,24], but the *Xist*-mediated barrier around the X chromosome seems to form at the same time as genes are silenced and as they relocate into a silent compartment (Fig. 1). The few X-linked genes that escape inactivation (that is, remain active on the inactive X chromosome) seem to be located at the edge of, or outside of, the *Xist* RNA compartment. Furthermore, if the A-repeat motif, which is required for silencing, is deleted from the *Xist* RNA, the Pol II exclusion compartment still forms, but genes usually scheduled for inactivation are not silenced or modified epigenetically and do not relocate to the inside of the *Xist* compartment. These results link the physical relocation of X-linked genes with the silencing mechanism and suggest that the silencing depends on the architecture of the nucleus and chromosome[22]. Although the strict order of events is not known, an intriguing possibility is that the A repeats, and the factors that interact with them, capture X-linked genes and consign them to the interior, thus leading eventually to epigenetic silencing.

Colocalization of active genes in the nuclear space

Are genes located at the periphery of, or outside of, chromosome territories just moving randomly in a different nuclear environment from those restricted to the chromosome interior, or are they interacting with, or tethered to, discrete sites? Actively transcribed genes localize at focal concentrations of Pol II called 'transcription factories'. The number of discrete factories visible in the nucleus seems to be lower than the number of expressed genes, suggesting that multiple genes share the same factory (Fig. 2). Indeed, both RNA FISH and 3C confirm the nuclear colocalization of active alleles of genes that are separated by tens of megabases in *cis*, or even of those located on different chromosomes[25]. The gene and factory may require a physical association for efficient transcription, since actively transcribed alleles are almost always found in the factories[25,26]. However, the process is not as simple as getting to a factory, becoming active and then staying there. Studies done in live cells have shown that individual genes are transcribed in bursts or pulses of production[27]. FISH studies support this transcriptional pulsing model and have shown that temporarily quiescent alleles of 'active' genes are located away from factories, suggesting that gene mobility is an important factor in gene control[25].

However, related studies have led to a different interpretation of the spatial colocalization of actively transcribed genes. The zone of spatial colocalization, or juxtaposition, of active gene loci was thought to be larger than the predicted size of transcription factories as measured by foci of bromodeoxyuridine UTP incorporation[28]. Rather, researchers have suggested that the active genes congregate at, or around, splicing-factor-enriched speckles — accumulations of messenger-RNA-splicing factors that have a diameter of 0.5-3.0 μm (Fig. 2)[14,29]. Some active genes are found at the speckles' boundaries, and specific pairs of genes in *cis* and *trans* have been shown to co-associate with the same speckle[14,29,30]. However, transcription sites are clearly distinct from splicing-factor-enriched speckles — nascent RNA can be detected at the edges, but most transcription occurs outside the speckles[31]. One suggestion is that some pre-mRNAs transit through the speckles as part of their processing and export[32,33].

The idea of nuclear zones in which very active genes are colocalized is compatible with the results from 4C analysis of the β-globin gene (*Hbb*) in fetal liver, and the ubiquitously expressed *Rad23a* gene in both liver and brain. In both cases, the active genes were associated with several clusters of other active genes. Each cluster was 150–200 kilobases in size and located mainly within a 70 megabase region in *cis*. But associations with gene clusters were also detected *in trans*[3] (Table 1). The 'interactions' were not restricted to a specific gene within each cluster nor to specific parts of genes such as promoters, and they were far too numerous to suggest that they all occurred simultaneously in a single cell. These findings raise the issue of whether the interactions detected by 4C originate from a probabilistic three-dimensional chromosome structure that differs from cell to cell or from multiple dynamic interactions that occur transiently in all or most cells. The multiple-interactions explanation is consistent with a study that used FISH to look at recognizable folding states of a 4.3 megabase region from mouse chromosome 14 that contains alternating gene-dense regions and gene deserts. FISH signals from the gene-dense regions were juxtaposed spatially in about 20% of the nuclei[34], and nuclear colocalization seemed not to be restricted to active gene regions.

Nuclear colocalization of genes and regulatory elements

In tissue in which *Hbb* is not expressed (brain), 4C showed that the interactions captured with *Hbb* were with other silent gene clusters, including clusters of olfactory-receptor genes that are *in cis* with, but distant from, *Hbb*[3] (Table 1). Although there is no evidence that in this instance the associations contribute functionally to the silent state of these gene clusters, the spatial organization of olfactory-receptor genes does seem to be involved in their extraordinary regulation in olfactory sensory neurons. Only 1 of the 1,300 olfactory-receptor genes is expressed in a given neuron, and then only from 1 allele of that gene. An enhancer element (*H*) required for expression of olfactory-receptor genes has been identified[35]. A quarter of sequences identified as interacting with this element by 4C analysis were immediately upstream of olfactory-receptor genes[36]. Like the 4C interactions described for *Hbb*, most (75%) of the olfactory-receptor genes captured with *H* were located *in cis*, although some interactions were *in trans* (Table 1). This predominance broadly reflects the relative frequency with which different olfactory-receptor genes are expressed and suggests that expression of an olfactory-receptor gene depends on its interaction with *H* in the nucleus (Fig. 3). Consistent with this idea, DNA FISH showed that *H* colocalizes with an allele of a specific olfactory-receptor gene in about 30% of the cells that express that gene. Since only one allele of *H* is thought to be active in this respect (the other is methylated, unusually at CpA sequences), expression of olfactory-receptor genes would be restricted to the allele that is in contact with *H*. Indeed, combined RNA and DNA FISH for *H* DNA and a specific olfactory-receptor RNA in *trans* showed that *H* is in contact with the actively transcribed allele in 85% of cells with a FISH signal for olfactory-receptor RNA. Taken together, these results suggest that *H* is in contact with the active olfactory-receptor gene specifically during a transcriptional pulse but may dissociate during periods of transcriptional inactivity.

Transient interactions between regulatory elements and genes in the nucleus have also been associated with coordinately regulated gene expression in other examples. Interchromosomal interactions between the interferon gamma (*Ifng*) gene and the locus control region of the T-helper 2 (T_H2) cytokine locus are found by 3C and FISH in the nuclei of naive $CD4^+$ T cells[37] (Table 1). This interaction is thought to hold the *Ifng* and T_H2 cytokine gene loci in a poised state that can respond rapidly to T-cell activation by expression of both gene loci, but only at very low levels. Later, after the decision to differentiate into T_H1 or T_H2 cells has been made, and expression of *Ifng* or T_H2 cytokines is very high, these interchromosomal associations are lost in favour of intrachromosomal ones. It will now be interesting to analyse the interaction of these loci with other regions of the genome during T-cell activation and differentiation by use of 4C approaches.

These studies of olfactory-receptor genes and cytokine genes clearly implicate nuclear interactions in gene-expression states that are then epigenetically stable, and may even implicate nuclear organization in epigenetic 'decisions'. The paradigms for investigation of epigenetic mechanisms that underpin allelic choice are X-chromosome inactivation and imprinting. Strikingly, nuclear interactions have been implicated in both. The two X chromosomes transiently colocalize, and the X-inactivation centres interact physically during the differentiation of female embryonic stem cells[9,10]. The timing of this nuclear interaction is concurrent with the onset of X-chromosome inactivation[38] (Fig. 1). It is also within the time frame that would implicate it in the intriguing process whereby the number of X chromosomes per nucleus is 'counted' and the 'choice' is made to inactive one of them.

The other system in which transient nuclear interaction happens on homologous chromosomes is imprinting[39]. In mouse neonatal liver, a 4C approach showed that the imprinting control region (ICR) of *H19* had many *trans* interactions in addition to its abundant *cis* interactions[2]. Importantly, sequences from multiple different chromosomes could be captured within the same 4C clone after intramolecular ligation, suggesting that the nuclear associations were not just pairwise, but that multiple genomic loci could co-associate simultaneously with the *H19* ICR in a single nucleus. In mice in which the maternally derived *H19* alleles could be distinguished from the paternally derived ones (*Mus musculus* versus *Mus spretus* origins), about 75% of the sequences captured were specific for the maternally derived allele. Hence, the epigenetic status of the *H19* ICR, which is methylated on the paternally derived allele, determines the patterns of most of its intra- and interchromosomal interactions. As well as maternal-specific interactions *in cis* with another imprinted domain on mouse chromosome 7, interactions were also detected with known, or predicted, imprinted regions on other chromosomes (Table 1). 3C analysis confirmed that the *H19* ICR interacted with a differentially methylated region on chromosome 18 that is implicated in the control of imprinting there. Hence, the nuclear interaction might not one be of gene with gene, or gene with regulatory element, but of two regulatory elements with each other. The functional significance of this finding was demonstrated by the altered expression of two genes, *Osbpl1a* and *Impact*, from within the chromosome 18 imprinted domain in mice

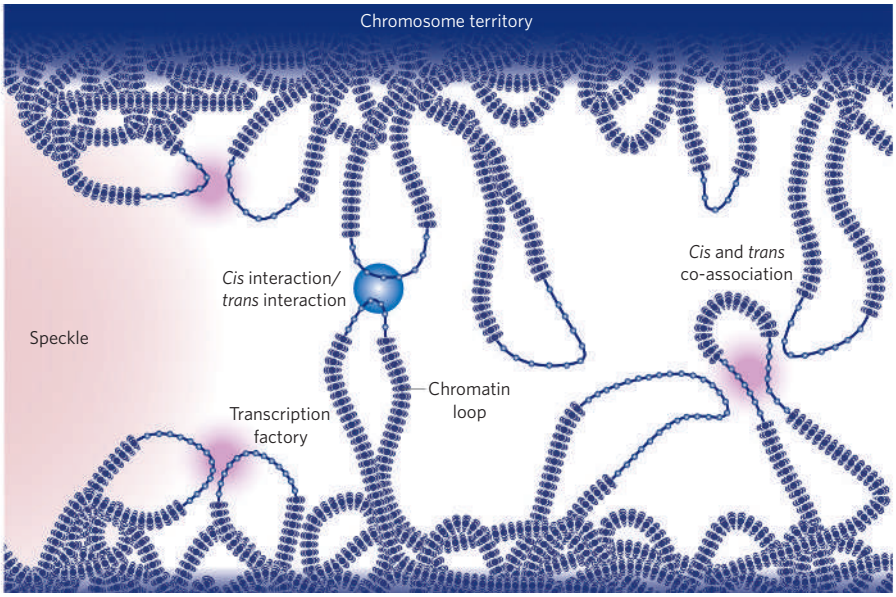

Figure 2 | Colocalization of genes in the nucleus for expression or coregulation. Active genes on decondensed chromatin loops that extend outside chromosome territories can colocalize both *in cis* and *in trans* at sites in the nucleus with local concentrations of Pol II (namely transcription factories; dark pink) and adjacent to splicing-factor-enriched speckles (pale pink). Interactions can also occur between regulatory elements and/or gene loci and lead to coregulation in *trans* (blue circle).

Table 1 | Long-range intrachromosomal and interchromosomal interactions detected by 3C and 4C

Starting locus	Captured interacting locus	Intra- or interchromosomal	Cell type	Reference
3C				
Hbb (active)	Erythroid associated factor (*Eraf*), uroporphyrinogen III synthase (*Uros*)	Intra	Fetal liver	25
T_H2 locus control region	*Ifng*	Inter	Naive $CD4^+$ T cells	37
Tsix (*Xic*)	Other Xinactive-specific transcript (*Tsix*) allele	Inter	Embryonic stem cells	9
H19 imprinting control region (maternal allele)	*Osbpl1a*, *Impact*	Inter	Neonatal liver	2
4C				
Rad23a (active)	Multiple active gene regions	Intra > inter	Fetal liver and brain	3
Hbb (active)	Multiple active gene regions including those containing *Eraf*, *Uros* and *Kcnq1*	Intra > inter	Fetal liver	3
Hbb (inactive)	Silent gene clusters: for example, olfactory-receptor genes	Intra	Fetal brain	3
H enhancer	Expressed olfactory-receptor allele	Intra > inter	Olfactory sensory neurons	36
H19 imprinting control region	*Wsb1*, *Nf1*	Intra-	Fibroblasts	40

with a mutated maternal *H19* ICR. The multiple (>100) nuclear interactions of *H19* ICR in this study[2] contrasted sharply with the results of a superficially similar experiment showing only one interaction *in cis* and two *in trans* for the maternal allele of the *H19* ICR[40]. Why such different pictures of the breadth of *H19* ICR interactions arise from these two experiments is unclear, but they might, for example, represent differences in the detailed experimental protocols, or in the cells and cell lines used in each case — liver and differentiating embryonic stem cells versus a fibroblast cell line.

Future directions and perspectives

The overall picture that is emerging, with the aid of rapid technological advances, is that genes have some independence in the nucleus but do not necessarily function in isolation from each other. They are taken to, or find themselves in, different nuclear environments, which are often shared by other genes. Although many genes may relocate for specific purposes in accordance with their own activation status, other genes nearby on the DNA sequence may be taken along for the ride. The constraints imposed by the functional organization of the nucleus could provide the selective pressure to maintain clusters of broadly expressed, apparently functionally unrelated, genes together on chromosomes during evolution[41,42]. In particular, multiple active genes and gene clusters are often located together at places in the nucleus that have high local concentrations of transcriptional and mRNA-processing machinery. A study of the spatial relationship of genes both with Pol II and with splicing-factor-enriched speckles will help to determine whether these nuclear zones are organized mainly by the preference for specific genes to be transcribed together (transcription factories) or to ensure efficient transcription and co-transcriptional mRNA processing (mRNA expression factories). An exciting new dimension to this area is added by the detection of interactions in the nucleus between regulatory elements. The colocalization of these elements is probably not because they are both recruited to a particular nuclear compartment, but because direct protein–protein interactions between the loci promote or restrain the genes' ability either to sample other nuclear environments necessary for their activation or repression or to directly modify the chromatin structure of the interacting alleles. To differentiate between these models we need to know more about the proteins that mediate the interactions — especially those that occur *in trans*. One clue that we have about this is that the interactions of the maternal *H19* ICR are dependent on the binding of the protein CTCF[2,40].

Two extreme camps have emerged about the functional relevance of nuclear organization. One camp thinks that nuclear organization merely reflects the functional processes occurring in the genome (for example, transcription). The other thinks that nuclear organization is a major factor in regulating the genome's function. The data we have reviewed here suggest that the dynamic spatial organization of the nucleus both reflects and shapes genome function[42]. There are also exciting examples of epigenetic 'decisions' that are made or influenced by *trans* interactions between specific loci, and these will continue to provide attractive model systems that further our understanding of the mechanistic importance of these levels of nuclear organization. We now have a picture of a genome that is 'structured', not in a rigid three-dimensional network, but in a dynamic organization with preferred or probabilistic conformations experienced by similarly programmed cells. This organization clearly changes during normal development and differentiation, and is itself 're-programmable' — for example, during the process of cloning by nuclear transfer[43,44]. The 'tough nut' of the nucleus is starting to be cracked as more complex molecular events are related to its three-dimensional functional organization. The studies reviewed here reveal that the scope of long-range *cis* and *trans* functional interactions is much greater than imagined, and there is no reason to think that this is the limit. Mobility is an important factor in genome function and could be a control point for several nuclear events. Thus, analysis in living cells will have a major role in future experiments. Exciting times lie ahead

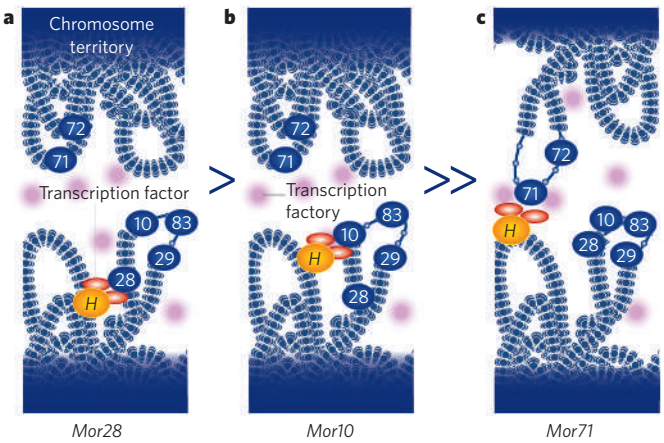

Figure 3 | *Cis* and *trans* interactions of the *H* enhancer and olfactory-receptor genes. A quarter of 4C products captured in the nuclei of sensory neurons with the olfactory-receptor *H* enhancer (orange) are olfactory-receptor genes themselves (numbered circles)[36]. More than half of these are the promoter of *Mor28*, which is adjacent to *H* on mouse chromosome 14 (**a**). The next most frequent interaction found was with the promoter of *Mor10*, which is also in *cis* with *H* (**b**). Much less frequent were interactions with olfactory-receptor genes (for example, *Mor71*) located on other mouse chromosomes, and so in *trans* with *H* (**c**). Each olfactory neuron expresses only one allele of one olfactory-receptor gene, and the frequencies of 4C interactions with *H* reflect the relative frequencies of expression of different olfactory-receptor genes in the population of sensory neurons. The *H* enhancer might function by recruiting transcription factors (red) and high concentrations of Pol II (present in transcription factories) to the expressed gene.

as the wealth of biochemical information known about specific nuclear processes is mapped onto the global coordinates of the nucleus to create a more holistic view of functional organization of the genome and its role in delivering gene-expression programmes. ■

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