Functional consequences of chromosomal rearrangements on gene expression: not so deleterious after all?

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Research Highlights:

- TADs are evolutionarily conserved regions largely overlapping with regulatory domains
- Deleting TAD boundaries only mildly affects gene expression
- Chromosomal inversions affecting TAD boundaries are usually more deleterious
- Enhancer redundancy and enhancer-promoter specificity might buffer the effect of chromosomal rearrangements

Declarations of interest: none

Abstract (max 250 words)

Chromosomes are folded and organized into topologically associated domains (TADs) which provide a framework for the interaction of enhancers with the promoter of their target gene(s). Structural rearrangements observed during evolution or in disease contexts suggest that changes in genome organization strongly affect gene expression and can have drastic phenotypic effects. In this review, I will discuss how recent genomic engineering experiments reveal a more contrasted picture, suggesting that TADs are important but not always essential for gene expression regulation.

Introduction

In metazoans, the expression of protein-coding genes is finely regulated both in space and time through the action of hundreds of thousands of regulatory elements, such as enhancers, scattered along the non-coding genome. Enhancers are short genomic regions containing DNA motifs, which act as binding sites for transcription factors. When ectopically inserted into the genome, enhancers can drive the expression of a reporter gene in an orientation- and context-independent manner^{1,2}. Active enhancers are located in open chromatin regions³ and are characterized by the presence of specific chromatin marks such as H3K4me1 and H3K27ac⁴⁻⁸. Importantly, enhancers can function either in an additive or partially redundant manner, collectively establishing the complex spatio-temporal expression pattern of their target gene(s)⁹. A particularly intriguing feature of enhancers is their ability to operate at a distance: they can be located at great linear distances from their target gene(s) but yet activate their expression via the formation of chromatin loop(s)⁹. While enhancer-promoter looping is essential to activate gene expression, enhancers can sometimes interact with gene promoters in tissues or at developmental stages when the gene is not active¹⁰⁻¹³, suggesting that preformed topologies might enable rapid and efficient transcriptional activation. Even more intriguing, a given enhancer might regulate the expression of a distal gene while skipping other genes located in its proximity¹⁴. This reflects a certain degree of specificity in enhancer-promoter interactions: the expression of a gene cannot be activated by just any enhancer. The exact mechanisms controlling this enhancer-promoter interaction specificity remain however largely elusive. While multiple factors are likely involved (reviewed by van Arensbergen *et al.*¹⁵), the evolutionary conservation of regulatory domains and the transcriptional consequences of their disruption during disease suggest that the three-dimensional (3D) organization of the genome might be of particular importance. These observations are however challenged by the advent of genome engineering techniques, allowing the engineering of very precise and focused *de-novo* rearrangements such as deletions and inversions. This review illustrates how these recent results are refining our perception of the link between genome topology, enhancer-promoter interactions, and gene expression.

3D genome organization

Chromatin structure: compartments, TADs and loops

To fit into the tiny cell nucleus, chromatin fibers need to be tightly folded. Classical 3D DNA fluorescent *in situ* hybridization (FISH)¹⁶ and the more recent Chromosome Conformation Capture (3C) technique and its derivatives (such as Hi-C¹⁷) have provided increasing evidence suggesting that chromatin folding is both highly organized and tightly linked to transcriptional regulation. While this review primarily focuses on results obtained using 3C-derived approaches, direct visualization of chromatin organization in single cells has become a valuable alternative, thanks to the development of new methods and improvements in imaging resolution¹⁸⁻²² (reviewed by Cardozo Gizzi *et al.* in this issue). Chromosomal domains are positioned according to their transcriptional activity, with repressed regions often associated with the nuclear lamina²³, while actively transcribed regions tend to be located at the center of the nucleus. Chromosomes are hierarchically organized into several types of domains. First, chromosomes can be divided into regions belonging to the A or B compartments, which broadly represent active and inactive chromatin respectively. These compartments can be identified by principal component analysis (PCA) of the normalized Hi-C matrix and visualized as a checkerboard pattern in Hi-C maps¹⁷. The sign of the first eigenvector (principal

component 1) indicates the compartment. This classification has subsequently been further refined in human and mouse cell lines into six sub-compartments (A1, A2, and B1 to B4) each associated with particular chromatin post-translational modifications²⁴. At a smaller scale, chromosomes are divided into domains of increased 3D proximity ranging from about 100 kb to a few megabases in size, named Topologically Associating Domains (TADs)^{12,25,26}. In vertebrates, the large majority of TADs are delimited by the binding of the CTCF insulator protein^{12,27} and have been proposed to form through a loop extrusion mechanism mediated by cohesin: the cohesin ring slides along the DNA until reaching two CTCF motifs positioned in a head-to-head orientation^{28–32}. This can be visualized in Hi-C maps by the presence of a strong signal (corner peak) at the tip of the TAD triangle, resulting from the high contact frequency between the CTCF-binding sites located at two adjacent TAD boundaries acting as loop anchors. However, it should be noted that not all loop anchors correspond to TAD boundaries, and that not all TAD domains display a strong corner peak²⁴. Besides, some genomic regions, in particular gene-rich regions, are less structured and lack clear boundaries³³, and a small proportion of TAD boundaries (10 to 20%) are not bound by CTCF and might thus form in a CTCF-independent manner, possibly through other mechanisms than loop extrusion³⁴. Accurately defining TADs is thus a complicated matter. A large variety of algorithms have been developed to identify TADs, resulting in a difference of up to one order of magnitude in the number of TADs called by different methods^{35,36}. Because TADs adopt a hierarchical tree-like structure composed of nested triangles, small changes in the parameters used to identify them (such as sequencing depth, resolution or cut-off) can greatly influence their size. In addition, Hi-C experiments themselves are not trivial: a variety of different protocols are available and reproducibility is a major issue. As a consequence, the structure and function of TADs and their potential role in transcriptional regulation are much debated.

TADs as functional regulatory domains?

It is generally admitted that TADs essentially restrict the activity of enhancers, preventing undesirable enhancer-promoter interactions across TAD boundaries. Indeed, the random insertion of a regulatory sensor acting as an enhancer-trap at various locations of the mouse genome revealed that the sensor only responds to the enhancers located within the same regulatory domain, and that these regulatory domains strongly correlate with TADs³⁷. Besides, the genes and enhancers located within the same TAD tend to be co-regulated^{38–41}. TADs can further be divided into “sub-TADs” or “sub-domains”. While TADs are generally invariants between cell types, sub-TADs are cell-type specific and mediated by cohesin and Mediator²⁷. Sub-TADs are also smaller (on average 32 kb versus 185 kb in lymphoblastoid cell lines⁴²) and driven by transcription²⁷. These initial studies suggest that TADs are functional regulatory units of the genome. However, recent evidence seems to contradict this view: TADs are lost upon CTCF or cohesin depletion but their loss only mildly affects gene expression^{34,43}, questioning their actual role in transcriptional regulation. This result might however be specific to resting cells, as recent work has highlighted that cohesin is essential for inflammatory gene expression in primary myeloid cells⁴⁴. A more direct method to establish TAD function is to examine the transcriptional consequences of their rearrangements, for example during evolution or in pathologies.

Chromosomal rearrangements during evolution and in disease contexts

Evolutionary conservation of TADs: proof of their importance or consequence of fragile TAD boundaries?

A particularly strong argument in favor of a functional role for TADs is their evolutionary conservation: about 50% of TADs are conserved when comparing human and mouse^{12,24}. In fact, TADs generally overlap with syntenic blocks, i.e. regions of DNA with conserved collinearity of genes and/or non-coding elements, and syntenic rearrangement breakpoints are enriched at TAD boundaries both in vertebrates and flies⁴⁵⁻⁴⁹. However, this overlap might simply be the consequence of TAD boundaries' relative "fragility" to double-strand breaks (DSB). DSBs are often found at loop anchors, suggesting that loop extrusion might create some torsional stress subsequently released by topoisomerase II when the cohesin complex encounters a CTCF site⁵⁰. In addition, TAD boundaries are enriched in open chromatin regions and active genes, which have an increased susceptibility to breakage. Damaged active genes tend to cluster in the nucleus⁵¹ where they get preferentially repaired with other regions in close 3D proximity. As a consequence, translocations tend to be enriched at TAD boundaries⁵²⁻⁵⁵. The remarkable evolutionary conservation of TADs also suggests that TAD boundary disruptions might be under purifying selection, i.e. that mutations affecting TAD boundaries are negatively selected during evolution. This is indeed the case: comparing human and ape populations revealed that deletions are depleted at TAD boundaries, but uniformly distributed in patients with developmental delay or autism⁵⁶. This could however just be a consequence to the overall depletion of deletions at active genes which tend to be located at TAD boundaries.

Chromosomal rearrangements in disease

Another strong argument in favor of a functional role for TADs is the effect of structural rearrangements affecting TADs and causing various diseases, such as developmental disorders and cancers. This topic has already been extensively reviewed⁵⁷⁻⁶⁰, and is based on the following assumption: if TADs are required for proper enhancer-promoter communication, disturbing TADs by deletions⁶¹⁻⁶⁵, duplications⁶⁶, or inversions⁶¹ should have deleterious effects. A particularly striking example supporting this model came from the analysis of a genomic inversion affecting a TAD boundary at the *Epha4* locus in mice. This inversion was shown to cause malformation syndromes in the developing limb bud due to the formation of *de-novo* interactions between *Epha4* enhancers and the *Wnt6* gene⁶¹. This mechanism, termed enhancer adoption⁶⁷ or enhancer hijacking⁶⁸, has been proposed to explain up to 12% of the deletions present in a database of rare genomic copy-number variants and their associated phenotypic data⁶⁹. Similarly, TAD boundary disruptions have been linked to a variety of cancers^{68,70-73}. For example, the expression of oncogenes in cancer cells can be upregulated due to the deletion or hypermethylation of cohesin and CTCF-binding sites at TADs boundaries^{71,73}. Finally, a recent study uncovered that nearly all disease-associated short tandem repeats (daSTRs) are located at the boundaries of TADs, in particular those featuring a high density of CpG islands⁷⁴.

These different examples suggest that altering TADs will necessarily cause adverse effects. Contrasting results have however emerged from two recent studies. First, Dong *et al.* compared the location of four translocations from the "1000 genomes" project with TAD calls from the human IMR fibroblast cell line. They found that eight TADs were disrupted, altogether containing 34 genes. Out of these 34 genes, 16 were expressed, but none differentially expressed⁷⁵. Therefore, TADs can be completely rearranged without causing transcriptional mis-expression, possibly because these translocations did not disconnect enhancers from their target genes. In a second study, Laugsch *et al.* used human induced pluripotent stem cells (hiPSCs) derived from a branchiooculofacial syndrome (BOFS) patient and differentiated into human neural crest cells (hNCCs) to analyze the effect of an 89

Mb inversion overlapping the *TFAP2A* gene⁷⁶. This inversion prevents *TFAP2A* from interacting with its cognate enhancers, causing the downregulation of *TFP2A*'s expression. As a consequence of the inversion, the *TFP2A* enhancers are in proximity to several other genes. This proximity did however not lead to the formation of new chromatin interactions, nor was the expression of these genes affected. Therefore, chromosomal inversions placing genes in proximity to different enhancers do not necessarily cause enhancer adoption⁷⁶, suggesting that enhancer-promoter interaction specificity and/or other mechanisms are involved.

Engineered chromosomal rearrangements

Deleting CTCF-binding sites at TAD boundaries

The functional consequences of structural rearrangements during evolution and disease have been very informative on the role of chromatin organization. However, in the case of disease, we are focusing on events that do cause pathologies and might represent exceptions (because they target a particular locus) rather than the rule. As most structural rearrangements have been identified as the cause of a disease, it is expected that they will display functional consequences. On the other hand, evolutionary studies focus on the result of a process where only the fittest solution has been selected. Most inconveniently, in both cases, we do not control the location of the structural rearrangements. To go beyond natural occurrences, the functional role of TADs has recently been investigated using a large variety of engineered chromosomal rearrangements. A first series of experiments evaluated the impact of selectively deleting CTCF-binding sites located at TAD boundaries. Intuitively, one might expect that if TAD boundaries are essential to insulate the regulatory landscapes of two adjacent TADs, deleting a TAD boundary would cause ectopic interactions between adjacent TADs and eventually fuse them. As a result, the expression of nearby genes should be affected. Experimental evidence however suggests that this model might be too simplistic. One of the earliest examples came from the seminal paper describing TADs on a portion of the X chromosome in mouse embryonic stem cells²⁶. Deleting a 58 kb-long region at the boundary between the *Xist* and *Tsix* TADs induced the formation of ectopic contacts across the deletion site. However, this deletion did not lead to a complete fusion between the two adjacent TADs: their global architecture was maintained and most contacts were still restricted to the original TADs. The effect on gene expression at the locus was however not assessed. The transcriptional effect of deleting or mutating CTCF-binding sites was investigated at other loci. For example, at the *b-globin* locus, the mutation of four nucleotides in a core CTCF-binding site (3'HS1) located downstream of the locus and overlapping a sub-TAD boundary⁷⁷ completely abolished CTCF binding and strongly affected the interaction of 3'HS1 with the *b-globin* locus⁷⁸. However, this mutation did not affect the expression of the *b-globin* genes. In addition, the loss of this boundary did not cause the inappropriate activation of other genes (mouse olfactory receptor genes) located across the boundary, as would be expected if the insulating property of the site was lost⁷⁸. Similarly, the deletion of a CTCF-binding site at the *Sox2* locus did not affect the expression of most genes in the vicinity, except for the *Fbn2* gene which is ~230 kb away³². Thorough dissection of the homeotic *homeobox* (*Hox*) gene cluster *HoxD* confirmed that the simple deletion of a CTCF-binding site is not sufficient to cause TAD fusion. The *HoxD* cluster is located at a boundary between two TADs, active in the proximal or distal limb buds respectively. This boundary consists of several adjacent CTCF-binding sites whose partial deletion did not significantly affect TAD insulation and gene expression. Only larger deletions, actually deleting a 400 kb region including the *HoxD* cluster itself, did result in

TAD fusion. And yet, despite the formation of this neo-TAD, only minor effects on gene expression were observed, and distally- and proximally-acting enhancers retained their specificity even when placed in the same TAD^{79,80}. As at the *HoxD* locus, TAD boundaries usually consist of a cluster of CTCF-binding sites. The deletion of a single site from this cluster generally has only local effects, the boundary been shifted to the next neighboring CTCF-binding site. In some cases, even large deletions preserve TAD boundaries. For example, deleting a ~82 kb region encompassing the highly conserved *Firre* locus, a non-coding RNA located on the X chromosome that forms a very strong TAD boundary with a cluster of 15 CTCF-binding sites, did not affect boundary formation⁸¹. This locus might however represent an extreme case. Indeed, the progressive deletion of four, eight, or nine adjacent CTCF-binding sites located at the boundary between the *Sox9* and *Kcnj2* TADs does eventually result in TAD fusion⁸², as observed at the *HoxD* locus^{79,80}. Again, this fusion has only mild effects on gene expression: the expression pattern of *Sox9* remains largely unaffected with only a slight down-regulation in the most severe deletion, and the deletion of eight or nine CTCF-binding sites causes a faint ectopic expression of *Kcnj2* in the digit anlagen, mimicking *Sox9*'s expression pattern⁸². Other loci seem however more sensitive to TAD boundary deletions. A particularly extreme example has been reported at the *HoxA* cluster during motor neuron differentiation⁸³. The deletion of a single CTCF-binding site (a nine bp deletion between *HoxA5* and *HoxA6*) results in the loss of the boundary and the expansion of the active chromatin domain into the repressed domain. Surprisingly, this deletion also affects the binding of CTCF to a nearby insulator site (between *HoxA6* and *HoxA7*). The boundary is therefore shifted to a more distal CTCF-binding site (between *HoxA7* and *HoxA9*). In addition, deleting this single CTCF-binding site in the *HoxA* cluster (as well as the equivalent deletion in the *HoxC* cluster) in motor neurons results in homeotic transformations typical of *Hox* gene mis-regulation⁸⁴. This increased sensitivity to CTCF-binding site deletion might be the consequence of a weaker and more plastic boundary. Indeed, a single CTCF-binding site separates some of the *HoxA* genes, and the location of this TAD boundary is cell-type specific: located between *HoxA5* and *HoxA6* in motor neuron, but between *HoxA10* and *HoxA11* in embryonic stem cells⁸⁴. It is thus possible that weaker TAD boundaries or boundaries between sub-TADs are more sensitive to deletions. In line with this hypothesis, the deletion of CTCF-binding sites at five super-enhancer domains (sub-TADs formed between a super-enhancer and its target genes linked by CTCF-binding sites) caused the down-regulation of genes located within the same domain and the up-regulation of genes located outside the domain. The up-regulated genes formed ectopic chromosomal interactions with regions located across the deleted CTCF-binding site, consistent with a classical insulation role of CTCF⁸⁵. Similarly, the deletion of the HS-38 and HS-39 CTCF-binding sites delimiting a ~ 80 kb sub-TAD at the mouse α -globin locus affected the expression of several genes located directly upstream and altered the 3D organization of the locus^{86,87}. Finally, an important consideration came from the analysis of deletions and inversions at the *HoxD* locus: all ectopic contacts observed after the rearrangements involved regions that were already weakly contacted in the wild-type situation⁸⁸. In conclusion, these studies suggest that TADs do favor the formation of local interactions within them, possibly through epigenetic-driven interactions^{89,90}, but that their boundaries do not act as extremely strict borders.

The example of the Shh locus

The *Sonic hedgehog* (*Shh*) locus in mouse has been the subject of thorough investigations and is therefore a good case study to assess the effect of structural rearrangements on gene expression. *Shh* has a critical role during development and has been involved in the patterning of several organs

including the limb. The limb-specific expression of *Shh* in the posterior limb bud mesenchyme is regulated by the Zone of Polarizing Activity (ZPA) Regulatory Sequence (ZRS) enhancer located about a megabase away within an intron of the unrelated *LMBR1* gene⁹¹. Single-nucleotide mutations in the ZRS affect *Shh* expression and can either cause preaxial polydactyly in mammals⁹¹ or be responsible for the absence of limbs in snakes⁹². *Shh* and its ZRS enhancer are located within the same megabase-sized TAD³⁷. Several recent studies^{93–95} used this very well-studied locus to generate deletions and inversions and thus assess their effects on chromatin organization and gene expression (Figure1).

First, the authors generated deletions within the *Shh* TAD, decreasing the size of the TAD and the linear distance between *Shh* and the ZRS without affecting TAD boundaries ($\Delta 700$ ⁹⁴ and $\Delta \text{DEL}(5-8)$ ⁹³). Despite a drastic change in the TAD's size, these deletions did not affect the expression of *Shh*. Second, the authors generated a series of deletions affecting CTCF-binding sites at the *Shh* TAD boundaries. As above, a single CTCF-binding site deletion had only a minor effect, merely shifting the TAD boundary to the next CTCF-binding site without affecting *Shh* expression. Surprisingly though, these single CTCF-binding site deletions caused a decrease in the 3D distance between *Shh* and the ZRS (as assessed by 3D DNA FISH). The expression of *Shh* was only affected when several adjacent CTCF-binding sites were deleted, as in the $\Delta i4:i5$ ⁹⁵ or ΔLmbr1P ⁹⁴ mutants. In addition, the deletion of different neighboring CTCF-binding sites is not equivalent: deleting the two CTCF-binding sites directly surrounding the ZRS is more deleterious than deleting a region encompassing the *Lmbr1* promoter. In both cases, the deletion of CTCF-binding sites did not lead to TAD fusion, but rather to the formation of ectopic contacts. On the same note, previous results showed that the deletion of the ZRS enhancer does not prevent the formation of interactions between the surrounding regions and the *Shh* promoter⁹⁶. Interestingly, in the $\Delta 35$ deletion affecting the promoter of *Lmbr1*, the ZRS can now contact some regions further to the right, including the *Mnx1* gene which becomes over-expressed in the limb. This ectopic expression is however specific to the ZRS, as other enhancers located in the same region (MFCS4 and MACS1) do not affect *Mnx1* expression. This suggests that enhancer adoption is specific to some enhancer-promoter pairs.

Finally, inversions placing a TAD boundary between *Shh* and the ZRS did affect the expression of *Shh*, confirming that TAD boundaries act as insulators. However, this insulation effect becomes milder as the linear distance between *Shh* and the ZRS decreases. Further characterization of this locus would require monitoring the expression of *Shh* at the single-cell level and simultaneously measuring the distance between *Shh* and the ZRS, as recently performed at the *even-skipped* locus in *Drosophila*⁹⁷. Indeed, a transient and dynamic interaction between *Shh* and the ZRS, potentially sufficient to trigger transcription, will be difficult to assess in a bulk heterogeneous population of cells. In addition, the role of cohesin in the organization of the locus should be clarified, as the presence of cohesin in the different mutants could explain their mild effects on gene expression.

Are inversions more deleterious than deletions?

The example of the *Shh* TAD suggests that an inversion affecting a TAD boundary has a stronger effect than the deletion of a TAD boundary. This difference has been confirmed at the *Sox9/Kcnj2*⁸² and *Tfap2c/Bmp7*⁹⁸ loci. However, recent work in *Drosophila* revealed that genomic inversions do not necessarily strongly affect gene expression⁹⁹. Allele-specific chromatin organization and gene expression were analyzed in heterozygote embryos carrying highly rearranged balancer chromosomes. Despite the presence of thousands of large and small structural variants differentiating the wild-type from the balancer alleles, remarkably little effect on transcription was

observed: only 10% of genes were differentially expressed. In particular, the expression of genes located around inversions breakpoints was not systematically affected, suggesting that enhancer adoption might be relatively infrequent. Moreover, the structural variants found in balancer chromosomes are not enriched at TAD boundaries nor in gene-poor regions, but rather distributed randomly. It is likely that several mechanisms such as enhancer redundancy, which is particularly pervasive in *Drosophila*¹⁰⁰, might buffer the effect of chromosomal rearrangements. In another study, the analysis of engineered inversions at three *Drosophila* testis-specific gene clusters¹⁰¹ also revealed little impact on the expression of neighboring genes. How can we explain this discrepancy between mammals and flies? First, it is possible that the effect of inversions is highly tissue-specific, and might be missed when analyzed in whole embryos. However, the transcriptional effect of inversions was not stronger in adult fly heads (a more homogeneous cell population largely composed of neurons) than in whole embryos⁹⁹. Besides, inversions affecting three testis-specific gene clusters did not significantly affect gene expression in the testes¹⁰¹. Second, the relatively smaller size of regulatory domains in *Drosophila* (resulting in enhancers located usually closer to their target genes than in vertebrates), and the smaller number of enhancers per gene could reduce the likelihood of observing an inversion breakpoint between an enhancer and its target gene. In addition, fly genomes may be somewhat more plastic than mammalian genomes. Indeed, the synteny of the *Hox* gene cluster is poorly conserved between several *Drosophilids*, contrary to mammals where this order is strongly conserved^{102,103}. It is also likely that the selection of the loci analyzed in mammals, biased towards important developmental genes or oncogenes, is enriched in regions that are more sensitive to chromosomal inversions. In fact, inversions are not always deleterious in mammals. As mentioned above, chromosomal inversions did not cause transcriptional mis-regulation by enhancer adoption at the *TFAP2A* locus in human cells⁷⁶. Finally, a potential concern might arise from the selection, in balancer chromosomes, of the least harmful mutations. However, the large number of variants found in the balancer chromosomes compared their wild type counterparts, their nature and location argue against such a strong selection⁹⁹. In addition, similar results were observed with *de novo* inversions¹⁰¹.

We therefore propose that at many (maybe most) loci, chromosomal inversions can be buffered by two mechanisms (Figure 2): First, disrupting the interaction between an enhancer and its target gene(s) by placing a TAD boundary in between can be compensated by the presence of one or multiple other redundant enhancers^{100,104,105}. Such redundant or “shadow” enhancers drive the same, or a similar, expression pattern. Their deletion (or relocation to a different TAD) would therefore not cause a visible phenotype^{106–108}. This compensatory mechanism can sustain a nearly normal expression level of the target gene. Second, while enhancer adoption is possible at some loci, it depends on parameters, such as enhancer-promoter specificity^{15,109}, still poorly understood. Placing an enhancer in a new regulatory domain will thus not necessarily create ectopic contacts and affect the expression of the genes located in that domain. In line with this hypothesis, a series of inversions at the *Epha4* locus in mouse did not affect the expression of all nearby genes to the same extent, and the most strongly mis-regulated gene was not necessarily the one directly adjacent to the inversion breakpoint³³.

Conclusion/Perspective

Despite years of research, the functional role of TADs remains unclear. TADs have been proposed to influence gene expression through two complementary mechanisms. On one hand, they can act as a

barrier to prevent unwanted interactions with regions located outside the domain. On the other hand, they can promote interactions between regions located within them. While these two activities can be observed at some loci, recent genomic engineering experiments suggest a more subtle function. At short distance, TAD boundaries are not required to promote intra-TAD interactions, but they become more crucial across large distances. Similarly, TAD boundaries do not act as strong borders, as their deletion only marginally affects the insulation of the domains. Instead, TAD boundaries might be important to provide precision and robustness to gene expression⁸². Overall, the organization of the genome in TADs is clearly an important and functional feature of the genome, but the strength of their boundaries seems to be largely locus-specific, if not condition- and species-specific. The discrepancy observed between studies assessing the functional consequences of TAD boundary modifications could indeed reflect biological differences. For example, it could be linked to differences in regulatory landscapes, with genes regulated by enhancers displaying different characteristics (distance, specificity, activity, redundancy, etc...). But this discrepancy might also to some extent simply be due to the poor definition of TADs. Finally, it is important to keep in mind that most available studies rely on bulk Hi-C data to define TAD boundaries. Indeed, recent single-cell Hi-C and imaging experiments uncovered extensive heterogeneity in TAD boundaries between conditions and cell-types at the single-cell level^{18,19,110,111}. This further supports the fact that other mechanisms beside chromatin organization into TADs are driving appropriate gene expression. Clearly, clarifying the link between chromatin organization and transcription regulation will require further investigations. These should in particular aim at better characterizing the rules governing enhancer-promoter interactions specificity, for example through the thorough dissection of specific loci and the precise live monitoring of chromatin organization and gene expression in single cells.

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Reference

1. Banerji, J., Rusconi, S. & Schaffner, W. Expression of a β -globin gene is enhanced by remote SV40 DNA sequences. *Cell* **27**, 299–308 (1981).
2. Moreau, P. *et al.* The SV40 72 base repair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. *Nucleic Acids Res* **9**, 6047–6068 (1981).
3. Gross, D. S. & Garrard, W. T. Nuclease Hypersensitive Sites in Chromatin. *Annual Review of Biochemistry* **57**, 159–197 (1988).
4. Creighton, M. P. *et al.* Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci USA* **107**, 21931 (2010).
5. Heintzman, N. D. *et al.* Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* **459**, 108–112 (2009).
6. Rada-Iglesias, A. *et al.* A unique chromatin signature uncovers early developmental enhancers in humans. *Nature* **470**, 279–283 (2011).
7. Zentner, G. E., Tesar, P. J. & Scacheri, P. C. Epigenetic signatures distinguish multiple classes of enhancers with distinct cellular functions. *Genome Res* **21**, 1273–1283 (2011).
8. Bonn, S. *et al.* Tissue-specific analysis of chromatin state identifies temporal signatures of enhancer activity during embryonic development. *Nat Genet* **44**, 148–156 (2012).
9. Long, H. K., Prescott, S. L. & Wysocka, J. Ever-Changing Landscapes: Transcriptional Enhancers in Development and Evolution. *Cell* **167**, 1170–1187 (2016).

10. Ghavi-Helm, Y. *et al.* Enhancer loops appear stable during development and are associated with paused polymerase. *Nature* **512**, 96–100 (2014).
11. de Laat, W. & Duboule, D. Topology of mammalian developmental enhancers and their regulatory landscapes. *Nature* **502**, 499–506 (2013).
12. Dixon, J. R. *et al.* Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature* **485**, 376–380 (2012).
13. Andrey, G. *et al.* Characterization of hundreds of regulatory landscapes in developing limbs reveals two regimes of chromatin folding. *Genome Res.* **27**, 223–233 (2017).
14. Sanyal, A., Lajoie, B. R., Jain, G. & Dekker, J. The long-range interaction landscape of gene promoters. *Nature* **489**, 109–113 (2012).
15. van Arensbergen, J., van Steensel, B. & Bussemaker, H. J. In search of the determinants of enhancer–promoter interaction specificity. *Trends in Cell Biology* **24**, 695–702 (2014).
16. Giorgetti, L. & Heard, E. Closing the loop: 3C versus DNA FISH. *Genome Biology* **17**, (2016).
17. Lieberman-Aiden, E. *et al.* Comprehensive Mapping of Long-Range Interactions Reveals Folding Principles of the Human Genome. *Science* **326**, 289–293 (2009).
18. Finn, E. H. *et al.* Extensive Heterogeneity and Intrinsic Variation in Spatial Genome Organization. *Cell* **176**, 1502–1515.e10 (2019).
19. Bintu, B. *et al.* Super-resolution chromatin tracing reveals domains and cooperative interactions in single cells. *Science* **362**, eaau1783 (2018).
20. Boettiger, A. N. *et al.* Super-resolution imaging reveals distinct chromatin folding for different epigenetic states. *Nature* **529**, 418–422 (2016).
21. Mateo, L. J. *et al.* Visualizing DNA folding and RNA in embryos at single-cell resolution. *Nature* **568**, 49–54 (2019).
22. Cardozo Gizzi, A. M. *et al.* Microscopy-Based Chromosome Conformation Capture Enables Simultaneous Visualization of Genome Organization and Transcription in Intact Organisms. *Molecular Cell* **74**, 212–222.e5 (2019).
23. Guelen, L. *et al.* Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. *Nature* **453**, 948–951 (2008).
24. Rao, S. S. P. *et al.* A 3D Map of the Human Genome at Kilobase Resolution Reveals Principles of Chromatin Looping. *Cell* **159**, 1665–1680 (2014).
25. Sexton, T. *et al.* Three-Dimensional Folding and Functional Organization Principles of the Drosophila Genome. *Cell* **148**, 458–472 (2012).
26. Nora, E. P. *et al.* Spatial partitioning of the regulatory landscape of the X-inactivation centre. *Nature* **485**, 381–385 (2012).
27. Phillips-Cremins, J. E. *et al.* Architectural Protein Subclasses Shape 3D Organization of Genomes during Lineage Commitment. *Cell* **153**, 1281–1295 (2013).
28. Sanborn, A. L. *et al.* Chromatin extrusion explains key features of loop and domain formation in wild-type and engineered genomes. *Proc Natl Acad Sci USA* **112**, E6456 (2015).
29. Fudenberg, G. *et al.* Formation of Chromosomal Domains by Loop Extrusion. *Cell Reports* **15**, 2038–2049 (2016).
30. Fudenberg, G., Abdennur, N., Imakaev, M., Goloborodko, A. & Mirny, L. A. Emerging Evidence of Chromosome Folding by Loop Extrusion. *Cold Spring Harb Symp Quant Biol* **82**, 45–55 (2017).
31. Guo, Y. *et al.* CRISPR Inversion of CTCF Sites Alters Genome Topology and Enhancer/Promoter Function. *Cell* **162**, 900–910 (2015).
32. de Wit, E. *et al.* CTCF Binding Polarity Determines Chromatin Looping. *Molecular Cell* **60**, 676–684 (2015).
33. Kraft, K. *et al.* Serial genomic inversions induce tissue-specific architectural stripes, gene misexpression and congenital malformations. *Nature Cell Biology* **21**, 305 (2019).
34. Nora, E. P. *et al.* Targeted Degradation of CTCF Decouples Local Insulation of Chromosome Domains from Genomic Compartmentalization. *Cell* **169**, 930–944.e22 (2017).

35. Forcato, M. *et al.* Comparison of computational methods for Hi-C data analysis. *Nat Methods* **14**, 679–685 (2017).
36. Zufferey, M., Tavernari, D., Oricchio, E. & Ciriello, G. Comparison of computational methods for the identification of topologically associating domains. *Genome Biology* **19**, 217 (2018).
37. Symmons, O. *et al.* Functional and topological characteristics of mammalian regulatory domains. *Genome Res.* **24**, 390–400 (2014).
38. Nora, E. P. *et al.* Spatial partitioning of the regulatory landscape of the X-inactivation centre. *Nature* **485**, 381–385 (2012).
39. Zhan, Y. *et al.* Reciprocal insulation analysis of Hi-C data shows that TADs represent a functionally but not structurally privileged scale in the hierarchical folding of chromosomes. *Genome Res* **27**, 479–490 (2017).
40. Le Dily, F. *et al.* Distinct structural transitions of chromatin topological domains correlate with coordinated hormone-induced gene regulation. *Genes Dev* **28**, 2151–2162 (2014).
41. Shen, Y. *et al.* A map of the cis-regulatory sequences in the mouse genome. *Nature* **488**, 116–120 (2012).
42. Delaneau, O. *et al.* Chromatin three-dimensional interactions mediate genetic effects on gene expression. *Science* **364**, eaat8266 (2019).
43. Schwarzer, W. *et al.* Two independent modes of chromatin organization revealed by cohesin removal. *Nature* **551**, 51–56 (2017).
44. Cuartero, S. *et al.* Control of inducible gene expression links cohesin to hematopoietic progenitor self-renewal and differentiation. *Nature Immunology* **19**, 932 (2018).
45. Krefting, J., Andrade-Navarro, M. A. & Ibn-Salem, J. Evolutionary stability of topologically associating domains is associated with conserved gene regulation. *BMC Biology* **16**, 87 (2018).
46. Vietri Rudan, M. *et al.* Comparative Hi-C Reveals that CTCF Underlies Evolution of Chromosomal Domain Architecture. *Cell Reports* **10**, 1297–1309 (2015).
47. Harmston, N. *et al.* Topologically associating domains are ancient features that coincide with Metazoan clusters of extreme noncoding conservation. *Nature Communications* **8**, 441 (2017).
48. Renschler, G. *et al.* Hi-C guided assemblies reveal conserved regulatory topologies on X and autosomes despite extensive genome shuffling. *bioRxiv* 580969 (2019). doi:10.1101/580969
49. Lazar, N. H. *et al.* Epigenetic maintenance of topological domains in the highly rearranged gibbon genome. *Genome Res.* **28**, 983–997 (2018).
50. Canela, A. *et al.* Genome Organization Drives Chromosome Fragility. *Cell* **170**, 507–521.e18 (2017).
51. Aymard, F. *et al.* Genome-wide mapping of long-range contacts unveils clustering of DNA double-strand breaks at damaged active genes. *Nature Structural & Molecular Biology* **24**, 353–361 (2017).
52. Zhang, Y. *et al.* Spatial Organization of the Mouse Genome and Its Role in Recurrent Chromosomal Translocations. *Cell* **148**, 908–921 (2012).
53. Engreitz, J. M., Agarwala, V. & Mirny, L. A. Three-Dimensional Genome Architecture Influences Partner Selection for Chromosomal Translocations in Human Disease. *PLOS ONE* **7**, e44196 (2012).
54. Berthelot, C., Muffato, M., Abecassis, J. & Roest Crolius, H. The 3D Organization of Chromatin Explains Evolutionary Fragile Genomic Regions. *Cell Reports* **10**, 1913–1924 (2015).
55. Roix, J. J., McQueen, P. G., Munson, P. J., Parada, L. A. & Misteli, T. Spatial proximity of translocation-prone gene loci in human lymphomas. *Nature Genetics* **34**, 287–291 (2003).
56. Fudenberg, G. & Pollard, K. S. Chromatin features constrain structural variation across evolutionary timescales. *PNAS* **116**, 2175–2180 (2019).
57. Lupiáñez, D. G., Spielmann, M. & Mundlos, S. Breaking TADs: How Alterations of Chromatin Domains Result in Disease. *Trends in Genetics* **32**, 225–237 (2016).
58. Spielmann, M. & Mundlos, S. Structural variations, the regulatory landscape of the genome and their alteration in human disease. *BioEssays* **35**, 533–543

59. Spielmann, M., Lupiáñez, D. G. & Mundlos, S. Structural variation in the 3D genome. *Nature Reviews Genetics* **19**, 453–467 (2018).
60. Kaiser, V. B. & Sempé, C. A. When TADs go bad: chromatin structure and nuclear organisation in human disease. *F1000Res* **6**, (2017).
61. Lupiáñez, D. G. *et al.* Disruptions of Topological Chromatin Domains Cause Pathogenic Rewiring of Gene-Enhancer Interactions. *Cell* **161**, 1012–1025 (2015).
62. Giorgio, E. *et al.* A large genomic deletion leads to enhancer adoption by the lamin B1 gene: a second path to autosomal dominant adult-onset demyelinating leukodystrophy (ADLD). *Hum Mol Genet* **24**, 3143–3154 (2015).
63. Flöttmann, R. *et al.* Microdeletions on 6p22.3 are associated with mesomelic dysplasia Savarirayan type. *Journal of Medical Genetics* **52**, 476–483 (2015).
64. Spielmann, M. *et al.* Homeotic Arm-to-Leg Transformation Associated with Genomic Rearrangements at the PITX1 Locus. *The American Journal of Human Genetics* **91**, 629–635 (2012).
65. Redin, C. *et al.* The genomic landscape of balanced cytogenetic abnormalities associated with human congenital anomalies. *Nature Genetics* **49**, 36–45 (2017).
66. Franke, M. *et al.* Formation of new chromatin domains determines pathogenicity of genomic duplications. *Nature* **538**, 265–269 (2016).
67. Lettice, L. A. *et al.* Enhancer-adoption as a mechanism of human developmental disease. *Human Mutation* **32**, 1492–1499
68. Northcott, P. A. *et al.* Enhancer hijacking activates GFI1 family oncogenes in medulloblastoma. *Nature* **511**, 428–434 (2014).
69. Ibn-Salem, J. *et al.* Deletions of chromosomal regulatory boundaries are associated with congenital disease. *Genome Biology* **15**, 423 (2014).
70. Northcott, P. A. *et al.* The whole-genome landscape of medulloblastoma subtypes. *Nature* **547**, 311–317 (2017).
71. Flavahan, W. A. *et al.* Insulator dysfunction and oncogene activation in *IDH* mutant gliomas. *Nature* **529**, 110–114 (2016).
72. Weischenfeldt, J. *et al.* Pan-cancer analysis of somatic copy-number alterations implicates *IRS4* and *IGF2* in enhancer hijacking. *Nature Genetics* **49**, 65–74 (2017).
73. Hnisz, D. *et al.* Activation of proto-oncogenes by disruption of chromosome neighborhoods. *Science* **351**, 1454–1458 (2016).
74. Sun, J. H. *et al.* Disease-Associated Short Tandem Repeats Co-localize with Chromatin Domain Boundaries. *Cell* **175**, 224–238.e15 (2018).
75. Dong, Z. *et al.* Identification of balanced chromosomal rearrangements previously unknown among participants in the 1000 Genomes Project: implications for interpretation of structural variation in genomes and the future of clinical cytogenetics. *Genetics in Medicine* **20**, 697 (2018).
76. Laugsch, M. *et al.* Modeling the Pathological Long-Range Regulatory Effects of Human Structural Variation with Patient-Specific hiPSCs. *Cell Stem Cell* (2019). doi:10.1016/j.stem.2019.03.004
77. Huang, P. *et al.* Comparative analysis of three-dimensional chromosomal architecture identifies a novel fetal hemoglobin regulatory element. *Genes Dev.* **31**, 1704–1713 (2017).
78. Splinter, E. *et al.* CTCF mediates long-range chromatin looping and local histone modification in the β -globin locus. *Genes Dev.* **20**, 2349–2354 (2006).
79. Rodríguez-Carballo, E. *et al.* The HoxD cluster is a dynamic and resilient TAD boundary controlling the segregation of antagonistic regulatory landscapes. *Genes Dev.* **31**, 2264–2281 (2017).
80. Rodríguez-Carballo, E., Lopez-Delisle, L., Yakushiji-Kaminatsui, N., Ullate-Agote, A. & Duboule, D. Impact of genome architecture on the functional activation and repression of Hox regulatory landscapes. *BMC Biol* **17**, (2019).

81. Barutcu, A. R., Maass, P. G., Lewandowski, J. P., Weiner, C. L. & Rinn, J. L. A TAD boundary is preserved upon deletion of the CTCF-rich *Firre* locus. *Nature Communications* **9**, 1444 (2018).
82. Despagne, A. *et al.* Functional dissection of the *Sox9* – *Kcnj2* locus identifies nonessential and instructive roles of TAD architecture. *Nat Genet* **51**, 1263–1271 (2019).
83. Narendra, V. *et al.* CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. *Science* **347**, 1017–1021 (2015).
84. Narendra, V., Bulajić, M., Dekker, J., Mazzoni, E. O. & Reinberg, D. CTCF-mediated topological boundaries during development foster appropriate gene regulation. *Genes Dev.* **30**, 2657–2662 (2016).
85. Downen, J. M. *et al.* Control of Cell Identity Genes Occurs in Insulated Neighborhoods in Mammalian Chromosomes. *Cell* **159**, 374–387 (2014).
86. Hanssen, L. L. P. *et al.* Tissue-specific CTCF–cohesin-mediated chromatin architecture delimits enhancer interactions and function *in vivo*. *Nature Cell Biology* **19**, 952–961 (2017).
87. Oudelaar, A. M. *et al.* A revised model for promoter competition based on multi-way chromatin interactions. (Genomics, 2019). doi:10.1101/612275
88. Fabre, P. J. *et al.* Large scale genomic reorganization of topological domains at the HoxD locus. *Genome Biol* **18**, (2017).
89. Ulianov, S. V. *et al.* Active chromatin and transcription play a key role in chromosome partitioning into topologically associating domains. *Genome Res.* **26**, 70–84 (2016).
90. Ghosh, S. K. & Jost, D. How epigenome drives chromatin folding and dynamics, insights from efficient coarse-grained models of chromosomes. *PLoS Comput Biol* **14**, (2018).
91. Lettice, L. A. *et al.* A long-range *Shh* enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet* **12**, 1725–1735 (2003).
92. Kvon, E. Z. *et al.* Progressive Loss of Function in a Limb Enhancer during Snake Evolution. *Cell* **167**, 633–642.e11 (2016).
93. Symmons, O. *et al.* The *Shh* Topological Domain Facilitates the Action of Remote Enhancers by Reducing the Effects of Genomic Distances. *Developmental Cell* **39**, 529–543 (2016).
94. Williamson, I. *et al.* Developmentally regulated *Shh* expression is robust to TAD perturbations. *bioRxiv* 609941 (2019). doi:10.1101/609941
95. Paliou, C. *et al.* Preformed chromatin topology assists transcriptional robustness of *Shh* during limb development. *PNAS* 201900672 (2019). doi:10.1073/pnas.1900672116
96. Amano, T. *et al.* Chromosomal Dynamics at the *Shh* Locus: Limb Bud-Specific Differential Regulation of Competence and Active Transcription. *Developmental Cell* **16**, 47–57 (2009).
97. Chen, H. *et al.* Dynamic interplay between enhancer-promoter topology and gene activity. *Nat Genet* **50**, 1296–1303 (2018).
98. Tsujimura, T. *et al.* A Discrete Transition Zone Organizes the Topological and Regulatory Autonomy of the Adjacent *Tfap2c* and *Bmp7* Genes. *PLOS Genetics* **11**, e1004897 (2015).
99. Ghavi-Helm, Y. *et al.* Highly rearranged chromosomes reveal uncoupling between genome topology and gene expression. *Nat Genet* **51**, 1272–1282 (2019).
100. Cannavò, E. *et al.* Shadow Enhancers Are Pervasive Features of Developmental Regulatory Networks. *Current Biology* **26**, 38–51 (2016).
101. Meadows, L. A., Chan, Y. S., Roote, J. & Russell, S. Neighbourhood Continuity Is Not Required for Correct Testis Gene Expression in *Drosophila*. *PLOS Biology* **8**, e1000552 (2010).
102. Negre, B. & Ruiz, A. HOM-C evolution in *Drosophila*: is there a need for Hox gene clustering? *Trends in Genetics* **23**, 55–59 (2007).
103. Drosophila 12 Genomes Consortium. Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* **450**, 203–218 (2007).
104. Frankel, N. *et al.* Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490–493 (2010).
105. Hong, J.-W., Hendrix, D. A. & Levine, M. S. Shadow Enhancers as a Source of Evolutionary Novelty. *Science* **321**, 1314–1314 (2008).

106. Cretekos, C. J. *et al.* Regulatory divergence modifies limb length between mammals. *Genes Dev.* **22**, 141–151 (2008).
107. Hay, D. *et al.* Genetic dissection of the α -globin super-enhancer *in vivo*. *Nature Genetics* **48**, 895–903 (2016).
108. Osterwalder, M. *et al.* Enhancer redundancy provides phenotypic robustness in mammalian development. *Nature* **554**, 239–243 (2018).
109. Zabidi, M. A. *et al.* Enhancer–core-promoter specificity separates developmental and housekeeping gene regulation. *Nature* **518**, 556–559 (2015).
110. Flyamer, I. M. *et al.* Single-nucleus Hi-C reveals unique chromatin reorganization at oocyte-to-zygote transition. *Nature* **544**, 110–114 (2017).
111. Nagano, T. *et al.* Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. *Nature* **502**, 59–64 (2013).

Figure legends

Figure 1: Effect of chromosomal rearrangements at the *Shh* locus.

A. Schematic representation of the mouse *Shh* locus displaying the location of the relevant genes (black), CTCF-binding sites (red) and of the ZRS. The approximate location of deletions (Δ Lmbr1P, Δ 35, Δ 700, DEL(5-8)) and inversions (INV(-500-C1), INV(6-C2), INV(4-C2), INV(2-C2)) assessed by Simmons *et al.*⁹³, Paliou *et al.*⁹⁵, and Williamson *et al.*⁹⁴ is indicated. **B.** Table summarizing the effect of the deletions and inversions represented in (A) on chromatin organization, 3D distance between *Shh* and the ZRS, *Shh* expression, and final phenotype.

Figure2: Schematic representation of a chromosomal inversion affecting a TAD boundary.

A chromosomal inversion can shift the location of an enhancer (purple box) from one TAD (orange triangle) to the next (pink triangle). The expression of the genes (green/red rectangles for transcriptionally active or inactive respectively) located in the pink TAD will be affected if their promoter is compatible with the inverted enhancer. The expression of the genes located in the orange TAD might not be affected if other redundant enhancers are located within the same TAD.

A

B

	Effect on chromatin organisation	Increased 3D distance between <i>Shh</i> and ZRS	Effect on <i>Shh</i> expression	Phenotype
$\Delta 700$	None	NA	No	No
DEL(5-8)	NA	NA	No	No
Δ CTCF1	shifts boundary to CTCF2	Yes	No	No
Δ CTCF2	some ectopic contacts and intra-TAD reorganization	Yes	No	No
Δ CTCF3	some intra-TAD reorganization	Yes	No	No
Δ CTCF4	increased intra-TAD interactions	No	No	No
Δ CTCF5	decreased interactions with <i>Mnx1</i> TAD	No	No	No
Δ Lmbr1P	shifts boundary to the left (i9)	NA	Yes - 20% reduction	No
Δ i4:i5	Loss of interaction between <i>Shh</i> and the <i>Lmbr1</i> boundary, and increased interactions with the <i>Mnx1</i> TAD	Yes	Yes - 51% reduction 98% in sensitized background	No, only in sensitized background
Δ i4:i5:ZRS	Loss of interaction between <i>Shh</i> and the <i>Lmbr1</i> boundary, and increased interactions with the <i>Mnx1</i> TAD	NA	Yes - 52% reduction	No
INV(-500-C1)	NA	NA	Yes	Yes in a sensitized background
INV(6-C2)	lost interaction between ZRS and <i>Shh</i>	NA	Yes	Yes in a sensitized background
INV(4-C2)	NA	NA	NA	Yes
INV(2-C2)	NA	NA	NA	Yes, but progressively milder as the distance between <i>Shh</i> and the ZRS decreases

buffering by enhancer redundancy

enhancer adoption only if compatibility