

Nanoscale spatial organization of the *HoxD* gene cluster in distinct transcriptional states

Pierre J. Fabre^a, Alexander Benke^b, Elisabeth Joye^a, Thi Hanh Nguyen Huynh^c, Suliana Manley^{b,1}, and Denis Duboule^{a,c,1}

^aSchool of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland; ^bLaboratory of Experimental Biophysics, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland; and ^cDepartment of Genetics and Evolution, University of Geneva, 1211 Geneva 4, Switzerland

Contributed by Denis Duboule, September 14, 2015 (sent for review July 21, 2015; reviewed by Victor Corces and Luca Giorgetti)

Chromatin condensation plays an important role in the regulation of gene expression. Recently, it was shown that the transcriptional activation of *Hoxd* genes during vertebrate digit development involves modifications in 3D interactions within and around the *HoxD* gene cluster. This reorganization follows a global transition from one set of regulatory contacts to another, between two topologically associating domains (TADs) located on either side of the *HoxD* locus. Here, we use 3D DNA FISH to assess the spatial organization of chromatin at and around the *HoxD* gene cluster and report that although the two TADs are tightly associated, they appear as spatially distinct units. We measured the relative position of genes within the cluster and found that they segregate over long distances, suggesting that a physical elongation of the *HoxD* cluster can occur. We analyzed this possibility by super-resolution imaging (STORM) and found that tissues with distinct transcriptional activity exhibit differing degrees of elongation. We also observed that the morphological change of the *HoxD* cluster in developing digits is associated with its position at the boundary between the two TADs. Such variations in the fine-scale architecture of the gene cluster suggest causal links among its spatial configuration, transcriptional activation, and the flanking chromatin context.

DNA FISH | super-resolution microscopy | limb development | topologically associating domains | *HoxD*

In the nuclei of mammalian cells, chromatin is packaged according to several levels of organization (1–3), which can either reflect or affect transcriptional regulation (e.g., ref. 4). By combining DNA FISH and microscopy, it was shown that chromatin decondensation occurs concomitantly with transcriptional activation (5), suggesting that the opening of chromatin makes gene promoters accessible for transcription. Recently, however, studies involving super-resolution microscopy have revealed a more complex relationship, showing that a higher compaction of chromatin can also be associated with an active state of transcription (6). In this latter case, the compaction of local regulatory elements allowed for a stronger enhancer effect, leading to a more robust activation.

Approaches based on chromosome conformation capture [and derivatives thereof (7)] at the mammalian *HoxD* locus have revealed that interactions between genes and their enhancers can occur not only during an active phase of transcription but also in the absence of transcriptional read out, in cells that do not necessarily express the related target genes (8). Such constitutive contacts covering large regulatory landscapes and their target gene or genes were found to be present in mammals genome-wide (9) and are referred to as topologically associating domains, or TADs (9, 10). TADs have been associated with a variety of regulatory functions (11), either in their implementation (e.g., ref. 12) or in their emergence during vertebrate evolution (13, 14).

Hox gene clusters have been successfully used to study the functional organization of TADs (15–17), as well as the relationship between the progressive decompaction of both genes and enhancers and their transcriptional read-out. Studies of the mouse *HoxD* cluster have provided insights into the global regulation of its nine consecutive genes during limb development, including the presence of multiple regulatory sequences spanning a

2-megabase large DNA interval (18). Recently, this gene cluster, similar to its *HoxA* relative (14, 15), was shown to reside at a boundary between two TADs (located ca. between *Hoxd11* and *Hoxd12*), with each TAD containing enhancers required to regulate different subgroups of genes in developing organs or structures (9, 16, 19) such as distal limbs, proximal limbs, genitals, or the cecum. Interestingly, all enhancers sharing a particular specificity are found within the same TAD, and thus far, no cell type or tissue was reported where these two opposite regulatory landscapes would operate concomitantly (8, 16, 17, 19), suggesting a functional switch occurs between these two TADs in their capacity to regulate subsets of target *Hoxd* genes.

TADs were originally defined by biochemical approaches (9, 10). The correspondence between the averaging of multiple interactions, some of them of unknown significance, on the one hand (see ref. 20), and a chromatin structure in the nuclear space of single cells, on the other hand, is of great interest and has recently come under discussion (see ref. 21). In this study, we used DNA FISH to show that the two TADs splitting the *HoxD* locus are distinct chromatin units, which rarely overlap despite their close association in space. Within this well-defined 3D organization, *Hoxd* genes can segregate over large distances. By using super-resolution microscopy, we observe that these large distances result from extensive elongation events over relatively short genomic distances, which appear to be maximal in tissue with high levels of *Hoxd* gene transcription. Our data suggest this elongation of the *HoxD* cluster is facilitated by its genomic position at the boundary between two TADs.

Significance

Ultrastructural chromatin dynamics may play a key role in regulating transcriptional activation. Here we have used super-resolution microscopy to study the folding mechanics of the *HoxD* cluster, as assayed by following the elongation of chromatin in single cells with different status of *Hox* gene activation. We observed that the spatial separation of *Hoxd* genes is strongest in those tissues where they are highly expressed. We also document that the opening of chromatin precedes transcription and that the strongest elongations are observed at the location of the boundary between two major topologically associating domains (TADs). These results shed light on how spatial compartmentalization is achieved, likely to accompany efficient chromatin reorganization upon activation of transcriptional switches.

Author contributions: P.J.F., S.M., and D.D. designed research; P.J.F., A.B., E.J., and T.H.N.H. performed research; S.M. and D.D. contributed new reagents/analytic tools; P.J.F., A.B., E.J., and S.M. analyzed data; and P.J.F., A.B., S.M., and D.D. wrote the paper.

Reviewers: V.C., Emory University; and L.G., Friedrich Miescher Institute for Biomedical Research.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The RNA-seq data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE72285).

¹To whom correspondence may be addressed. Email: Denis.Duboule@epfl.ch or suliana.manley@epfl.ch.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1517972112/-DCSupplemental.

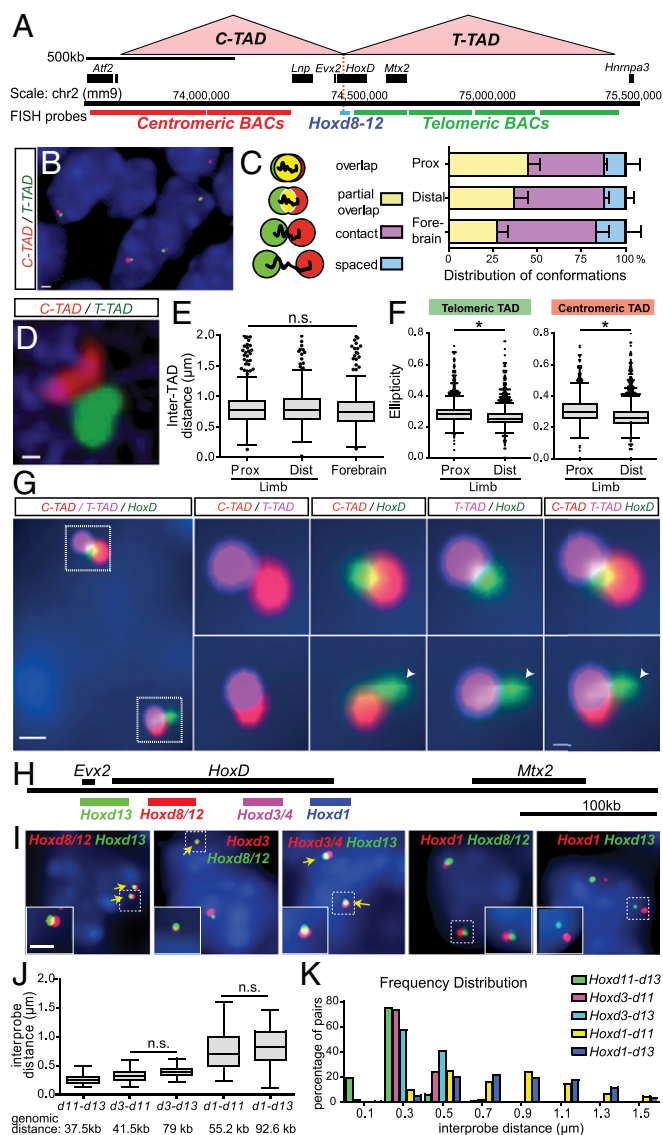


Fig. 1. The *HoxD* cluster is at the interface between two TADs. (A) Schematic of the centromeric and telomeric TADs (C-TAD; T-TAD) using public data available under ref. 9 with genes as black boxes below and the relative localization of the probes (in red, blue and green) used in the DNA FISH experiments. The red dotted line represents the TAD boundary within *HoxD*. (B) E12.5 distal forelimb nuclei stained with DAPI (blue) and the centromeric (red) and telomeric (green) TADs. (Scale bar, 1 μ m.) (C) Schematic (Left) and distributions (Right) of the various configurations observed by FISH in B. Prox, Dist, proximal and distal limb cells, respectively. (D) An example of structured illumination microscopy showing the absence of overlap between the two TADs. (Scale bar, 500 nm.) (E and F) Quantifications of the parameters observed under B. (E) Distances between the centers of both TADs. n.s., nonsignificant, using a Kruskal-Wallis test followed by Dunn's multiple comparison posttest. (F) Ellipticity measured for both the telomeric (left) and centromeric (right). * $P < 0.0001$, using unpaired t tests. (G) Position of the *HoxD* cluster for two alleles of a representative distal forelimb cell, using a fosmid probe specific for the genes *Hoxd8* to *Hoxd12* (green). The signal is scored between the centromeric (red) and telomeric (magenta) TADs. (Scale bar, 500 nm.) (Right) Close-ups of both alleles with white arrowheads showing an elongated *HoxD* cluster. (Scale bar, 200 nm.) (H–K) Distances between various probes localized within the *HoxD* cluster. (I) Forelimb cells nuclei stained with DAPI (blue) and DNA FISH (red and green) for different combinations of *Hoxd* fosmids (Top). (Scale bar, 500 nm.) (J) Quantification of the distribution of interprobe distances between selected pairs of probes. The statistical significance between datasets was tested using Kruskal-Wallis test followed by Dunn's multiple comparison posttest. All were significantly different ($P < 0.05$) except the ones indicated with n.s. See SI Appendix, Table S2 for details. (K) Frequency distribution of the measurements shown in J.

of the cluster and either its transcriptional activity or the presence of polycomb-associated proteins. For instance, the probe containing *Hoxd1* is covered by H3K27me3 marks in distal cells, whereas the *Hoxd8* to *Hoxd12* fragment shows a moderate coverage. In contrast, the probes encompassing either the *Hoxd11* to *Hoxd13* or the *Hoxd13* to *Evx2* DNA intervals are free of H3K27me3 in embryonic day (E) 12.5 distal and active cells, likely as a consequence of high transcriptional activity (Fig. 3A).

Each fosmid clone was labeled with the same Alexa 647 fluorophore and its signal analyzed by STORM microscopy. By comparing the signal morphologies (Fig. 3B), we noticed that the *Hoxd1* signals did not appear significantly more compact than the others (Fig. 3B; representative examples). If anything, this area of the *HoxD* cluster, which is densely covered by H3K27me3 marks, was slightly less circular than images obtained from the fosmids covering regions with lower amounts of H3K27me3 and highly expressed genes (Fig. 3C). To complement this observation, we labeled the two fosmid clones containing either the *Hoxd1* or the *Hoxd8* to *Hoxd12* regions, using Alexa 647 and Alexa 555, respectively, to directly assess their relative morphologies within the same cells. These experiments confirmed that these two DNA regions were decompacted to similar extents in the same cells (SI Appendix, Fig. S2B). The most extensive elongation observed (*Hoxd8-d12*, Fig. 3B and C) matches the set of genes that was shown to have cell-specific long-range DNA contacts in the developing forelimb (16). To test whether decompaction could change with a modification of long-range contacts, we compared the two tissues in which the pattern of long-range interactions differs drastically: the proximal and distal forelimbs (Fig. 3D). Here we observed similar structures, both of them significantly more dispersed than that which was observed in the forebrain, used as a negative control (Fig. 3D).

We next investigated whether such a decompaction would also be observed in another tissue in which the *HoxD* cluster is strongly activated, and thus imaged the *Hoxd8-d12* region in the developing trunk. We compared forebrain cells in which the cluster is completely inactive, anterior trunk cells in which transcription occurs from *Hoxd1* to *Hoxd8* only, and posterior trunk cells in which *Hoxd1* to *Hoxd12* are activated. We observed the strongest decompaction in those tissue with the highest level of transcription (Fig. 3E), suggesting the existence of a link between transcription and the unfolded structure of *Hox* genes.

Effect of the Gene Deserts on Compaction. 4C studies using microdissected limb samples have indicated that parts of the *HoxD* cluster are strongly contacted by enhancer sequences located in both the centromeric and telomeric TADs (8, 16). Such strong enhancer contacts may exert forces upon the *HoxD* cluster, leading to variations in its global chromatin architecture. We assessed this possibility by analyzing three genetic perturbations in which *Hoxd* genes were separated from their respective enhancers by large targeted inversions (31, 32). The first allele was an inversion separating *HoxD* from its telomeric TAD by displacing the latter by 28 Mb (*Inv(attP-cd44)*; Fig. 3F), which switched off *Hoxd9-11* in proximal limb cells and decreased their transcription in distal limb cells. The morphologies of signals detected when using this large inversion were as elongated as in noninverted cells, showing that the immediate regulatory neighborhood, even though it contains sequences interacting with the probe, was not critical in the shaping of the cluster, at least in these particular conditions. In both the inverted and wild-type loci, a low circularity index was determined for the *Hoxd8* to *Hoxd12* DNA region (Fig. 3G and H), suggesting that the presence of genuine enhancer sequences and constitutive interactions (16) is not necessary for the decompaction of the *HoxD* cluster.

We also assessed the *Inv(Nsi-Iga6)* centromeric inversion (SI Appendix, Fig. S3A and B), which repositions the centromeric regulatory sequences several Mb away from the cluster, thus abrogating all specific enhancer–promoter interactions occurring during digit development and, consequently, turning off *Hoxd*

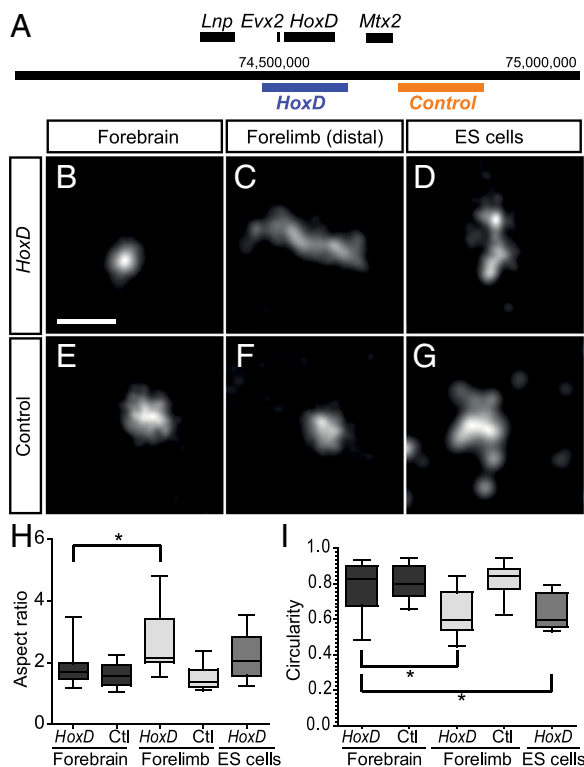


Fig. 2. Distinct conformations of the *HoxD* cluster visible by STORM microscopy. (A) Schematic view of the two BAC clones, 160 and 175 kb large, respectively, used to visualize the *HoxD* cluster and part of the telomeric gene desert, using STORM imaging. (B–G) DNA FISH using Alexa 647 and resolved through STORM, using either “inactive” forebrain (B and E), “active” distal forelimb (C and F), or synchronized ES (D and G) cells. (Scale bar, 200 nm.) (H and I) For the same cells, quantifications of aspect ratio (H) and circularity (I) are shown, where the asterisk indicates $P < 0.01$, using a Kruskal–Wallis test followed by Dunn’s multiple comparison posttest (see *SI Appendix, Materials and Methods*).

genes transcription (33). Again, the decompacted aspect of the *HoxD* cluster was not dramatically modified after STORM imaging (*SI Appendix, Fig. S3B*). Finally, we used a large inversion, with a breakpoint located between *Hoxd10* and *Hoxd11*, thus splitting the *HoxD* cluster into two parts, leaving in place only from *Hoxd11* to *Hoxd13* and their centromeric gene desert (ref. 31; scheme in *SI Appendix, Fig. S3*). We monitored the aspect ratio of the short remaining cluster, using the fosmid probe covering from *Hoxd11* to *Hoxd13*, and found no difference in elongation (*SI Appendix, Fig. S3C*), suggesting a full *HoxD* cluster is not critical to spatially organizing chromatin in its various parts.

Discussion

The organization of chromatin in the nuclear space is a critical parameter for the proper control of transcription, and the extent of chromatin elongation at a given genetic locus may reflect its capacity to be efficiently transcribed (34). We previously determined that the *HoxD* gene cluster was the target of a bimodal type of global regulation, exerted from either the telomeric or centromeric gene deserts, two regulatory landscapes matching TADs, and separated by the *HoxD* cluster itself (9, 16). However, chromosome conformation capture-based experiments average the treatment of several million independent cells, and the presence of dense and compact chromatin architectures flanking the *HoxD* cluster remained to be shown at the cellular level. Here, by concomitantly labeling series of BACs spanning specifically the two TADs, we demonstrate that such configurations indeed exist as dense and separate objects in individual cells, as

previously seen on a specific locus at the X-chromosome (10), with a low level of partial overlap consistent with the reduced number of interactions observed in 4C between the two TADs (16, 17). This observation was confirmed by structured illumination microscopy. The distances between TADs were comparable between cells, whether or not they expressed *Hoxd* genes, supporting the existence of a poised regulatory structure already present in the absence of transcription (8). However, the direct visualization of TADs at this locus is not informative regarding potential fluctuations in contacts within each TAD, from one cell to another (20). It nevertheless demonstrates that such dense structures exist in all cells and on both alleles.

By using super-resolution microscopy (STORM), we scored the most extensively elongated forms in cells from either the developing distal forelimb or the posterior trunk, two tissues in which several *Hoxd* genes are strongly active. In contrast, dense and compact structures were scored either in brain cells, negative for all *Hox* gene transcription at this stage, or when using a control probe located outside the gene cluster. These elongated morphologies generally occurred along a major axis and appeared continuous, either when using large probes covering the whole cluster or with smaller probes detecting only one or a few genes. In distal forelimb cells, however, where the *Hoxd1* to *Hoxd4* region is covered by H3K27me3 chromatin marks (16), we did not observe any significant asymmetric compaction. In contrast, the inactive part of the *HoxD* cluster was at least as elongated as the transcriptionally active region. One explanation for this unexpected observation may be the robust and nonproductive contacts established by *Hoxd1* with the telomeric TAD (16), which may lead to tensions elongating the gene cluster (see following).

A significant elongation of the gene cluster was also scored in ES cells, even though the entire *HoxD* cluster is labeled with both H3K4me3 and H3K27me3 chromatin marks (22, 28, 30) associated with the formation of local chromatin domains (23, 35), in contrast with the idea that *Hox* clusters adopt a “closed” conformation whenever genes are silent (23). In ES cells, however, the 4C interaction patterns are much weaker than in brain cells (35), probably because of the presence of bivalent chromatin marks and the concomitant reduced amount of H3K27me3 modifications [Fig. 1 and figure S1 in ref. 35, the latter being even weaker when ES cells were cultured in 2i medium (36)]. It was also noticed that *Hoxd* genes established many more interactions with the neighboring gene deserts in ES cells than in brain cells (35), where most interactions involved the gene cluster itself, further suggesting a more decompacted configuration of the locus in ES cells. Therefore, the vast majority of ES cells may display decompacted chromatin at their *Hox* loci. The presence of some H3K27me3-labeled nucleosomes may induce enough transitory interactions to be translated into read counts after deep sequencing of 4C products.

Altogether, these data suggest that in the developing limbs, the *HoxD* cluster is decompacted, with an elongated shape including both active and inactive genes. Therefore, the coverage of specific parts of the cluster by polycomb complexes, depending on the proximal versus distal position of the cells (8, 16) and observed in other functional contexts (30, 37, 38), does not seem to significantly influence its level of compaction. The distances apparently associated with these elongated forms correspond to previous distance predictions established by mathematical modeling (29). Stretching events associated with transcription were also reported, which seem to occur within this range of elongation lengths (5), and several recent studies either describe or predict distances ranging from 300 to more than 1,000 nm (39, 40). The elongation of the *HoxD* cluster in limb cells despite its partial labeling by H3K27me3 may reflect an influence from the flanking TADs in extending the structure in both directions, following either productive or constitutive interactions. We evaluated this possibility by using three large inversions modifying the global relationships between the gene cluster and its two regulatory landscapes. However, none of these rearrangements did affect the capacity of the cluster to elongate. If anything, elongation

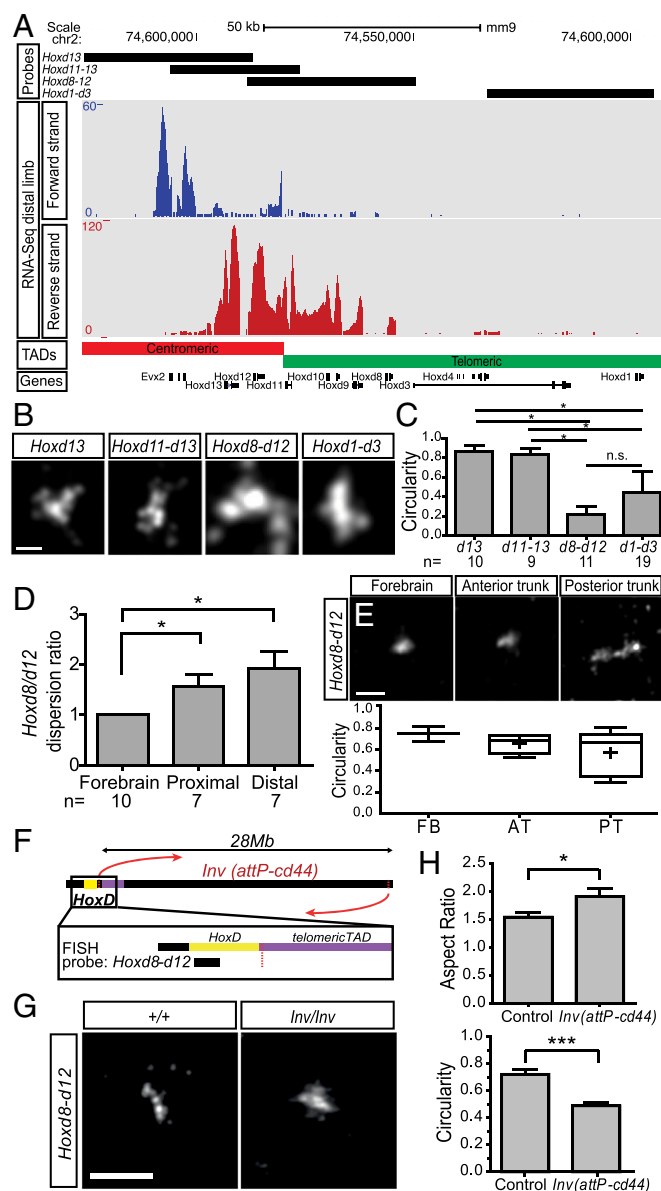


Fig. 3. STORM analysis using smaller probes in different control and mutant tissues. (A) RNA profile of distal forelimb cells showing the expression of *Hoxd8* to *Hoxd13* (red) aligned with the positions of four fosmid probes (black, Top). The boundary between the centromeric (red) and telomeric (green) TADs is shown below, as well as the transcription of *Evx2* from the other DNA strand (blue). (B) STORM imaging using the four probes under A and their relative circularity index (C). (Scale bar, B, 100 nm.) (D) Refined analysis of the *Hoxd8* to *Hoxd12* probe in forebrain, proximal limb, and distal limb cells, quantifying the level of dispersion as depicted in C and SI Appendix, Fig. S2B. (Scale bar, 200 nm.) (E) STORM imaging of the *Hoxd8* to *Hoxd12* probe in E10.5 embryos in cells positioned along the anterior to posterior axis. AT, anterior trunk; FB, forebrain; PT, posterior trunk. (Scale bar, 200 nm.) (F) Scheme of the 28-Mb large *Inv(attP-cd44)* inversion (red arrows) removing the telomeric TAD (purple), and location of the *Hoxd8* to *Hoxd12* probe (black). (G) STORM imaging of the *Hoxd8* to *Hoxd12* region in E12.5 distal forelimb cells from either a homozygous *Inv(attP-cd44)* embryo (Right) or control littermate (Left). (Scale bar, 200 nm.) (H) Quantification of the aspect ratio and circularity in both configurations shown under G. * $P < 0.05$ and *** $P < 0.001$, using Mann-Whitney U test. In C and D, ** $P < 0.01$, using a Kruskal-Wallis test followed by Dunn's multiple comparison posttest. n.s., not significant.

was more pronounced and the circularity index lower, suggesting the telomeric gene desert may participate in the compaction of the system, rather than its elongation.

In this context, it is noteworthy that the part of the cluster displaying the highest level of decompaction was the *Hoxd8* to *Hoxd12* region, which precisely matches with the inter-TADs boundary (9, 16). This particular region of *HoxD*, located between genes strongly and constitutively interacting with either the centromeric (*Hoxd13*) or the telomeric (*Hoxd1*, *Hoxd4*) TADs, may thus display more flexibility, perhaps reflected by the capacity of *Hoxd8* or *Hoxd12* to switch their contacts from one TAD to the other in various limb cell-types (16). Interestingly, this region is strongly enriched in binding sites for architectural proteins such as CCCTC-binding factor (CTCF) and cohesin (41, 42), which are known for their capacity to organize genomic boundaries and domains, in particular at TAD boundaries (43). Whether the presence of several sites bound by CTCF in the sequence targeted by the *Hoxd8* to *Hoxd12* fosmid play a role in the elongation observed by STORM remains to be tested, however, as CTCF-driven interactions would be expected to increase compaction, rather than the opposite. An alternative explanation is that the resolution of our STORM approach, although allowing a clear distinction between fully compacted and decompacted *Hox* clusters, may not easily detect mixed configurations, in particular when only a small part of the cluster differs in shape from the rest.

In conclusion, these data suggest the structural organization of the *HoxD* gene cluster may predate transcriptional activation and may subsequently be rather independent from its transcriptional status. Perhaps such an elongated structure is maintained in cells expressing subsets of *Hoxd* genes, whereas compaction occurs in those cells where genes are silent, such as the fetal brain. In this latter tissue indeed, an elongated structure was never observed. By using 2D and 3D FISH, it was previously reported that a decompaction of the *HoxD* cluster occurs in ES cells that are differentiating in vitro, and that this process is linked to the progressive reduction in levels of PRC1 components (22). With the resolution of our STORM approach, the *HoxD* cluster did not appear to adopt a compact structure, which would be released on differentiation. It is possible that the spatial architecture of a decondensed cluster differs only slightly between the active and inactive states, leading to the observation of a range of distances between localized probes used for FISH. The presence of polycomb complexes may, for instance, impose such distinct organizations of the *HoxD* cluster in space, while having little influence on its general level of compaction. In this context, a detailed analysis of these structures by super-resolution microscopy in ES cells mutant for components of either the PRC1 or PRC2 complexes may be informative.

Materials and Methods

All experiments involving animals were performed in agreement with the Swiss law on animal protection, with the appropriate legal authorizations to D.D. Tissues were isolated from E10.5 or E12.5 embryos, either wild-type or mutant for the *Inv(Nsi-Itga6)*, *Inv(attP-cd44)*, and *Inv(HoxD^{RVIII}-Cd44)* inversions (31). Mouse ES cells were grown in serum with 1,000 U/mL leukemia inhibitory factor under feeder-free conditions and G1-synchronized through mitotic shake off. 3D DNA FISH was as in ref. 5. Structured illumination images were acquired using a Nikon structured illumination microscopy setup (Eclipse T1 microscope fitted with a super-resolution Apochromat total internal reflection fluorescence 100 \times /1.49 NA objective IXON3 camera; Andor Technology). For STORM imaging, DNA FISH samples were imaged using a UPlanSApo 100 \times /1.40 oil objective (Olympus), and typically 10,000–15,000 snapshot images with a pixel size of 100 nm and an exposure time of 0.03 s were acquired to create one super-resolution (SR) image. SR images were reconstructed with the Octane software (44), and statistical differences between samples were evaluated with the Kruskal-Wallis test, followed by Dunn's multiple comparison posttest, with the exception of Fig. 3H (Mann-Whitney U test). RNA-Seq was performed according to the TruSeq Stranded Illumina protocol, with polyA selection. The reads were mapped to Ensembl Mouse assembly National Center for Biotechnology Information Mouse Assembly 37 (mm9) and translated into reads per gene (RPKM) using the Bioinformatics and Biostatistics Core Facility (BBCF), High-Throughput Sequencing station (available at htstation.epfl.ch). An extended description of the materials, methods, and data analysis is provided in the SI Appendix.

ACKNOWLEDGMENTS. We thank the members of the D.D. laboratories for discussions and S. Boyle and W. Bickmore (Medical Research Council Edinburgh) for their help with setting up the FISH procedures. We also thank O. Burri and R. Guet [Ecole Polytechnique Fédérale de Lausanne (EPFL)] for assistance with image analysis and T. Laroche for help with structured illumination microscopy, as well as B. Mascrez and S. Gitto for help with mutant stocks. We thank the

Geneva Genomics Platform (University of Geneva) and the Bioinformatics and Biostatistics Core Facility (EPFL) in Lausanne for their assistance with RNA-Seq. This work was supported by funds from the EPFL, the University of Geneva, the Swiss National Research Fund, the European Research Council (ERC) Grant SystemsHox.ch, and the Claraz Foundation (to D.D.), as well as Swiss National Research Fund Grant CR33I2_149850 and ERC Grant PALMassembly (to S.M.).

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