Visualizing the *HoxD* Gene Cluster at the Nanoscale Level

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Transcription of *HoxD* cluster genes in limbs is coordinated by two topologically associating domains (TADs), neighboring the cluster and containing various enhancers. Here, we use a combination of microscopy approaches and chromosome conformation capture to assess the structural changes occurring in this global architecture in various functional states. We observed that despite their spatial juxtaposition, the TADs are consistently kept as distinct three-dimensional units. *Hox* genes located at their boundary can show significant spatial segregation over long distances, suggesting that physical elongation of the *HoxD* cluster occurs. The use of superresolution imaging (STORM [stochastic optical reconstruction microscopy]) revealed that the gene cluster can be in an either compact or elongated shape. The latter configuration is observed in transcriptionally active tissue and in embryonic stem cells, consistent with chromosome conformation capture results. Such morphological changes at *HoxD* in developing digits seem to be associated with its position at the boundary between two TADs and support the idea that chromatin dynamics is important in the establishment of transcriptional activity.

As a substrate for gene regulation, chromatin organization is tightly regulated during development (Bickmore and van Steensel 2013; de Laat and Duboule 2013; Gorkin et al. 2014). For example, it was shown to decompact during early transcriptional activation (Morey et al. 2007), suggesting that its unfolding makes gene promoters more accessible for transcription. Recently, however, superresolution imaging has revealed a more complex relationship, whereby a closed chromatin conformation can be somewhat permissive for gene expression (van de Corput et al. 2012). In the latter case, an increased compaction of regulatory elements with their target sequences may allow for a stronger transcriptional enhancing effect.

Chromosome conformation capture techniques at the genome-wide level such as 4C and Hi-C reveal the map of interactions established by any given genomic locus and thus provide global views of chromatin topology (de Laat and Dekker 2012; Dixon et al. 2012). However, these approaches represent the averaging of a million cells at a given time point, and hence they may not reflect events occurring locally or transiently. Likewise, they cannot discriminate between various possible conformations occurring in distinct cells at different times (see Giorgetti et al. 2014). At the *HoxD* cluster, which is a model system to study the impact of chromatin organization during developmental decisions, strong interactions between *Hoxd* genes and their regulatory islands have been observed in both active and inactive cells (Montavon et al. 2011). These constitutive contacts are found over several hundreds of kilobases and have been shown by Hi-C to be a rather general feature of chromatin compartmentaliza-

tion (Dixon et al. 2012). These higher-order structures are referred to as topologically associating domains (TADs; Dixon et al. 2012; Nora et al. 2012). TADs are associated with several potential regulatory functions, either during development (Nora et al. 2013; Schwarzer and Spitz 2014) or in the course of vertebrate evolution (Woltering et al. 2014; Lonfat and Duboule 2015).

Hox genes are widely implicated in the formation of many structures during development, and the functional characterization of the associated TADs in vivo starts to be well documented (Andrey et al. 2013; Berlivet et al. 2013; Lonfat et al. 2014). Tissue-specific, global regulations at the *HoxD* cluster involve a progressive decompaction of its genes, likely because of the increase in dynamic contacts established with their long-range enhancers. Genetic approaches used to perturb their transcriptional activity during limb development showed that most (if not all) strong regulatory sequences were engaged with either one of the two neighboring TADs (Montavon and Duboule 2013). Therefore, the *HoxD* cluster is located at the precise boundary between two TADs, each of them including regulatory sequences necessary to control subgroups of *Hoxd* genes in developing organs or structures (Dixon et al. 2012; Andrey et al. 2013; Delpretti et al. 2013) such as distal limbs, proximal limbs, genitals, or the cecum. Notably, enhancers sharing specificity for a particular structure are always located within the same TAD, and these two opposite regulatory landscapes have not yet been found to operate concomitantly (Montavon et al. 2011; Andrey et al. 2013; Delpretti et al. 2013; Lonfat et al. 2014). This latter observation suggests that a global switch

occurs between TADs in their ability to regulate their own sets of target *Hoxd* genes.

Biochemical data on chromatin organization are highly suggestive of a spatial arrangement, which can be more directly validated at the single-cell level by fluorescence microscopy (e.g., see Williamson et al. 2014), and hence we looked at the dynamic rearrangements of *HoxD* architecture and the spatial independence of TADs at the *HoxD* locus using DNA FISH (fluorescence in situ hybridization). We also evaluated the well-defined three-dimensional organization of *Hoxd* genes by using superresolution microscopy. The large distances that separate the two TADs were reflected by extensive elongation over relatively short genomic distances, which appeared maximal in tissues displaying high levels of *Hoxd* transcription. Overall, a correspondence emerged between superresolution microscopy and high-resolution C-seq sequencing techniques and, altogether, the chromatin transitions observed with both techniques suggested that the dynamic elongation of the *HoxD* cluster was facilitated by its genomic position between these TADs.

VISUALIZATION OF TADs AT THE *HoxD* CLUSTER

TADs were initially characterized by biochemical approaches (Dixon et al. 2012; Nora et al. 2012), and Hi-C performed by using embryonic stem (ES) cells as starting material showed that the *HoxD* cluster is flanked by two well-defined TADs (Fig. 1A, top). Even in different tissues such as proximal and distal developing forelimbs, *Hoxd* genes located at the boundary can display preferential interactions with either one of the TADs (Fig. 1A, bottom; Andrey et al. 2013). This apparent compartmentalization of the TADs nevertheless is derived from averaging snapshots of multiple distinct interactions, and hence the question can be raised as to how such chromatin structures appear—if at all—in the nuclear space of single cells.

We assessed the chromatin topology surrounding the *HoxD* cluster using 3D-DNA FISH by labeling consecutive bacterial artificial chromosomes (BACs) in both active (forelimb) and inactive (forebrain) cells. When labeling various combinations of BACs belonging to the same TAD (as defined by Hi-C), mixing between the signals from each probe was consistently observed (Fig. 1B,C, conditions 2–4). However, when the labeled BAC clones were taken from different sides of the TAD boundary, clear mixing of signals was never observed (condition 1), as shown in similar contexts (Nora et al. 2012; Crane et al. 2015). Finally, the differential labeling of several consecutive BACs belonging to both TADs (Fig. 1E) showed that such structures exist and are organized as two dense and distinct units, poorly interconnected but closely associated in the nuclear space. This was confirmed by using structured illumination microscopy (SIM; Fig. 1F) and quantified with standard 3D microscopy (Fig. 1G,H). This bimodal configuration was observed routinely and in many different cell types, regardless of the transcriptional activity of the locus

(Fig. 1H, left). However, their structures of low ellipticity appeared somewhat flexible and potentially variable in cellular domains displaying different *Hoxd* gene expression patterns (Fig. 1H, right).

Within this rather stable two-TAD structure, the relative position of *HoxD* was determined using a 39-kb-large fosmid probe covering from the *Hoxd8* to *Hoxd12* genes (blue segment in Fig. 1E). The signal was consistently located at the TAD junction (Fig. 1I), with, in some cases, a somewhat elongated aspect (Fig. 1I; arrowheads). These stretched conformations may reflect the multiple interactions encountered by the gene cluster with its regulatory environment (Montavon et al. 2011; Andrey et al. 2013).

SPATIAL SEGREGATION WITHIN *HoxD*

Compartmentalization within the *HoxD* cluster was previously observed by DNA FISH (Eskeland et al. 2010). The *Hoxd1*–*Hoxd3* genes were found at a distance from *Hoxd13* during the differentiation of ES cells, suggesting that the gene cluster can be split in the nuclear space. We tested several combinations of probes in embryo, in transcriptionally active, developing digit cells (Fig. 2A–D). The probes covered either *Hoxd1* and *Hoxd3/4* (i.e., genes included within the telomeric TAD), *Hoxd8* to *Hoxd12* (i.e., genes located around the TAD boundary), or *Hoxd13*, which resides in the centromeric TAD (Fig. 2A; Andrey et al. 2013). Systematic measurements of distances between various combinations of pairs of probes revealed that parts of the *HoxD* cluster could be separated from one another by >500 nm (Fig. 2B,C). Although the distances measured between the centers of the two signals generally correlated with the genomic distance (Fig. 2C), they were typically shorter between genes displaying similar expression patterns.

In contrast, distances were rather large whenever the inactive *Hoxd1* gene was tested with any other part of *HoxD* (see Fig. 4A). In this context, the spatial distances between the different parts of *HoxD* revealed that its apparent split tends to occur between active and inactive sets of genes (Noordermeer et al. 2011), rather than at a fixed TAD boundary as described in ES cells, for example (Fig. 2C). The separation between *Hoxd1* and the rest of the cluster was not systematic and followed a broad distribution (Fig. 2D). Interestingly, several 4C profiles corroborated the “looping out” of *Hoxd1*, which is a member of the *HoxD* cluster showing distinct expression specificities when compared with its neighbors (Zakany et al. 2001). In forelimb cells, only rare interactions were observed between *Hoxd1* and *Hoxd11*, in both proximal and distal cellular domains (Fig. 2E), as in the developing cecum or in brain cells where *Hoxd1* contacted neither *Hoxd4* nor *Hoxd11* (Delpretti et al. 2013; see also Noordermeer et al. 2011). This separation between *Hoxd1* and the rest of *HoxD* may have a functional relevance and be mediated by unique and strong interactions of the former with a specific part of the telomeric TAD (Andrey et al. 2013).

Figure 1. Direct visualization of topologically associating domains (TADs) at the *HoxD* locus. (A) TAD structures as seen from Hi-C and 4C data. (Top) The Hi-C heatmap showing the centromeric and telomeric TADs bordering the *HoxD* cluster (data from Dixon et al. 2012). (Below) The 4C profiles *Hoxd11* as a viewpoint in two different tissues, showing the changes in *Hoxd11* contacts within each TAD (adapted from Andrey et al. 2013). (B) (Top) A scheme of the probes used in 3D-DNA FISH. The dotted line represents the TAD boundary as defined in Dixon et al. (2012) and Andrey et al. (2013). (Bottom) Each image shows the overlap observed for the tested combinations of probes. Scale bar, 200 nm. (C) Quantification of the distances between two probes for each combination. The average distance in three different embryonic tissues is shown: E12.5 forebrain (dark gray), where *Hoxd11* is inactive, and proximal (light gray) and distal (white) forelimb, two tissues where *Hoxd11* is active. $N > 100$ for each pair. Error bars denote SD. (D) Blinded and qualitative measurement showing the percentage of overlapping pairs (70–100 pairs). The errors bars denote SD ($n = 2$ for each, except condition 4: $n = 1$). (E) Schematic of C-TAD and T-TAD, with genes as black boxes and the relative localization of the probes used in the DNA FISH experiments (red, blue, and green). The red dotted line is the TAD boundary. (F) Example of SIM showing the absence of overlap between the two TADs. Scale bar, 500 nm. (G) E12.5 distal forelimb nuclei stained with DAPI (blue) and the centromeric (red) and telomeric (green) TADs. Scale bar, 1 μ m. (H) Quantifications of the parameters observed under G. (Left) Distances between the centers of both TADs. n.s.: nonsignificant using a Kruskal–Wallis test followed by Dunn's multiple comparison posttest. (Right) Ellipticity measured for both the telomeric (left) and centromeric (right). * denotes $P < 0.0001$ using unpaired t -tests. (I) Positions of the *HoxD* cluster for two alleles of a representative distal forelimb cell using a fosmid probe specific for the genes *Hoxd8–Hoxd12* (green). The signal is scored between the centromeric (red) and telomeric (magenta) TADs (scale bar, 500 nm). Images on the right are close-ups of both alleles with white arrowheads showing an elongated *HoxD* cluster (scale bar, 200 nm). (B–I, Adapted from Fabre et al. 2015.)

Figure 2. Spatial segregation of *Hoxd1*. (A) Position of the probes within the *HoxD* cluster. (B) Forelimb cells nuclei stained with DAPI (blue) and DNA FISH (red and green) for different combinations of *Hoxd* probes shown in A. Scale bar, 500 nm. (C) Distances between various probes localized within the *HoxD* cluster. (D) Quantification of the distribution of interprobe distances between selected pairs of probes. The statistical significance between data sets was tested using a Kruskal–Wallis test followed by Dunn’s multiple comparison posttest. All were significantly different ($P < 0.05$) except those indicated with “n.s.” (not significant). (D) Frequency distribution of the measurements shown in C. (A–D, Adapted from Fabre et al. 2015.) (E,F) Quantitative local 4C profiles in five different tissues showing weak interactions involving *Hoxd1*, whose position is emphasized by the red box. The locations of *Hoxd* genes are shown below. (E) 4C profiles with *Hoxd13* as a viewpoint in distal (blue) and proximal (purple) forelimb cells. (F) 4C profiles from cecum (*Hoxd3* to *Hoxd11* active) and brain (*Hoxd* genes inactive) with three different viewpoints (*Hoxd4*, *Hoxd11*, and *Hoxd13*) showing weak interactions involving *Hoxd1* (adapted from Delpretti et al. 2013). (G) 4C profiles with *Hoxd13*, *Hoxd9*, and *Hoxd4* as viewpoints in ES cells (*Hoxd* genes inactive). The H3K27me3 and H3K4me3 chromatin marks are shown below (adapted from Noordermeer et al. 2014).

Figure 3. Distinct conformations of the *HoxD* cluster are visible by STORM microscopy. (A) Scheme of the centromeric (C-) and telomeric (T-) TADs (data from Dixon et al. 2012) with the *HoxD* cluster as a black box. (B,C) 4C-seq signals for the *Hoxd13* viewpoint in ES and forebrain cells or from proximal (purple) and distal forelimb (blue) (C), with orange boxes showing the external interactions. *Hox* genes (red) and other transcripts (black) are shown below (B). The boundaries of the inactive *Hoxd* gene compartments are indicated by dashed lines. (B, Adapted from Noordermeer et al. 2014.) For the ES and forebrain tracks, the H3K27me3 and H3K4me3 chromatin marks are shown in black. (D) Super-resolution DNA FISH on the *HoxD* cluster. (Top) 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 by STORM. The pictures below represent DNA FISH resolved through STORM, using “inactive” forebrain, “active” distal forelimb, or synchronized ES cells. Scale bar, 200 nm (adapted from Fabre et al. 2015). (E) Quantification of the 4C signals from B and C to show the ratio of signals that map outside versus inside the *HoxD* compartments (within the dotted lines in B and C). (F) Quantifications of the circularity of the images shown in D, where the asterisk indicates $P < 0.01$, using a Kruskal–Wallis test followed by Dunn’s multiple comparison posttest (adapted from Fabre et al. 2015).

THE *HoxD* LOCUS AT SUPERRESOLUTION

During the early activation of the *HoxD* cluster, active and inactive genes form distinct interaction groups, as seen by dynamic subdomains using 4C (Noordermeer et al. 2011, 2014). Comparison of 4C profiles revealed that *Hoxd13* establishes more contacts outside the *HoxD* cluster in ES cells than in forebrain cells where the structure seems to be more compact (Fig. 3B; see ratio (log 2) and Fig. 3E). In transcriptionally active forelimb cells, an open cluster was observed, similar to ES cells (Fig. 3C). A comparison between the four indices (Fig. 3E) confirmed that the transcriptional activity of the cluster is not directly associated with external contacts. Instead, it was proposed that H3K27me3 marks help stabilize interactions, which could then be captured by 4C (Vieux-Rochas et al. 2015). H3K27me3 marks over *HoxD* are weaker in ES cells than in brain cells (Fig. 3), which may explain the observed difference in compaction. As ES cells cultured in 2i medium have even fewer H3K27me3 marks (Marks et al. 2012), it would be interesting to evaluate the 3D state of *HoxD* in such a context.

To obtain a direct visualization of the gene cluster, we performed DNA FISH using a labeled BAC whose shape was resolved using stochastic optical reconstruction microscopy (STORM). STORM involves the se-

quential blinking and localization of single-molecule signals to reconstruct superresolution images (Fig. 3D; Huang et al. 2008; Manley et al. 2011; Benke and Manley 2012). The control was a BAC of similar size located nearby in the telomeric gene desert (Fig. 3D). In forebrain cells where the *HoxD* cluster is silent, both control and *HoxD* probes appeared as compacted objects (Fig. 3D). In contrast, developing distal forelimb cells (high transcription of *Hoxd13* to *Hoxd9*) (see Fig. 4A; Montavon et al. 2008), as well as ES cells displayed more variable morphologies (Fig. 3D). Although the average cluster area did not change significantly, signals detected in forelimb and ES cells were often substantially more elongated or less “circular,” consistent with the higher degree of external contacts observed in ES cells and forelimb cells than in forebrain cells, in our 4C experiments (Fig. 3B,C,E).

Quantification of these morphologies showed a circularity significantly lower in both active forelimb and ES cells than in inactive forebrain cells (Fig. 3F), suggesting that chromatin may open to prepare and maintain transcription at *HoxD* (Fig. 3B). Finally, we measured the extent of the signals, which could span more than 500 nm, consistent with previous estimations (Fig. 2C; Papageorgiou 2006).

Elongation of *HoxD* in ES cells was previously triggered by in vitro differentiation (Eskeland et al. 2010) and

reported to depend on a progressive reduction in levels of PRC1 components. In contrast, our data from STORM showed that *HoxD* does not appear as a compact structure, which would be further released upon differentiation. It is however possible that a “decondensed” cluster differs only slightly between the active and inactive states, leading to a range of distances between probes used for FISH. Polycomb complexes may impose such distinct organizations of the *HoxD* cluster in space, while having little influence upon its general level of compaction. A detailed analysis of these structures by superresolution microscopy in ES cells mutant for either PRC1 or PRC2 components may be informative in this respect.

The elongation of *HoxD* in limb cells, regardless of their H3K27me3 levels, may reflect the impact of the flanking TADs in extending the structure in both directions, following either productive or constitutive interactions. Three large inversions disrupting the TAD structures were used to challenge this idea (Fabre et al. 2015), and none of these alleles modified the elongating capacity of the cluster. If anything, elongation was more pronounced, meaning that TADs may help to compact the system rather than to elongate it.

MAXIMAL CHROMATIN ELONGATION AT THE TAD BOUNDARY

Chromatin unfolding has been suggested to increase accessibility for the transcriptional machinery, thus triggering active transcription (Morey et al. 2007; Bell et al. 2011). It was proposed early on to have a key role in the collinear regulation of the *HoxD* cluster (Dolle et al. 1989), which is enriched in Polycomb-associated chromatin marks such as H3K27me3 before it starts to operate. Such marks may participate in its compaction via the recruitment of the PRC1 complex (Bernstein et al. 2006; Soshnikova and Duboule 2009; Eskeland et al. 2010). In ES cells, H3K27me3 marks cover the entire cluster but with a much lower density than in forebrain (Fig. 3B). This particular chromatin status of ES cells may thus mediate a state of decompaction comparable with that of transcriptionally active chromatin. In this view, an unfolded chromatin state may not always reflect active transcription.

To better evaluate the association between the presence of H3K27me3 marks and gene transcription, we performed STORM using 40-kb FISH probes covering subregions of the *HoxD* cluster in various functional states (Fig. 4A). For example, the probe containing *Hoxd1* is rich in H3K27me3 marks in distal cells, whereas an *Hoxd8–Hoxd12* DNA probe of similar size showed a reduced coverage. On the other hand, the probes including either the *Hoxd11–Hoxd13* or the *Hoxd13–Evx2* DNA regions are mostly free of H3K27me3 in distal cells, associated with high transcriptional activities (Fig. 4A). By comparing signal morphologies (Fig. 4B,C), the *Hoxd13* signal was significantly more compact than the others (Fig. 4B; representative examples). If anything, this region of the *HoxD* cluster, which is densely covered

by H3K27me3 marks, was less circular than other highly transcribed regions covered with lower amounts of H3K27me3 (Fig. 4C). In fact, the most extensive elongation observed was located at the TAD boundary, a region labeled with a probe covering *Hoxd8–Hoxd12*—that is, containing those genes that establish versatile interactions with either the telomeric or the centromeric TADs (Andrey et al. 2013).

Further comparison of the morphology of this hinge region between two tissues in which the pattern of long-range interactions differs drastically (proximal and distal forelimbs) revealed similar structures, both being more dispersed than that observed in forebrain cells (Fabre et al. 2015). This particular region of transition within *HoxD*, separating genes constitutively interacting with either the centromeric (*Hoxd13*) or the telomeric (*Hoxd1*, *Hoxd4*) TADs, may thus display more flexibility in its spatial structure, perhaps associated with the capacity of some genes to switch their contacts from one TAD to the other in various limb cell types (Andrey et al. 2013). Interestingly, this region is strongly enriched in binding sites for architectural proteins such as CTCF and cohesin (Soshnikova et al. 2010; Gomez-Diaz and Corces 2014), which are known for their potential capacity to organize genomic boundaries and domains (Van Bortle et al. 2014). Whether or not the presence of several CTCF sites in this fosmid probe plays a role in the elongation observed by STORM remains to be tested since CTCF-driven interactions are generally considered to increase compaction rather than the opposite. Alternatively, the resolution of our STORM approach, although allowing a clear distinction between fully compacted and decompacted elongated *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 limbs, the *HoxD* gene cluster is regulated by two flanking TADs (Dixon et al. 2012; Andrey et al. 2013). Our results show that these TADs exist as dense and separate objects, either by imaging the DNA sequence spanning specifically the two TADs (FISH) in individual cells, as shown previously in a different context (Nora et al. 2012), or by using 4C over large cellular populations. The distances between both TADs were comparable in cells, independent of whether they did or did not express or *Hoxd* genes, further supporting the existence of a poised regulatory structure (Montavon et al. 2011). The direct visualization of TADs at this locus, however, cannot provide information about potential fluctuations within each TAD (Giorgetti et al. 2014).

By using STORM, the most elongated DNA FISH signals were scored in cells from either the developing distal forelimb or the posterior trunk, two tissues where several *Hoxd* genes are strongly active. In forebrain cells where these genes are inactive, signals were relatively compact and circular. These dense structures were also always observed using a control probe located outside the gene cluster, irrespective of the tissue imaged. In distal forelimb cells, however, where the *Hoxd1–Hoxd4* region is covered by H3K27me3 chromatin marks (Andrey et al.

Figure 4. The strongest elongations occur at the TAD boundary. (A) RNA profile of distal forelimb cells showing the expression of *Hoxd8–Hoxd13* (red), aligned with four fosmid probes (black, on 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.

2013), 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 (Andrey et al. 2013), which may lead to tensions elongating the gene cluster.

CONCLUSION

Recent advances in imaging and genomic technologies provide a more comprehensive analysis of the chromatin transitions taking place during transcriptional regulation. In this context, the link between chromatin modifications and transcription is particularly clear for the *HoxD* cluster. In forelimb cells, depending on their proximal versus distal position (Montavon et al. 2011; Andrey et al. 2013), or in tissues with different transcriptional regulations (Soshnikova and Duboule 2009; Ferraiuolo et al. 2010; Noordermeer et al. 2011; Phillips-Cremins et al. 2013), the decoration by histone marks can change drastically. The use of superresolution imaging, such as STORM, now allows observation of the corresponding shape of the gene cluster itself at high resolution such that the association between the chromatin status and

the spatial organization can be reevaluated with more precision.

Chromatin organization at and around the *HoxD* gene cluster may be established before transcriptional activation, as observed in ES cells with both 4C and STORM imaging, and thus may be rather independent from its transcriptional status. The presence of a TAD boundary within the *HoxD* cluster may confer a greater flexibility to regulate compaction and to compartmentalize the genes in their responses to multiple regulatory elements located in different TADs.

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