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The remote transcriptional control of *Hox* genes

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ABSTRACT Since the discovery by Ed Lewis that the order of *Hox* genes on the chromosome reflects the partitioning of their patterning function along the anterior-posterior axis of the developing fruit fly embryo, extensive efforts have been dedicated to uncovering the regulatory events underlying the collinear expression of *Hox* genes. These studies have revealed various aspects of *Hox* regulation, including short-range and long-range transcriptional enhancers, insulator elements and non-coding RNAs. With the development of technologies allowing for high resolution probing of chromatin architecture, notably Chromosome Conformation Capture (3C)-based techniques, a clear relationship is emerging between long-range regulation of *Hox* genes and the three-dimensional organization of the genome. Here, we provide an overview of these studies and in particular we discuss the functional relevance of genome compartmentalization, CTCF- mediated insulation and the Polycomb Repressive Complexes in the remote control of *Hox* genes.

KEY WORDS: *Hox gene, long-range regulation, chromatin conformation, TAD, polycomb group protein*

Introduction

Hox genes encode transcription factors which are essential in the intricate processes that pattern the developing embryo. In mammals and most vertebrates, *Hox* genes are located in four genomic clusters, *HoxA*-*HoxD*, which are found on four different chromosomes. These four *Hox* clusters likely arose from two duplication events of an ancestral cluster (Hart *et al*., 1987; Holland *et al*., 2008), consistent with the fact that genes with the same relative position within the different clusters, referred to as paralogues, share more sequence similarity and function than neighboring *Hox* genes in the same cluster (Krumlauf, 1994). *Hox* genes are categorized in 13 groups of paralogous genes (*Hox1* to *Hox13*) but each cluster contains a varying number of *Hox* genes. Interestingly, within a *Hox* cluster, all genes are transcribed from the same DNA strand and in the same orientation, resulting in an overall 5' to 3' orientation for each cluster, with paralogous group 1 being located at the 3'end of the clusters and paralogous group 13 at the 5'end. A fascinating feature of *Hox* gene clustering is the correlation between their physical position within the cluster and their sequential expression both in time and space, a feature known as temporal and spatial collinearity (Gaunt *et al*., 1988; Dollé *et al*., 1989). In mice and chick embryos, temporal *Hox* gene collinearity is best visualized during early axial extension events, where sequential transcription of *Hox* genes determines the regional identity of the cells generated from the primitive streak, which give rise to the axial and paraxial tissues (Forlani *et al*., 2003; Deschamps and van Nes, 2005; Iimura and Pourquie, 2006). During the extension of the embryonic axis, via posterior elongation, the first set of cells to leave the primitive streak express exclusively 3' *Hox* genes (e.g. *Hoxa1*) and contribute to the formation of anterior structures. Subsequently, the more 5'*Hox* genes become sequentially activated in cells leaving the primitive streak to form more posterior embryonic structures. The sequential activation of *Hox* genes thus results in nested expression patterns of the *Hox* genes, and thereby a succession of distinct combinations of *Hox* proteins along the A-P axis (*Hox* code), which generate morphological diversity along this axis (Kessel and Gruss, 1991; Hunt *et al*., 1991; see reviews by Krumlauf, 1994; Kmita and Duboule, 2003; Mallo *et al*., 2010). While the early signaling events regulating the initial activation of *Hox* genes during gastrulation are the first steps in the partitioning of the main body axis into domains of varying sets of *Hox* transcription factors (Neijts *et al*., 2016; reviewed in Deschamps and Duboule, 2017), the subsequent regulatory processes eventually refine *Hox* expression patterns in a tissue specific manner. These two phases of *Hox* expression have been comprehensively reviewed recently by Deschamps and Duboule (2017). Since the initial discovery by Ed Lewis that *Hox* gene order on the chromosome is directly

Abbreviations used in this paper: 3C, chromosome conformation capture (3C) PcG, polycomb group; PRC1/2, polycomb repressive complex 1/2; TAD, topologically associating domain.

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related to the embryonic domains along the A-P axis patterned by the individual *Hox* genes (Lewis, 1978), major efforts have been dedicated to understand the coordinated control of *Hox* expression. In this review, we discuss recent studies investigating the relationship between the three-dimensional architecture of the genome and the regulation of *Hox* genes.

Remote transcriptional control and *Hox* cluster organization

Early *in vivo* studies in mice identified cis-regulatory elements within the *Hox* clusters contributing to the regulation of *Hox* genes. In several cases, transgenes carrying individual *Hox* genes are capable of recapitulating their endogenous expression pattern when inserted randomly into the genome, indicating that the necessary cis-regulatory element(s) required for their proper gene expression are located either immediately upstream, downstream or within the gene itself. For example, transgenic analysis of the *Hoxd4* locus identified elements 3' and 5' of *Hoxd4*, which were capable of recapitulating the endogenous *Hoxd4* expression in the hindbrain to the proper A- P boundary (Morrison *et al*., 1996; 1997). Similarly, an 18kb DNA fragment encompassing the *Hoxb1* coding sequence was sufficient to recapitulate the endogenous *Hoxb1* expression pattern (Marshall *et al*., 1992; 1994) and retinoic acid-response elements located between *HoxB3-HoxB4* and between *HoxB4-B5* were shown to control the expression of *HoxB5* to *HoxB9* during CNS development (Ahn *et al*., 2014).

Targeted rearrangements at *Hox* clusters revealed the importance of the cluster organization for the coordinated expression of *Hox* genes. For instance, relocalization of *Hoxd9*- and *Hoxd11*-lacZ transgenes into a 5' position within the *HoxD* cluster resulted in their delayed activation as compared to activation of the endogenous *Hoxd9* and *Hoxd11* and the posteriorization of their expression domain along the A-P axis (van der Hoeven *et al*., 1996). In contrast, random insertion of these same transgenes was able to recapitulate the early expression of *Hoxd9* and *Hoxd11* in the tail bud, resulting in a rostral limit of their expression domain reminiscent of the endogenous *Hoxd9* and *Hoxd11* expression (van der Hoeven *et al*., 1996). This raised the possibility that the collinear expression of *Hox* genes is associated with the existence of a repressive mechanism at the 5' end of *Hox* clusters preventing the activation of 5' *Hox* genes during early development. This model was further supported by a series of deletions and inversions within the *Hox*D cluster (e.g. Kondo and Duboule, 1999; Zakany *et al*., 2004; Tarchini and Duboule, 2006). However, the relocation of a *Hoxb1*-lacZ transgene at the 5'end of the *HoxD* cluster resulted in the precocious activation of *Hoxd13* indicating that the insertion of an early enhancer at the 5'end of the cluster can locally disrupt the collinear activation of *Hox* genes (Kmita *et al*., 2000). A local break in collinearity was also observed upon a targeted deletion that resulted in a chimeric *Hoxd13-Hoxd12* gene, which was expressed with a pattern reminiscent of the *Hoxd11* expression pattern (Kondo *et al*., 1998). Together these studies suggested that the collinear expression of *Hox* genes is not simply achieved through a passive spreading of transcriptional activation. The analysis of histone marks for active and repressive chromatin states revealed a progressive transition from a repressive to an active state associated with the sequential activation of *Hox* genes (Soshnikova and Duboule, 2009; Neijts *et al*., 2016). Importantly, the progressive modifications of histone marks coincide with step-wise changes in the 3D conformation of the cluster itself, whereby active genes are physically segregated from inactive ones (Ferraiuolo *et al*., 2010; Noordermeer *et al*., 2011; Noordermeer *et al*., 2014). Accordingly, a series of analyses based on DNA fluorescent *in situ* hybridization showed that the sequential transcriptional activation of *Hoxb* genes correlates with the progressive looping out of these genes from their chromosome territories, whereas the inactive genes remain located within their chromosome territories (Chambeyron and Bickmore, 2004; Chambeyron *et al*., 2005).

While several examples of random relocation of *Hox* transgenes suggest that local cis- regulatory sequences are, in some cases, sufficient to recapitulate the correct spatial distribution of *Hox* expression along the A-P axis, numerous analyses of transgenic lines pointed to the existence of transcriptional enhancers, which are not part of the *Hox* cluster itself. Various targeted modifications within the *HoxD* cluster as well as BAC transgenesis provided the initial evidence that expression in developing limbs and genitalia relies on regulatory sequences located outside the gene cluster (van der Hoeven *et al*., 1996; Zakany *et al*., 1997; Spitz *et al*., 2001). This was subsequently confirmed with the identification of a series of distal limb enhancers located within the large gene desert 5' of the *HoxD* cluster (Spitz *et al*., 2003; Gonzalez *et al*., 2007; Montavon *et al*., 2011) and proximal limb enhancers in the gene desert 3' of the *HoxD* cluster (Andrey *et al*., 2013). The enhancers in the 3' gene desert were also characterized to control *Hoxd* genes in the cecum and in the developing kidneys (Delpretti *et al*., 2013; Di-Poi *et al*., 2007). Although extensive studies regarding long-range regulation of *Hox* genes used the *HoxD* cluster as a model system, evidence for remote control of *Hox* genes have also been obtained for the other *Hox* clusters. For instance, the initial activation of *Hoxa* genes at early stages of gastrulation relies on a series of enhancers (referred to as Ades enhancers) located outside the cluster, in the large intergenic region between *Hoxa1* and *Skap2* (Neijts *et al*., 2016), while expression of *Hox*a genes in developing distal limbs is associated with a series of enhancers located within the 800kb region flanking *Hoxa13* (Berlivet *et al*., 2013). Similarly, BAC transgenic assays probing the 3' region flanking the *HoxA* and *HoxB* clusters lead to the identification of enhancers capable of driving *Hoxa* and *Hoxb* gene expression in the endoderm as well as in heart tissue (Nolte *et al*., 2013). These examples illustrate the importance of remote transcriptional control of *Hox* genes for their coordinated expression and thereby for *Hox*-mediated patterning of developing embryos.

The physical segregation of active and inactive *Hox* genes, together with the long-range activation of *Hox* transcription that requires physical proximity between the remote enhancers and the *Hox* promoters, pointed to the existence of an intimate link between *Hox* regulation and 3D chromatin conformation. With the emergence of experimental tools for probing the 3D architecture of the genome at high resolution, it has become technically possible to investigate the relationship between genome topology and *Hox* regulation. In the following paragraphs, we review the significant advances already achieved and their impact on our understanding of *Hox* regulation.

Layers of genome organization

To unravel the 3D organization of the genome, one must be

able to study the nature of interactions that occur within chromosomes (intrachromosomal) as well as between chromosomes (interchromosomal) and analyze the underlying tendency in contact frequencies. The development of the Chromosome Conformation Capture (3C) technology, designed to assess the frequency of long-range DNA-DNA contacts within a cell population (Dekker *et al*., 2002), has provided a powerful tool to do exactly this. In fact, the use of the Hi-C technique, a modified version of 3C that allows for the unbiased identification of interactions occurring across the entire genome, enabled the discovery of various types of spatial compartmentalization of the genome. Analysis of Hi-C contact maps in human cell lines, at the megabase resolution, revealed that the genome is partitioned into two spatial compartments, an "active" compartment and a "silent" compartment, whereby regions on the same chromosome or on different chromosomes have a significantly higher probability of spatially clustering if they belong to the same compartment (Lieberman-Aiden *et al*., 2009). In agreement with what was described for the fly genome (Sexton *et al*., 2012), these compartments or physical domains correlate with the underlying epigenetic state of the chromatin (Lieberman-Aiden *et al*., 2009). The active compartment is characterized by high levels of transcriptional activity, chromatin accessibility and the enrichment of active histone marks. On the other hand, the silent compartment is more densely packed and transcriptionally repressed as indicated by the trimethylation of lysine 27 of Histone H3 (H3K27me3). Higher resolution Hi-C subsequently identified highly self-interacting regions, named topologically associating domains or TADs (Dixon *et al*., 2012). Interestingly, the structural organization of the genome into TADs is a conserved property across many cell types as well as between mice and humans, suggesting that this organizational framework is largely invariant (Dixon *et al*., 2012). Importantly, disruption of TAD organization through depletion of CTCF in mouse ES cells demonstrated that the structural organization of the genome into TADs is unrelated to the compartmentalization of the genome into active and inactive chromatin regions (Nora *et al*., 2017). The evidence that non-adjacent regions physically interact at a significantly higher frequency within TADs than between TADs, irrespective of the distance separating the two regions, prompted the investigation of insulator or barrier elements at these borders and the possible constraints of the TAD organization on transcriptional control. Indeed, the insulator protein CTCF is significantly enriched at TAD boundaries along with additional factors including, but not exclusively, housekeeping genes and SINE elements (Dixon *et al*., 2012). While the relatively invariant TAD structure limits the range of regulatory contacts between enhancers and promoters (e.g. Nora *et al*., 2012), cell-type specific changes in chromatin contacts occur within TADs (Phillips-Cremins *et al*., 2013). Interestingly, high resolution 5C uncovered a sub-TAD organization, with both invariant sub-TADs as well as tissue-specific sub-TADs existing within the larger context of an invariant TAD structure (Phillips-Cremins *et al*., 2013). Importantly, the occupancy of architectural proteins, such as CTCF, within TADs and not only at the TAD boundaries has been found to be implicated in the finer scale sub- TAD organization of the genome (Phillips-Cremins *et al*., 2013; Vietri Rudan *et al*., 2015). In agreement, comparative studies of human cell lines representing all germ layers revealed that this sub-compartmentalization involves CTCF- and cohesin- mediated chromatin contacts and also coincides with domains bearing distinct chromatin signatures (Rao *et al*., 2014).

Topology and long-range *Hox* regulation

The observation that a TAD boundary resides within the *HoxA* and the *HoxD* clusters (Dixon *et al*., 2012), together with the evidence that, at other loci, disrupting TAD boundaries results in rewiring of enhancer-promoter contacts (Nora *et al*., 2012; Lupianez *et al*., 2015; Franke *et al*., 2016), led to the hypothesis that these intra-cluster TAD boundaries could impose regulatory constraints on the *HoxA* and *HoxD* clusters. This hypothesis was consistent with the bi-modal organization of the *HoxD* cluster observed during limb development (Andrey *et al*., 2013). Indeed, limb development occurs in two phases of transcriptional activation at the *HoxD* cluster: the first phase is controlled by regulatory contacts between the *3'Hoxd* genes and enhancers located in the same TAD (3'TAD), which triggers expression of the *3'Hoxd* genes in early/proximal limbs, followed by a switch to the second phase of regulation by the 5'TAD (on the *Hoxd13* side) and activation of 5'*Hoxd* genes in distal limbs. During the first phase of limb development, the 3'TAD containing 3'*Hoxd* genes is active and covered with active histone marks whereas the 5'TAD is inactive. Then during the second phase, the 3'TAD gets silenced and covered with the repressive H3K27me3 mark while the 5'TAD becomes active and gains the active H3K27ac mark (Andrey *et al*., 2013). Studies of long-range interactions occurring during distal limb development at the *HoxA* cluster led to similar findings whereby the active *5'Hoxa* genes contact distal limb enhancers located in the 5'TAD (Berlivet *et al*., 2013). Similarly, the contacts between the Ades enhancers driving the initial expression of *Hoxa* genes at gastrulation are restricted to genes located in the same TAD as the enhancers, i.e. the 3'TAD (Neijts *et al*., 2016). These studies reveal that the intra-cluster TAD boundary coincides with the partitioning of the cluster into genes controlled by the remote enhancers and those that are not, consistent with the evidence that intra-TAD contacts are favored. Interestingly, the finding that disrupting enhancer activity did not affect the contacts between *5'Hoxa* genes and their limb enhancers, suggested that enhancer activity is not a pre-requisite for long-range enhancer-promoter interactions (Berlivet *et al*., 2013). Accordingly, 4C-seq analyses at the *HoxD* cluster led to the observation that some enhancer-promoter interactions are established regardless of the activity of the enhancers whereas other interactions are set up in conjunction with enhancer activity (Andrey *et al*., 2013; Delpretti *et al*., 2013). For example, in early limb buds, the transcriptionally inactive *Hoxd13* gene interacts with several enhancers in the 5'TAD despite the transcriptionally inactive state of this enhancer landscape and upon activation of the 5'TAD in distal limbs, additional enhancer-promoter contacts are formed (Andrey *et al*., 2013). Evidence for pre-existing interactions between enhancers and promoters was also reported at other loci (e.g. Jin *et al*., 2013; Phillips-Cremins *et al*., 2013; Melo *et al*., 2013) and it was proposed that pre-set enhancer-promoter contacts contribute to defining the set of genes which are targeted by specific transcription factors (Jin *et al*., 2013). On the other hand, a recent study has also shown that upon mouse neuronal differentiation, a large majority of enhancer- promoter contacts are established concomitantly with gene activation (Bonev *et al*., 2017). Together these data suggest that dynamic *Hox* regulation involves both pre-set chromatin contacts, possibly for a rapid induction of gene activation in a cell-type specific manner, as well as a reorganization of chromatin conformation to establish de novo

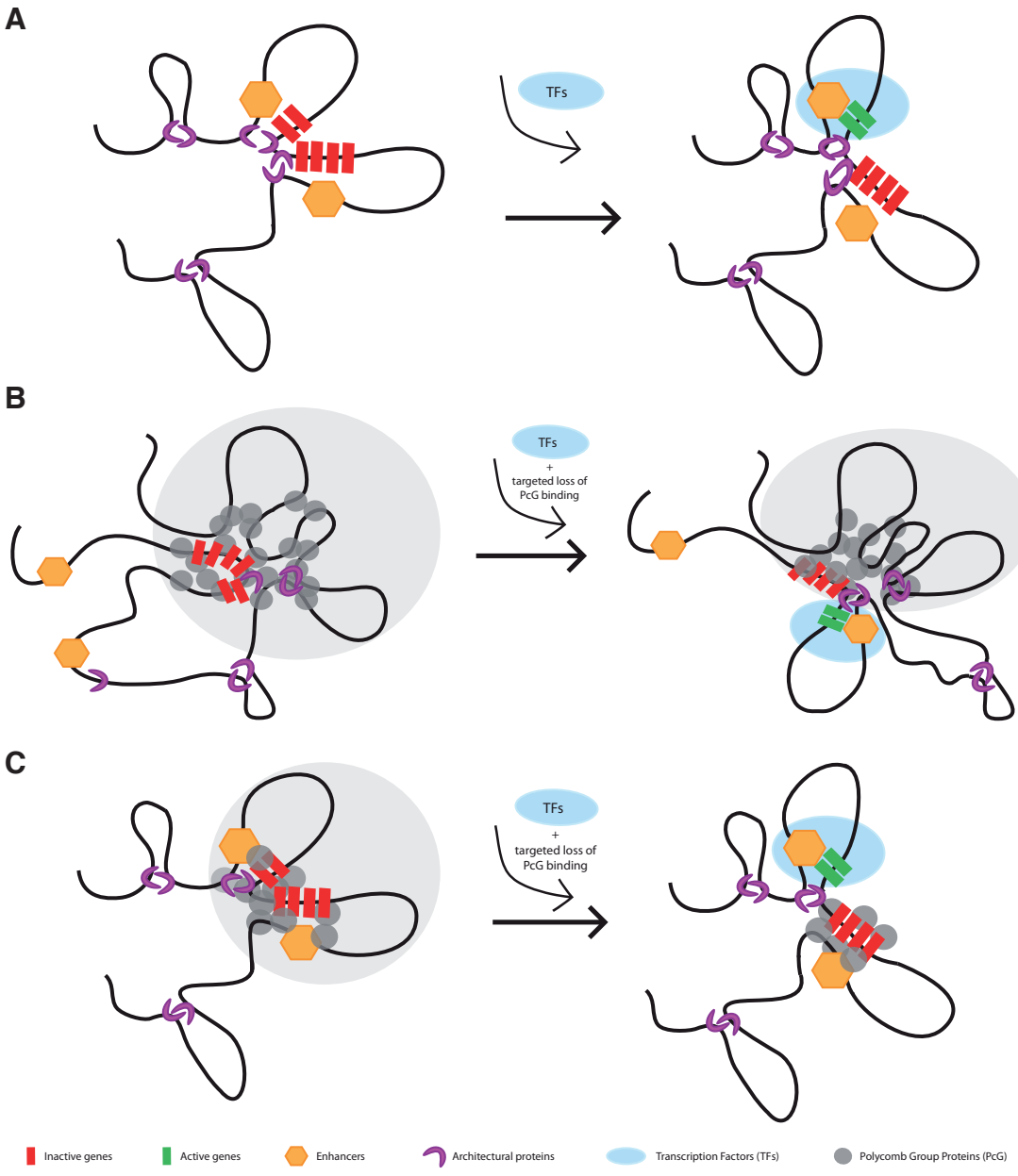

Fig. 1. Chromatin conformation constraints in long-range activation of *Hox* genes. *Schematic representations of three possible scenarios illustrating how chromatin conformation could impact on long-range activation of* Hox *genes. In the first scenario* **(A)**, *the enhancer-promoter contact is pre-set as a result of chromatin folding mediated by architectural proteins (purple), which allows for subsequent rapid gene activation by cell-type specific TFs (blue oval). In the second scenario* **(B)**, *PcG binding creates a repressive PcG hub (light grey circle) which sequesters inactive genes away from their enhancers that may already be in an active chromatin state. Tissue-specific local loss of PcG-repression allows for a novel interaction between two sites bound by architectural proteins that results in enhancer-promoter contact and gene activation. In the third scenario* **(C)**, *PcG binding at enhancer elements and promoters creates a poised configuration as a result of PcG capacity to establish long-range contacts. Upon targeted loss of PcG-mediated repression, a swift activation of the promoters is achieved in the presence of the appropriate transcription factors.*

enhancer-promoter interactions (Fig. 1).

Although long-range enhancer-promoter contacts are largely confined within TADs, whether the TAD boundary is a cause or a consequence of the partitioning of *Hox* clusters into active and inactive domains is unclear. Notably, following the initial identification of a TAD boundary within the *HoxA* and *HoxD* clusters, a more precise assessment of the intra-cluster TAD boundary revealed that its position actually varies in a tissue-specific manner. At the *Hox*A cluster, the TAD boundary was initially located between *Hoxa7* and *Hoxa9* both in mice and human embryonic stem cells (ESCs) and this boundary coincides with a previously validated insulator/CTCF site (Dixon *et al.*, 2012; Kim *et al.*, 2007). However, studies from the Reinberg group showed that the TAD boundary at the *HoxA* cluster is not rigid. Comparative 5C (chromosome conformation capture carbon copy), which measures contact frequency at high resolution (Dostie *et al.*, 2006), revealed that the TAD boundary in mouse

ESCs (mESCs) is located between *Hoxa10* and *Hoxa11*, while in motor neurons (MNs), it is positioned between *Hoxa5* and *Hoxa6* (Narendra *et al*., 2016). As for the *HoxD* cluster, the initial Hi-C analyses in mESCs reported the boundary to fall between *Hoxd9* and *Hoxd10* (Dixon *et al*., 2012). However, 4C-seq data revealing the contact pattern of a regulatory element in the 3'TAD and one in the 5' TAD, suggested that the position of the TAD boundary within the *HoxD* cluster is around *Hoxd10/d11* in distal limb and between *Hoxd11* and *Hoxd12* in proximal limb (Rodriguez-Carballo *et al*., 2017). These tissue-specific variations in the position of the intra-cluster TAD boundary suggests that the positioning of the intra-cluster TAD boundary is modulated by tissue- specific factors/ regulatory events. While this does not exclude the possibility that the boundary imposes some structural constraints to long-range regulation of *Hoxa* and *Hoxd* genes, it raises the possibility of a reciprocal influence between the intra-cluster TAD boundary and transcriptional regulatory events.

CTCF in long-range *Hox* regulation

The various positions of the intra-cluster TAD boundary at the *HoxA* and *HoxD* clusters identified so far coincide with CTCF bound loci, consistent with CTCF being one of the hallmarks of TAD boundaries (Dixon *et al*., 2012; Sexton *et al*., 2012; Van Bortle *et al*., 2014). Interestingly, the deletion of the CTCF sites between *Hoxa5* and *Hoxa6* and between *Hoxa7* and *Hoxa9* in MNs resulted in the repositioning of the TAD boundary to the next intact CTCF site located between *Hoxa9* and *Hoxa10* and resulted in the mis-expression of *Hoxa7*, *Hoxa9* and *Hoxa10* (Narendra *et al*., 2015; Narendra *et al*., 2016). While this shows the importance of CTCF- mediated DNA looping in positioning the TAD boundary, numerous studies (comprehensively reviewed in Phillips-Cremins and Corces, 2013), indicate that CTCF is unlikely to be the only player in the process. At the *HoxA* cluster, CTCF bound loci are identical in mESCs and MNs and yet the intra-cluster TAD boundary is located at a distinct position in these two cell types (Narendra *et al*., 2016). While structural proteins, such as cohesin, contribute to chromosome looping (Phillips-Cremins *et al*., 2013), tissue-specific factors are likely important players in establishing the responsive versus non-responsive part of the *Hox* cluster to remote regulation. Interestingly, genome-wide analyses of the dynamics of genome topology during neuronal differentiation from mESCs suggested that insulation might be influenced by enhancer-promoter contacts and specific transcription factors (Bonev *et al*., 2017). A series of deletions within the *HoxD* cluster also highlighted the importance of CTCF in establishing regulatory insulation within the *HoxD* cluster and demonstrated that deleting the intra- cluster TAD boundary had relatively minor outcomes as long as alternative CTCF bound loci remain (Rodriguez-Carballo *et al*., 2017). However, upon a 400kb deletion encompassing the entire *HoxD* cluster, the 3' and 5' TADs merged despite the presence of two CTCF bound sites, in opposite orientation, remaining in the *Hoxd9-lacZ* transgene (Rodriguez-Carballo *et al*., 2017), further supporting the idea of a multifactorial based mechanism underlying boundary positioning. Moreover, the conditional inactivation of CTCF in limb mesenchyme did not trigger ectopic expression of *Hoxd* genes as expected if there was a loss of boundary (Soshnikova *et al*., 2010). For instance, in CTCF mutant limb buds, the expression of *Hoxd12* and *Hoxd13* remains distally restricted indicating that despite the absence of CTCF, proximal enhancers were not able to activate *Hoxd12* and *Hoxd13* (Soshnikova *et al*., 2010). Thus, while CTCF contributes to partitioning the *Hox* clusters with respect to their responsiveness to long-range enhancers, there are clearly other factors involved.

Polycomb in *Hox* regulation: it might not all be about repression...

Extensive studies have shown that the function of the multi-protein Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) is associated with silencing of *Hox* genes and mediate post- translational histone modifications that in turn affect chromatin compaction. PRC2-mediated trimethylation of H3K27 (H3K27me3) is highly associated with gene repression and has been shown to decorate silent *Hox* gene clusters in mESCs and generate a highly compact structure (Eskeland *et al*., 2010). Analyses of chromatin architecture at the *HoxA*, *B*, *C* and *D* clusters in mouse and human cell lines revealed a dynamic compartmentalization of the clusters associated with differentiation, where active genes are spatially segregated from silent genes (Fraser *et al*., 2009; Rousseau *et al*., 2014), which suggested that Polycomb group (PcG) protein mediated repression of transcription is associated with PcG-dependent compartmentalization of silenced genes (Ferraiuolo *et al*., 2010; Rousseau *et al*., 2014). The dynamic partitioning of *Hox* clusters in active and inactive compartments was also observed *in vivo* during mouse embryogenesis, by comparing chromatin architecture in different tissues (Noordermeer *et al*., 2011) and at various developmental stages in the pre-somitic mesoderm (Noordermeer *et al*., 2014). Interestingly, the tissue-specific variations in the position of the intra-cluster TAD boundary coincides with the dynamic partitioning of the *Hox* cluster in active and inactive compartments, which raises the possibility that PcG-mediated chromatin compaction modulates the position of the TAD boundary. Accordingly, the variation in the position of the TAD boundary in the *HoxA* cluster between mESCs and MNs, despite having similar CTCF binding patterns, was proposed to be associated with qualitative and quantitative differences in CTCF-mediated interactions when CTCF-bound loci are embedded in a Polycomb domain (Narendra *et al*., 2016). As such, this effect of Polycomb presence could also impact enhancer-promoter interactions. For instance, PcG-binding at promoters could sequester genes away from enhancers and upon loss of PcG-binding, quantitative and/ or qualitative variations in CTCF/cohesin looping, would bring the gene into close proximity to the active enhancer, eventually leading to gene activation (Fig. 1B).

The role of PcG proteins in higher-order gene regulation has been an area of increasing interest, leading to some exciting findings. Landmark studies in *Drosophila* demonstrated a role for PcG in mediating long-range interactions required for stabilizing *Hox* gene silencing (Bantignies *et al*., 2011). When genes from the two distinct *Hox* gene clusters ANT-C and BX-C are co-repressed by PcG, they spatially interact in a PcG-dependent manner. In addition, the Polycomb response element (PRE) Fab-7, which regulates *Abd-B* expression, also engages in long-range chromatin interactions with the silent *Hox* genes, highlighting the functional role of PcG at regulatory elements. Similarly, DNA FISH and 4C-seq analyses in different mammalian cell types revealed that H3K27me3-marked loci, notably at *Hox* clusters, physically interact to form both long-range intra- and inter-chromosomal contacts (Vieux-Rochas *et*

al., 2015). In addition, studies in mESCs showed that loci bound by the PRC1 protein RING1B frequently interact, especially the PRC1- bound *Hox* clusters, and these long-range inter- and intra-chromosomal PRC1-mediated contacts are lost upon knockout of *Ring1B* (Schoenfelder *et al.*, 2015). Thereby, in addition to CTCF/cohesin-mediated looping, PcG-associated long-range interactions contribute to the overall topology of the genome. Accordingly, high resolution Hi-C maps from mESCs, neural progenitors and cortical neurons revealed that there is a significant decrease in long-range interactions involving PcG during neuronal differentiation (Bonev *et al.*, 2017). The evidence for long-range interactions between PcG-occupied regions suggests that Polycomb group proteins likely create structural constraints on the 3D genome architecture that are not solely due to PcG-mediated compaction. For instance, PcG-mediated long-range interactions could be one of the mechanisms which pre-sets enhancer-promoter proximity. PcG binding at both an enhancer and promoter (or nearby) could trigger spatial proximity between the enhancer and the promoter, thereby allowing for a rapid activation of the genes upon loss of PcG binding and provided that the appropriate trans-acting factors are present (Fig. 1C). Evidence for PcG-dependent poised enhancer-promoter contacts was obtained both for PRC1 and PRC2 (Kondo *et al.*, 2014; Schoenfelder *et al.*, 2015; Cruz-Molina *et al.*, 2017). Overall, the series of evidence showing that Polycomb group proteins influence genome topology opens a novel perspective on the role of PcG in transcriptional regulation, whereby in addition to their well-known repressive role, PcG proteins also positively impact enhancer-promoter connectivity as a consequence of PcG-mediated long-range chromatin contacts.

Conclusion

Since the initial studies aimed at understanding *Hox* collinearity, changes in higher order structure of the *Hox* clusters has been proposed as a mechanism underlying the collinear expression of *Hox* genes. A link between chromatin architecture and *Hox* regulation was further supported with the evidence of remote enhancers controlling *Hox* genes in diverse tissues/organs. With the development of molecular tools allowing for high resolution probing of genome topology, an unprecedented assessment of chromatin architecture in the context of *Hox* regulation is ongoing. Addressing the role of chromatin topology in *Hox* regulation undeniably relies on our comprehension of the mechanistic factors establishing and modulating the 3D architecture of the genome. Cell-type specific changes in long-range interactions have been shown to occur primarily within TADs and have led to the model whereby TAD boundaries are implicated in restricting remote enhancers' activity over *Hox* clusters. On the other hand, the tissue-specific modulation of the position of the TAD boundary within *Hox* clusters raises the possibility that TADs correspond more to a permissive chromosome compartment than a structural constraint for long-range regulation, at least at *Hox* loci. The evidence that looping, involving CTCF sites within *Hox* clusters, can get rewired and varies in strength/frequency in a cell type/tissue-specific manner suggests a functional cooperation and/or interference between architectural proteins and tissue- specific factors in spatially organizing the genome. The challenge will be to establish the extent to which the functional interplay between tissue-specific factors and architectural proteins impact the remote control of *Hox* genes.

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