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At the base of colinear Hox gene expression: *cis*-features and *trans*-factors orchestrating the initial phase of Hox cluster activation

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A B S T R A C T

Hox genes are crucial players in the generation and pattering of the vertebrate trunk and posterior body during embryogenesis. Their initial expression takes place shortly after the establishment of the primitive streak, in the posterior-most part of the mouse embryo and is a determinant step for setting up the definitive Hox expression boundaries along the antero-posterior body axis. The developmental signals and epigenetic mechanisms underlying this early activation remained unsolved until recently. The development of novel embryo-derived model systems, combined with methods that examine chromatin status and chromosome conformation, led to deeper understanding of the process of Hox activation in the early embryo. Here we summarize how the early Hox *cis*-regulatory landscape becomes active upon receiving the appropriate developmental signal, and we discuss the importance of the local topological segmentation of the HoxA cluster during early Hox activation.

1. Introduction

One of the most fascinating gene regulatory processes in developmental biology is the onset of temporal and spatial colinear expression of Hox genes (Kmita and Duboule, 2003; Krumlauf, 1994). Each of the four mammalian Hox clusters – A, B, C and D – starts to be activated at its 3' side. The process gradually extends to the middle of the clusters in register with developmental time, until it reaches the 5'-most genes, the Hox13 paralogs. The colinear relationship between the position of a given Hox gene on the chromosome and its spatial domain of activity was first observed in *Drosophila* (Lewis, 1978). Since then, spatially colinear expression of Hox genes has been demonstrated to be widespread in the animal kingdom, whereas temporal colinearity was shown to be restricted to bilaterians that have maintained their Hox clusters in a relatively intact organization (Duboule, 2007; Noordermeer and Duboule, 2013). The sequential turning on of Hox genes over developmental time provides precursors of embryonic tissues with position-specific Hox information along the trunk axis and along appendicular axes such as the limb and external genitals. Timing of initial Hox gene expression is intimately linked to the later spatial expression domains of these genes. These domains – and therefore the timing – of Hox gene expression are crucial for normal embryonic development, and ectopically expressed Hox genes cause severe developmental abnormalities. A striking example of failure to obey this requirement is the premature arrest of posterior axial growth by precociously expressed Hox13 genes (Young et al., 2009).

In this review we focus on the initial onset of Hox gene expression that shortly follows the specification of the primary body axis. The emergence of novel model systems to study early embryogenesis, like epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007) and other embryonic stem cell (ESC)-derived systems (Etoc et al., 2016; Henrique et al., 2015), accelerated our understanding of the cellular, genetic and epigenetic aspects of the regulation of early developmental genes like Hox genes. These new models combined with the application of chromosome conformation capture-based technologies, and methods that examine the chromatin status (like ChIP-seq and ATAC-seq) revealed molecular genetic mechanisms that were thus far not easy to explore in early embryos. We discuss the developmental signals and epigenetic events that are at the basis of the transcriptional initiation of the earliest Hox gene expression. We describe the interplay between *trans*-acting factors and *cis*-regulatory elements of the polarized Hox landscape guiding progression of Hox gene expression towards the precursors of the tissues that will generate the vertebrate axial structures.

2. Early induction signals and initial Hox activation in the gastrulating embryo

2.1. Priming, initiation and spreading of early Hox gene transcription

In the mouse embryo, gastrulation starts in the proximo-posterior

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epiblast, at a site demarcated by expression of *T Brachyury* and *Wnt3* at embryonic day (E)6.2 (Rivera-Perez and Magnuson, 2005). During early gastrulation the primitive streak gradually extends towards the distal tip of the embryonic egg cylinder. The cells that first ingress through the streak do not contribute to the embryo proper, but to extraembryonic tissues among which the allantois (Lawson et al., 1991). The first Hox-positive region in the embryo is the very posterior part of the fully extended primitive streak at E7.2 (Deschamps and Wijgerde, 1993; Forlani et al., 2003; Gaunt and Strachan, 1994). It was shown that the earliest Hox gene is primed for expression one full day before transcripts can be detected by *in situ* hybridization (Forlani et al., 2003). After the 3'-most Hox gene is turned on in the posterior streak area, its expression domain spreads anterior-wards by a process that does not involve cell migration (Deschamps and Wijgerde, 1993; Forlani et al., 2003; Gaunt and Strachan, 1994). The transcript domain then reaches the anterior part of the streak that is now known to harbor axial progenitors among which long term bipotent neuromesodermal progenitors (NMPs) (Wilson et al., 2009; Wymeersch et al., 2016). More 5'-located Hox genes start to be transcribed in the posterior streak area subsequently to the 3' genes. Their expression domain in its turn spreads anteriorly. The anterior streak region, corresponding to the NMP-containing growth zone from which the trunk axial tissues will be formed, thus sequentially expresses more and more posterior Hox genes (Fig. 1) (Deschamps and van Nes, 2005). Although this first wave of Hox expression does not yet concern differentiated tissues from the definitive embryonic germ layers, timing defects at this early stage of Hox transcription result in phenotypic abnormalities. Deletion of an early *Hoxc8* enhancer, which is active during the initial phase of the gene expression, results in homeotic transformations along the vertebral column later on (Juan and Ruddle, 2003). The Hox expression domains later extend more anteriorly than the node and reach their spatial boundaries in the paraxial mesoderm and, independently, in the neurectoderm (Deschamps and van Nes, 2005; Forlani et al., 2003). The precise timing of early Hox initiation in the primitive streak is a first and determinant step for the later setting up of the Hox expression boundaries in the embryo.

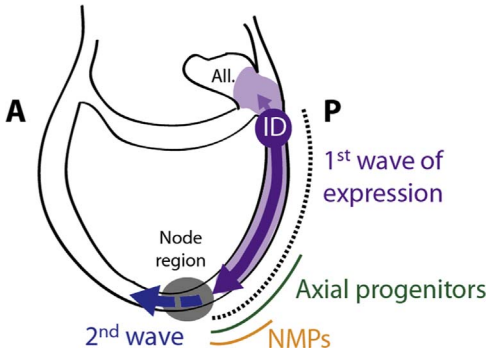

Fig. 1. Schematic representation of the two waves of Hox expression in the early posterior embryo. *Hoxa1* expression (in purple) starts in the posterior-most part of the streak (ID, induction domain) at E7.2. From the ID, cells expressing Hox contribute to extra-embryonic tissue (the allantois, all.; small purple arrow) and *Hoxa1* expression spreads anterior-wards towards (large purple arrow). This spreading signal reached the anterior streak and node region, which harbor axial progenitors including NMPs. The more posterior Hox genes sequentially follow the same expression dynamics. As a consequence these progenitors are sequentially expressing more and more posterior Hox genes with time. In the second wave, the Hox expression acquired by the axial progenitors in the node region is transmitted to the descendants of these progenitors and regulated independently in neurectoderm and mesoderm contributing to the elongating axis. The process started at the ID in prospective extraembryonic mesoderm ends up patterning the embryonic tissues along the antero-posterior axis. The primitive streak is indicated by a dashed line. A, anterior. P, posterior.

2.2. Hox initiation signals: Wnt ligands and other candidates

2.2.1. Wnt as instructive Hox initiation signal

An involvement of Wnt/β-catenin in antero-posterior polarity of the body axis predates the bilaterian ancestor, since a Wnt-dependence in the primary axis patterning is observed amongst cnidarians (Petersen and Reddien, 2009). In mouse embryos, early anterior-posterior regionalization is manifested by asymmetrical expression of *Wnt3* (Rivera-Perez and Magnuson, 2005), which is restricted to the proximal and posterior epiblast. *Wnt3* has been shown to be essential for the formation of the primitive streak and gastrulation. *Wnt3* mutant embryos do not express any Hox genes (Liu et al., 1999). Involvement of Wnt signals in the initiation 3' Hox genes was suggested by Forlani et al. (2003). Pre-gastrulation embryos exposed to the Wnt agonist Chiron precociously express *Hoxa1* and *Hoxb1* (Neijts et al., 2016) demonstrating the early responsiveness of 3' Hox genes to Wnt signals. Epiblast stem cells (EpiSCs) generated from wild type or *Wnt3* mutant epiblast start to express Hox genes as soon as Wnt signaling is provided. Moreover, deletion of Wnt-sensitive enhancers in the 3' regulatory landscape of HoxA prevent Wnt-induced activation of *Hoxa1* (Neijts et al., 2016).

The signal inherent to posteriorization in bilaterians – the Wnt-dependent stabilization of β-catenin – thus functions as a master regulator to initiate Hox gene expression at a suitable time point in the embryo. It allows the Hox transcription domains to reach the axial progenitor region at the time when the hindbrain to caudal-most tissues are laid down from progenitors in the posterior embryonic growth zone. Interestingly, it has been hypothesized that the Hox gene regulation was co-opted with the Wnt regulatory network in animals predating the last common ancestor of cnidarian and bilaterian animals (Ryan and Baxevanis, 2007). From that co-option on, Wnt signaling and Hox expression would be at work in concert in a posterior genetic network underlying body extension and patterning.

2.2.2. Is RA required for Hox initiation?

Numerous lines of evidence, *in vivo* and in embryo-derived model systems, have shown that retinoids are able to influence Hox gene expression during embryogenesis (Gavalas and Krumlauf, 2000; Oosterveen et al., 2003). RA is a well-known inducer of colinear Hox gene expression in pluripotent mouse embryonal carcinoma cells, embryonic stem cells (ESCs) and human pluripotent cells (Agger et al., 2007; Breier et al., 1986; Chambeyron and Bickmore, 2004; Colberg-Poley et al., 1985; Simeone et al., 1990). Despite the ability of RA to induce Hox genes in pluripotent cell lines and its effect on Hox expression in late developing embryos, the role of endogenous retinoids in the initial activation of the clusters in vertebrates remains uncertain.

In murine early somite embryos, significant levels of retinoids have been found in the node region (Hogan et al., 1992). RA was not found where and when Hox gene transcription is primed and initiated, in the posterior part of the primitive streak. Embryos deficient for the RA-producing enzyme Aldh1a2 (or Raldh2) could still initiate expression of the 3' Hox gene *Hoxb1* (Roelen et al., 2002) and were able to gastrulate and to generate some anterior somites (Niederreither et al., 1999). In addition, the deletion of a proximal RA-responsive element (RARE) at the *Hoxa1* locus affects the gene expression level at E7.5, but it does not prevents the initial transcriptional induction (Dupe et al., 1997). Deletion of a RARE 3' near *Hoxb1* leads to the absence of *Hoxb1* transcription in the posterior part of the E8.25 embryos, but the situation was not examined at the time of endogenous *Hoxb1* initiation (around E7.2) (Marshall et al., 1994).

In summary, the Hox clusters are responsive to exogenous RA signals from early stages on. But despite this RA sensitivity and the fact that retinoids induce colinear Hox gene expression in pluripotent cell lines, a role for endogenous RA in initial Hox induction remains unestablished.

2.2.3. Are Cdx genes involved in Hox transcriptional initiation?

Cdx and Hox genes are evolutionarily linked as they both derive from an ancient protoHox gene or gene cluster (Pollard and Holland, 2000). The similar *in vivo* expression dynamics of 3' Hox and Cdx genes are obvious. Both gene subfamilies start to be expressed in the posterior primitive streak around E7.2 and their transcription similarly spreads anterior-wards (Young and Deschamps, 2009). Like the 3' Hox genes, *Cdx2* is initially induced by Wnt signals in epiblast-derived EpiSCs (Amin et al., 2016). Cdx genes were found to regulate trunk Hox genes and to modify the identity of axial trunk tissues accordingly (Charite et al., 1998; Gaunt et al., 2004; Shashikant and Ruddle, 1996; Tabaries et al., 2005). Cdx mutants that over-express trunk Hox genes were rescued in their posterior truncation phenotype (Young et al., 2009); both Cdx and Hox genes are involved in the maintenance of NMPs during axial elongation (Amin et al., 2016; Neijts et al., 2014).

Our laboratory recently identified the direct downstream targets of Cdx2, including Hox genes, by ChIP-seq experiments in Wnt-stimulated EpiSCs (Amin et al., 2016). We have found that Cdx is required for making the DNA accessible at several *cis*-elements within the middle part the Hox clusters (Neijts et al., 2017). In contrast, the chromatin at the 3' parts of the HoxA and HoxB clusters is open and decorated by active histone mark H3K27ac independently of Cdx. These findings point to a function of Cdx during the activation of the middle/trunk Hox genes in the post-initiated clusters (Neijts et al., 2017). In accordance, Cdx triple mutant embryos could still initiate the early *Hoxb1* gene like wild type embryos (van Rooijen et al., 2012). Therefore it is unlikely that Cdx transcription factors are involved in the initial transcription of 3' Hox genes. Rather they control the activation of the subsequent genes in the middle of the cluster (Neijts et al., unpublished; van Rooijen et al., 2012).

3. Dissecting the Hox locus: *cis*-regulatory modules

3.1. The Hox regulatory landscape is subdivided over topological domains

The expression of a developmental gene is regulated by its *cis*-regulatory landscape consisting of enhancers, insulators and other architectural elements which can be located proximal to the gene or dispersed over large genomic distances [reviewed by Spitz (2016)]. Besides numerous regulatory elements within the Hox clusters themselves, the large gene-poor regions that flank the clusters on both sides – in particular HoxA and HoxD – harbor various elements that influence Hox spatiotemporal expression during development (Montavon and Duboule, 2013). Over the years, the long-range regulatory potential of these genomic landscapes has been intensively studied using a comprehensive collection of mouse strains carrying genomic rearrangements (Tschopp and Duboule, 2014). More recently genome-wide approaches such as ChIP-seq and ATAC-seq (Buenrostro et al., 2013) made it possible to identify and map different *cis*-regulatory elements in and around the clusters.

In addition to proximal and distal *cis*-regulatory sequences and their chromatin, the three-dimensional organization and the spatial compartmentalization of loci play a major role in developmental gene regulation (de Laat and Duboule, 2013). Initially, DNA-FISH has been the most pertinent method to study the physical architecture of the Hox loci. Using this method it was observed that transcriptionally induced Hox genes could loop out of their 'chromosome territory' during HoxB activation (Chambeyron and Bickmore, 2004). This chromatin dynamics of HoxB was first observed in RA-induced ESCs, and later confirmed in gastrulating embryos (Chambeyron et al., 2005). The introduction of chromosome conformation capture-based approaches, like 3 C, 4C-seq and HiC [reviewed by Denker and de Laat (2016)] in developmental biology allowed the in-depth study of the genome architecture, chromatin compartments and gene-enhancer contacts – and their dynamics – over developmental time. Using 4C-seq, the Duboule laboratory observed that the activated genes shift from an inactive chromatin domain to an active domain during colinear activation of Hox gene expression between E8.5 and E12.5 in tissues along the embryonic axis, (Noordermeer et al., 2011). DNA-FISH and 4C-seq independently established the concept that the conformation of Hox loci is very dynamic during progressive Hox gene activation, and that higher order re-arrangements take place over time as colinear Hox expression continues.

The discovery of the partition of chromosomes into large segments called 'topologically associating domains' (TADs) (Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012) was very important to understand the regulatory logic of the Hox *cis*-landscape. The HoxA and HoxD clusters were found to lie at the junction of two topological domains (Dixon et al., 2012). It is this boundary position that probably allowed a bimodal and stepwise regulation to control the expression of 3' Hox genes separately from their 5' neighbors during mouse limb outgrowth, and probably during axial development as well (Andrey et al., 2013; Beccari et al., 2016; Darbellay and Duboule, 2016; Sheth et al., 2016). Such a TAD-based bipartite regulatory mode of subsets of genes was not seen for the HoxB and HoxC clusters (Dixon et al., 2012). Interestingly, these latter clusters have an inherent *cis*-organization imposing a physical separation of the 3' and 5' Hox genes, *Hoxb13* is located so remotely that it is isolated from the *Hoxb1-Hoxb9* genes, and HoxC lacks the 3' part of the cluster.

The distribution of the HoxA and HoxD clusters over two different TADs appeared in an ancestor in the vertebrate lineage. The bipartite architecture is present in snakes and teleosts (Guerreiro et al., 2016; Woltering et al., 2014), whereas only a single (3') TAD exists on the Hox locus of the early chordate amphioxus (Acemel et al., 2016). It is possible that the origin of the 5' TAD is linked to the appearance of novel body appendages at the root of the vertebrate lineage, among which the fin. Evolutionarily conserved functional regulatory elements within the 5'Hox TAD have been found in teleosts (zebra fish) and in a more primitive bony fish (the spotted gar) (Gehrke and Shubin, 2016). Strikingly the 5' elements active in the fin of the spotted gar drive gene expression in the mouse distal limb (Gehrke et al., 2015) similarly to the corresponding mouse elements residing in the HoxA and HoxD 5' TADs (Andrey et al., 2013; Berlivet et al., 2013; Montavon et al., 2011). The presence of a functional second Hox TAD seems to correlate with the evolutionary emergence of a secondary axis. It is not ruled out that the bimodal regulation alternatively might have arisen for appropriate controle of the vertebrate primary axis, since tight and differential regulation of posterior *versus* early (anterior) Hox genes is primordial for building the axial body structures from anterior to posterior.

3.2. The early 3' Hox cis*-regulatory landscape*

The molecular events underlying the earliest onset of Hox gene expression were recently studied in Wnt-stimulated epiblast-derived stem cells (EpiSCs) representing the posterior post-implantation epiblast (Neijts et al., 2016). A bipartite distribution of contacts was observed by 4C-seq between genes of the HoxA cluster located within the intersection of the two large TADs (Fig. 2A). The 3' part of the cluster (*Hoxa1-Hoxa3*) interacts heavily with a proximal Hox flanking region including the neighboring gene *Skap2* (Neijts et al., 2016). This region was identified as being a 3' subTAD (Fig. 2)(Neijts et al., 2016). In this subTAD, several Wnt responsive (called 'Ades') enhancers are active in the posterior-most part of the streak at the moment of Hox initiation. The compact conformation of the HoxA early 3' neighborhood together with the 3' side of the cluster, their chromatin structure and the presence of multiple Wnt responsive enhancers, constitute an environment that is primed for gene expression on that side in response to incoming Wnt signals. The trunk Hox genes and the late *Hoxa13* lack intense contacts with the early 3' subTAD and are thus isolated from the Wnt-activated enhancers.

Besides the above described enhancers, the 3' flanking region of

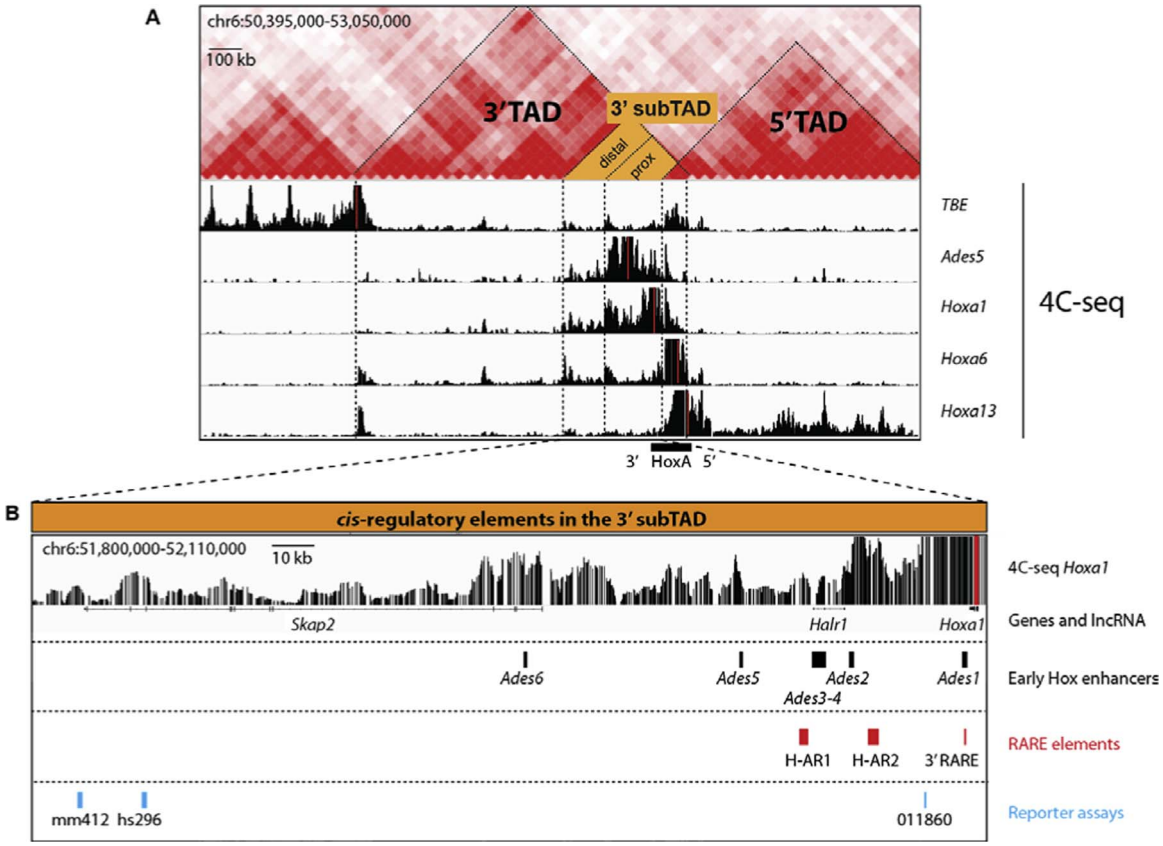

Fig. 2. The architecture of the HoxA locus and its 3' flanking region.A) Global *cis* conformation of the HoxA locus, which is located at boundary domain of a 3' TAD and a 5' TAD. 4C-seq viewpoints are taken from the *TBE* element at the 3' boundary of the 3' TAD, the *Ades5* enhancer and *Hoxa1*, both within the 3' subTAD (indicated in yellow), and *Hoxa6* and *Hoxa13*. HiC data from Dixon et al. (2012). B) The 3' flanking region of the HoxA cluster, *Skap2* and lncRNA *Halr1*. Regulatory functions of Ades enhancers were described by in Neijts et al. (2016). *Halr1* functions and dynamics are described in Guttman et al. (2011), Maamar et al. (2013), De Kumar et al. (2015), Yin et al. (2015), Liu et al. (2016). The early 3' RARE was described by Langston and Gudas (1992). H-AR1 and H-AR2 domains are in De Kumar et al. (2015). Public databases VISTA (Visel et al., 2007) and TRACER (Chen et al., 2013) revealed additional elements (mm412 and hs296, VISTA) and regulatory response, respectively (011860, TRACER). 4C-seq viewpoints are indicated with red lines.

HoxA harbors additional regulatory information. Krumlauf and colleagues investigated a 40 kb-large portion of the proximal HoxA 3' subTAD (Nolte et al., 2013). The proximal-most 10 kb, containing the *Ades1* enhancer, contains a 3' RARE (Dupe et al., 1997; Langston and Gudas, 1992), and generates a pattern that is very similar to endogenous *Hoxa1* expression (Nolte et al., 2013). Moreover, the 3' subTAD harbors the long non-coding RNA (lncRNA) *Halr1* that resides in the *Heater* locus (De Kumar and Krumlauf, 2016; De Kumar et al., 2015). Several groups have been dissecting the function of this region in ESCs (De Kumar et al., 2015; Guttman et al., 2011; Liu et al., 2016; Maamar et al., 2013; Yin et al., 2015). *Halr1* acts as a transcriptional enhancer and is activated upon RA exposure. This lncRNA acts on the HoxA locus *in trans* to repress gene expression by preventing H3K27me3 demethylation (Liu et al., 2016; Yin et al., 2015). Although *Halr1* was show to be important for proper activation of the HoxA locus in ESCs, mice lacking the lncRNA had no reported developmental abnormalities (Lai et al., 2015; Sauvageau et al., 2013), suggesting a role for *Halr1* in later fine-tuning rather than in any crucial early transcriptional control. Besides different isoforms of *Halr1* from both strands, unspliced short transcripts are also produced from the *Heater* region (De Kumar et al., 2015). As *Ades3-4* and *Ades2* enhancers are located in this interval, they could be responsible for the transcription of 'enhancer-RNA' (eRNA) (Li et al., 2016) that include unspliced short fragments. It is not known whether these additional regulatory inputs impact on the initiation of the 3' Hox gene expression. Whatever the case, the *Heater* transcripts do not respond to Wnt signals in EpiSCs (our unpublished data). In addition, the absence of *Halr1* transcripts in differentiated ESCs expressing the nascent mesodermal marker *T Brachyury* (Yin et al., 2015), suggests that *Halr1* and the Ades enhancers follow different regulatory rules and answer to different regulatory input. The most important regulatory elements which are identified in the early regulatory landscape flanking HoxA are summarized in Fig. 2B.

3.3. The topological segmentation of Hox restricts regulatory input in time and space

Besides a 3' subTAD we could identify additional segments in HoxA (see Fig. 3) (Neijts et al., 2016). 5' to the early HoxA genes, a middle segment within the cluster harbors *cis*-elements are dependent on Cdx transcription factors for their accessibility and activity (Neijts et al., 2017). Since expression of Cdx genes is induced by Wnt as well, this transcription factors inherently function as secondary activators. Lastly, a 5' segment contains *Hoxa13* and its associated enhancers. Upon Wnt-stimulation of EpiSCs we observed that the cluster topology becomes reorganized in the vicinity of the boundaries of these segments (Neijts et al., 2016). The compartmentalization of the HoxA cluster and its flanking regulatory domains allows a segmental-wise – and relatively independent – activation of the different sets of Hox genes. It seems to constitute a good strategy to restrict regulatory impact to genes within each segment, without jeopardizing the regulation of the other gene subsets.

The 3' subTAD may have formed an evolutionary playground for early and anterior regulation. In the topologically isolated 3' subTAD a diversity of transcriptional enhancers would have arisen that act later in anterior axial tissues like the hindbrain, cranial neural crest cells and branchial arches (Maconochie et al., 1999; McEllin et al., 2016; Neijts et al., 2016; Parker et al., 2014). In addition, the topological segmenta-

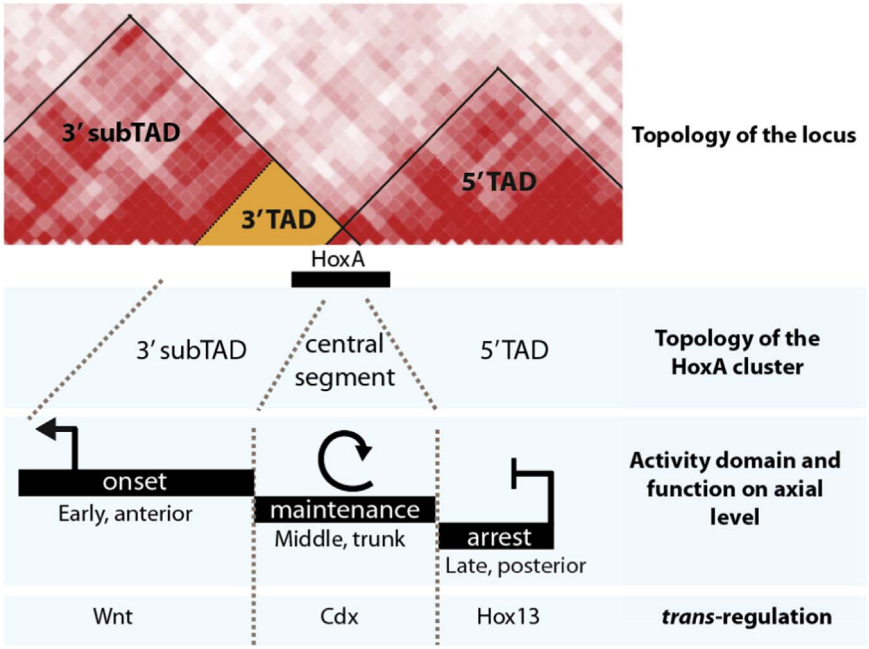

Fig. 3. *Cis* and *trans*-regulatory features of the HoxA locus from transcriptional initiation to the completion of axial elongation. Upper panel, The HoxA locus is lying across the junction between the 3' TAD, that included a proximal 3' subTAD (in yellow), and the 5' TAD. HiC data from Dixon et al. (2012). Lower panel, Partition of the HoxA cluster and its flanking regions into three segments, respectively the 3' subTAD, a central part, and the 5' TAD. The 3' subTAD is activated early by Wnt signals in the induction domain of the anterior-most HoxA genes (see Fig. 1). The central part of HoxA is activated by Cdx transcription factors following the initial Wnt-mediated induction of 3' HoxA genes. It ensures central Hox gene expression to maintain axial progenitors of the trunk and to pattern trunk tissues. The 5' part of HoxA harbors and regulates *Hoxa13*. It is expressed relatively late and is responsible for the arrest of axial elongation – and for the transition between proximal and distal Hox expression programme in the limb buds (Beccari et al., 2016; Sheth et al., 2016).

tion allows a 3' enhancer residing outside the 3' subTAD, the very distant *TBE* (1 Mb away from *Hoxa1*), to exert its regulatory function on trunk and posterior HoxA cluster members while ignoring the 3' segment (Neijts et al., 2017).

The molecular basis of the ontogenesis of the 3' TAD, and the HoxA organization into segments are not yet understood. Binding of the structural protein CTCF is generally associated with the boundaries of TAD domains (Dixon et al., 2012; Yaffe and Tanay, 2011). CTCF is involved in defining the activity domains on the chromosome of ESC-derived motor neurons, as deletion of intra-cluster CTCF sites results in an undue spreading of chromatin modification (Narendra et al., 2015). Whether CTCF plays a role in the stepwise activation of the Hox cluster in the early embryo is unknown. The initiation of Hox gene transcription is very likely independent of CTCF binding as CTCF occupancy is mainly found at the 5' half of the cluster and not at the 3' end in ESCs and in human pluripotent cell lines (Ferraiuolo et al., 2010; Narendra et al., 2015). The important CTCF site *CBS5* between *Hoxa7* and *Hoxa9* (Kim et al., 2011) marks a topological boundary in ESCs (Dixon et al., 2012) and in EpiSCs, and demarcates the 5'-limit of the Cdx2 binding domain (Neijts et al., 2017). Therefore, CTCF might rather play a role during the post-initiation Hox regulation, as supported by a recent study (Narendra et al., 2016).

4. Final remarks

The topological organization of the HoxA cluster stands at the basis of 3'oriented polarity in Hox cluster activation by incoming developmental signals. The battery of Wnt-responsive enhancers at the early side of the cluster elicits a robust transcriptional activity, the action of which is very locally restricted to the genes and elements present in the 3' subTAD. The 5' TAD does not contain early Wnt-responsive enhancers. Genes in the middle segment of the cluster do not rapidly react to the initial Wnt signals like 3' Hox genes do. They were found to be initially fully covered by repressive histone modifications and depend on Cdx-driven chromatin opening for activation.

The three Hox clusters with an intact 3' region (*i.e.* A, B and D) are responding to Wnt exposure by activating the transcription of their 3' genes in EpiSCs (Neijts et al., 2016). However, they each must use different or additional strategies to bring about 3'-polarized Wnt-dependent transcriptional initiation of the locus. The HoxB cluster presents a localized depletion of PcG-PRC1 Polycomb component Ring1b coverage at the 3'-most gene, Hoxb1 (Neijts et al., 2016). Upon Wnt-exposure, the *Hoxb1* region – and its 3' enhancers (Marshall et al., 1994; Nolte et al., 2013) – may function as an exposed 'docking' region for the transcriptional machinery. In conclusion, evolution has modelled the 3' landscape and structural features of the Hox clusters in varying ways ending up with their sensitivity to transcription activating Wnt signals in the gastrulating embryo, unleashing the cascade of events that will allow trunk and posterior tissues to be correctly laid down and patterned.

Acknowledgments

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