Developmental Biology 422 (2017) 146–154

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Cdx is crucial for the timing mechanism driving colinear Hox activation and defines a trunk segment in the Hox cluster topology

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ARTICLE INFO

Keywords:
Hox gene regulation
cis-regulatory elements
Chromatin organization
DNA accessibility
Cdx
Trunk development

ABSTRACT

Cdx and Hox transcription factors are important regulators of axial patterning and are required for tissue generation along the vertebrate body axis. Cdx genes have been demonstrated to act upstream of Hox genes in midgestation embryos. Here, we investigate the role of Cdx transcription factors in the gradual colinear activation of the Hox clusters. We found that Hox temporally colinear expression is severely affected in epiblast stem cells derived from Cdx null embryos. We demonstrate that after initiation of 3′ Hox gene transcription, Cdx activity is crucial for H3K27ac deposition and for accessibility of *cis*-regulatory elements around the central – or 'trunk' – Hox genes. We thereby identify a Cdx-responsive segment of HoxA, immediately 5′ to the recently defined regulatory domain orchestrating initial transcription of the first Hox gene. We propose that this partition of HoxA into a Wnt-driven 3′ part and the newly found Cdx-dependent middle segment of the cluster, forms a structural fundament of Hox colinearity of expression. Subsequently to initial Wnt-induced activation of 3′ Hox genes, Cdx transcription factors would act as crucial effectors for activating central Hox genes, until the last gene of the cluster arrests the process.

1. Introduction

Hox and Cdx genes, derived from an ancient ProtoHox gene cluster, are pivotal regulators of axial patterning and tissue generation along the vertebrate body axis (Kmita and Duboule, 2003; Krumlauf, 1994; Young and Deschamps, 2009). Mutations in Cdx genes lead to arrest of axial extension (Chawengsaksophak et al., 2004, 1997; van Rooijen et al., 2012) as a result of the exhaustion of axial progenitors in the posterior growth zone (Amin et al., 2016; Neijts et al., 2014; Young et al., 2009). Cdx genes are key regulators of Hox genes, as their inactivation affects central Hox gene expression at midgestation, which in turn leads to homeotic transformations in the vertebral column (Subramanian et al., 1995; van den Akker et al., 2002). Strikingly, central – or 'trunk' – Hox genes *Hoxa5* and *Hoxb8* are able to rescue Cdx mutant truncation phenotypes (Young et al., 2009).

Recently we have used epiblast stem cells (EpiSCs), derived from the epiblast of pre-gastrulation embryos (embryonic day 6.0, E6.0), to study the molecular mechanism of Hox gene induction (Neijts et al., 2016). We have demonstrated that the HoxA cluster, and its primed early *cis*-regulatory landscape, is transcriptionally activated upon Wnt stimulation. Interestingly, after the initial activation of the earliest (3′) Hox genes, a subsequent temporal colinear activation of the rest of cluster members takes place, down to the 5′-most Hox13 genes (Neijts et al., 2016). This temporal transcriptional dynamics is similar to the sequential activation observed in embryos between E7.2 and E9.0 [reviewed in (Deschamps and van Nes, 2005)]. Temporal Hox colinearity is widely present in the animal kingdom (Duboule, 2007). Different parameters have been proposed to contribute to bilaterian colinear Hox activation, including the intrinsic Hox locus organization (the cluster), the surrounding *cis*-regulatory landscape (enhancers, lncRNAs), active and inactive chromatin marks (histone modifications), boundary elements (like CTCF binding regions), and chromatin conformation (Noordermeer and Duboule, 2013).

We recently observed that, concurrently with 3′ Hox activation, *Cdx2* becomes transcriptionally active in EpiSCs stimulated by Wnt signaling (Amin et al., 2016). By performing ChIP-seq experiments on stimulated EpiSCs, we could identify regions bound by Cdx2 genome-wide. In agreement with their sensitivity to regulation by Cdx gene products, all four Hox clusters were found to be direct target of Cdx2, and trunk Hox gene expression was demonstrated by RNA-seq to be downregulated in mouse embryos lacking all three Cdx genes ('Cdx triple null') (Amin et al., 2016).

Here, we investigate the role of Cdx in the temporal colinearity of Hox gene expression. As Cdx triple null embryos do not generate any

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http://dx.doi.org/10.1016/j.ydbio.2016.12.024
Received 4 November 2016; Received in revised form 9 December 2016; Accepted 27 December 2016
Available online 29 December 2016
0012-1606/

post-occipital tissue as a result of failing to maintain their posterior growth zone and axial progenitor population (van Rooijen et al., 2012), it is difficult to study the Cdx-Hox interaction in the mutant embryo. Previously we have generated EpiSCs from Cdx null embryos (Amin et al., 2016). In the present work we used these cells to study the transcription and chromatin dynamics of Hox loci during their Wnt-triggered activation in the absence of Cdx. We now find that Cdx genes are required for temporal colinear Hox gene expression, and are crucial for the opening and activation of *cis*-elements proximal to trunk Hox genes. Cdx gene products exert their function in a genomic segment that is different from two other distinguishable segments, the Wnt-dependent 3′ part, and a 5′ posterior part containing the late Hox13 gene.

2. Material and methods

2.1. EpiSCs

The generation and culture of wild type (WT) and Cdx triple null EpiSCs, and Wnt activation by Chiron, are described elsewhere (Amin et al., 2016; Neijts et al., 2016).

2.2. LacZ reporter assay

To generate a *TBE-lacZ* construct, a 6.2kb fragment was cloned upstream of a minimal *Hsp68* promoter (Pennacchio et al., 2006). Forward primer: CACCGAGGTCCAGAAACGGGATTT. Reverse primer: AGAATTCGCCATCAGGAGAC. Micro-injection and X-gal staining were performed as described elsewhere (Neijts et al., 2016).

2.3. RT-qPCR

RNA isolation, cDNA synthesis and RT-qPCR methods are describes elsewhere (Neijts et al., 2016). Primer sequences are listed in Supplemental Table 1.

2.4. 4C-seq

The 4C-seq procedure was described elsewhere (Neijts et al., 2016). For the *TBE* viewpoint, the following primers were used. Forward primer: AATGATACGGCGACCACCGAGATCTAC-ACTCTTTCCCTACACGACGCTCTTCCGATCTTGGTGACTGGAACCGTGAT. Reverse primer: CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCT-CTTCCGATCTCATAAAAGGGAACTATGCGT.

2.5. ChIP-qPCR and ChIP-seq

H3K27ac ChIP-seq was performed on non-induced and induced (24 h Chiron) WT and Cdx null EpiSCs as described elsewhere (Neijts et al., 2016). ChIP against Menin (antibody from Bethyl, A300-105A) was performed as described in Amin et al. (2016). ChIP-qPCR primer sequences are listed in Supplemental Table 2.

2.6. Data sets

Data sets from ChIP-seq, ATAC-seq and 4C-seq experiments deposited in Gene Expression Omnibus (GEO) series GSE91046, or are obtained from GSE81203 (Neijts et al., 2016), GSE84899 (Amin et al., 2016) and GSE39435 (Mahony et al., 2014). HiC data is obtained from promoter.bx.psu.edu (Dixon et al., 2012). Experimental procedures are described in above mentioned references.

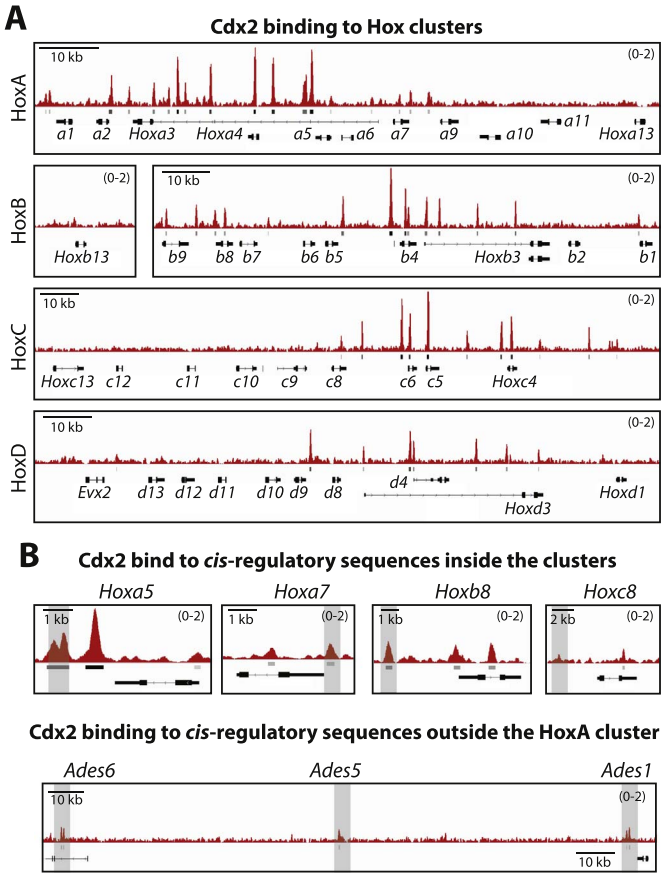

Fig. 1. Distribution of Cdx2 binding throughout the Hox clusters in induced EpiSCs (data from Amin et al., 2016). **A)** Binding of Cdx2 on HoxA, HoxB, HoxC and HoxD clusters. **B)** Upper panels, Cdx2 binds to Cdx-dependent *cis*-regulatory elements in different Hox clusters (references in text). Lower panel, early HoxA enhancers *Ades1*, *Ades5* and *Ades6* are bound by Cdx2. The bars underneath the Cdx2 binding data represent the peaks that are identified by the MACS peak calling algorithm.

3. Results

3.1. Cdx2 binds the Hox clusters and their regulatory landscapes in Wnt-stimulated EpiSCs

Cdx and 3′ Hox genes are initially expressed in the posterior streak region of the E7.2 mouse embryo and follow the same spatiotemporal dynamics during axial elongation (Young and Deschamps, 2009). In EpiSCs, both 3′ Hox genes and *Cdx2* start to be expressed upon Wnt stimulation (using the Wnt3a protein or the Wnt agonist CHIR99021 – hereafter referred to as 'Chiron') (Amin et al., 2016; Neijts et al., 2016). We previously performed ChIP-seq experiments against Cdx2, the most dominant Cdx member during embryonic development, on Chiron/Fgf8-treated EpiSCs (24 h of stimulation). We could identify almost 4000 Cdx2-bound regions, genome-wide. All four Hox clusters were found to be heavily occupied by Cdx2 (see Fig. 1A) (Amin et al., 2016).

Several of the sequences bound by Cdx2 have been demonstrated to be important during axial patterning, like elements close to *Hoxa5* (Tabaries et al., 2005), *Hoxa7* (Gaunt et al., 2004), *Hoxb8* (Charite et al., 1998) and *Hoxc8* (Shashikant and Ruddle, 1996) (Fig. 1B). Specific deletion of the latter enhancer was shown to affect the timing of *Hoxc8* initial expression (Juan and Ruddle, 2003).

In addition to sequences within the four clusters, Cdx2 occupancy was also observed at elements that are part of the Hox *cis*-regulatory landscapes. Some of the early Hox enhancers at the 3′ side of HoxA

(*Ades1*, *Ades5* and *Ades6*) (Neijts et al., 2016) are bound by Cdx2, albeit to a lesser extend (Fig. 1B). LacZ reporter assays demonstrated that these elements are all active in the posterior growth zone of the embryo, at least until E9.0-E9.5 (Neijts et al., 2016). In addition to these early enhancers, some late enhancers are also occupied by Cdx2. For instance, we find that the *e4* enhancer, 280 kb upstream of *Hoxa13* and active in limb and genitals (Berlivet et al., 2013), is bound by Cdx2 (data not shown). Moreover, the *cs38* element, 380 kb away from *Hoxd1* (Beccari et al., 2016) is occupied by Cdx2 as well (data not shown). This element is a transcription start site of *Hotdog* and *Twin of Hotdog* lncRNAs, important for emergence of the endoderm-derived caecum (Delpretti et al., 2013).

We next compared our Cdx2 binding data from induced EpiSCs with published ChIP-seq data obtained from Cdx2 overexpressing embryonic stem cells (ESCs)(Mahony et al., 2014). As expected, Cdx binding sites over the central Hox genes are present in both situations (Supplemental Fig. S1). However, we observed that overexpressed Cdx2 in ESCs – and also in ESC-derived endoderm and motor neurons (Mahony et al., 2014; Mazzoni et al., 2013) – binds proximal to late trunk Hox genes like *Hoxa7, Hoxa9, Hoxb8, Hoxc8 and Hoxc10* relatively heavily compared to the binding in EpiSCs (Supplemental Fig. S1). On the contrary, induced EpiSCs show Cdx2 binding at the 3′ part of the clusters which are not occupied in ESCs (Supplemental Fig. S1, in orange bars).

A very contrasted situation therefore opposes the clear Cdx2 binding profile in the 3′ part of HoxA in EpiSCs, to that in ESCs where Cdx2 does not bind. This is likely to be a consequence of the Wnt-driven activation of the 3′ side of the cluster that would make Cdx2 binding possible. Strikingly, numerous Cdx2 binding sites are observed in the interval between *Hoxa3* and *Hoxa9* in both induced EpiSCs and Cdx2-overexpressing ESCs, with relatively more intense binding in the *Hoxa7-Hoxa9* interval in the ESC situation. A similar dichotomy is observed for the HoxB cluster (Supplemental Fig. S1). This suggests that the middle part of the Hox cluster has a different status than the 3′ part, which needs to have been sensitized by an initial Wnt activation (Neijts et al., 2016) before it accommodates Cdx2 binding.

3.2. Hox cluster colinearity of expression is impaired in Cdx mutant EpiSCs

Considering the functional evidence of Cdx regulatory activity on Hox gene expression during development, and Cdx2 binding on the clusters, we tested whether Cdx2 is involved in temporal colinear activation of Hox genes. Although we had previously used Chiron and Fgf8 in the EpiSCs to identify Cdx2 targets in the Wnt and Fgf pathways (Amin et al., 2016), we used Chiron only in the experiments described in the Hox induction studies described from this point on. We had formerly verified that the transcriptional induction profiles of Hox genes in EpiSCs stimulated by Chiron and Fgf8 and by Chiron only were very similar and that the pattern of locus activation witnessed by H3K27 acetylation (H3K27ac) profile, were similar as well in both conditions for *Cdx2*, and for the HoxA locus (data not shown).

We previously did not observe any defects in the onset of *Hoxb1* in Cdx triple null embryos at E7.5 (van Rooijen et al., 2012), whereas the more 5′ located genes were not activated or were affected in their expression levels. However, this impaired expression was believed to be the result of the exhaustion of the posterior progenitor population in these mutant embryos occurring after E7.5. We now compared the inducibility of the HoxA genes upon Wnt pathway activation in Cdx triple null and wild type (WT) EpiSCs. Treatment of WT EpiSCs by Chiron over a time course of 72 h caused colinear activation of HoxA genes. There is a striking difference between the induction profile of the 3′ Hox genes and that of the central and posterior Hox genes in WT cells. Hox1 and Hox2 are strongly and rapidly induced by Chiron and their level of expression decreases thereafter with induction time (see *Hoxa1* and *Hoxa2* in Fig. 2A), whereas the central Hox genes (see *Hoxa7, Hoxa9* and *Hoxa13* in Fig. 2A) are induced with a colinearly increasing delay and reach higher and higher expression levels with induction time.

The comparison between the non-induced levels of HoxA transcripts in wild type and Cdx null EpiSCs revealed a higher level of initial basal expression of *Hoxa5* in mutant cells (Fig. 2A). The cause of this elevated basal expression in the Cdx null cells is elusive, and seems to correlate to the existence of a peak of H3K27 acetylation flanking but not overlapping with a Cdx2 binding position in the *Hoxa5* region (arrow in Fig. 2B), both in Cdx null EpiSCs and in Chiron induced WT cells. *Hoxa5* is the only central HoxA gene that exhibits this unexpected high expression in the absence of Cdx in uninduced conditions. The other central Hox genes do not manifest this feature. Apart from this *Hoxa5* exception, the central/trunk Hox genes clearly exhibit a strongly diminished response to Wnt activation in the Cdx null EpiSCs (see *Hoxa7*, *Hoxa9* and *Hoxa13*, *Hoxb4*, *Hoxb8* and *Hoxb9* shown in Fig. 2A and in Supplementary Fig. S2A). Cdx is thus needed for colinear transcriptional activation of the clustered Hox genes.

The levels of H3K27 acetylation along the HoxA cluster were increased after Wnt stimulation, and the activation domain spread towards both the 3′ and the 5′ side in WT cells (Fig. 2B). In the absence of Cdx, several positions that normally bind Cdx2 in the HoxA locus remain depleted of H3K27ac, particularly in the middle of the cluster. These positions are marked with asterisks in Fig. 2B. For the HoxB cluster the situation is even more striking (Supplemental Fig. S2B).

The data therefore reveal that Cdx inactivation leads to disturbed colinear induction of the clustered Hox genes, and to a diminished H3K27ac deposition in the central part of the clusters, under Wnt-induced conditions.

3.3. Wnt-induced Hox activation opens Cdx2-binding positions in the middle part of the Hox clusters

To understand the mechanism by which Cdx2 influences colinear Hox gene activation, we examined the chromatin opening at Hox loci during Wnt stimulation. We performed an ‘assay for transposase-accessible chromatin followed by next generation sequencing’ (ATAC-seq) (Buenrostro et al., 2013) in non-induced WT EpiSCs. Multiple elements appear to already be accessible throughout the clusters, including in the gene body of Hox13 genes (see Fig. 3A, for HoxA) (Neijts et al., 2016). The ATAC-seq profile over the cluster is very similar to the distribution of H3K4me3 – except for the 5′-most *Hoxa13* peak that lacks the active histone methylation mark. Apart from this *Hoxa13* exception, the deposition of active histone mark H3K4me3 by the trithorax group methyltransferases therefore correlates with the opening of the chromatin at these positions. The 5′ boundary of the H3K4me3 decoration – and of the opened chromatin domain – marks the boundary of the Cdx2 binding domain determined in induced EpiSCs (Fig. 3A). The location of this virtual boundary is a well-conserved sequence (*CTCF Binding Site 5*, *CBS5*) shown to bind CTCF and Oct4 in ESCs and to play a role in Hox boundaries in motor neurons (Kim et al., 2011; Narendra et al., 2015).

Stimulation of EpiSCs by Wnt (Chiron for 48 h), leads to further opening of the HoxA cluster and of the early enhancers *Ades1* and *Ades2* (Neijts et al., 2016). Comparison of the sequences opened upon this Wnt exposure with the regions bound by Cdx2 revealed a striking overlap (Fig. 3B). In the *Ades1-CBS5* interval, the sequences that become accessible upon induction appear to be bound by Cdx2 (green regions in Fig. 3B). This suggests that Cdx2 immediately binds to newly opened chromatin, or alternatively, that Cdx2 acts as a pioneer transcription factor (Zaret and Mango, 2016) opening up these positions.

3.4. Chromatin opening around trunk Hox genes is Cdx-dependent

To functionally test whether Cdx is required for the opening of Hox

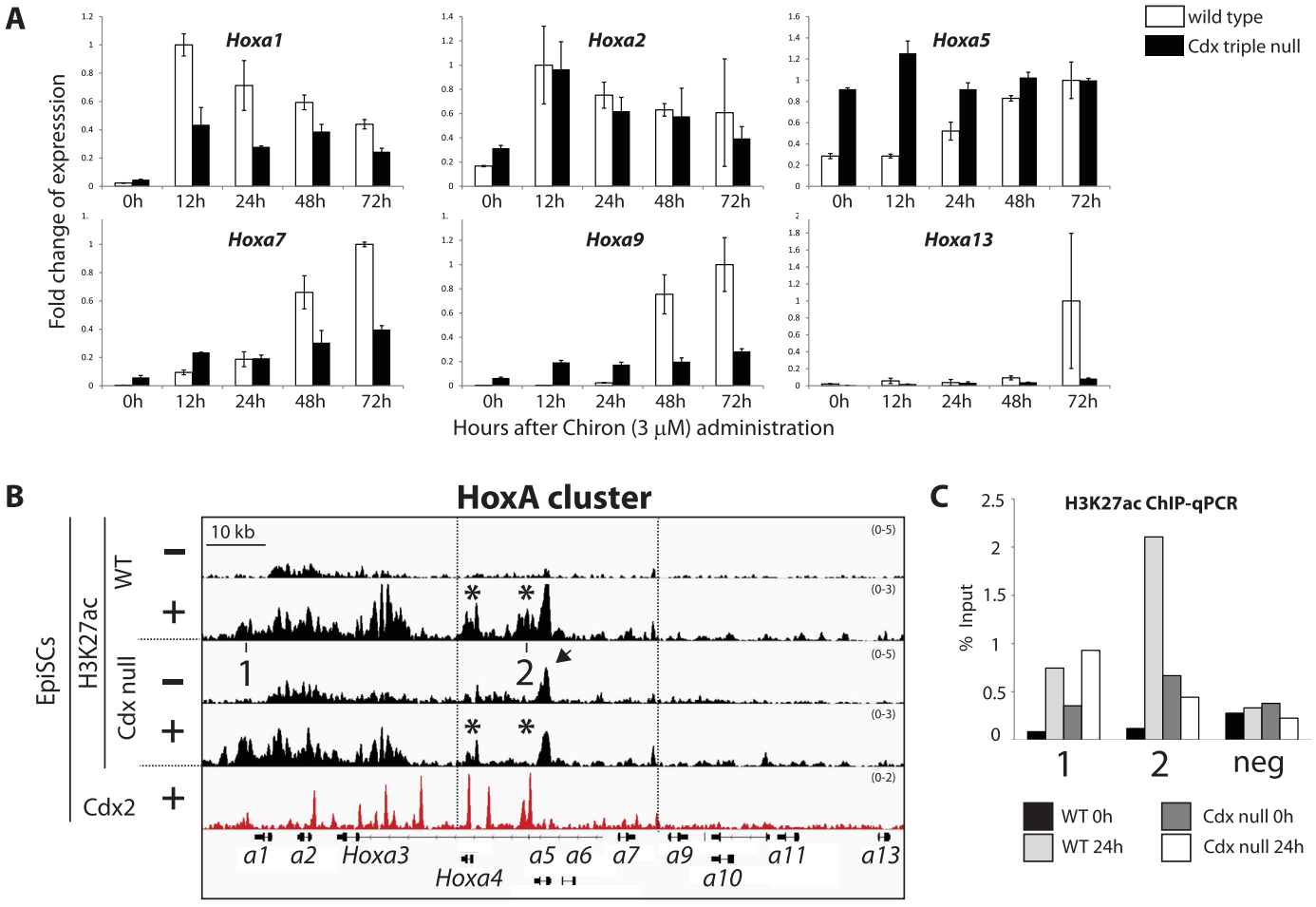

Fig. 2. Temporal colinearity of Hox activation is affected in the absence of Cdx. **A)** Expression of 3′, central and posterior HoxA genes in WT and Cdx null EpiSCs. Central genes *Hoxa7, Hoxa9* and 5′ gene *Hoxa13* are not colinearity upregulated in Cdx triple null EpiSCs (black), as they are in WT EpiSCs (white). Error bars indicate ± S.D. **B)** H3K27ac and Cdx2 binding through the HoxA cluster in Cdx null mutant EpiSCs, compared to WT EpiSCs in uninduced (-) and induced (+) conditions. After 24 h of stimulation, H3K27ac levels at Cdx2 binding sites in the middle of the cluster increase in WT cells and less or not at all in Cdx null cells. Asterisks indicate that acetylation at these Cdx2-binding positions is Cdx-dependent. Numbers 1 and 2 indicate regions used for ChIP-qPCR quantification of H3K27ac in C. The arrows point to an acetylation peak adjacent to a Cdx2 binding region in the *Hoxa5* vicinity in induced WT EpiSCs and in Cdx null EpiSCs induced and uninduced. **C)** Validation of H3K27ac binding profiles by ChIP-qPCR of regions 1 (*Ades1*) and 2 (Cdx2 binding site 3′ to *Hoxa5*) indicated in B, in non-induced (0 h) and Chiron-induced (24 h) WT and Cdx null EpiSCs. Neg, negative control region on chromosome 17.

cis-sequences, we performed ATAC-seq on Cdx null EpiSCs. ATAC-seq results on non-induced Cdx mutant cells show the same distribution of opened chromatin positions throughout the four Hox clusters as in non-induced WT EpiSCs (data not shown).

Upon stimulating Cdx null EpiSCs for 48 h with Chiron, we compared the chromatin opening profile with that in induced WT cells. Several positions within the Hox clusters are dependent on Cdx to be opened (Supplementary Fig. S3A) [data from (Amin et al., 2016)]. Interestingly, we observed a dichotomy in the opening of Hox regions. In Cdx null EpiSCs, the 3′ part of the HoxA cluster is open similarly and at the same positions as in WT cells (Fig. 4, orange bars). This is true even for sequences that are bound by Cdx2. The 3′ part of the Hox cluster is thus not dependent on Cdx to become accessible. In contrast, elements in the middle part of the clusters are dependent on the presence of Cdx to become accessible. All positions that are bound by Cdx2 in the *Hoxa3-CBS5* interval in the WT cells have reduced levels of chromatin opening in Cdx mutant EpiSCs (Fig. 4, purple bars), and some of these positions are not open at all in the absence of Cdx (asterisks). Such a Cdx-dependence for chromatin opening was previously seen for several non-Hox Cdx2-bound enhancers (79% of these enhancers were found to be dependent of Cdx2) (Amin et al., 2016). As expected, H3K27ac data show that some of the Cdx-dependent regions are affected in their acetylation levels in Cdx mutant cells (arrow in Fig. 4, lower lanes). Noteworthy, all elements that are bound by Cdx2 in *Cdx2*-overexpressing ESCs (Mahony et al., 2014) within the trunk HoxA and HoxB areas, seem to be at least partially dependent on Cdx for opening and activation (Fig. 4 and Supplemental Fig. S3B). Cdx2 might be able to bind to positions slightly more posterior than *Hoxa9* in embryos, as ESCs overexpressing *Cdx2* exhibit Cdx2 binding down to *Hoxa10* (Mahony et al., 2014). For the HoxB cluster we find similar striking effects of Cdx-dependence. Our data reveal a need of Cdx for maximal DNA opening and H3K27ac deposition for all genes located 5′ of *Hoxb3* (arrows and purple bars in Supplemental Fig. S3B). Collectively, the ATAC-seq data indicate that Cdx transcription factors are required for trunk Hox gene opening and activation, and suggest that the Cdx gene products may act as pioneer transcription factors at some at least of these positions. The 3′ part of the cluster is independent of Cdx for DNA accessibility.

We looked for potential co-factors for Cdx2 at Cdx2 binding positions. Among the factors which we investigated is Men1 (Menin), responsible for methylation of lysine 4 of histone H3 (Hughes et al., 2004), and involved in the regulation of Hox gene expression (Chen et al., 2006; Yan et al., 2006). As shown in Supplemental Fig. S4, we provide evidence that binding of Menin is enriched at a number of Cdx2-bound sites in HoxA and HoxB. Central Hox genes of clusters A and B are not the only ones that do significantly bind Menin and Cdx2.

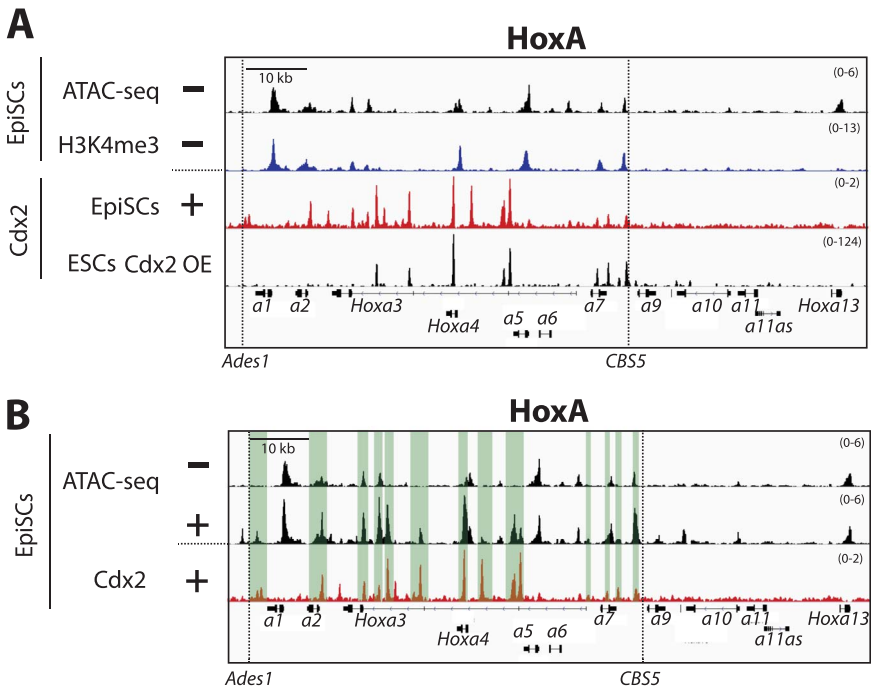

Fig. 3. DNA accessibility and Cdx2 distribution before and during Hox activation in EpiSCs. **A)** In non-induced WT EpiSCs, the DNA accessibility (ATAC-seq) profile (black) is similar to the distribution of H3K4me3 (blue), except for *Hoxa13*, in non-induced EpiSCs. The distribution of Cdx2 binding (red for induced EpiSCs and black for Cdx2-overexpressing ESCs) and of H3K4me3 decoration and DNA accessibility, share the same 5′ boundary (dashed line) at CTCF binding site 5 (*CBS5*) (Kim et al., 2011). **B)** During Wnt activation (Chiron, 48 h) DNA accessibility increases at the HoxA cluster. The positions that become accessible are the same that are bound by Cdx2 (in red) in induced EpiSCs (regions indicated by green bars).

The anterior Hox genes *Hoxa1* and *Hoxb1* also do. This means that Menin is a candidate co-factor mechanistically involved in transcriptional activation of Hox genes, but is not responsible for the differential response of central and anterior Hox genes to Cdx.

3.5. Segmental distribution of Cdx2 binding and of Cdx-dependent chromatin opening in the HoxA cluster

Examination of the higher order architecture of the HoxA cluster, by chromosome conformation capture techniques as 4C-seq and HiC revealed that the locus is located at the boundary of two topologically associating domain (TADs) (Dixon et al., 2012). We previously characterized a series of enhancers in the 3′ flanking region of HoxA and found that they reside in a 3′ subTAD, together with 3′ HoxA genes (Neijts et al., 2016). We also showed that HoxA is subdivided in several subdomains (Fig. 5A). In addition to the 3′ part of the cluster (*Hoxa1-Hoxa4*) in the 3′ subTAD, the central Hox genes (*Hoxa4-Hoxa10/11*) form a second segment. *Hoxa13* form a third HoxA domain and is mainly in contact with the large 5′ TAD (Neijts et al., 2016), which also contains the distant 5′ enhancers involved in late Hox regulation in limb and genitals (Berlivet et al., 2013; Lonfat et al., 2014) (not shown in Fig. 5A).

Interestingly, we observed that trunk Hox genes (like *Hoxa6*) that are poor in contacts with the 3′ subTAD, could interact with a very distant (1 mega base remote) region at the 3′ boundary of the 3′ TAD (hereafter: '*TAD boundary element*', *TBE*) (Fig. 5A). A 4C-seq view from the *TBE* reveals that it indeed interacts with the trunk and posterior Hox segment, and less with the 3′ Hox genes and their 3′ subTAD (Fig. 5A). Interestingly, the activity domain of *TBE* in the embryo is very similar to that of trunk Hox genes, as it is initiated relatively late in the posterior streak and remains active in the posterior growth zone (Fig. 5B). Notably, the *TBE* is not bound by Cdx2,

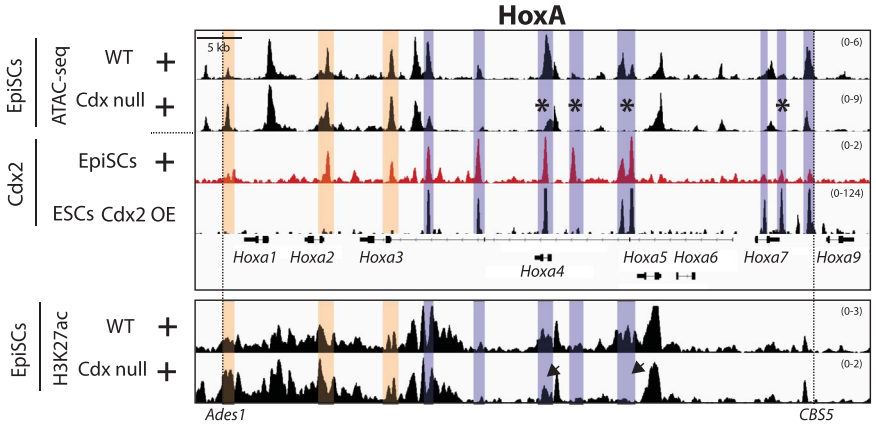

Fig. 4. Cdx is required for DNA accessibility of regulatory elements around trunk Hox genes. **A)** Upper panel, In induced WT EpiSCs, genomic positions at both 3′ and trunk Hox genes are open (see also Fig. 3). In Cdx triple null EpiSCs, the 3′ positions are open (orange bars) whereas elements around trunk Hox genes (purple bars) are not (asterisks) or less. These Cdx-dependent positions overlap with regions that bind Cdx2 in Cdx-overexpressing ESCs (lower lane, in black). Lower panel, H3K27ac profiles of WT and Cdx null EpiSCs at HoxA show decreased levels of acetylation in Cdx mutant cells, at the positions that are dependent on Cdx for DNA accessibility. Asterisks indicate the positions where chromatin opening and acetylation depend on the presence of Cdx.

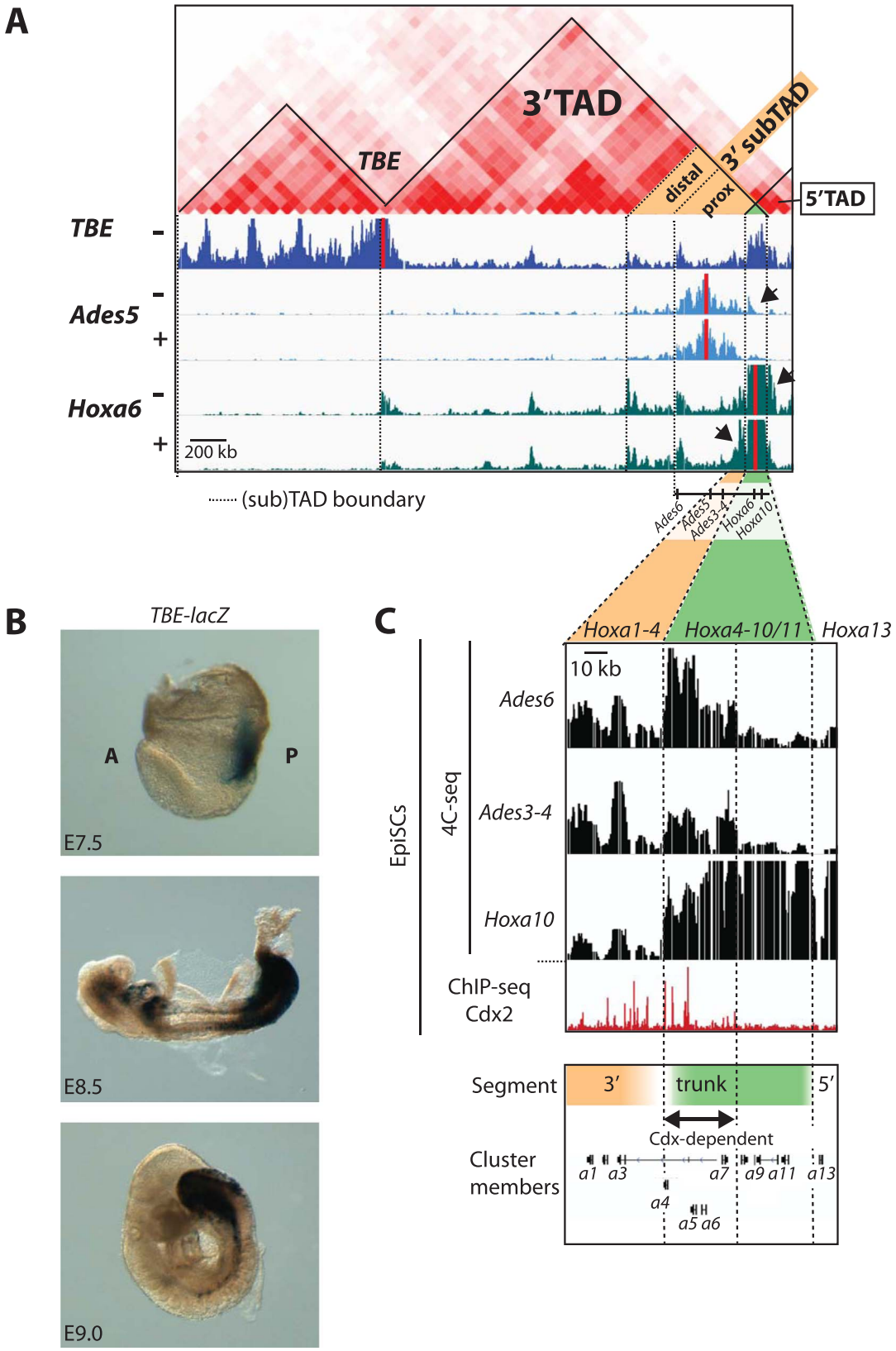

Fig. 5. The HoxA locus can virtually be partitioned in several segments, differentially regulated by Cdx2. **A)** Broad overview of the HoxA segments; interactions between HoxA and its 3′ surrounding including the distant element *TBE,* seen from several viewpoints, in uninduced (-) and Wnt-induced (+) WT EpiSCs. The 3′ subTAD (yellow) forms a segment distinct from the central segment (in green). The *Hoxa6* and *Ades5* data are from Neijts et al., 2016. The HiC data are from Dixon et al., 2012. Red lines indicate 4C-seq viewpoint positions. **B)** LacZ staining of embryos expressing the *TBE-lacZ* transgene. A, anterior. P, posterior. **C)** More detailed mapping of the proposed genomic segments, and of the documented Cdx2 binding sites in the HoxA cluster. The regulation of genes in the trunk segment is dependent on Cdx, whereas the genes and regulatory elements in 3′ subTAD are independent of Cdx.

suggesting the existence of additional input besides Cdx in the regulation of these Hox genes.

We wondered whether the distribution of the *cis*-elements that respond to Cdx is organized in a similar segmented manner as the locus. Therefore, we examined the interactions seen from 4C-seq viewpoints located in the same TAD in which the Cdx2 binding sites are located. To avoid a too high number of interactions due to proximity, we selected three viewpoints relatively distant from the trunk Hox sequences. At the 5′ side, we selected *Hoxa10* – which lies at the 5′ limit of the 3′ TAD. At the 3′ side we selected the *Ades3-4* and *Ades6* enhancers – which are within the 3′ subTAD, and at the 5′ boundary of the 3'subTAD, respectively (Neijts et al., 2016).

Seen from *Ades6* and *Ades3-4*, a dense interaction domain overlaps with the Cdx2 binding domain between *Hoxa4* and *CBS5* in uninduced conditions (Fig. 5C). Not much interaction was scored 5′ of *CBS5,* indicating that boundary might be functional in EpiSCs as it is in ESCs (Kim et al., 2011). From the *Hoxa10* viewpoint, we observed the same sharp boundary around *Hoxa4*. At the 3′ side of this boundary, a relatively low amount of contacts are observed along a short 'low intensity' domain. Further 3′, a higher intensity of contacts is observed, also seen from the other viewpoints. The latter domain includes the 3′ part of the HoxA cluster, in which *cis*-elements do not require Cdx to be opened. 5′ of the *Hoxa4* boundary, extremely high interactions with the *Hoxa10* viewpoint are observed, throughout the entire *Hoxa4-Hoxa13* interval (Fig. 5C) – as observed from the *TBE* viewpoint (Fig. 5A).

In conclusion, we can distinguish differentially Cdx-bound domains in HoxA that correspond to the topological segments. At the 3′-most end of the cluster, a Cdx-independent domain is located spanning *Hoxa1* to *Hoxa3* (Fig. 4B). Between *Hoxa4* and *Hoxa9* a central domain is identified, with sharp topological boundaries and with discrete *cis*-elements that are fully dependent on Cdx for opening (see Fig. 4). The most 5′ part of the HoxA cluster (*Hoxa10-Hoxa13*) is activated much later than the 3′ and central Hox genes. Its dependence on Cdx, shown in Cdx null EpiSCs, is probably secondary to the activation of central Hox genes, as no pre-existing binding sites are detectable at 24 h of induction in EpiSCs.

4. Discussion

The progressive expression in time of the genes of the Hox clusters is of essential importance for the correct generation and antero-posterior patterning of the embryonic trunk in vertebrates. We previously deciphered the mechanism of initial activation of the anterior (3′) part of the HoxA cluster by early embryonic Wnt signals (Neijts et al., 2016). We made use of epiblast stem cells (EpiSCs) which, when induced by Wnt, are a good model for the posterior part of the early embryo where Hox genes are activated. In the present work, we address the question of the mechanism underlying the subsequent colinear induction of clustered Hox genes. We therefore compared the expression and the epigenetic and architectural features of the clustered Hox genes in wild type and Cdx null EpiSCs. We find that Cdx gene products intervene to shape the epigenetic landscape of the middle part of the HoxA cluster and to activate central Hox genes after the 3′ genes have been turned on. This stepwise activation of the clustered Hox genes appears as at least one of the molecular genetic fundaments of the temporal colinearity of Hox gene expression. Early embryonic signaling by Wnt3 in the posterior embryonic epiblast induces the expression of 3′ Hox and of Cdx genes. Cdx gene products then activate the expression of the central and more 5′ located Hox genes. The genomic area of action of the Wnt signal is the *cis*-regulatory landscape forming a 3′ Hox subTAD (Neijts et al., 2016), whereas the domain activated by Cdx proteins is the central segment of the cluster, 5′ to *Hoxa4*, where many Cdx2 binding sites have been detected.

These successive phases of the Hox gene regulation are at least partially independently driven, as transcriptional initiation of *Hoxa1* is independent of Cdx, and the central HoxA genes need Cdx to be activated. The dichotomy in the kinetics of induction of 3′ versus central Hox genes emphasizes the singularity of each of the two regulatory phases. Wnt signals induce the 3′ Hox genes rapidly and strongly, and their effects progressively decrease after 24 h of induction. On the contrary, Cdx induces the central Hox genes slowly and in a colinear crescendo. This dichotomy already constitutes evidence for a biphasic upregulation of the clustered Hox genes.

The sequentiality of expression of the 3′ and central Hox genes is crucial for the correct differential morphogenetic activity the Hox genes. The timing of initial transcription of *Hoxa1* and of the central Hox genes in the posterior-most epiblast is a determinant of the subsequent expression of the genes in the anterior part of the primitive streak where progenitors for the axis are located (Wilson et al., 2009). A gene initially transcribed early will be expressed early in the axial progenitor region, and thus will instruct the emerging axial structures at a more anterior level than in the case of a gene initially transcribed later (Deschamps and van Nes, 2005; Forlani et al., 2003).

The biological activity of Cdx gene products on trunk axial tissues is two-fold. Central Hox genes and their Cdx activators ensure posterior growth by maintaining axial progenitors active in the growth zone. Partial or complete inactivation of the Cdx genes causes arrest of axial extension at levels posterior to the occipital part of the axis, and central Hox genes can rescue this effect (van Rooijen et al., 2012; Young et al., 2009). Cdx genes are also modulating antero-posterior identity of the tissues derived from the trunk progenitors at least in part by regulating central Hox genes (Charite et al., 1998; Tabaries et al., 2005; van den Akker et al., 2002). Some at least of the *cis*-elements activated by Cdx factors within the middle segment of the HoxA cluster correspond to identified transcriptional enhancers, as it is the case for a Cdx-responsive *Hoxa5* element and for *Hoxb8* upstream sequences (Charite et al., 1998; Tabaries et al., 2005). These elements, as we now show, require Cdx proteins for their accessibility and activation, and thus constitute the controllers of the central Hox gene activation necessary for regulating posterior growth and patterning of the trunk.

One particular central Hox gene controller is the inter-TAD boundary element *TBE*, marking the 3′ end of the 3′ HoxA TAD. This element which lies 1 mega base 3′ of the HoxA cluster does specifically contact the central and posterior HoxA genes, as revealed in 4C-seq experiments, and it does exhibit enhancer activity reproducing the expression of trunk HoxA genes in lacZ transgenic embryos in vivo. As it is accessible already in uninduced EpiSCs and was not seen to bind Cdx2 in our experimental conditions (data not shown), it may belong to an additional regulatory circuit exerting Cdx-independent control of central HoxA genes from the remote inter-TAD position.

The genomic domain corresponding to the central Hox genes where Cdx2 was shown to bind and where it modifies the epigenetic status, extends between *Hoxa4* and *Hoxa9* in our experiments with Wnt-induced EpiSCs. We cannot rule out that genomic positions in HoxA posterior to *Hoxa9* also would bind Cdx2 in conditions of longer exposure to Wnt or other posterior signals in embryos in vivo. These positions would activate more 5′ HoxA genes to sustain growth and patterning of more caudal trunk tissues. The *Hoxa13* gene, in any case, plays a distinct role, as Hox13 proteins antagonize Cdx2 by binding to the same targets, and so doing arrests axial elongation (Amin et al., 2016; Young et al., 2009). The *Hoxa13* gene and the Hoxa13 protein therefore constitute the most posterior module ending central Hox gene temporally colinear activation, possibly by direct competition with the trunk effector Cdx. This event also has to be timely regulated to operate the trunk to tail transition at the correct axial level. We present a model of the gradual regulation of the different architectural units by their corresponding *trans*-acting effectors, which together direct the

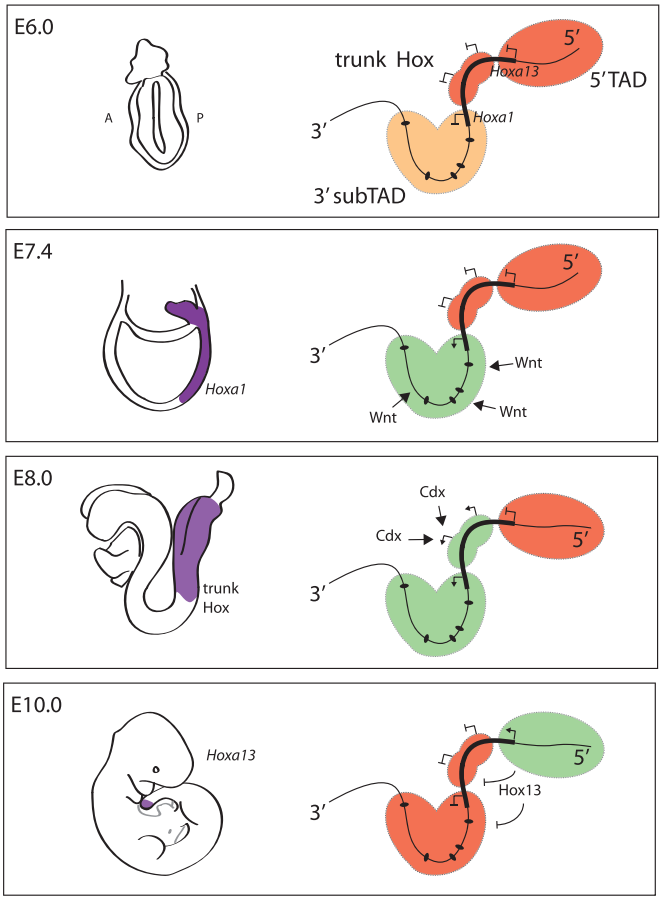

Fig. 6. The gradual activation of HoxA is progressively regulated by Wnt and Cdx in a stepwise manner, segment by segment. Pre-gastrulation E6.0 embryos do not express any Hox gene; the 3′ subTAD including the 3′ regulatory domain (with enhancers, black dots) and *Hoxa1* are primed to become active (in orange). After gastrulation has started, Wnt signals are present posteriorly, inducing *Hoxa1* from the activated 3′ subTAD (shown here at about E7.4) in the primitve streak region and allantois (in purple). At E8.0, trunk Hox genes are expressed. They correspond to the central HoxA segment (green in Fig. 5). Later on (E10.0), the 5′-most Hox segment is activated, and *Hoxa13* antagonizes the inducing effect of Cdx on trunk Hox genes, either upon competing with Cdx2 at their common target sites (Amin et al., 2016), or by a repression mechanism similar to the way Hox13 represses more anterior HoxD genes in the limb (Beccari et al., 2016). Purple, Hox gene expression domain. Orange, region primed for activity. Red, inactive. Green, active. Thicker black line, HoxA cluster.

temporally colinear turning on of HoxA genes until *Hoxa13* arrests the process (Fig. 6).

Acknowledgments

The authors Sander Tan and Geert Geeven for bioinformatical support on the H3K27ac ChIP-seq and 4C-seq data, respectively. We thank Menno P. Creyghton and Wouter de Laat for their expert advice on the ChIP-seq and 4C-seq approach, respectively. We thank Jeroen Korving for micro-injection of the *TBE-lacZ* construct. This work was supported by the Netherlands Institute for Regenerative Medicine (NIRM; grant FES0908).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.12.024.

References

Amin, S., Neijts, R., Simmini, S., van Rooijen, C., Tan, S., Kester, L., van Oudenaarden, A., Creyghton, M.P., Deschamps, J., 2016. Cdx and T Brachyury co-activate growth signaling in the embryonic axial progenitor niche. Cell Reports 17, 3165–3177, in press.

Beccari, L., Yakushiji-Kaminatsui, N., Woltering, J.M., Necsulea, A., Lonfat, N., Rodriguez-Carballo, E., Mascrez, B., Yamamoto, S., Kuroiwa, A., Duboule, D., 2016. A role for HOX13 proteins in the regulatory switch between TADs at the HoxD locus. Genes Dev. 30, 1172–1186.

Berlivet, S., Paquette, D., Dumouchel, A., Langlais, D., Dostie, J., Kmita, M., 2013. Clustering of tissue-specific sub-TADs accompanies the regulation of HoxA genes in developing limbs. PLoS Genet. 9, e1004018.

Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y., Greenleaf, W.J., 2013. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat. Methods 10, 1213–1218.

Charite, J., de Graaff, W., Consten, D., Reijnen, M.J., Korving, J., Deschamps, J., 1998. Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. Development 125, 4349–4358.

Chawengsaksophak, K., de Graaff, W., Rossant, J., Deschamps, J., Beck, F., 2004. Cdx2 is essential for axial elongation in mouse development. Proc. Natl. Acad. Sci. USA 101, 7641–7645.

Chawengsaksophak, K., James, R., Hammond, V.E., Kontgen, F., Beck, F., 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. Nature 386, 84–87.

Chen, Y.X., Yan, J., Keeshan, K., Tubbs, A.T., Wang, H., Silva, A., Brown, E.J., Hess, J.L., Pear, W.S., Hua, X., 2006. The tumor suppressor menin regulates hematopoiesis and myeloid transformation by influencing Hox gene expression. Proc. Natl. Acad. Sci. USA 103, 1018–1023.

Delpretti, S., Montavon, T., Leleu, M., Joye, E., Tzika, A., Milinkovitch, M., Duboule, D., 2013. Multiple enhancers regulate Hoxd genes and the Hotdog LncRNA during cecum budding. Cell Rep. 5, 137–150.

Deschamps, J., van Nes, J., 2005. Developmental regulation of the Hox genes during axial morphogenesis in the mouse. Development 132, 2931–2942.

Dixon, J.R., Selvaraj, S., Yue, F., Kim, A., Li, Y., Shen, Y., Hu, M., Liu, J.S., Ren, B., 2012. Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature 485, 376–380.

Duboule, D., 2007. The rise and fall of Hox gene clusters. Development 134, 2549–2560.

Forlani, S., Lawson, K.A., Deschamps, J., 2003. Acquisition of Hox codes during gastrulation and axial elongation in the mouse embryo. Development 130, 3807–3819.

Gaunt, S.J., Cockley, A., Drage, D., 2004. Additional enhancer copies, with intact cdx binding sites, anteriorize Hoxa-7/lacZ expression in mouse embryos: evidence in keeping with an instructional cdx gradient. Int. J. Dev. Biol. 48, 613–622.

Hughes, C.M., Rozenblatt-Rosen, O., Milne, T.A., Copeland, T.D., Levine, S.S., Lee, J.C., Hayes, D.N., Shanmugam, K.S., Bhattacharjee, A., Biondi, C.A., Kay, G.F., Hayward, N.K., Hess, J.L., Meyerson, M., 2004. Menin associates with a trithorax family histone methyltransferase complex and with the hoxc8 locus. Mol. Cell 13, 587–597.

Juan, A.H., Ruddle, F.H., 2003. Enhancer timing of Hox gene expression: deletion of the endogenous Hoxc8 early enhancer. Development 130, 4823–4834.

Kim, Y.J., Cecchini, K.R., Kim, T.H., 2011. Conserved, developmentally regulated mechanism couples chromosomal looping and heterochromatin barrier activity at the homeobox gene A locus. Proc. Natl. Acad. Sci. USA 108, 7391–7396.

Kmita, M., Duboule, D., 2003. Organizing axes in time and space; 25 years of colinear tinkering. Science 301, 331–333.

Krumlauf, R., 1994. Hox genes in vertebrate development. Cell 78, 191–201.

Lonfat, N., Montavon, T., Darbellay, F., Gitto, S., Duboule, D., 2014. Convergent evolution of complex regulatory landscapes and pleiotropy at Hox loci. Science 346, 1004–1006.

Mahony, S., Edwards, M.D., Mazzoni, E.O., Sherwood, R.I., Kakumanu, A., Morrison, C.A., Wichterle, H., Gifford, D.K., 2014. An integrated model of multiple-condition ChIP-Seq data reveals predeterminants of Cdx2 binding. PLoS Comput. Biol. 10, e1003501.

Mazzoni, E.O., Mahony, S., Peljto, M., Patel, T., Thornton, S.R., McCuine, S., Reeder, C., Boyer, L.A., Young, R.A., Gifford, D.K., Wichterle, H., 2013. Saltatory remodeling of Hox chromatin in response to rostrocaudal patterning signals. Nat. Neurosci. 16, 1191–1198.

Narendra, V., Rocha, P.P., An, D., Raviram, R., Skok, J.A., Mazzoni, E.O., Reinberg, D., 2015. CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. Science 347, 1017–1021.

Neijts, R., Amin, S., van Rooijen, C., Tan, S., Creyghton, M.P., de Laat, W., Deschamps, J., 2016. Polarized regulatory landscape and Wnt responsiveness underlie Hox activation in embryos. Genes Dev. 30, 1937–1942.

Neijts, R., Simmini, S., Giuliani, F., van Rooijen, C., Deschamps, J., 2014. Region-specific regulation of posterior axial elongation during vertebrate embryogenesis. Dev. Dyn.: Off. Publ. Am. Assoc. Anat. 243, 88–98.

Noordermeer, D., Duboule, D., 2013. Chromatin architectures and Hox gene collinearity. Curr. Top. Dev. Biol. 104, 113–148.

Pennacchio, L.A., Ahituv, N., Moses, A.M., Prabhakar, S., Nobrega, M.A., Shoukry, M., Minovitsky, S., Dubchak, I., Holt, A., Lewis, K.D., Plajzer-Frick, I., Akiyama, J., De Val, S., Afzal, V., Black, B.L., Couronne, O., Eisen, M.B., Visel, A., Rubin, E.M., 2006. In vivo enhancer analysis of human conserved non-coding sequences. Nature 444, 499–502.

Shashikant, C.S., Ruddle, F.H., 1996. Combinations of closely situated cis-acting elements determine tissue-specific patterns and anterior extent of early Hoxc8 expression. Proc. Natl. Acad. Sci. USA 93, 12364–12369.

Subramanian, V., Meyer, B.I., Gruss, P., 1995. Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. Cell 83, 641–653.

Tabaries, S., Lapointe, J., Besch, T., Carter, M., Woollard, J., Tuggle, C.K., Jeannotte, L., 2005. Cdx protein interaction with Hoxa5 regulatory sequences contributes to Hoxa5 regional expression along the axial skeleton. Mol. Cell. Biol. 25, 1389–1401.

van den Akker, E., Forlani, S., Chawengsaksophak, K., de Graaff, W., Beck, F., Meyer, B.I., Deschamps, J., 2002. Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. Development 129, 2181–2193.

van Rooijen, C., Simmini, S., Bialecka, M., Neijts, R., van de Ven, C., Beck, F., Deschamps, J., 2012. Evolutionarily conserved requirement of Cdx for post-occipital tissue emergence. Development 139, 2576–2583.

Wilson, V., Olivera-Martinez, I., Storey, K.G., 2009. Stem cells, signals and vertebrate body axis extension. Development 136, 1591–1604.

Yan, J., Chen, Y.X., Desmond, A., Silva, A., Yang, Y., Wang, H., Hua, X., 2006. Cdx4 and menin co-regulate Hoxa9 expression in hematopoietic cells. PloS One 1, e47.

Young, T., Deschamps, J., 2009. Hox, Cdx, and anteroposterior patterning in the mouse embryo. Curr. Top. Dev. Biol. 88, 235–255.

Young, T., Rowland, J.E., van de Ven, C., Bialecka, M., Novoa, A., Carapuco, M., van Nes, J., de Graaff, W., Duluc, I., Freund, J.N., Beck, F., Mallo, M., Deschamps, J., 2009. Cdx and Hox genes differentially regulate posterior axial growth in mammalian embryos. Dev. Cell 17, 516–526.

Zaret, K.S., Mango, S.E., 2016. Pioneer transcription factors, chromatin dynamics, and cell fate control. Curr. Opin. Genet. Dev. 37, 76–81.