Developmental Biology 410 (2016) 178–189

Contents lists available at ScienceDirect

Developmental Biology

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

CDX4 and retinoic acid interact to position the hindbrain–spinal cord transition

Jessie Chang [a], Isaac Skromne [b], Robert K. Ho [c,*]

[a] Committee on Developmental Biology, University of Chicago, Chicago, IL 60637, USA
[b] Department of Biology, University of Miami, Coral Gables, FL 33146, USA
[c] Department of Organismal Biology and Anatomy, University of Chicago, 1027 E. 57th St, Chicago, IL 60637, USA

ARTICLE INFO

Article history:
Received 28 May 2015
Received in revised form
17 December 2015
Accepted 23 December 2015
Available online 6 January 2016

Keywords:
Cdx
Retinoic acid
Hox genes
Hindbrain
Spinal cord

ABSTRACT

The sub-division of the posterior-most territory of the neural plate results in the formation of two distinct neural structures, the hindbrain and the spinal cord. Although many of the molecular signals regulating the development of these individual structures have been elucidated, the mechanisms involved in delineating the boundary between the hindbrain and spinal cord remain elusive. Two molecules, retinoic acid (RA) and the Cdx4 transcription factor have been previously implicated as important regulators of hindbrain and spinal cord development, respectively. Here, we provide evidence that suggests multiple regulatory interactions occur between RA signaling and the Cdx4 transcription factor to establish the anterior–posterior (AP) position of the transition between the hindbrain and spinal cord. Using chemical inhibitors to alter RA concentrations and morpholinos to knock-down Cdx4 function in zebrafish, we show that Cdx4 acts to prevent RA degradation in the presumptive spinal cord domain by suppressing expression of the RA degradation enzyme, Cyp26a1. In the hindbrain, RA signaling modulates its own concentration by activating the expression of *cyp26a1* and inhibiting the expansion of *cdx4*. Therefore, interactions between Cyp26a1 and Cdx4 modulate RA levels along the AP axis to segregate the posterior neural plate into the hindbrain and spinal cord territories.

1. Introduction

Following the initial induction of neural tissue, molecular cues act to partition the neural tube along the anterior–posterior (AP) axis to delineate four discrete territories of the nascent nervous system (Stern, 2001; Melton et al., 2004), the forebrain, midbrain, hindbrain and spinal cord. In contrast to the forebrain and midbrain, which are segregated by junctions that also serve as organizing centers (Martinez-Barbera et al., 2001; Rhinn and Brand, 2001), no morphological boundaries have been detected that separate the hindbrain territory from the spinal cord. However, the transition zone between the hindbrain and spinal cord territories can be distinguished by the position of tissue specific neurons and the expression of *Hox* genes, with the group 1–4 *Hox* genes expressed within the native hindbrain region and the group 5–13 *Hox* genes expressed throughout regions of the spinal cord (Gaunt et al., 1989; Gruss and Kessel, 1991; Hunt et al., 1991; Nolte et al., 2006; Prince et al., 1998a, 1998b).

Due to their collinear and nested expression in the hindbrain and spinal cord and their established importance in conferring identity to various tissues, the regulatory mechanisms governing regionalization of the hindbrain and spinal cord have been largely attributed to the function of Hox genes (Reviewed in Krumlauf et al., 1993; Schilling and Knight, 2001). However, alteration in hindbrain and spinal cord size or shifts of the hindbrain–spinal cord transition has so far not been reported in functional studies of Hox genes (Carpenter et al., 1993; Chisaka and Capecchi, 1991). Consequently, these findings suggest that additional mechanisms regulate the subdivision of the neural tissue into the domains of the hindbrain and spinal cord.

In contrast, perturbation of Retinoic Acid (RA) signaling, a crucial regulator of *Hox* gene expression in the hindbrain affects both hindbrain and spinal cord development. This is observed in embryos exposed to excess RA where the rostral expansion of posterior hindbrain *Hox* gene expression is also accompanied by a rostral expansion of spinal cord motor neurons (Emoto et al., 2005; Hernandez et al., 2007; Marshall et al., 1992; Wood et al., 1994) suggesting that the underlying phenotype of elevating RA signaling could be a rostral shift of the hindbrain–spinal cord (Hb–Sc) transition. Alternatively, these results could also indicate that increased RA levels may induce a transformation of cell fate from

* Corresponding author.
E-mail address: rkh@uchicago.edu (R.K. Ho).

http://dx.doi.org/10.1016/j.ydbio.2015.12.025
0012-1606/

hindbrain to spinal cord. Previous findings employing RA reporters have suggested that, in the neural tube, RA signaling is present in the spinal cord at a higher level than in the hindbrain (Maden et al., 1998; Shimozono et al., 2013; Wagner et al., 1992). Since cells of both the posterior hindbrain and spinal cord are located adjacent to the somitic mesoderm source of RA, the concentration difference between these two regions has been partly attributed to the function of Cyp26, a family of RA degradation enzymes expressed exclusively in the hindbrain (Gu et al., 2005; Hernandez et al., 2007; White et al., 2007). For example, Cyp26-deficient embryos have a smaller hindbrain territory and a rostrally expanded spinal cord (Emoto et al., 2005; Hernandez et al., 2007), a similar phenotype found in embryos treated with excess RA (Durston et al., 1989; Marshall et al., 1992).

In addition to RA signaling, perturbation of CDX/Caudal, a family of transcription factors in vertebrates having at least one family member expressed in the spinal cord (Bel-Vialar et al., 2002; Charité et al., 1998; Isaacs et al., 1998; Shimizu et al., 2006; Skromne et al., 2007), also leads to AP positional shifts of the Hb-Sc transition. For example, *cdx*4 zebrafish mutants and morphants have caudally expanded hindbrains and reduced spinal cords (Shimizu et al., 2006; Skromne et al., 2007), congruent with a caudal-ward shift of the Hb–Sc transition. In contrast, loss of Cdx1a (Skromne et al., 2007) does not result in the mis-alignment of the Hb–Sc transition. Therefore, this Hb–Sc transition shift observed in Cdx4 deficient embryos suggests that Cdx4 may also be involved in delineating the sizes of the hindbrain versus spinal cord territories; however the regulatory role of Cdx4 and how it interacts with RA signaling in directing this process remains to be established.

Using various chemical inhibitors and genetic manipulations to alter RA signaling and Cdx4 function, we show that coordinated modulation of RA signaling at the Hb–Sc transition is required for proper AP localization of the Hb–Sc transition. Through gene expression analysis, we show that this is achieved through reciprocal regulation between Cdx4, which inhibits the expression of the RA degradation enzyme, *cyp26a1*, in the spinal cord, and RA, which suppresses *cdx*4 expression in the hindbrain. Further comparison between the arrangement of *hox* gene expressions and motor neuron positions in our epistasis experiments indicates that RA signaling and Cdx4 act to set the position of the Hb–Sc transition and do so independently from their roles in regulating *hox* gene expression.

2. Materials and methods

2.1. Fish husbandry, microinjections and pharmacological treatments

Zebrafish (*Danio rerio*) were raised and handled using standard protocols. Embryos were collected from *AB stock fish, *Tg(isl1:gfp)* (Higashijima et al., 2000) or gir^{rw716} (Cyp26a1 mutant, Emoto et al., 2005), grown at 28 °C in embryo media and staged following Kimmel (2005). Microinjections of *cdx*4 morpholinos were performed at the 1 cell stage as previously described using standard injection protocol (20 ng/μl; Skromne et al., 2007). Uninjected and Cdx4 morphants were incubated in the dark with 10 μM DEAB (inhibitor of RA synthesis; Aldrich) starting at 5.3 h post-fertilization (hpf) or 10 μM R115866 (Hernandez et al., 2007; Cyp26 inhibitor; JanssenPharmaceutica) starting at 4.3 hpf. Control embryos were incubated with the vehicle, 0.1% DMSO in embryo media.

2.2. Whole mount in situ hybridization and antibody staining

Gene expression was detected by standard *in-situ* protocol using NBT/BCIP and Fast Red as the enzyme substrate. Antisense RNA labeled with DIG or FITC were generated for *cdx*4 (Skromne et al., 2007); *cyp26a1*, *cyp26b1*, *cyp26c1* (Hernandez et al., 2007); *hoxa2b*, *hoxb1a*, *hoxb3a*, *hoxb4a*, *hoxd4a*, *hoxb8a*, (Prince et al., 1998a, 1998b); *krx*20 (Oxtoby and Jowett, 1993); *myoD* (Weinberg et al., 1996); *radical fringe* (Cheng et al., 2004); *mafb* (Moens et al. 1998). Antibody staining was performed following previously described method (Skromne et al., 2007). Mouse anti-myosin heavy chain (A4.1025, Developmental Studies, Hybridoma Bank, IA, USA); mouse anti-neurofilament 169 K (RMO44, Zymed, CA, USA); mouse zn5 antibody (Trevarrow et al.,1990); polycolonal rabbit anti-GFP (Invitrogen); mouse anti-acetylated tubulin (Sigma-Aldrich); were used at a dilution of 1:100, 1:50, 1:1000, 1:500 and 1:500 respectively. Anti-mouse Alexa-488 and Alexa-546 secondary antibodies were used at a concentration of 1:2000 (Skromne et al., 2007).

2.3. Imaging and embryo processing

Embryos processed for *in-situ* hybridization and antibody staining were deyolked and flat mounted on slides. Fluorescent images were taken using a Zeiss LSM 710 confocal microscope and images were processed using ImageJ. Additional images were collected on a Zeiss compound microscope using a Nikon-5000 camera. Images were compiled using Adobe Photoshop CS5.1.

3. Results

3.1. Cdx4 and RA have opposite activities in the specification and patterning of hindbrain and spinal cord territories

Although previous papers have reported shifts in *hox* gene expression in Cdx4 and RA deficient embryos, no findings so far have measured and carefully compared the shifts in patterning gene expression to changes in the size of the hindbrain and spinal cord territory. To begin to understand how Cdx4 and RA regulate AP specification and patterning of the hindbrain and spinal cord territories, we first analyzed the spatial distribution of hindbrain and spinal cord *hox* gene transcripts and motor neuron populations relative to somite landmarks in embryos deficient for Cdx4 or RA signaling.

As previously reported (Davidson and Zon, 2006; Shimizu et al., 2006; Skromne et al., 2007; Hayward et al., 2015), loss of Cdx4 had no effect on the expression of anterior *hox* genes (*hoxa2b,* and *hoxb3a*; Fig. 1A, B, E, F), but the expression of a posterior hindbrain gene (*hoxb4a,* Fig. 1C, G) is caudally expanded by 2-somite lengths into what is normally the spinal cord domain. Concurrently, loss of Cdx4 results in caudally shifted spinal cord *hoxb8a* expression (Fig. 1D, H), suggesting a caudal-ward expansion of the hindbrain with a concurrent caudal reduction of the spinal cord. These early changes in *hox* expression were mirrored by late changes in motor neuron architecture. For example, loss of Cdx4 caused a 2-somite length caudal expansion in the distribution of *isl*1-positive hindbrain vagal motor neurons (Fig. 2C), and an equal 2-somite length caudal shift in the position of Zn5-positive spinal motor neurons (Fig. 2D). Thus, Cdx4 is necessary for the anterior placement of the Hb–Sc transition.

Next we examined the function of RA on the Hb–Sc transition by exposing embryos to the RA synthesis inhibitor DEAB starting at the beginning of gastrulation (5.3 hpf). Consistent with previous reports (Begemann et al., 2001; Grandel et al., 2002), RA deficient embryos had a slight caudal expansion of anterior hindbrain gene

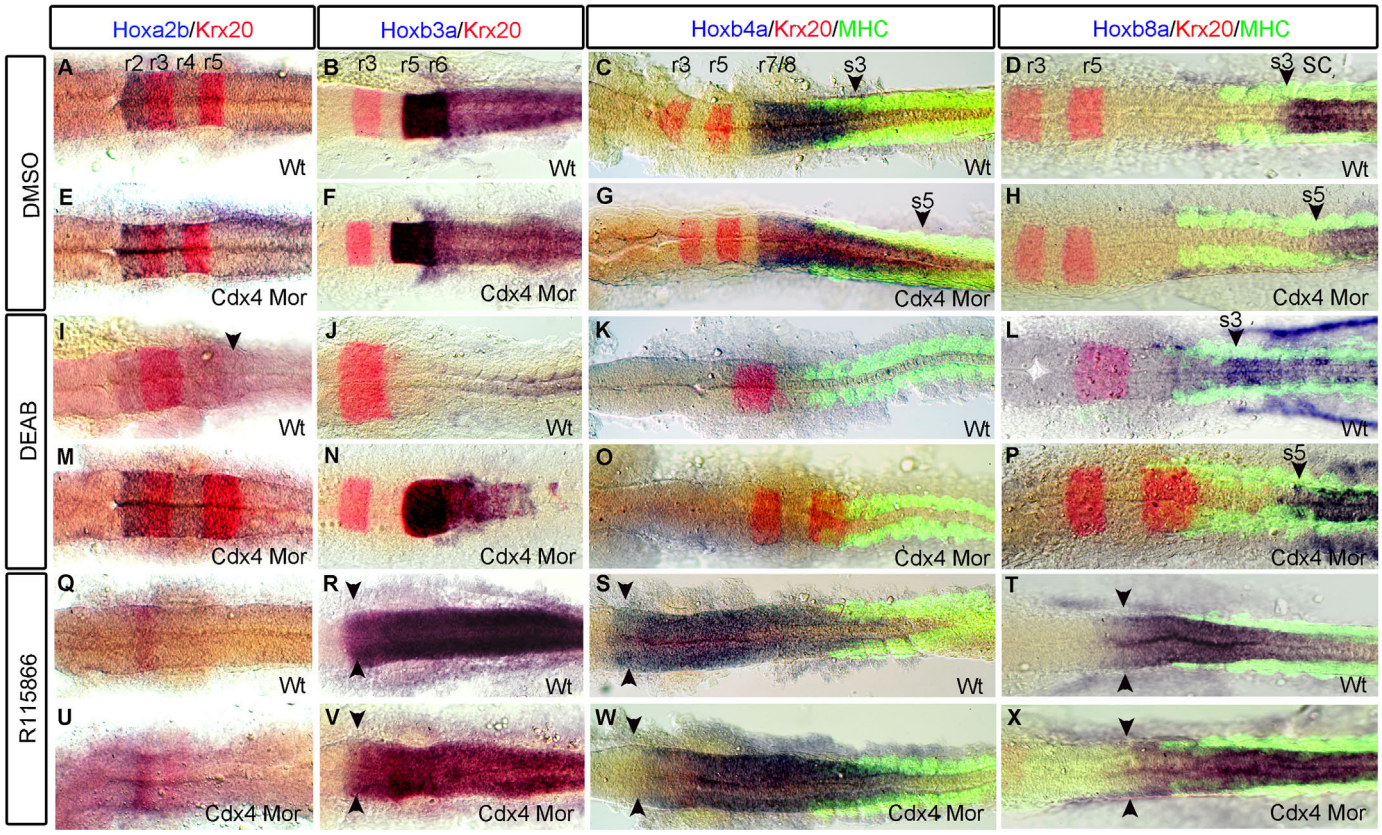


Fig. 1. Hindbrain and spinal cord *hox* gene expression shifts along the AP-axis when Cdx4 or RA functions are perturbed. Expression of *hoxa2b* in r2–4 and *hoxb3a* in r5–6 is the same in Cdx4-deficient embryos (E, F) and wild-types (A, B). *hoxb4a* expression in r7/8 is caudally expanded in Cdx4-deficient embryos (G arrowhead) compared to wildtypes (C arrowhead). In RA-deficient embryos *hoxb3a* (J) and *hoxb4a* (K) expression is lost, while *hoxa2b* expression in r4 is caudally expanded (I arrowhead). In RA/Cdx4 deficient embryos, *hoxb3a* expression is rescued (N) but not *hoxb4a* (O). In both wild type and Cdx4 deficient embryos treated with R115866 *hoxa2b* (Q, U) expression is lost while *hoxb3a* (R, V) and *hoxb4a* (S, W) expression are both rostrally expanded. The anterior expression limit of spinal cord *hoxb8a* is located at somite 3 in wildtypes (D arrowhead) and RA deficient embryos (L arrowhead) but its expression is caudally shifted to somite 5 in Cdx4 deficient embryos (H arrowhead) and RA/Cdx4 deficient embryos (P arrowhead). *hoxb8a* expression expands into the hindbrain in both wild type (T arrowheads) and Cdx4 deficient embryos (X arrowhead) treated with R115866. (A–X) are co-stained with *krx*20 (r3,r5). (C, D, G, H, K, L, O, P, S, T, W, X) co-stained with myosin heavy chain antibody (somites). (A–X) flat-mounted embryos at 20 somite stage $n=45/45$ per condition.

hoxa2b, and the loss of more posterior hindbrain genes *hoxb3a* and *hoxb4a* (Fig. 1I–K), indicating the loss of posterior hindbrain. These changes in hindbrain patterning were confirmed by the failure of facial motor neurons to migrate out of r4 and by the loss of vagal motor neurons in r7/8 (Figs. 2E and 3V). RA-inhibition treatments, however, did not change spinal cord *hoxb8a* expression, which remained at somite 3 (Figs. 1L and S1C, D), suggesting that RA may not be necessary for specifying the AP position of the spinal cord territory. In order to test this, we analyzed the position of the spinal motor neurons. However, spinal motor neurons do not develop in these embryos, as RA is essential for spinal motor neuron development (Novitch et al., 2003; Pierani et al., 1999). To circumvent this limitation we restricted DEAB treatments to gastrulation, as this treatment impacts specification of tissues without blocking spinal motor neuron development (Lee and Skromne, 2014). Loss of RA under these conditions likewise failed to change *hoxb8a* expression at 24 and 48 hpf but shifted the position of the first spinal cord motor neuron rostrally from somite 3 to 2 (Figs. 1L, S1A–D), suggesting that, contrary to the *hox* expression analysis, the spinal cord territory was indeed shifted rostrally. This phenotype is the opposite to what is seen in embryos lacking Cdx4, indicating that Cdx4 and RA have antagonistic effects in regulating the AP position of the Hb–Sc transition: along the primary axis, Cdx4 functions to position the transition more rostrally, and RA functions to place the transition more caudally.

3.2. *Cdx4 and RA signaling interactions specify and pattern the hindbrain territory*

Our analyses of Cdx4 and RA deficient embryos indicate that these two factors have opposing effects on hindbrain–spinal cord development and AP axial positioning of the Hb–Sc transition. Therefore, we next addressed whether these two factors genetically interact in regulating the development of these two tissues. To investigate the relationship between Cdx4 and RA, we treated Cdx4-deficient embryos with DEAB and then analyzed the spatial distribution of *hox* transcripts and neuronal populations. In the hindbrain, loss of Cdx4 partially rescued the defects associated with the loss of RA, in that *hoxb3a* expression is restored, while *hoxb4a* is not (Fig. 1I–K, M–O). This was also seen at the morphological level with the reappearance and expansion of several branchial motor neuron populations (Fig. 2G). Importantly, the territory occupied by these hindbrain neuronal populations is expanded caudally into the spinal cord domain, similar to the expansion observed in Cdx4-deficient embryos indicating a caudal shift in the Hb–Sc transition. Consistent with this shift was the observation that in Cdx4/RA deficient embryos, the expression of spinal cord marker *hoxb8a* was also shifted caudally (Fig. 1P). The observation that the loss of Cdx4 caused a similar posterior expansion of the hindbrain and reduction of the spinal cord irrespective of the presence or absence of RA suggests that Cdx4 is epistatic to RA signaling in establishing the AP position of the Hb–Sc transition. In addition, loss of Cdx4 partially rescues

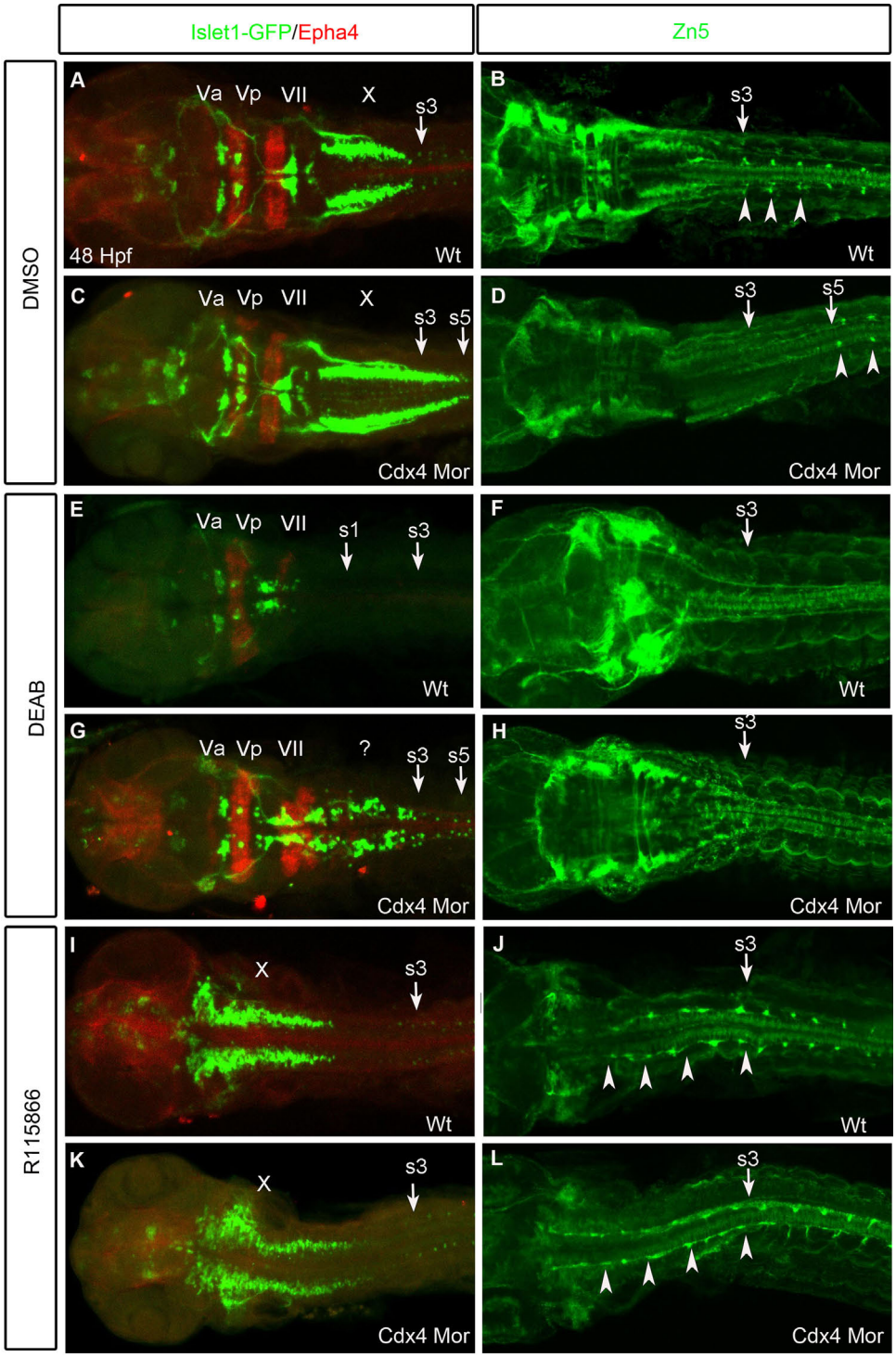
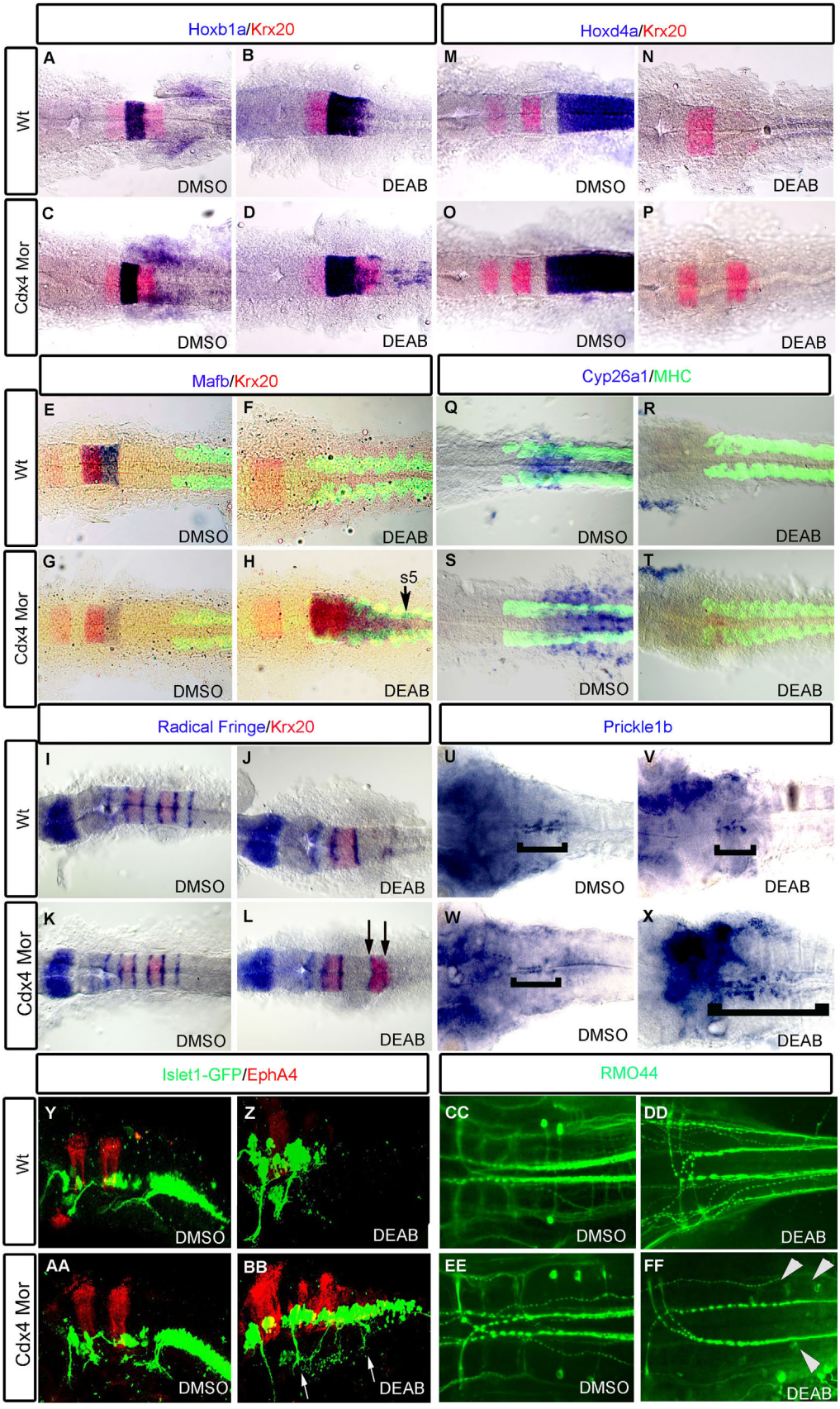


Fig. 2. Hindbrain and spinal cord neural populations shift along the AP-axis when Cdx4 or RA functions are perturbed. (A,C,E,G,I,K) Branchial motor neurons marked by Islet1-Gfp and r3,r5 visualized by anti-Epha4. The trigeminals(Va/Vp) in r2–3 and facials(VII) in r6 are unchanged but the vagals(X) in r7/8 are caudally expanded in Cdx4-deficient embryos (C) compared to wildtypes (A). Vagals (r7/8) and Epha4 (r5) are lost in RA-deficient embryos (E). Epha4 in r5 and Islet1-Gfp cells in r5–8 are rescued in RA/Cdx4 deficient embryos (G). Vagals are rostrally shifted while trigeminals and facials are absent in both wildtypes (I) and Cdx4-deficient embryos (K) treated with R115866. (B,D,F,H,J,L) Spinal cord motor neurons marked by Zn5 antibody. The first spinal cord motor neuron arises at the level of somite 3 in wildtypes (B arrow) but at the level of somite 5 in Cdx4-deficient embryos (D arrow). Spinal cord motor neurons are lost in RA-deficient (F) and RA/Cdx4 deficient (H) embryos. Spinal cord motor neurons expand rostrally in both wildtypes (J) and Cdx4-deficient embryos (L) treated with R115866. (A–L) Flat-mounted embryos at 48 hpf. (A,C,E,G,I,K) n=30/30 per condition and (B,D,F,H,J,L) $n=15/15$ per condition.

rhombomere patterning defects associated with the loss of RA, suggesting that RA and Cdx4 have antagonistic functions in regulating hindbrain patterning.

In the hindbrain, RA is essential for rhombomere formation and patterning (Begemann et al., 2001; Grandel et al., 2002). Therefore, it was significant to find that loss of Cdx4 could partially rescue the defects induced by loss of RA (Fig. 1M–P). To further characterize the extent of this rescue and better understand Cdx4 and RA interactions during rhombomere formation and patterning, we assayed Cdx4/RA deficient embryos with a repertoire of rhombomere


Hoxb1a/Krx20
Hoxd4a/Krx20
Wt
Cdx4 Mor
A
B
C
D
M
N
O
P
DMSO
DEAB
Mafb/Krx20
Cyp26a1/MHC
E
F
G
H
Q
R
S
T
s5
Radical Fringe/Krx20
Prickle1b
I
J
K
L
U
V
W
X
Islet1-GFP/EphA4
RMO44
Y
Z
AA
BB
CC
DD
EE
FF

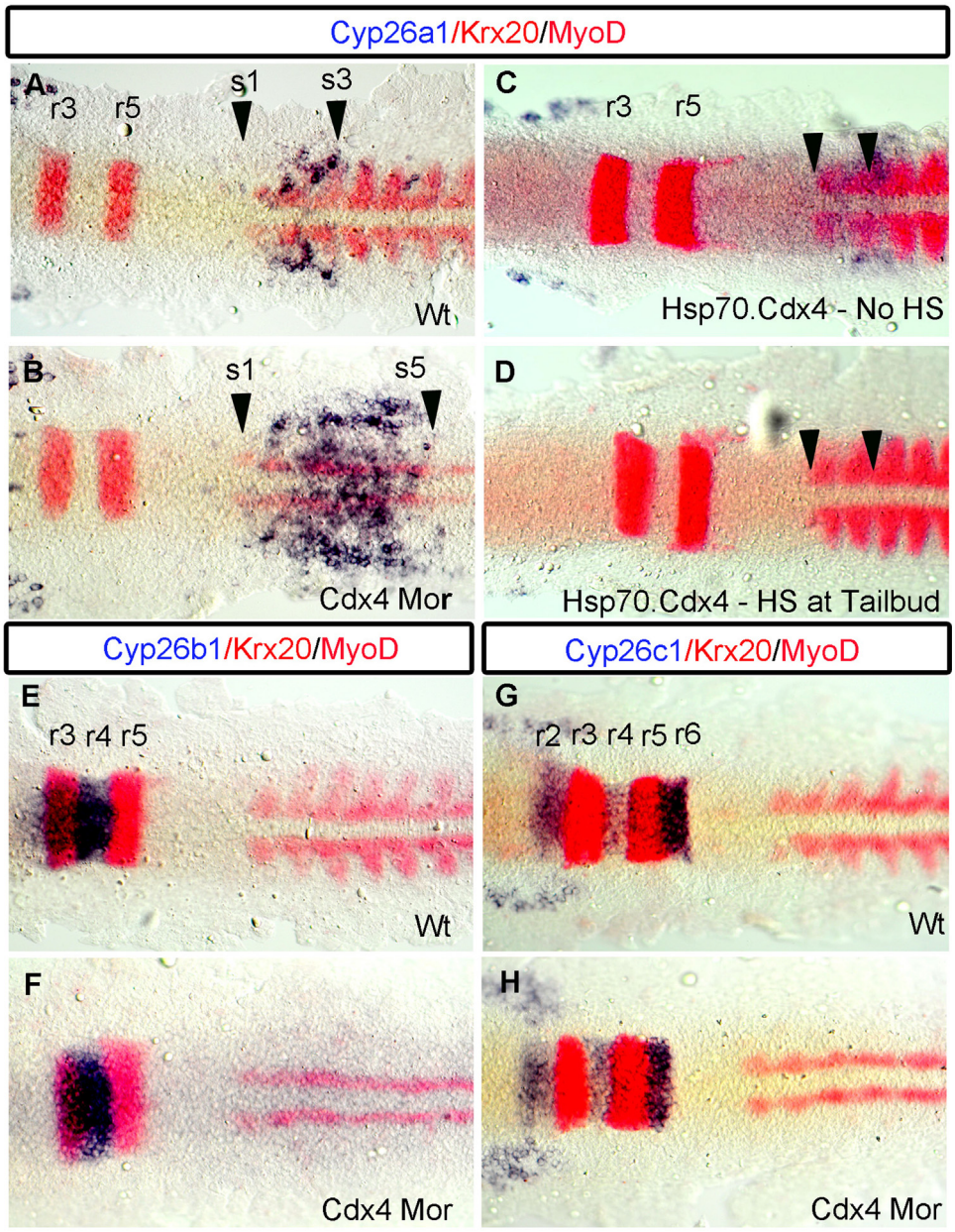


Fig. 4. *Cyp26a1* expression at the Hb-Sc transition is under the regulation of Cdx4. In wild-types, *cyp26a1* is expressed in r7/8, and in the adjacent somites 1–3 (A arrowhead). In Cdx4-deficient embryos (B), *cyp26a1* expression is expanded into the spinal cord domain and terminates at the level of somite 5 (B arrowhead). Over-expression of *cdx4* using *Tg(hsp70:cdx4)* line (C) leads to abrogation of *cyp26a1* expression (D arrowheads). *cyp26b1* expression (E) in r3–4 and *cyp26c1* expression (G) in r2, r4,r6 remain unaffected in Cdx4-deficient embryos (F, H). (A–H) Embryos are co-stained with *krx20* (r3,r5), *myoD* (somites) and flat-mounted at the 10 somite stage. (A, B) $n=30/30$ (C, D) $n=15/15$, (E, H) $n=30/30$, (I, J) $n=10/10$ per condition.

markers. In RA-deficient embryos, *hoxb1a* expression in r4 expanded caudally while *krx20* expression in r5 and *mafb* in r5–6 were lost (Fig. 3B, F). However, in Cdx4/RA deficient embryos, *krx20* expression in r5 and *mafb* expression in r5–6 was rescued while *hoxb1a* expression was moderately reduced (Fig. 3D, H). Furthermore, rescued *mafb* expression was caudally expanded with its expression terminating at the level of somite 5, suggesting that the rescued hindbrain is expanded beyond its normal size (Fig. 3H arrow).

Fig. 3. Knockdown of Cdx4 in RA-deficient embryos rescues the posterior hindbrain. *hoxb1a* expression in r4 is slightly expanded in RA-deficient embryos (B) compared to wildtypes (A) and Cdx4-deficient embryos (C). Expression of *krx20* in r5; *mafb* in r5,r6; *hoxd4a* and *cyp26a1* in r7/8 are lost in RA-deficient embryos (F, N, R) compared to wildtypes (E, M, Q) and Cdx4-deficient embryos (G, O, S). In RA/Cdx4 deficient embryos *hoxb1a* (D) expression is reduced, *krx20* in r5 (D, H, L, P), *mafb* in r5,r6 (H) is rescued but *hoxd4a* (P) and *cyp26a1* (T) expression in r7/8 is still absent. Six *radical fringe* (*rf*) bands marking rhombomere boundaries are found in wildtypes (I) and Cdx4-deficient embryos (K). Three *rf* bands are detected in RA-deficient embryos (J), while two additional bands, flanking *krx20* expression in r5, return in RA/Cdx4 deficient embryos (L arrows). *Prickle1b* expression is in facial motor neurons of r4–6 in wildtypes (U bracket), *cdx4* morphants (W bracket) and RA deficient embryos (V bracket). Prickle1b expression is expanded into the r7/8 position in Cdx4/RA double deficient embryos (X bracket). (Y–Z, AA, BB) Lateral view of hindbrain motor neurons marked by Islet:gfp and r3,5 marked with *epha4*. Branchal motor neurons in *cdx4* morphants (AA) appear similar to wildtypes (Y). Vagal motor neurons of r7/8 are absent in RA deficient embryos (Z). Islet:gfp neurons return to r7/8 in Cdx4/RA double deficient embryos (BB arrows). RMO44 antibody marks reticulospinal neurons in wild-types (CC) and Cdx4-deficient embryos (EE). Mi and CaD are absent in RA-deficient embryos (DD) but return in Cdx4/RA deficient embryos (FF arrowheads). (A–P) are co-stained with *krx20* (r3, r5) and (E–dH, Q–T) are co-stained with myosin heavy chain antibody (somites). All embryos are flat-mounted, (A–T) at 20 somite stage and (U–Z, AA–FF) at 48 hpf. (A–D) $n=30/30$, (E–H) $n=15/15$, (I–T) $n=15/15$, (U–X) $n=20/20$, (Y–Z, AA–BB) $n=15/15$, (CC–FF) $n=13/15$ per condition.

Expression analysis of the r7/8 markers *hoxd4a* and *cyp26a1* showed that Cdx4 failed to rescue their transcription in RA-deficient embryos (Fig. 3M, P, Q, T), indicating that the rescue was limited to r5/r6 genes. To corroborate the rhombomere rescue, we analyzed the expression of *radical fringe (rfng)*, which in wild-type embryos is expressed in six distinctive stripes marking boundaries between each rhombomere (Fig. 3I). In RA-deficient embryos, *rfng* was expressed in three stripes that correspond to the boundaries between r1–4 (Fig. 3J). In Cdx4/RA deficient embryos, two of the three posterior boundaries lost in RA-deficient embryos are rescued (Fig. 3J, L arrows), suggesting the formation of all rhombomeres except r7/r8. This rescue was also observed at the morphological level. Using the *Tg(isl:gfp)* line we found that the rescued motor neurons, despite occupying the position of r7/8 and the anterior spinal cord, appear to resemble r4-6 facial motor neurons in their morphology and neuron like projections (Fig. 3BB arrows). To confirm that these neurons were facial motor neurons we stained Cdx4/RA deficient embryos with the facial motor neuron specific marker, *prickle1b* (Rohrschneider et al., 2007), finding that *prickle1b* expression was ectopically expressed in the rescued hindbrain region (Fig. 3U–X) further indicating that these rescued hindbrain segments develop r5–6 like properties. Additional motor neuron analysis shows that RMO44-positive Mi3 reticulospinal cord neurons located in wild type r5–r6 (Mendelson, 1986a), but lost in RA-deficient embryos (Fig. 3DD), were rescued by the additional loss of Cdx4 (Fig. 3FF arrowheads). Together, these results show that loss of Cdx4 partially alleviates the hindbrain phenotypes caused by the loss of RA, namely, the mid and some of the posterior hindbrain patterning defects, and consequently leads to the partial restoration of lost rhombomeres. However, despite the absence of the last rhombomere in Cdx4/RA deficient embryos, the hindbrain territory itself is still caudally expanded at the expense of the spinal cord indicating that the interaction between RA signaling and Cdx4 regulates the size of the hindbrain territory and the AP positioning of the Hb–Sc transition, while additional signaling mechanisms are required for directing complete patterning of the hindbrain. Thus, these results suggest that specification and patterning of the hindbrain and spinal cord regions are directed by separable regulatory mechanisms.

3.3. *Cdx4 and RA signaling pathway components cross regulate each other's transcription*

The opposite activities of Cdx4 and RA in hindbrain-spinal cord specification raised the possibility that they cross-regulate each other's expression or activity. To explore this possibility we examined the impact of eliminating Cdx4 upon components of the

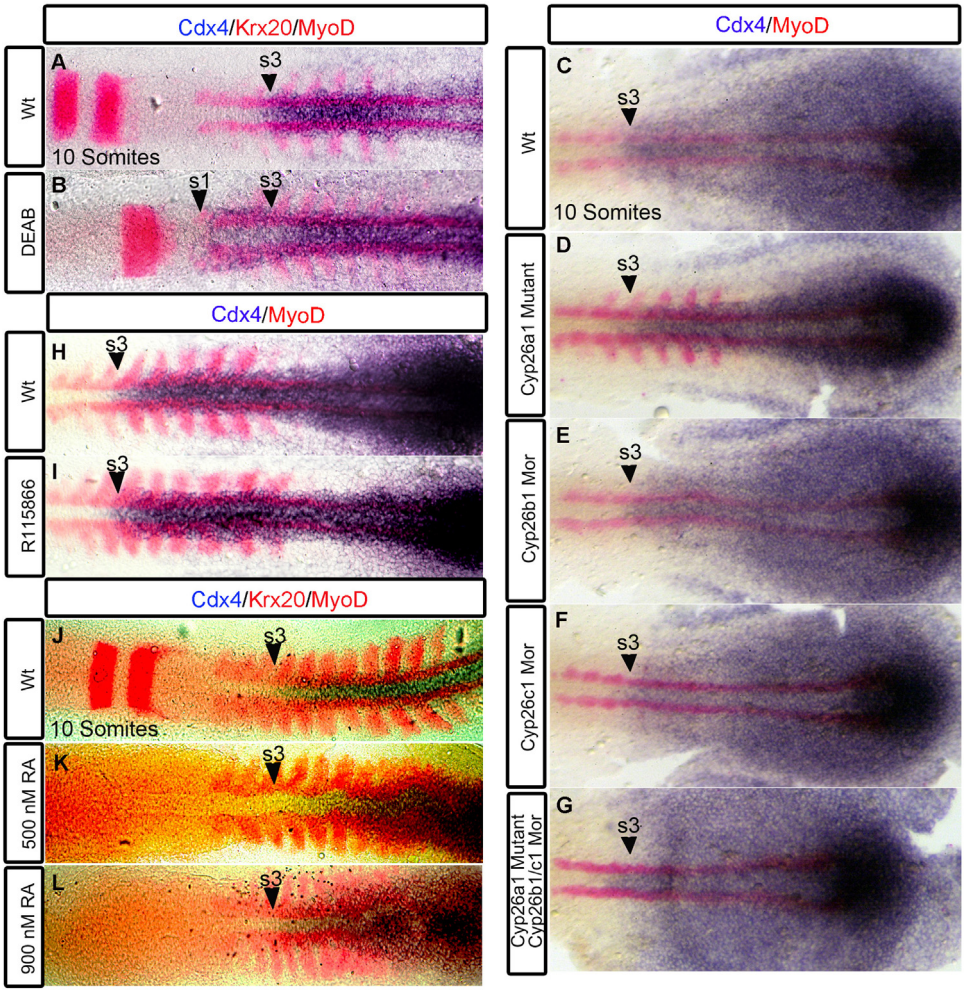


Fig. 5. Tissue specific regulation of *cdx4* expression by RA signaling. In wildtypes, *cdx4* anterior expression limit corresponds to the beginning of the spinal cord located at the level of somite 3 (A, C, H, J, arrowheads). *cdx4* expression is rostrally expanded to the level of somite 1 (B arrowhead) in RA-deficient embryos. Expression of *cdx4* is unchanged in R115866 treated embryos (I arrowhead), in embryos treated with 500 nM RA (K arrowhead) and 900 nM RA (L arrowhead). *cdx4* expression remains unchanged in *cyp26a1* mutants (D), *cyp26b1* morphants (E), *cyp26c1* morphants (F), and *cyp26a1* mutants injected with *cyp26b1* and *cyp26c1* morpholinos (G). (A–G, J–L) Embryos are co-stained with *myoD* (somites) and *krx20* (r3,r5). (H–J) Embryos co-stained with *myoD*. (A–L) Embryos are at 10 somite stage and flat-mounted. (A–B, H–I) $n=30/30$, (C–G) $n>12$, (J–L) $n=20/20$ per condition.

RA signaling pathway and the impact of altering RA signaling on *cdx4* expression. We first focused on the effect Cdx4 activity has upon the RA signaling pathway by analyzing the expression of enzymes involved in RA synthesis and degradation. RA is synthesized in the paraxial mesoderm by the enzyme *aldh2a* (Begemann et al., 2001; Grandel et al., 2002) and degraded in the hindbrain by the enzymes *cyp26a1*, *cyp26b1* and *cyp26c1* (Emoto et al., 2005; Hernandez et al., 2007; White et al., 2007). In Cdx4-deficient embryos, we corroborated that *aldh2a* expression domain was at the same axial level as in wild types, with a slight caudal expansion (Shimizu et al., 2006; Skromne et al., 2007; Wingert et al., 2007). In contrast, the expression domain of *cyp26a1*, but not *cy26b1* or *cyp26c1*, was expanded caudally into what ought to be the spinal cord territory in Cdx4-deficient embryos (Fig. 4A, B, E–H). *cyp26a1* is the RA degradation enzyme that is most caudally expressed in r7/8 of the hindbrain and its shift in expression suggests that Cdx4 represses its transcription (Fig. 4A, B). To further test this, we ectopically induced *cdx4* expression from a heat inducible transgene (Skromne et al., 2007). Transgenic induction of *cdx4* at tailbud stage caused the loss of *cyp26a1* expression at 10-somite stage, confirming that Cdx4 represses *cyp26a1* expression (Fig. 4C, D). These results suggest that Cdx4 inhibits the expression of the RA inhibitor *cyp26a1*, thus allowing high levels of RA signaling in the spinal cord.

To determine if RA regulates *cdx4* transcription, we examined *cdx4* expression in RA-deficient embryos at 10-somite stage, when *cdx4* expression is normally restricted to the prospective spinal cord territory between somite 3 to the tailbud (Fig. 5A; Skromne et al., 2007). Loss of RA caused the rostral expansion of *cdx4* expression domain into an embryonic region that in the wild type embryo would become hindbrain (Fig. 5B). This result suggests that RA normally inhibits *cdx4* expression in the hindbrain. To test this idea, we characterized the expression of *cdx4* in embryos overexpressing RA by systematically eliminating *cyp26* activities by using previously published combinations of mutant and morpholino approaches (Emoto et al., 2005; Hernandez et al., 2007). Significantly, the individual or combined elimination of Cyp26-a1, -b1, and -c1 did not alter the expression domain of *cdx4* (Fig. 5C–G). Similar findings to the individual loss of Cyp26 activities were obtained by exposing embryos to the pan-Cyp26 inhibitor R115866 to increase endogenous RA levels (Fig. 5H, I) or by treating the embryos with exogenous RA (Fig. 5J–L), Together these gain and loss of function experiments suggest a context-dependent regulation of *cdx4* by RA whereby RA is necessary for restricting *cdx4* expression to the prospective spinal cord territory and preventing its expansion into the hindbrain, but is not sufficient to repress *cdx4* expression within the spinal cord.

3.4. RA, Cyp26a1 and Cdx4 establish a feedback loop to regulate hox gene expression and establish the axial position of the Hb–Sc transition

It has been shown previously that excess RA leads to a posteriorization of the hindbrain by activating posterior hindbrain *hox* genes in anterior hindbrain regions (Durston et al., 1989; Emoto et al., 2005; Grandel et al., 2002; Hernandez et al., 2007). In this paper, we show that excess RA perturbs the position of the Hb–Sc transition without affecting the *cdx4* expression domain in the spinal cord (Figs. 2I, J, and 5I, K, L). In contrast, loss of Cdx4 both perturbs *cyp26a1* expression (Fig. 4B) and expands the hindbrain region at the expense of the spinal cord. These observations raise the question of what regulatory impact Cdx4 has on RA signaling in establishing hindbrain and spinal cord identities. To answer this question, we compared the spatial distribution of *hox* gene transcripts and motor neuron populations in wild type and Cdx4-deficient embryos treated with a pan-Cyp26 inhibitor to block RA degradation. We opted to increase RA levels by this method since it directly changes RA levels in the neural tissue while leaving the paraxial mesoderm, a topographically important structure for our analysis, morphologically unaffected. As previously reported (Emoto et al., 2005; Hernandez et al., 2007), increase in RA signaling due to Cyp26 inhibition caused the loss of *hoxa2b* expression in the anterior hindbrain (Fig. 1Q), and rostrally expanded the expression of posterior hindbrain markers *hoxb3a* and *hoxb4a* (Fig. 1R, S). Cyp26 inhibition also caused the anterior expansion of the spinal cord marker *hoxb8a* (Fig. 1T). These changes at the gene expression level were also observed at the morphological level, with the loss of trigeminal (r2/r3) and facial (r4–r6) motor neurons and the rostral expansion of vagal (r7/8) and spinal motor neurons (Fig. 2I, J). Thus, failure to restrict RA signaling in the hindbrain causes this tissue to develop improperly, suggesting that Cyp26 activity is required for maintaining separate hindbrain and spinal cord territories.

To test if the shift in *hoxb8a* expression and spinal motor neurons was dependent on Cdx4, we simultaneously elevated RA levels while eliminating Cdx4. Loss of Cdx4 did not change the phenotypes associated with the loss of Cyp26: we observed equivalent changes in *hox* gene expression and motor neuron distribution in Cyp26 and Cyp26/Cdx4-deficient embryos (Figs. 1Q–X and 2I–L). The observation that loss of Cyp26/Cdx4 mirrors Cyp26 loss of function phenotype suggests that Cyp26 is epistatic to Cdx4 in defining the AP position of the Hb–Sc transition. Furthermore, these results indicate that increased RA levels through inhibition of Cyp26 can compensate for the reduction of the spinal cord territory observed in Cdx4-deficient embryos. As we have shown that Cdx4 normally acts to inhibit *cyp26a1* expression in the anterior spinal cord (Fig. 4B, D), these results imply that Cdx4 has a positive modulatory effect on RA levels to establish the anterior spinal cord and limit the extent of the hindbrain territory. These interactions normally play an important role in the proper positioning of the hindbrain–spinal cord boundary.

4. Discussion

Our findings show that cross-regulatory interactions between RA signaling and Cdx4 are crucial for the correct partitioning of the posterior neural plate into separate domains of the hindbrain and spinal cord. By comparing the AP position of patterning gene expression and motor neuron architecture in RA and Cdx4 single and double deficient embryos, we show that these two molecular pathways interact antagonistically to regulate hindbrain and spinal cord territory size. This is in addition to the individual roles played by RA and Cdx4 during the organization of the hindbrain and spinal cord, suggesting that the processes specifying territory size and identity of the posterior neural plate are under the control of distinct regulatory mechanisms. Significantly, epistatic experiments analyzing RA signaling and Cdx4 interactions reveal that feedback regulatory loops determine the location of the hindbrain–spinal cord (Hb–Sc) transition. This feedback regulation provides a testable model of interactions for the partitioning of large territories into smaller domains (Fig. 6A).

4.1. Separate processes direct specification versus patterning of the hindbrain and spinal cord

Loss and gain of function studies of RA signaling and Cdx4 have suggested that these factors contribute to hindbrain and spinal cord development, respectively, through the regulation of *hox* gene expression (Gould et al., 1998; Marshall et al., 1994; Shimizu et al., 2006; Skromne et al., 2007; Hayward et al., 2015). Here, we provide evidence for a novel interaction of RA signaling and Cdx4

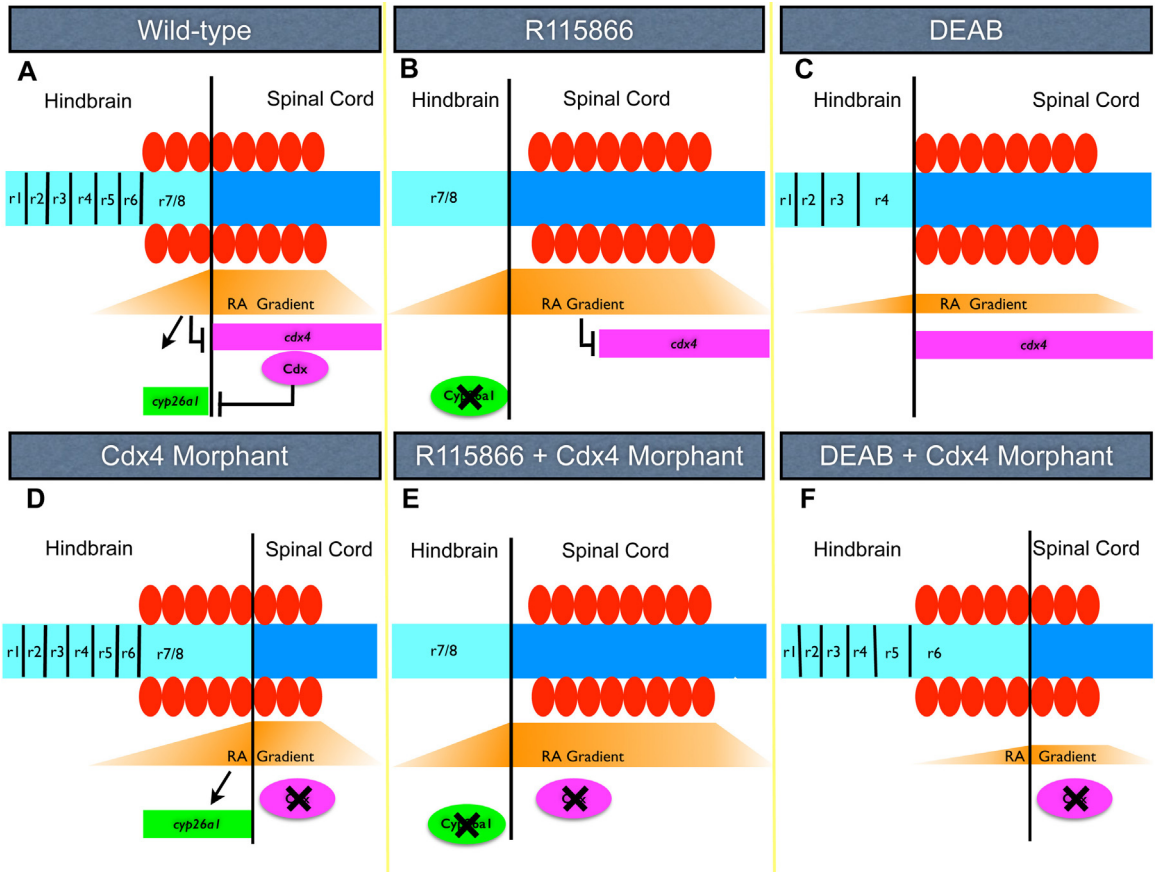


Fig. 6. Model summarizing the genetic interaction between Cyp26a1, Cdx4 and RA signaling with the AP-position of the Hb–Sc transition. (A) In wild-type embryos, the hindbrain (light blue) ends, and the spinal cord (dark blue) begin at the level of somite 3 (red ovals). In the posterior hindbrain, *cyp26a1* expression required for posterior rhombomere formation (Emoto et al., 2005; Hernandez et al., 2007) is under the dual regulation of RA working as an activator, and Cdx4 working as a repressor. RA also represses *cdx4* transcription, but only in the posterior hindbrain and not in the spinal cord. Maintenance of *cdx4* expression in the spinal cord may be due to the activity of other somite-derived signals (e.g., FGF, Wnts; Lee and Skromne, 2014). (B) Treatment of embryos with the Cyp26 inhibitor R115866 elevates RA levels and shifts the Hb–Sc transition rostrally; however *cdx4* expression in the spinal cord remains unchanged suggesting that RA signaling is not sufficient to inhibit *cdx4* expression in the spinal cord. (C) Embryos treated with DEAB are RA deficient and both *cdx4* expression and the Hb–Sc transition are shifted rostrally to the level of somite 1 in these embryos suggesting that RA inhibits the expansion of *cdx4* expression into the hindbrain. (D) In the absence of Cdx4, *cyp26a1* expression and the hindbrain domain is expanded and the Hb–Sc transition is shifted caudally. (E) Cyp26/Cdx4 double deficient embryos phenocopy Cyp26 deficient embryos with an expanded spinal cord territory and a rostrally shifted Hs–Sc transition. This indicates that Cyp26 is epistatic to Cdx4. (F) Cdx4/RA double deficient embryos have an expanded hindbrain territory and a caudally shifted Hb–Sc transition. The AP position of the Hb–Sc transition in Cdx4/RA double deficient and Cdx4 deficient embryos are both located at the level of somite 5 suggesting that Cdx4 is epistatic to RA signaling.

function in establishing hindbrain and spinal cord territories independent of their *hox* regulatory roles. Of significance is the rostral shift in position of spinal motor neurons without the alteration of *hox* gene expression in RA-deficient embryos (Figs. 1L and S1B,D), indicating that the size of the spinal cord territory can be modified without shifting *hox* gene expression. Further support for the idea that territory specification and patterning are independent events, comes from the finding that the elimination of Cdx4 in an RA-deficient embryo rescues the r5–6 cranial motor neurons lost by the lack of RA without rescuing the expression of caudal hindbrain *hox* genes (Figs. 2G and 3P, T, BB, FF). Thus, the correlated expression of *hox* patterning genes is not required for the specification of the posterior neural plate into separate spinal cord and hindbrain territories.

The rescue of posterior hindbrain neuronal populations in Cdx4/RA-deficient embryos that do not express characteristic *hox* genes in the posterior hindbrain is difficult to reconcile with the extensive evidence supporting RA's role in regulating posterior hindbrain *hox* gene expression (reviewed in Gavalas and Krumlauf, 2000; Niederreither et al., 2000), and the role of posterior hindbrain *hox* genes in specifying posterior hindbrain identities (Dollé et al., 1993; Lufkin et al., 1991; Marshall et al., 1992). We propose that loss of Cdx4 function can compensate for the lack of RA signaling required for the activation of r5–6 patterning gene expression (Figs. 1N and 3H) and suggest that activation of these patterning genes by RA signaling could be mediated, at least in part, through the repression of Cdx4. Consistent with this hypothesis, our expression analyses of RA deficient embryos suggest that RA signaling inhibits *cdx4* expression in the posterior hindbrain (Fig. 5B). Cdx transcription factors have been shown to inhibit hindbrain development (Shimizu et al., 2006; Skromne et al., 2007) and in mouse there is evidence to suggest that Cdx factors directly limit the expression of the r5–6 patterning gene *Mafb* (Sturgeon et al., 2011). Therefore, exclusion of Cdx4 from the hindbrain would be necessary for this tissue's development. Whether RA relieves the repressive effect that Cdx has on hindbrain patterning genes remains to be determined. It is also possible that part of this effect is through Cdx4-dependent regulation of other RA pathway genes, as shown by the ability of Cdx4 protein to bind to these genes' enhancer regions (Paik et al., 2013). In this case, attenuation of Cdx4 function in RA-deficient embryos may de-repress factors controlling RA signaling, thus lowering the threshold requirement and making cells more sensitive to RA (Cai et al., 2012). Consequently, cells would still be able to detect and interpret low levels of RA signaling sufficiently to activate some RA response genes. This could explain why expression of genes found

in r5–6 cells, which have been shown to require less RA, are rescued, but not that of r7/8 cells which require a higher concentration of RA for rhombomere-specific gene activation (Dupé and Lumsden, 2001; Gould et al., 1998; Maves and Kimmel, 2005).

Alternatively, the rescue and expansion of the hindbrain territory could indirectly influence the return of r5–6 in Cdx4/RA deficient embryos. In this scenario, hindbrain size reduction caused by the loss of RA may limit the development of posterior hindbrain rhombomeres because not enough territory is available for the allocation of all rhombomere fates. By expanding the hindbrain territory through inhibiting Cdx4 function in RA deficient embryos, adequate space is now available to accommodate r5–6 formation. However in this case, even though the hindbrain territory is greatly expanded to beyond wild-type levels (Figs. 1N, 2G, and 3H, X), we note that the extent of the rescue in hindbrain fate is limited to r5–6 only and does not include the r7/8 region. Previous studies have shown that RARE sites are required to drive expression of r7/8-specific *hox* genes (Gould et al., 1998; Nolte et al., 2006; Punnamoottil et al., 2010). The requirement for direct signaling by RA may explain why rescue of r7/8 *hox* gene expression is not observed in Cdx4/RA deficient embryos despite the rescue of hindbrain territory size (Figs. 2G and 3P, T). Alternatively the signaling pathways required for r7/8 formation may continue to be affected in Cdx4/RA deficient embryos. Since no reports to date have shown that RA signaling regulates r5–6 patterning gene expression directly (Hernandez et al., 2004), in this scenario, RA could be indirectly regulating r5–6 development by maintaining the size of the hindbrain territory. In either case, these findings show that a regulatory interaction between Cdx4 and RA signaling is necessary to establish the territory size of the hindbrain, while RA signaling regulates patterning of r7/8 independently of Cdx4. Thus, these data again suggest that there are distinct mechanisms mediating patterning versus specification of the hindbrain and spinal cord territories.

4.2. Regulatory feedback loop between RA, Cdx4 and Cyp26 establishes hindbrain and spinal cord territories

Previous reports have demonstrated that RA signaling modulates its own pathway in the hindbrain by activating the expression of its degradation enzyme, *cyp26a1* (Dobbs-McAuliffe et al., 2004; Kinkel et al., 2008; Kudoh et al., 2002; White et al., 2007). This regulatory interaction was suggested to regulate the size of the hindbrain–spinal cord territories (Emoto et al., 2005; Lee and Skromne, 2014). Our results support and expand upon previous findings by examining the molecular interactions between RA signaling and Cdx4 function, to demonstrate that *cyp26a1* expression is under the dual regulation of Cdx4 and RA signaling, and that these interactions are essential for establishing the position of the Hb–Sc transition.

Through performing genetic interaction experiments, we identify Cyp26a1 as a key RA signaling pathway component whose regulation by Cdx4 is essential for establishing the hindbrain territory and Hb–Sc transition. In the posterior neural plate, Cyp26 gene expression is normally limited to the hindbrain, where it promotes anterior and suppresses posterior neural gene expression (Kudoh et al., 2002). In loss of function experiments, Cyp26 elimination results in the rostral expansion of the spinal cord territory at the expense of the hindbrain (Figs. 1T and 2J; Hernandez et al., 2007). In contrast, knock down of Cdx4 function expands both the caudal expression of *cyp26a1* (Fig.4B) and caudal extent of the hindbrain territory (Figs. 1G and 2C) at the expense of the spinal cord, suggesting that suppression of *cyp26a1* expression by Cdx4 is normally necessary to establish the spinal cord territory. Simultaneous inhibition of both Cyp26 and Cdx4 function (Figs. 1X and 2L) results in the rescue of the lost spinal cord territory characteristic of Cdx4-deficient embryos, confirming that Cyp26 is epistatic to Cdx4 in regulating the size of the spinal cord region (Fig. 6E).

In contrast to *cyp26*, *cdx4* expression is confined to the spinal cord territory where it promotes spinal cord formation and suppresses hindbrain fates (Fig. 5A; Shimizu et al., 2006; Skromne et al., 2007; Sturgeon et al., 2011). Here we show that, in the absence of RA signaling, *cdx4* expression is expanded rostrally, mirroring the loss of hindbrain territory and the expansion of spinal cord territory in these embryos (Figs. 5B and S1B) suggesting that an inhibitory relationship between RA signaling and Cdx4 is involved in establishing the Hb–Sc boundary. Further confirmation of this interaction is shown through analyses of Cdx4/RA double deficient embryos (Figs. 1N, 2G, and 3H, L), which display an expanded hindbrain phenotype similar to Cdx4-deficient embryos, indicating that Cdx4 is epistatic to RA signaling in determining the size of the hindbrain territory. As we have shown that *cyp26a1* is under the repression of Cdx4, these data show that RA signaling may additionally be restricting *cdx4* expression to ensure proper activation of *cyp26a1* transcription in the hindbrain territory.

Our findings suggest that, in the posterior neural tube, RA signaling limits *cdx4* expression to the presumptive spinal cord. How RA signaling selectively inhibits *cdx4* expression in the hindbrain and not in the spinal cord remains to be determined. RA signaling has been shown to spatially restrict the expression of downstream target genes through acting as both an activator and a repressor. For example, in the case of *hoxb1* transcription RA signaling first establishes a broad expression domain, which it then refines by suppression of *hoxb1* expression in rhombomeres adjacent to r4 (Studer et al., 1994). Two RAREs in the regulatory region of *hoxb1* have been shown to mediate these different responses (Studer et al., 1994). RAREs have also been identified in the regulatory region of Cdx and can drive *cdx* expression in cell culture assays and mouse embryos (Gaunt et al., 2003; Houle et al., 2000). However, inhibitory factors, activated by RA signaling in the anterior region of the embryo, have also been shown to competitively bind to the RARE suggesting that regulation of *cdx* expression by RA is likely context dependent (Béland and Lohnes, 2005). Additional analysis of the Cdx4 regulatory region will help clarify the regulatory role of RA signaling on *cdx4* expression in the neural tube.

A recent paper (Lee and Skromne, 2014) showed that loss of RA signaling alone or in combination with loss of FGF selectively alters the anterior expression limit of *cdx4*. Lee and Skromne (2014) additionally showed that expression of *cdx4* in the spinal cord can be severely perturbed when RA-deficient embryos are also made Wnt deficient. Their findings suggest that Wnt and Fgf signaling may also be modifying the regulatory effect of RA signaling on *cdx4* expression. Multiple signaling pathways, including Wnts, FGFs and RA, have been shown to regulate *cdx4* expression (Gaunt et al., 2003; Houle et al., 2000; Isaacs et al., 1998; Shimizu et al., 2005). Our results expand upon these previous findings by characterizing the epistatic relationships between RA, Cyp26 and Cdx4 at the Hb–Sc junction, thereby demonstrating that cross regulation of *cdx4* expression by molecular components of the RA signaling pathway is part of a regulatory feed-back loop involved in directing the precise compartmentalization and development of the hindbrain and spinal cord. In addition, we examined the impact of these interactions on the global expression of rhombomere segmentation markers (Fig. 3I–L), patterning genes (M–P) and motor neuron subtype specific genes (U–X); our comprehensive approach has led us to suggest that the initial specification of the hindbrain and spinal cord territories versus the patterning of these two areas may be regulated by distinct processes, albeit likely involving the same signaling molecules utilized at different times of development or with different downstream effectors. In

particular, we find that the hindbrain territory size does not necessarily specify the number of formed hindbrain segments. For example, in Cdx4/RA double deficient embryos, we show that the rescued Islet1-Gfp neurons only express r4–6 neuron specific genes and that the r7/8 segment fails to form even within a larger than normal sized hindbrain region (summarized in Fig. 6F).

Having established that RA and Cdx4 have functions that are separate from their regulatory roles on patterning gene expression, we propose a model where the territories of the hindbrain and spinal cord are established by a feedback regulatory loop involving RA, Cyp26a1 and Cdx4 (Fig. 6A). In this model (Fig. 6A), RA signaling excludes *cdx4* expression from the future hindbrain territory while Cdx4 limits the expression of the RA degradation enzyme, *cyp26a1*, from the spinal cord. The cross-regulatory interaction between these factors is necessary to ensure that cells receive the correct levels of RA and Cdx4 required to specify the hindbrain versus spinal cord territories since perturbation of either RA signaling (Fig. 6B, C) or Cdx4 function (6D) causes the Hb–Sc transition to be mis-aligned. Through these interactions, Cdx4 and RA function are spatially regulated to distinguish the posterior neural tissue into two separate structures of the hindbrain and spinal cord.

Acknowledgments

We thank members of the V. Prince and R.K.H laboratories for helpful comments; V. Prince for critically reading the manuscript; A. Kuuspalu for technical assistance and fish care; and members of the zebrafish community for reagents.

This study was supported in part by NIH Molecular and Cellular Biology Training Grant T32 GM007183 (J.C.), the Helen Hay Whitney Foundation (I.S.) and NSF Grant IOS-090449 (I.S.), and NIH Grant GM067714 (R.K.H.).

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.bios.2014.05.063.

References

Begemann, G., Schilling, T.F., Rauch, G.J., Geisler, R., Ingham, P.W., 2001. The zebrafish neckless mutation reveals a requirement for raldh2 in mesodermal signals that pattern the hindbrain. Development 128, 3081–3094.

Béland, M., Lohnes, D., 2005. Chicken ovalbumin upstream promoter-transcription factor members repress retinoic acid-induced Cdx1 expression. J. Biol. Chem. 14, 13858–13862.

Bel-Vialar, S., Itasaki, N., Krumlauf, R., 2002. Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. Development 129, 5103–5115.

Cai, A.Q., Radtke, K., Linville, A., Lander, A.D., Nie, Q., Schilling, T.F., 2012. Cellular retinoic acid-binding proteins are essential for hindbrain patterning and signal robustness in zebrafish. Development 12, 2150–2155.

Carpenter, E.M., Goddard, J.M., Chisaka, O., Manley, N.R., Capecchi, M.R., 1993. Loss of *Hoxa-1 (Hox-1.6)* function results in the reorganization of the murine hindbrain. Development 118, 1063–1075.

Charité, 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.

Chisaka, O., Capecchi, M., 1991. Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene *hox1.5*. Nature 350, 473–479.

Cheng, Y.C., Amoyel, M., Qiu, X., Jiang, Y.J., Xu, Q., Wilkinson, D.G., 2004. Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain. Dev. Cell 6, 539–550.

Davidson, A.J., Zon, L.I., 2006. The caudal-related homeobox genes cdx1a and cdx4 act redundantly to regulate hox gene expression and the formation of putative hematopoietic stem cells during zebrafish embryogenesis. Dev. Biol. 2, 506–518.

Dobbs-McAuliffe, B., Zhao, Q., Linney, E., 2004. Feedback mechanisms regulate retinoic acid production and degradation in the zebrafish embryo. Mech. Dev. 4, 339–350.

Dollé, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D., Chambon, P., 1993. Local alterations of Krox-20 and Hox gene expression in the hindbrain suggest lack of rhombomeres 4 and 5 in homozygote null Hoxa-1 (Hox-1.6) mutant embryos. Proc. Natl. Acad. Sci. U.S.A. 16, 7666–7670.

Dupé, V., Lumsden, A., 2001. Hindbrain patterning involves graded responses to retinoic acid signalling. Development 128, 2199–2208.

Durston, A.J., Timmermans, J.P., Hage, W.J., Hendriks, H.F., de Vries, N.J., Heideveld, M., Nieuwkoop, P.D., 1989. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 340, 140–144.

Emoto, Y., Wada, H., Okamoto, H., Kudo, A., Imai, Y., 2005. Retinoic acid-metabolizing enzyme Cyp26a1 is essential for determining territories of hindbrain and spinal cord in zebrafish. Dev. Biol. 278, 415–427.

Gaunt, S.J., Krumlauf, R., Duboule, D., 1989. Mouse homeo-genes within a subfamily, Hox-1.4, -2.6 and -5.1, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation. Development 107, 131–141.

Gaunt, S.J., Drage, D., Cockley, A., 2003. Vertebrate caudal gene expression gradients investigated by use of chick cdx-A/lacZ and mouse cdx-1/lacZ reporters in transgenic mouse embryos: evidence for an intron enhancer. Mech. Dev. 120, 573–586.

Gavalas, A., Krumlauf, R., 2000. Retinoid signalling and hindbrain patterning. Curr. Opin. Genet. Dev. 4, 380–386.

Gould, A., Itasaki, N., Krumlauf, R., 1998. Initiation of rhombomeric Hoxb4 expression requires induction by somites and a retinoid pathway. Neuron 21, 39–51.

Grandel, H., Lun, K., Rauch, G.J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Küchler, A.M., Schulte-Merker, S., Geisler, R., Holder, N., Wilson, S.W., Brand, M., 2002. Retinoic acid signalling in the zebrafish embryo is necessary during presegmentation stages to pattern the anterior–posterior axis of the CNS and to induce a pectoral fin bud. Development 12, 2851–2865.

Gruss, P., Kessel, M., 1991. Axial specification in higher vertebrates. Curr. Opin. Genet. Dev. 1, 204–210.

Gu, X., Xu, F., Wang, X., Gao, X., Zhao, Q., 2005. Molecular cloning and expression of a novel CYP26 gene (cyp26d1) during zebrafish early development. Gene Expr. Patterns 5, 733–739.

Hayward II, A.G., Joshi, P., Skromne, I., 2015. Spatiotemporal analysis of zebrafish hox gene regulation by Cdx4. Dev. Dyn. 244, 1564–1573.

Hernandez, R.E., Rikhof, H.A., Bachmann, R., Moens, C.B., 2004. vhnf1 integrates global RA patterning and local FGF signals to direct posterior hindbrain development in zebrafish. Development 131, 4511–4520.

Hernandez, R.E., Putzke, A.P., Myers, J.P., Margaretha, L., Moens, C.B., 2007. Cyp26 enzymes generate the retinoic acid response pattern necessary for hindbrain development. Development 134, 177–187.

Higashijima, S., Hotta, Y., Okamoto, H., 2000. Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter. J. Neurosci. 20, 206–218.

Houle, M., Prinos, P., Iulianella, A., Bouchard, N., Lohnes, D., 2000. Retinoic acid regulation of Cdx1: an indirect mechanism for retinoids and vertebral specification. Mol. Cell. Biol. 20, 6579–6586.

Hunt, P., Wilkinson, D., Krumlauf, R., 1991. Patterning the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating cranial neural crest. Development 112, 43–50.

Isaacs, H.V., Pownall, M.E., Slack, J.M., 1998. Regulation of Hox gene expression and posterior development by the Xenopus caudal homologue Xcad3. EMBO J. 17, 3413–3427.

Kinkel, M.D., Eames, S.C., Alonzo, M.R., Prince, V.E., 2008. Cdx4 is required in the endoderm to localize the pancreas and limit beta-cell number. Development 135, 919–929.

Krumlauf, R., Marshall, H., Studer, M., Nonchev, S., Sham, M.H., Lumsden, A., 1993. Hox homeobox genes and regionalisation of the nervous system. J. Neurobiol. 10, 1328–1340.

Kudoh, T., Wilson, S.W., Dawid, I.B., 2002. Distinct roles for Fgf, Wnt and retinoic acid inposteriorizing the neural ectoderm. Development 129, 4335–4346.

Lee, K., Skromne, I., 2014. Retinoic acid regulates size, pattern and alignment of tissues at the head-trunk transition. Development 141, 4375–4384.

Lufkin, T., Dierich, A., LeMeur, M., Mark, M., Chambon, P., 1991. Disruption of the Hox-1.6 homeobox gene results in defects in a region corresponding to its rostral domain of expression. Cell 6, 1105–1119.

Maden, M., Sonneveld, E., van der Saag, P.T., Gale, E., 1998. The distribution of endogenous retinoic acid in the chick embryo: implications for developmental mechanisms. Development 125, 4133–4144.

Marshall, H., Nonchev, S., Sham, M.H., Muchamore, I., Lumsden, A., Krumlauf, R., 1992. Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. Nature 360, 737–741.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S., Krumlauf, R., 1994. A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. Nature 370, 567–571.

Martinez-Barbera, J.P., Signore, M., Boyl, P.P., Puelles, E., Acampora, D., Gogoi, R., Schubert, F., Lumsden, A., Simeone, A., 2001. Regionalisation of anterior neuroectoderm and its competence in responding to forebrain and midbrain inducing activities depend on mutual antagonism between OTX2 and GBX2. Development 128, 4789–4800.

Maves, L., Kimmel, C.B., 2005. Dynamic and sequential patterning of the zebrafish

posterior hindbrain by retinoic acid. Dev. Biol. 285, 593–605.
Melton, K.R., Iulianella, A., Trainor, P.A., 2004. Gene expression and regulation of hindbrain and spinal cord development. Front. Biosci. 9, 117–138.
Mendelson, B., 1986a. Development of reticulospinal neurons of the zebrafish. II. Early axonal outgrowth and cell body position. J. Comp. Neurol. 251, 172–184.
Moens, C.B., Cordes, S.P., Giorgianni, M.W., Barsh, G.S., Kimmel, C.B., 1998. Equivalence in the genetic control of hindbrain segmentation in fish and mouse. Development 125, 381–391.
Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P., Dollé, P., 2000. Retinoic acid synthesis and hindbrain patterning in the mouse embryo. Development 127, 75–85.
Nolte, C., Rastegar, M., Amores, A., Bouchard, M., Grote, D., Maas, R., Kovacs, E.N., Postlethwait, J., Rambaldi, I., Rowan, S., Yan, Y.L., Zhang, F., Featherstone, M., 2006. Stereospecificity and PAX6 function direct Hoxd4 neural enhancer activity along the antero-posterior axis. Dev. Biol. 299, 582–593.
Novitch, B.G., Wichterle, H., Jessell, T.M., Sockanathan, S., 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. Neuron 40, 81–95.
Oxtoby, E., Jowett, T., 1993. Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain develiopment. Nuclei Acids Res. 21, 1087–1095.
Paik, E.J., Mahony, S., White, R.M., Price, E.N., Dibiase, A., Dorjsuren, B., Mosimann, C., Davidson, A.J., Gifford, D., Zon, L.I., 2013. A Cdx4–Sall4 regulatory module controls the transition from mesoderm formation to embryonic hematopoiesis. Stem Cell. Rep. 5, 425–436.
Pierani, A., Brenner-Morton., S., Chiang, C., Jessell, T.M., 1999. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell 97, 903–915.
Prince, V.E., Moens, C.B., Kimmel, C.B., Ho, R.K., 1998a. Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, valentino. Development 125, 393–406.
Prince, V.E., Joly, L., Ekker, M., Ho, R.K., 1998b. Zebrafish hox genes: genomic organization and modified colinear expression patterns in the trunk. Development 125, 407–420.
Punnamoottil, B., Herrmann, C., Pascual-Anaya, J., D'Aniello, S., Garcia-Fernàndez, J., Akalin, A., Becker, T.S., Rinkwitz, S., 2010. Cis-regulatory characterization of sequence conservation surrounding the Hox4 genes. Dev. Biol. 340, 269–282.
Rhinn, M., Brand, M., 2001. The midbrain–hindbrain boundary organizer. Curr. Opin. Neurobiol. 11, 34–42.
Rohrschneider, M.R., Elsen, G.E., Prince, V.E., 2007. Zebrafish Hoxb1a regulates multiple downstream genes including prickle1b. Dev. Biol. 2, 358–372.
Schilling, T.F., Knight, R.D., 2001. Origins of anteroposterior patterning and Hox gene regulation during chordate evolution. Philos. Trans. R. Soc. Lond. B Biol. Sci. 356, 1599–1613.
Shimizu, T., Bae, Y.K., Muraoka, O., Hibi, M., 2005. Interaction of Wnt and caudal-related genes in zebrafish posterior body formation. Dev. Biol. 1, 125–141.
Shimizu, T., Bae, Y.K., Hibi, M., 2006. Cdx–Hox code controls competence for responding to Fgfs and retinoic acid in zebrafish neural tissue. Development 133, 4709–4719.
Shimozono, S., Iimura, T., Kitaguchi, T., Higashijima, S., Miyawaki, A., 2013. Visualization of an endogenous retinoic acid gradient across embryonic development. Nature 496, 363–366.
Skromne, I., Thorsen, D., Hale, M., Prince, V.E., Ho, R.K., 2007. Repression of the hindbrain developmental program by Cdx factors is required for the specification of the vertebrate spinal cord. Development 134, 2147–2158.
Stern, C.D., 2001. Initial patterning of the central nervous system: how many organizers? Nat. Rev. Neurosci. 2, 92–98.
Studer, M., Pöpperl, H., Marshall, H., Kuroiwa, A., Krumlauf, R., 1994. Role of a conserved retinoic acid response element in rhombomere restriction of Hoxb-1. Science 265, 1728–1732.
Sturgeon, K., Kaneko, T., Biemann, M., Gauthier, A., Chawengsaksophak, K., Cordes, S.P., 2011. Cdx1 refines positional identity of the vertebrate hindbrain by directly repressing Mafb expression. Development 138, 65–74.
Trevarrow, B., Marks, D.L., Kimmel, C.B., 1990. Organization of hindbrain segments in the zebrafish embryo. Neuron 4, 669–679.
Wagner, M., Han, B., Jessell, T.M., 1992. Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. Development 116, 55–66.
Weinberg, E.S., Allende, M.L., Kelly, C.S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O.G., Grunwald, D.J., Riggleman, B., 1996. Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. Development 122, 271–280.
White, R.J., Nie, Q., Lander, A.D., Schilling, T.F., 2007. Complex regulation of cyp26a1creates a robust retinoic acid gradient in the zebrafish embryo. Plos. Biol. 5, e304.
Wingert, R.A., Selleck, R., Yu, J., Song, H.D., Chen, Z., Song, A., Zhou, Y., Thisse, B., Thisse, C., McMahon, A.P., Davidson, A.J., 2007. The cdx genes and retinoic acid control the positioning and segmentation of the zebrafish pronephros. PLoS Genet. 3, 1922–1938.
Wood, H., Pall, G., Morriss-Kay, G., 1994. Exposure to retinoic acid before or after the onset of somitogenesis reveals separate effects on rhombomeric segmentation and 3′ HoxB gene expression domains. Development 120, 2279–2285.