

Repression of the hindbrain developmental program by Cdx factors is required for the specification of the vertebrate spinal cord

Isaac Skromne*, Dean Thorsen, Melina Hale, Victoria E. Prince and Robert K. Ho

The spinal cord is a unique vertebrate feature that originates, together with the hindbrain, from the caudal neural plate. Whereas the hindbrain subdivides into rhombomeres, the spinal cord remains unsegmented. We have identified Cdx transcription factors as key determinants of the spinal cord region in zebrafish. Loss of Cdx1a and Cdx4 functions causes posterior expansion of the hindbrain at the expense of the unsegmented spinal cord. By contrast, *cdx4* overexpression in the hindbrain impairs rhombomere segmentation and patterning and induces the expression of spinal cord-specific genes. Using cell transplantation, we demonstrate that Cdx factors function directly within the neural ectoderm to specify spinal cord. Overexpression of 5' Hox genes fails to rescue hindbrain and spinal cord defects associated with *cdx1a/cdx4* loss-of-function, suggesting a Hox-independent mechanism of spinal cord specification. In the absence of Cdx function, the caudal neural plate retains hindbrain characteristics and remains responsive to surrounding signals, particularly retinoic acid, in a manner similar to the native hindbrain. We propose that by preventing the posterior-most region of the neural plate from following a hindbrain developmental program, Cdx factors help determine the size of the prospective hindbrain and spinal cord territories.

KEY WORDS: Cdx, Caudal, Hox, Retinoic acid, Segmentation, Rhombomeres, Hindbrain, Spinal cord, Central nervous system, Chordates, Vertebrates, Evolution

INTRODUCTION

One of the most prominent characteristics that distinguishes the rostral and caudal regions of the vertebrate central nervous system (CNS) is its segmental nature. Both forebrain and hindbrain have been shown to be organized into boundary-restricted, clonally related groups of cells known as neuromeres (Figdor and Stern, 1993; Fraser et al., 1990; Orr, 1887). The forebrain neuromeres (or prosomeres) and the more familiar hindbrain rhombomeres are important for proper neuronal organization and structural patterning of the adult brain and brain stem (reviewed by Borday et al., 2004; Kiecker and Lumsden, 2005; Puelles and Rubenstein, 2003). In the hindbrain, extensive genetic and molecular data have shown the existence of a regulatory network of transcription and secreted factors that specify the identity and order of rhombomere formation (Waskiewicz et al., 2002) (reviewed by Moens and Prince, 2002). In sharp contrast, the spinal cord remains unsegmented, although an anterior-posterior iterative arrangement of various neuronal populations can be seen to different degrees in *Amphioxus*, zebrafish and in certain tetrapods (Bone, 1960; Fetcho, 1987; Forehand and Farel, 1982; Myers, 1985; Roberts and Clarke, 1982; Stern et al., 1991). However, these iterated arrangements are not the consequence of cryptic segmentation, as demonstrated by the extensive mixing of cells in clonal analysis experiments (Morin-Kensicki and Eisen, 1997; Stern et al., 1991), but are the result of spinal cord neurons responding to paraxial mesoderm-derived signals (Detwiler, 1934; Eisen and Pike, 1991; Ensini et al., 1998; Keynes and Stern, 1984; Lewis and Eisen, 2004). Despite the

striking difference in segmentation, the hindbrain and spinal cord share a number of characteristics: both derive from the caudal neural plate (Brown and Storey, 2000; Muhr et al., 1997; Schoenwolf, 1992), are patterned along their anterior-posterior axis by Hox transcription factors (Deschamps and van Nes, 2005; Krumlauf et al., 1993; Lumsden and Krumlauf, 1996) and have a common evolutionary origin (Ghysen, 2003; Hirth et al., 2003). Therefore, we set out to investigate the mechanisms that direct the caudal neural plate to develop as hindbrain or as spinal cord.

Cdx (Caudal) transcription factors have been implicated in the development of trunk and tail structures across all major animal groups by controlling the sequential addition and identity of body segments (Copf et al., 2004). Within vertebrates, the three family members *Cdx1*, *Cdx2* and *Cdx4* are expressed in nested domains in the trunk and tail of the embryo (Davidson et al., 2003; Frumkin et al., 1993; Gamer and Wright, 1993; Joly et al., 1992; Lohnes, 2003; Marom et al., 1997; Meyer and Gruss, 1993; Pillemer et al., 1998; Reece-Hoyes et al., 2002). Most of our understanding of Cdx function is restricted to their role in paraxial mesoderm in mouse, where they have been shown to integrate FGF, retinoic acid and Wnt signals into coherent Hox gene expression (reviewed by Deschamps and van Nes, 2005; Lohnes, 2003). This role seems to be conserved in zebrafish and *Xenopus* (Davidson et al., 2003; Davidson and Zon, 2006; Isaacs et al., 1998; Pownall et al., 1998; Pownall et al., 1996; Shimizu et al., 2006; Shimizu et al., 2005). The function of Cdx genes in CNS development, however, is poorly understood, despite the fact that expression of Cdx genes in the caudal neural plate is highly conserved across vertebrates (Ehrman and Yutzey, 2001; Frumkin et al., 1993; Joly et al., 1992; Marom et al., 1997; Meyer and Gruss, 1993; Nordstrom et al., 2006; Pillemer et al., 1998; Reece-Hoyes et al., 2002).

Using a variety of morphological, cellular and molecular criteria we present evidence that spinal cord specification in zebrafish is dependent on the partially redundant functions of Cdx1a and Cdx4.

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In agreement with a previous study (Shimizu et al., 2006), we show that zebrafish embryos lacking full *Cdx1a* and *Cdx4* functions develop an expanded hindbrain. In addition, we show that this expanded hindbrain is organized into segmental units arranged in a mirror-image duplicated pattern of ectopic rhombomeres within the trunk region of the embryo. We also show that *Cdx* factors can induce the development of spinal cord cell types and posterior *Hox* gene expression, when misexpressed in rostral regions of the CNS. We propose that *Cdx* transcription factors normally function to prevent rhombomere formation in the caudal neural plate and that by preventing the posterior-most region of the neural plate from following a segmented developmental program, *Cdx* transcription factors help determine the size of the prospective hindbrain and spinal cord territories. We hypothesize that this newly proposed function of *Cdx* transcription factors allowed the development of the dorsal, hollow and unsegmented caudal neural tube that is characteristic of the vertebrate lineage.

MATERIALS AND METHODS

Fish care, microinjection, cell transplantation and pharmacological treatments

Zebrafish (*Danio rerio*) were raised and handled following standard techniques (Westerfield, 1994). Embryos from wild-type AB stock, *kgg^{hi2188A}* (Golling et al., 2002), *Tg[isl1:GFP]* (Higashijima et al., 2000) and *Tg[βactin:GFP]* (Gillette-Ferguson et al., 2003) were obtained from natural spawning, grown at 28°C and staged as described (Kimmel et al., 1995). Injections were carried out at the one-cell stage. Antisense *cdx1a* (5 ng) (Shimizu et al., 2005) and *cdx4* (20 ng) (Davidson et al., 2003) morpholino oligonucleotides (Gene Tools LLC) were injected alone or in combination. For mRNA overexpression, 25 pg of *hoxc6a*, *hoxb8a* or *hoxa9a* (Prince et al., 1998a), or 40 pg of *gap43-RFP* (cell membrane marker, gift of E. Amaya, University of Cambridge, Cambridge, UK) capped sense mRNA (SP6 mMessage mMachine Kit, Ambion) was injected using a standard injection protocol (Bruce et al., 2001). Retinoic acid signaling was blocked by incubating embryos in 1 μM BMS493 in the dark.

Cell transplantation was performed as previously described (Ho and Kane, 1990). Donor embryos were injected at the one-cell stage with 40-kDa lysine-fixable fluorescein (Invitrogen). Donor cells were collected at sphere stage and approximately 20 cells were transplanted 2- to 5 cell-diameters away from the margin of a stage-matched unlabeled host.

Transgenic constructs, genotyping and heat shock

To generate a heat-inducible *cdx4* expression construct, full-length zebrafish *cdx4* was PCR amplified using the following primers: mCdx5'-forward, 5'-CGATTCGGGATCCACCGGTCGCCACCATGTATGGATCGTGT-TTGCTCGAAAAAGAGGCAAGCATGTATCACCAA-3' (*Bam*HI and *Age*I sites underlined, translation initiation site in bold); and 3'MCScdx-reverse, 5'-TTGCTAGAAAGCTTGGTACCGATCGATAGTTTGTA-ATCCTTTTGGACCAC-3' (*Xba*I site underlined). The forward primer changes the 5' end of the gene to that of mouse *Cdx4*, rendering it unrecognizable to the zebrafish *cdx4* morpholino. *Bam*HI/*Xba*I-digested PCR product was cloned in-frame into pcDNA3.1(-)/Myc-His B (Invitrogen). The modified mouse 5'-zebrafish *cdx4-myc-his* (*m5'zcdx4*) gene was digested with *Sph*I, blunt ended, digested with *Age*I and gel purified. Separately, the zebrafish *hsp70* promoter (Halloran et al., 2000) and the pBS-ISce-II KS vector (Thermes et al., 2002) were digested with *Sac*I/*Age*I and *Sac*I/*Eco*RV, respectively, and purified. A double ligation was then set up using the purified fragments to generate *hsp70:m5'zcdx4-ISce*. All constructs were confirmed by sequencing.

Stable transgenic fish were generated by injecting 1 μg *hsp70:m5'zcdx4-ISce* plasmid and 1 unit of ISce-I meganuclease in 1× ISce-I buffer (New England Biolabs) into fertilized eggs during the first 15 minutes of development (Thermes et al., 2002). Embryos were grown to adulthood, pairwise crossed and their embryos genotyped using the 3*hsp70*-forward (5'-GTATTACTTTGTTAACGTGGC-3') and BGHrev (5'-TGAAAGBCACAGTCGAGG-3') primers (IDT). As positive PCR

controls, Wnt5a-12 (5'-CAGTTCTCACGTCTGCTACTTGCA-3') and Wnt-21 (5'-ACTTCCGGCGTGTGGAGAATTC-3') primers were included in all reactions. Founder fish whose progeny produced diagnostic bands were out-crossed to wild-type fish. F1 fish were genotyped and individuals carrying the transgene were used as the *Tg[hsp70:cdx4]* line founders. A standard heat-shock protocol was used to induce transient *cdx4* expression (Halloran et al., 2000).

Whole-mount in situ hybridization and immunocytochemistry

Detection of *cdx1a* (Shimizu et al., 2005); *cdx4* (Joly et al., 1992); *cyp26a1* (Kudoh et al., 2002); *epha4a* (Xu et al., 1994); *foxb1.2* (*mar*) (Odenthal and Nusslein-Volhard, 1988); *islet1* (Inoue et al., 1994); *krx20* (Oxtoby and Jowett, 1993); *hoxa2b*, *hoxb1a*, *hoxb3a*, *hoxb8a*, *hoxc4a*, *hoxd3a* and *hoxd4a* (Prince et al., 1998a; Prince et al., 1998b); *myod* (Weinberg et al., 1996); *olig2* (Park et al., 2002); *radical fringe* (*rfg*) (Cheng et al., 2004); *raldh2* (also known as *aldh1a2* – ZFIN) (Begemann et al., 2001); *retinoic acid receptor alpha* (*RARα*; also known as *raara* – ZFIN) (Hale et al., 2006); and *valentino* (*val*) (Moens et al., 1998) expression by in situ hybridization was carried out as previously described (Bruce et al., 2001), using NBT/BCIP or Fast Red as the enzyme substrate.

Antibody labeling was performed as previously described (Svoboda et al., 2001). Monoclonal mouse anti-acetylated Tubulin (Sigma-Aldrich), mouse anti-myosin HC (A4.1025, Developmental Studies Hybridoma Bank, IA, USA), mouse anti-neurofilament 160k (RMO44, Zymed, CA, USA) and polyclonal rabbit anti-GFP conjugated to FITC (Invitrogen) were used at 1:500, 1:100, 1:5000 and 1:1000, respectively. Goat anti-mouse [Alexa Fluor 647 (Invitrogen) and/or FITC (Jackson ImmunoResearch)] or goat anti-rabbit (Alexa Fluor 488, Invitrogen) secondary antibodies were used at 1:2000.

Image processing

Deyolked embryos were manually sectioned using a scalpel. Specimens were photographed with a Nikon D1 digital camera mounted on a Leica MZFL III or Zeiss Axioskop microscope. For confocal microscopy, single optical sections and image stacks were obtained using a Zeiss laser-scanning confocal imaging system (LSM 510). Three-dimensional reconstructions were produced with the Zeiss LSM 510 software and ImageJ 1.32 (NIH). Figure panels were constructed using Photoshop 7.0 (Adobe).

RESULTS

Cdx4 is required for proper anterior-posterior position of the hindbrain-spinal cord transition

In zebrafish, the *Cdx* genes *cdx1a* and *cdx4* are expressed in the posterior portion of the embryo (see Fig. S1 in the supplementary material) (Davidson et al., 2003; Davidson and Zon, 2006; Joly et al., 1992; Shimizu et al., 2005). We tested the function of *Cdx4* in zebrafish CNS development by examining the distribution of hindbrain and spinal neuronal populations in wild-type and *cdx4* morpholino-injected embryos (Fig. 1A-J). These embryos, referred to hereafter as *cdx4*-deficient embryos, are phenotypically indistinguishable from *cdx4* loss-of-function mutants *kgg^{hi2188A}* and *kggⁿ²⁰⁵* (Davidson et al., 2003; Golling et al., 2002). We used the vagal motor neurons and the T reticular interneurons as landmarks for the posterior hindbrain. These neurons are located in the spinobulbar junction, and they have a characteristic organization in vertebrates that is evolutionarily conserved (Fetcho, 1987; Kimmel et al., 1985; Wake, 1993). We found that in *cdx4*-deficient embryos, the size of the vagus expanded posteriorly by two somites as compared with wild-type siblings (Fig. 1A,F). Similarly, the RMO44-immunopositive T reticular interneurons also expanded posteriorly in these embryos (Fig. 1B,G). Other hindbrain-specific populations, such as the branchiomotor and reticulospinal neurons of rhombomeres (r) 1-6, appeared unaffected (Fig. 1B,G and data not shown). We also examined three distinct spinal cord populations in *cdx4*-deficient embryos: motor neurons and their exit roots,

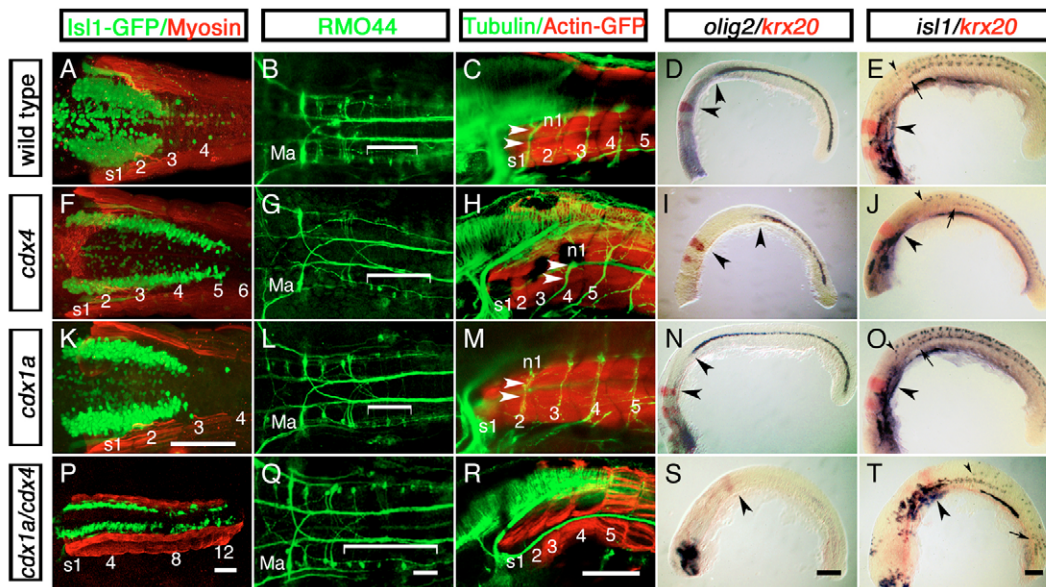


Fig. 1. Hindbrain expansion and spinal cord reduction or loss in zebrafish embryos lacking Cdx function. Distribution of hindbrain and spinal cord cell populations in wild-type embryos (A-E) and in *cdx4*- (F-J), *cdx1a*- (K-O) and *cdx1a/cdx4*- (P-T) deficient embryos. (A,F,K,P) Distribution of *isl1*:GFP-positive vagal motor neurons (green) in relation to adjacent somites (s, red) at 50 hours post-fertilization (hpf). (B,G,L,Q) Distribution of RMO44-immunopositive reticulospinal neurons at 50 hpf. The region occupied by T reticular interneurons is indicated with a bracket. r2 Mauthner neurons are also indicated (Ma). (C,H,M,R) Distribution of spinal motor neurons visualized with an anti-acetylated Tubulin antibody (n, green) in relation to adjacent somites (s, red) at 72 hpf. Axons of the first spinal motor neuron pool are indicated with arrowheads. (D,I,N,S) Distribution of *olig2*-expressing spinal cord oligodendrocytes (purple) with respect to *krx20*-expressing r3 and r5 (red) at the 20-somite stage (19 hpf). Distances between r5 and the rostral-most *olig2*-positive cells are indicated by arrowheads. Forebrain was removed for mounting purposes except in S, where it was retained to show that the in situ hybridization worked in these embryos. (E,J,O,T) Distribution of spinal cord *isl1*-positive (purple) motor neurons and Rohon-Beard sensory neurons as compared with *krx20* expression in r3 and r5 (red) at 20 hpf. The position of r5 (large arrowheads), the most-rostral spinal motor neurons (arrows) and Rohon-Beard sensory neurons (small arrowhead) are indicated. For each condition, a minimum of 40 embryos from five independent experiments were analyzed. A,B,F,G,K,L,Q, dorsal view; K, oblique view; remaining embryos, lateral view. Scale bars: 100 μ m for each column, except in K, for A,F,K.

oligodendrocytes and Rohon-Beard sensory neurons. In wild-type embryos, the axons of the first spinal motor neurons exit at the level of somite 2 (Fig. 1C). By contrast, these axons exited at the level of somite 4 in *cdx4*-deficient embryos (Fig. 1H), correlating with the two-somite posterior expansion of the hindbrain neuronal populations (Fig. 1F,G). The distribution of *olig2*-expressing primary motor neurons and oligodendrocytes and *isl1*-positive Rohon-Beard sensory neurons also shifted posteriorly in *cdx4*-deficient embryos (Fig. 1D,E,I,J), indicating a generalized posterior shift of the spinal cord territory. Together, these data suggest that Cdx4 is necessary in zebrafish for the proper determination of the axial location of the transition from hindbrain to spinal cord.

Cdx1a and Cdx4 act redundantly in the specification of the prospective spinal cord territory

Cdx transcription factors have been shown to act redundantly in the patterning of the mouse paraxial mesoderm (reviewed by Deschamps and van Nes, 2005; Lohnes, 2003), raising the possibility that *cdx1a* and *cdx4* might cooperate in determining the hindbrain-spinal cord transition in zebrafish. To test this hypothesis, we injected wild-type and *cdx4*-deficient embryos with a *cdx1a* morpholino (Shimizu et al., 2005) and examined the distribution of cell populations described above (Fig. 1K-T). Whereas wild-type embryos injected with a *cdx1a* morpholino showed no CNS defects (Fig. 1A-E,K-O), reduction of *cdx1a* function in a *cdx4*-deficient background led to an almost complete loss of spinal cord identities:

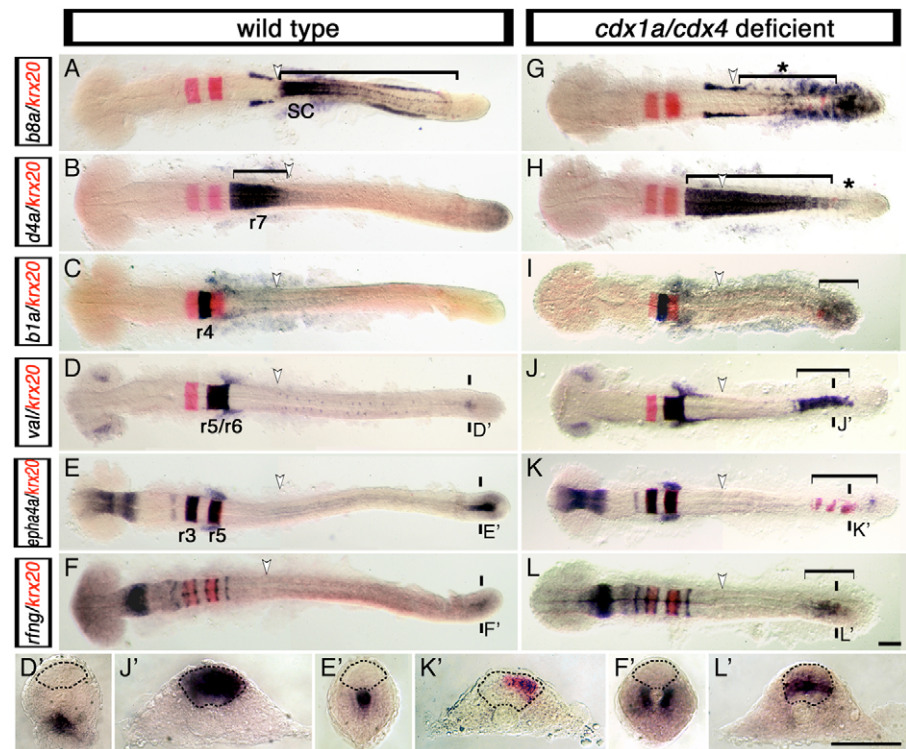
spinal motor neurons and oligodendrocytes were absent, and the number of *isl1*-positive Rohon-Beard sensory neurons was greatly reduced (Fig. 1R-T). Conversely, there was a posterior expansion of r7 and r8 in these embryos, as shown by the trunk and tail distribution of *isl1*-GFP-positive branchiomotor neurons (Fig. 1P). Most of these branchiomotor neurons had axonal projections and morphologies characteristic of vagal cells, although separate and distinct neuronal clusters were also observed near the tail of these embryos (Fig. 1P). A posterior expansion of T reticular interneurons was also observed in these embryos (Fig. 1Q). Together, these data suggest that Cdx1a and Cdx4 function redundantly in the specification of spinal cord-specific neuronal identities, and in their absence most of the caudal neural plate takes on an expanded hindbrain fate.

The hindbrain patterning and segmentation program is improperly induced in the CNS of *cdx1a/cdx4*-deficient embryos

We next examined the expression of Hox patterning genes in the CNS of *cdx1a/cdx4*-deficient embryos (i.e. in embryos deficient for both genes). In these embryos, the r7 and anterior spinal cord marker *hoxd4a* was found to be expressed throughout the posterior CNS, whereas expression of the spinal cord markers *hoxb6a*, *hoxb8a* and *hoxb10a* was notably absent (Fig. 2A,B,G,H and data not shown). Within the hindbrain, *cdx1a/cdx4*-deficient embryos showed no changes in the anterior limit of *hoxa2b* (r2), *hoxb1a* (r4), *hoxb3a* (r5) and *hoxd4a* expression as compared with wild-type embryos (Fig.

Fig. 2. Loss of Cdx function activates the expression of hindbrain genes in the posterior CNS.

Expression of hindbrain markers (purple) in wild-type (A-F, D'-F') and *cdx1a/cdx4*-deficient (G-L, J'-L') zebrafish embryos counterstained for the r3 and r5 marker *krx20* (red signal). (A, G) Spinal cord *hoxb8a* expression (bracket in A) is lost in *cdx1a/cdx4*-deficient embryos (bracket with asterisk in G). (B, H) r7/8 *hoxd4a* expression (bracket in B) is expanded caudally in *cdx1a/cdx4*-deficient embryos (bracket in H) except for the most caudal tip of the CNS (asterisk in H). (C, I) In addition to its normal domain of expression in r4, *hoxb1a* expression can also be seen in the posterior CNS of *cdx1a/cdx4*-deficient embryos (bracket in I). (D, J) In the hindbrain, *val* is expressed in r5 and r6 of wild-type (D) and *cdx1a/cdx4*-deficient (J) embryos. In the tail region, *val* is also expressed in the posterior CNS of *cdx1a/cdx4*-deficient (bracket in J, J') but not wild-type (D') embryos. (E, K) Overlapping expression of *epha4a* (purple) and *krx20* (red) are visualized in r3 and r5 of wild-type (E) and *cdx1a/cdx4*-deficient (K) embryos. In the tail region, *epha4a* and *krx20* are expressed in the posterior CNS of *cdx1a/cdx4*-deficient (bracket in K, K') but not wild-type (E') embryos. (F, L) In the hindbrain, *radical fringe* (*rfng*) is expressed in seven stripes at the rhombomere boundaries in wild-type (F) and *cdx1a/cdx4*-deficient embryos (L). In the tail region, *rfng* is also expressed in the posterior CNS of *cdx1a/cdx4*-deficient (bracket in L, L') but not wild-type (F') embryos. For each condition, a minimum of 44 embryos from at least three independent experiments were analyzed, with more than 82% of embryos displaying the phenotype shown. Representative 20-somite, stage-matched, whole-mounted embryos are shown in dorsal view, anterior to the left. The position of somite 3, the hindbrain-spinal cord transition in wild-type embryos, is indicated with a white arrowhead. The planes of section are indicated with two short vertical bars. Sections are dorsal to the top, with the neural rod delineated by the dashed line. Scale bars: 100 μ m.



2B, C, H, I and data not shown) suggesting that only spinal cord fates fail to be specified in these embryos. Together with our morphological data, these results show that the native hindbrain region in embryos lacking Cdx1a and Cdx4 activities has a normally ordered and nested set of rhombomeric identities and that the increase in hindbrain size seen in these embryos is mostly owing to an expansion of r7 and r8.

We also noticed that in *cdx1a/cdx4*-deficient embryos, the expanded *hoxd4a*-positive r7/8 region did not extend along the entire length of the posterior CNS (Fig. 2H, asterisk). Instead, ectopic expression of more-anterior rhombomere-specific genes such as the r4-specific marker *hoxb1a* was seen within the posterior CNS (Fig. 2I, bracket). This phenomenon was not restricted to Hox genes. The gene *valentino* (*val*; also known as *maf* and *kr*), which is normally expressed in the eyes, hindbrain r5 and r6 and in the tail mesenchyme (Moens et al., 1998), was also found to be expressed in the posterior CNS of these embryos (Fig. 2D, D', J, J'). Similarly, *krx20* (*egr2b* – ZFIN) expression, normally confined to r3 and r5 (Oxtoby and Jowett, 1993), was now present in the posterior CNS (Fig. 2G-I, K, K'). This shows that in *cdx1a/cdx4*-deficient embryos, several members of the regulatory network controlling rhombomere patterning are ectopically expressed outside of their native hindbrain region.

The regulatory network controlling hindbrain patterning also controls its segmentation into rhombomeres (Moens and Prince, 2002; Waskiewicz et al., 2002). Therefore, we examined the expression of *epha4a*, *radical fringe* (*rfng*) and *foxb1.2* (also known

as *mar*) in *cdx1a/cdx4*-deficient embryos, as these genes have been shown to be involved in rhombomere cell-sorting and boundary formation (Cheng et al., 2004; Cooke et al., 2001; Cooke et al., 2005; Odenthal and Nusslein-Volhard, 1988). In wild-type embryos, *epha4a* is expressed within odd-numbered rhombomeres as well as in the forebrain, midbrain and tail notochord, whereas expression of *rfng* and *foxb1.2* in the CNS is restricted to the boundaries between rhombomeres (Fig. 2E-F'). In *cdx1a/cdx4*-deficient embryos, these genes were ectopically expressed in small discontinuous domains in the posterior CNS (Fig. 2K-L'), showing the induction of hindbrain segmentation genes beyond their normal domain of expression. Together, these data suggest that in the absence of Cdx1a and Cdx4 activities, the caudal neural plate not only fails to acquire spinal cord characteristics, but it also becomes competent to initiate the molecular program leading to the formation of supernumerary hindbrain segments and boundaries.

Development and mirror-image patterning of supernumerary rhombomeres in the CNS of *cdx1a/cdx4*-deficient embryos

Despite the fact that several hindbrain- and boundary-specific genes were ectopically expressed in the posterior CNS of *cdx1a/cdx4*-deficient embryos, we noted the absence of definitively sized additional rhombomeres in this region. We hypothesized that the expansion of the native r7/8 region in *cdx1a/cdx4*-deficient embryos could be inhibiting the formation of correctly sized rhombomeres within the transformed caudal neural

Fig. 3. Development of supernumerary rhombomeres and their mirror-image patterning in *cdx1a/cdx4*-deficient zebrafish embryos with compromised RA signaling.

Changes in *rfng*, *foxb1.2* (*mar*), *hox* (purple) and *krx20* (red) gene expression visualized in wild-type (A-H) and *cdx1a/cdx4*-deficient (I-P) embryos treated with the retinoic acid (RA) receptor inhibitor BMS493 (BMS) at mid-gastrulation (75% epiboly, 8 hpf). (A,I) *hoxc4a* expression in r8 is lost in wild-type (bracket with asterisk in A) and *cdx1a/cdx4*-deficient (I) embryos with compromised RA signaling. (B,J) RA inhibition reduced expression of the r7/8 marker *hoxd4a* in wild-type (bracket in B) and *cdx1a/cdx4*-deficient (J) embryos.

(C,K) *hoxd3a* expression in the hindbrain posterior to r5 is reduced in wild-type embryos (bracket in C) and is limited to a central domain of the CNS in *cdx1a/cdx4*-deficient embryos (K). (D,L) *hoxb3a*, which is normally expressed posterior to the r4/r5 boundary (bracket in D), is expressed in a central domain in the CNS of *cdx1a/cdx4*-deficient embryos that includes the *krx20* r5 and ectopic expression domains (L). (E,M) *hoxb1a* is expressed in r4 of wild-type (E) and *cdx1a/cdx4*-deficient (M) embryos. The latter also shows an additional *hoxb1a*-positive domain of expression in the posterior CNS. (F,N) *hoxa2b* is strongly expressed in r2 and r3 and weakly in r4 in wild-type (F) and *cdx1a/cdx4*-deficient (N) BMS-treated embryos. (G,O) *rfng* is expressed in six and nine boundary-like stripes (arrowheads) in wild-type (G) and *cdx1a/cdx4*-deficient (N) BMS-treated embryos, respectively. (H,P) *foxb1.2* is expressed in nine boundary-like stripes (arrowheads) in *cdx1a/cdx4*-deficient BMS-treated embryos (P), compared with the six stripes seen in their wild-type siblings (H). For each condition, a minimum of 44 embryos from at least three independent experiments was analyzed at the equivalent of the 20-somite stage. More than 82% of embryos displayed the phenotype shown. Representative embryos were dorsal flat-mounted, anterior to the left. Ectopic rhombomere-like *krx20* domain of expression is labeled with an arrow. Position of somite 3, the hindbrain-spinal cord transition in wild-type embryos, is indicated with a white arrowhead. Supernumerary rhombomeres are labeled r'. Scale bar: 100 μ m.

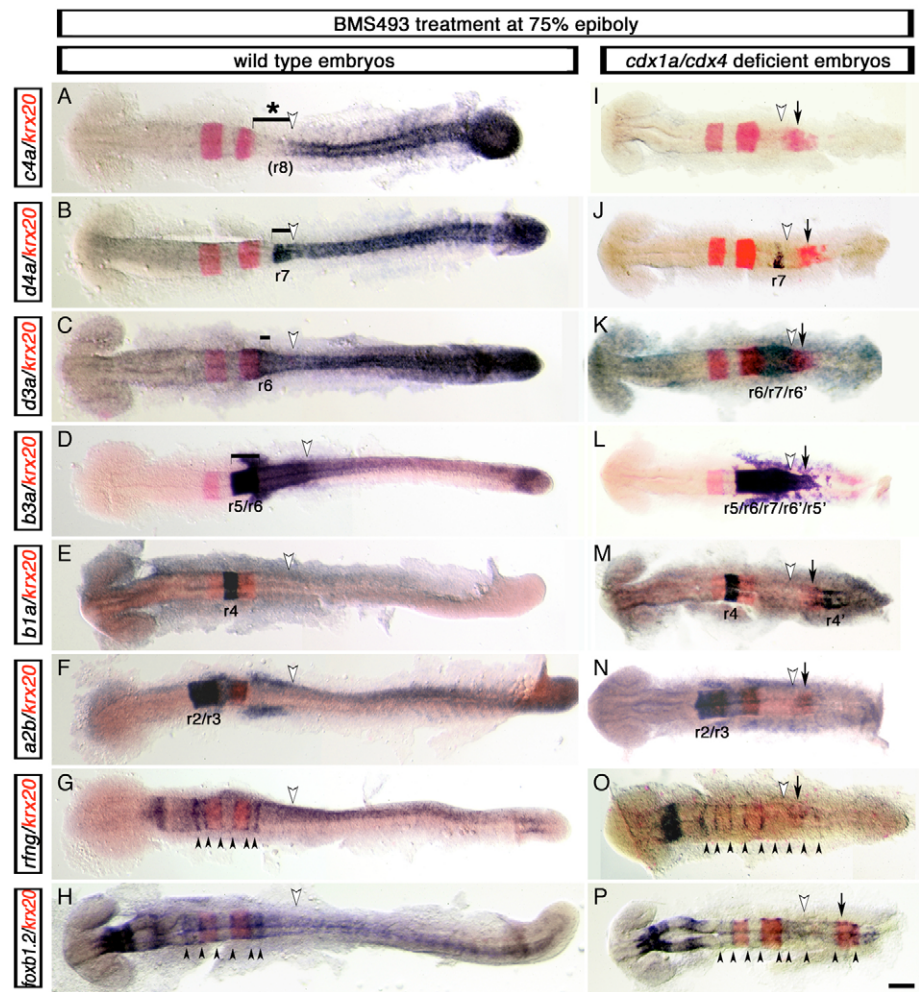


plate. In order to experimentally reduce the size of the r7/8 region, we took advantage of the observation that retinoic acid (RA) signaling inhibition results in the loss of r6-8 markers in wild-type (Begemann et al., 2004; Maves and Kimmel, 2005) and *cdx1a/cdx4*-deficient embryos (Shimizu et al., 2006). We treated embryos with the pan-RA receptor inhibitor BMS493 at mid-gastrulation to reduce the size of r7/8 without affecting the development of more-rostral rhombomeres (Begemann et al., 2004; Maves and Kimmel, 2005). As predicted, these treatments led to the loss of the r8 marker *hoxc4a* and an altered expression limit of the r7/8 marker *hoxd4a* (Fig. 3A,B,I,J). We then examined the expression of more-anterior Hox genes including *hoxa2b* (r2/r3), *hoxb1a* (r4), *hoxb3a* (r5/r6) and *hoxd3a* (r6) in comparison with the r3 and r5 marker *krx20* (Fig. 3C-F,K-N). Excluding *hoxa2b*, whose expression domain was confined to r2/3 as in wild-type embryos (Fig. 3N) (Prince et al., 1998b), ectopic expression of these markers was observed in rhombomere-sized domains in the posterior CNS (Fig. 3K-M). These embryos expressed *krx20* in three definitive stripes (Fig. 3I-N), contrasting with the more loosely organized expression seen in the posterior of *cdx1a/cdx4*-

deficient embryos without BMS493 treatment (Fig. 2). Within the second and third *krx20* stripes, we observed broad *hoxb3a* expression (r5/r6 marker, Fig. 3L) and, nested within it, smaller *hoxd3a* (r6/7 marker, Fig. 3K) and *hoxd4a* (r7 marker, Fig. 3J) expression domains. The r4 marker *hoxb1a* was expressed in two rhombomere-like domains, between the first and second stripe of *krx20* expression and posterior to the third *krx20*-positive domain (Fig. 3M). In addition, we examined the expression of the rhombomere boundary markers *rfng* and *foxb1.2* (Cheng et al., 2004; Odenthal and Nusslein-Volhard, 1988) and found that *cdx1a/cdx4*/RA-deficient embryos had nine evenly spaced, boundary-like stripes (Fig. 3O,P), instead of the six seen in wild-type embryos (Fig. 2F and data not shown). Together, these results show that upon RA-pathway inhibition, *cdx1a/cdx4*-deficient embryos can develop three supernumerary rhombomeres in the posterior CNS in addition to the normal seven. These supernumerary rhombomeres express Hox identity genes in a reverse anterior-posterior orientation in what seems a mirror-image duplication of the hindbrain, as follows: r2, r3, r4, r5, r6, r7, r6, r5, r4 (Fig. 3I-L, summarized in Fig. 4).

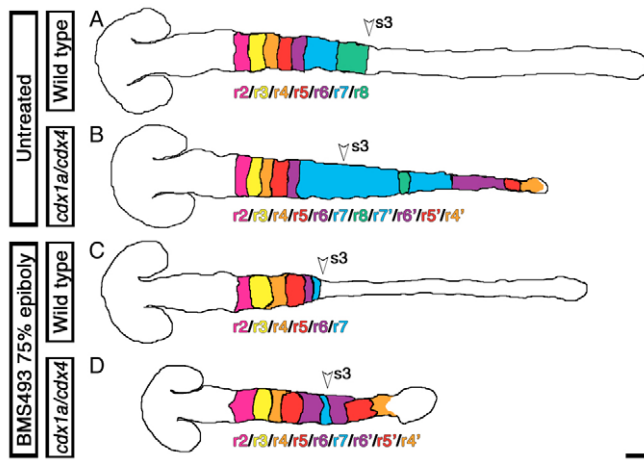


Fig. 4. Summary of CNS transformations caused by the loss of Cdx function. Summary diagrams showing the hindbrain and spinal cord transformation seen in wild-type (A,C) and *cdx1a/cdx4*-deficient (B,D) zebrafish embryos with (A,B) or without (C,D) an intact retinoic acid signaling pathway. Diagrams of 20-somite, stage-matched embryos are based on Figs 2 and 3. Rhombomeres are color coded according to their identity; the location of the third somite, the normal position of the hindbrain-spinal cord transition in wild-type embryos, is indicated by an arrowhead. Dorsal views, anterior to the left. Scale bar: 100 μ m.

The formation of supernumerary segments in the posterior CNS region of *cdx1a/cdx4*-deficient embryos is likely to be owing to the loss of Cdx1a and Cdx4 activity rather than RA signaling, as inhibition of the RA pathway in wild-type embryos results in the loss of posterior rhombomeres (Begemann et al., 2004; Begemann and Meyer, 2001; Begemann et al., 2001). In support of this, close re-examination of hindbrain-specific gene expression in *cdx1a/cdx4*-deficient embryos not treated with BMS493 showed that these embryos had signs of incipient mirror-image duplication in the CNS, despite having normal expression levels of genes involved in the synthesis, reception and degradation of RA (see Fig. S2 in the supplementary material). For example, the expanded *hoxd4a*-positive r7/8 territory was flanked by the r5/6 marker *val*, which in turn was bordered by the r4-marker *hoxb1a* (Fig. 2H-J, summarized in Fig. 4). Together, these results suggest that the absence of Cdx1a and Cdx4 functions allows the posterior CNS to adopt a segmented pattern of development with the potential to develop supernumerary, hindbrain rhombomere-like fates.

Cdx function is required in the CNS to prevent hindbrain expansion

It has been suggested that spinal cord fate may depend on paraxial mesoderm-derived signals (Ensini et al., 1998; Muhr et al., 1999; Muhr et al., 1997; Nordstrom et al., 2002; Nordstrom et al., 2006), raising the question of whether Cdx function is required in the CNS, paraxial mesoderm, or in both tissues, to specify spinal cord. We transplanted *cdx1a/cdx4*-deficient, fluorescein-labeled cells into the CNS of wild-type host embryos and analyzed the expression of the r5/6 marker *val* ($n=8$; Fig. 5A-E), as this gene is ectopically expressed in the posterior CNS of *cdx1a/cdx4*-deficient but not wild-type embryos (Fig. 2D,J). As shown in Fig. 5, transplanted *cdx1a/cdx4*-deficient cells were typically found populating the entire length of the CNS by the 20-somite stage. In rostral regions, transplanted *cdx1a/cdx4*-deficient as well as wild-type host cells

located in the native r5/6 territory expressed *val* (Fig. 5C). In the posterior CNS, only the transplanted *cdx1a/cdx4*-deficient cells, but not surrounding wild-type cells, expressed *val*, even when in isolation ($n=8$; Fig. 5D,E, arrowheads). In reciprocal experiments, transplantation of wild-type cells into the paraxial mesoderm of a *cdx1a/cdx4*-deficient host failed to prevent *val* expression within the posterior CNS ($n=2$; Fig. 5F-H). Taken together, these transplant experiments indicate that Cdx function is required autonomously within neural ectoderm for correct spinal cord fate specification.

We also transplanted wild-type cells into the CNS of *cdx1a/cdx4*-deficient embryos ($n=5$; Fig. 5G,I,J). Wild-type cells were able to contribute to the entire length of the CNS. In rostral regions, wild-type and *cdx1a/cdx4*-deficient cells were evenly distributed within the tissue, with transplanted cells expressing *val* only when located in the native r5/6 territory (Fig. 5I). Interestingly, in posterior regions, wild-type cells were found segregating from host *cdx1a/cdx4*-deficient cells and did not express *val* (black arrowhead). However, in the posterior CNS, isolated wild-type cells surrounded by *cdx1a/cdx4*-deficient cells were occasionally seen expressing *val* (Fig. 5J, white arrowheads). Together, these results suggest that the expression of the hindbrain-specific *val* gene can be controlled by the level of Cdx function within the CNS.

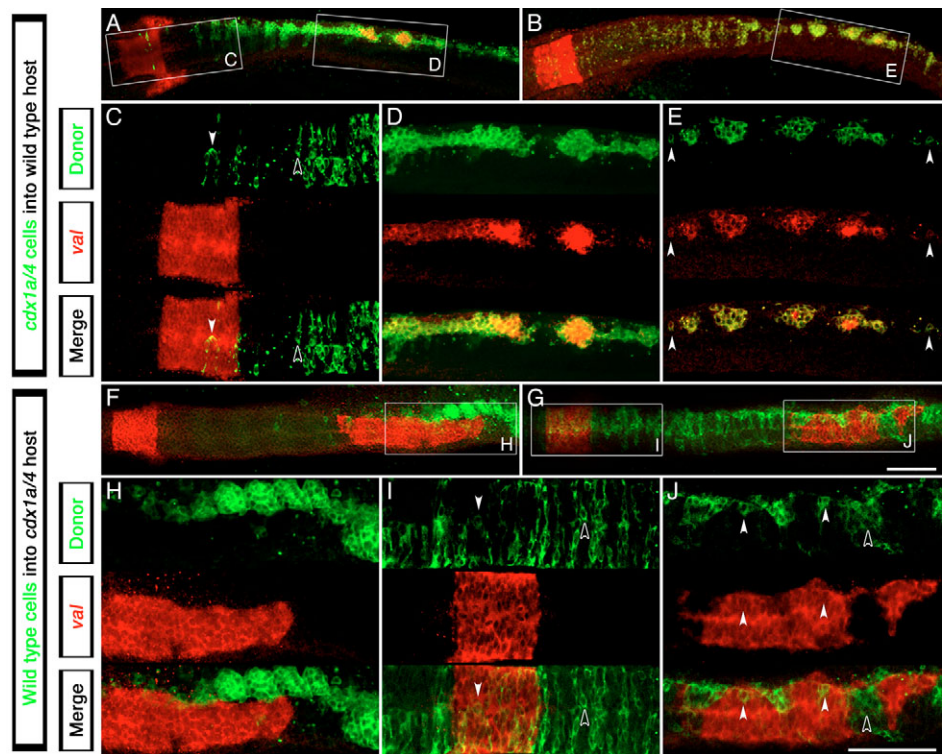
cdx4 overexpression in the hindbrain induces spinal cord development

Our experiments indicate that Cdx activity is required for the caudal neural plate to develop as spinal cord instead of as segmented hindbrain. This hypothesis predicts that *cdx4* overexpression in the hindbrain should: (1) interfere with the segmentation of this region; (2) change hindbrain neuronal identities; and (3) induce spinal cord neuronal markers. *cdx4* overexpression by mRNA injection at the one-cell stage causes severe gastrulation defects (Davidson et al., 2003) (data not shown). To overcome this limitation, we generated a transgenic fish line, *Tg[hsp70:cdx4]*, carrying a 5'-end modified zebrafish *cdx4* gene under the control of the heat-inducible *hsp70* promoter, which enables the rapid and ubiquitous induction of transgene expression at any point during development by incubating the embryos for 1 hour at 37°C (Halloran et al., 2000) (see Fig. S3A,B in the supplementary material and data not shown).

To study the effects of *cdx4* overexpression in rhombomere formation, one-cell stage *Tg[hsp70:cdx4]* embryos were injected with *gap43-RFP* mRNA, a membrane-tagged red fluorescent protein, to follow the formation of rhombomere boundaries after heat shocking the embryos at the three-somite stage. This labeling method has been used to reveal the rhombomere boundaries before they become morphologically distinct (Moens et al., 1998). In transgenic heat-shocked and control embryos, boundary formation initiated at the six-somite stage (data not shown) and visible boundaries were apparent by the 14-somite stage, although less well defined in embryos carrying the *cdx4* transgene (Fig. 6A,B). By the 20-somite stage, however, the characteristic rhombomere bulges seen in wild-type embryos were not present in their transgenic siblings (Fig. 6C,D). At this stage, heat-shocked embryos also showed loss of *rfng* and *foxb1.2* expression at the rhombomere boundaries, and downregulation of the cell adhesion molecule-encoding *epha4a* gene in r1, r3 and r5 (Fig. 6E-H). Less severe defects were obtained when the transgene was induced at other developmental stages [from 75% epiboly to the ten-somite stage, 8-14 hours post-fertilization (hpf), data not shown]. These results show that Cdx4 interferes with rhombomere cell sorting and boundary formation.

Fig. 5. Autonomous requirement of Cdx factors in the zebrafish CNS for hindbrain and spinal cord specification.

Expression analysis of the r5/6 marker *val* (red staining) in clones of *cdx1a/cdx4*-deficient cells transplanted into wild-type hosts (A-E) or wild-type cells transplanted into *cdx1a/cdx4*-deficient host embryos (F-J) (transplanted cells in green). (A,B) *cdx1a/cdx4*-deficient cells can incorporate into the wild-type host CNS at all axial levels. The boxed regions are shown at higher magnification in C-E. (C) *cdx1a/cdx4*-deficient cells are evenly distributed in hindbrain and spinal cord regions of the CNS, only expressing *val* when located in the r5/6 territory (white arrowhead compared with black arrowhead). (D,E) *cdx1a/cdx4*-deficient cells located in the caudal spinal cord tend to form clusters of cells that express *val* ($n=8$). Surrounding wild-type cells do not express this marker. Isolated cells also express this gene (arrowheads). (F,H) *cdx1a/cdx4*-deficient embryos show ectopic *val* expression in the posterior CNS despite the presence of wild-type cells in the paraxial mesoderm ($n=2$). (G) Incorporation of wild-type cells throughout the CNS of *cdx1a/cdx4*-deficient hosts. The boxed regions are shown at higher magnification in I and J. (I) Uniform distribution of wild-type cells in the hindbrain and surrounding regions of *cdx1a/cdx4*-deficient host embryos. Cells located within the r5/6 region express the marker *val* (white arrowheads compared with black arrowheads). (J) In the posterior CNS, most wild-type cells segregate in clusters that fail to express *val* (black arrowhead). When in isolation, wild-type cells express *val* (white arrowheads, $n=5$). Confocal 3 μm sections of dorsal flat-mounted embryos, anterior to the left. Scale bars: in G, 100 μm for A,B,F,G; in J, 100 μm for C-E,H-J.



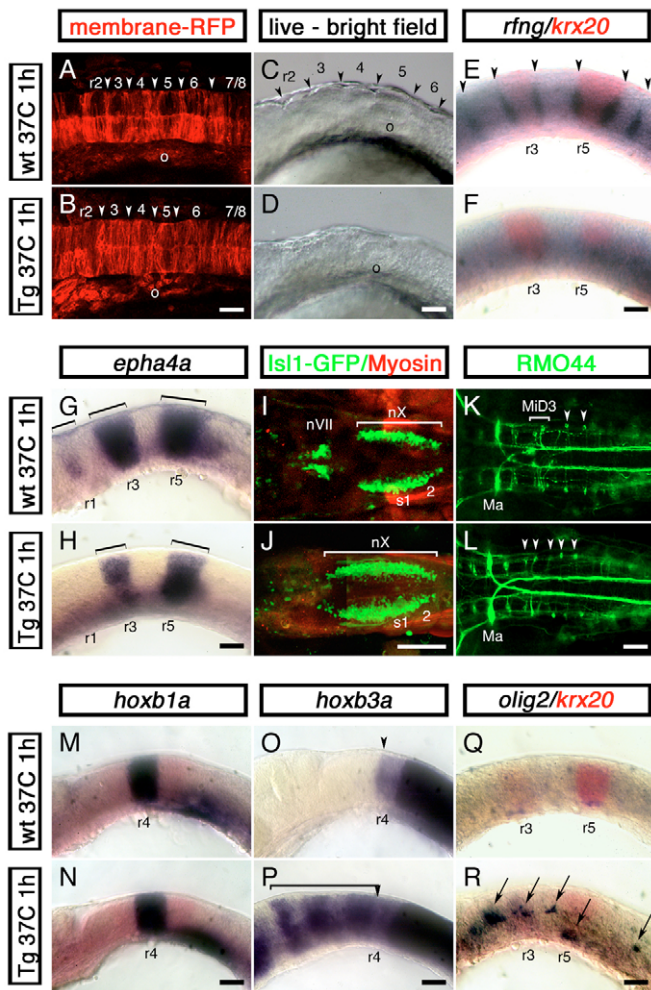
We then examined the effects of *cdx4* overexpression on hindbrain neuronal populations and patterning. *cdx4* overexpression caused the anterior expansion of r7/8 neuronal populations such as the vagal motor neurons (nX) and the T reticular interneurons (Fig. 6I-L). Furthermore, rostral neuronal populations, including the trigeminal (nV in r2) and facial (nVII in r4) motor neurons as well as the MiD2 (r5), MiD3 (r6) and in some cases Mauthner (r4) reticulospinal neurons, were lost in these embryos (Fig. 6I-L and data not shown). The posteriorization of hindbrain neuronal populations was also reflected at the level of Hox gene expression. Although *cdx4* overexpression had no effect on Hox genes normally expressed in r2-4 (*hoxa2b* in r2/r3, *hoxb1a* in r4, *hoxb2a* in r3/r4; Fig. 6M,N and data not shown), it induced generalized *hoxb3a*, *hoxb5a*, *hoxb8a* and *hoxb10a* hindbrain expression (Fig. 6O,P and data not shown). Despite the variability in response of different rhombomeres to *cdx4* overexpression, these results corroborate that Cdx4 has the ability to posteriorize the hindbrain.

Although these results suggest that Cdx4 can interfere with normal hindbrain development, they do not address whether Cdx4 is sufficient to initiate the development of spinal cord neuronal populations. We examined the expression of *olig2*, a marker for spinal cord primary motor neurons and oligodendrocytes (Park et al., 2002), after *cdx4* overexpression. We found that this gene was ectopically expressed in the hindbrain of heat-shocked transgenic but not wild-type embryos (Fig. 6Q,R), suggesting that Cdx4 can divert the development of hindbrain cells to a spinal cord fate. Taken together, these results suggest that Cdx factors normally promote spinal cord development by inducing and later patterning

neuronal cell types specific to this region, and by interfering with molecular pathways leading to hindbrain patterning and segmentation.

Hox-independent specification of spinal cord fates by Cdx factors

Hindbrain expansion in *cdx1a/cdx4*-deficient embryos was accompanied by the loss of posterior Hox gene expression (Fig. 2). Since posterior Hox genes are under direct Cdx regulation (Charite et al., 1998) and their activity is required to override anterior Hox function in caudal regions of the embryo (Duboule, 1991), it was important to know whether this hindbrain expansion was due to the lack of posterior Hox activity or more directly to the loss of Cdx function itself. We overexpressed the posterior Hox genes *hoxc6a*, *hoxb8a* and *hoxa9a* by injecting 25 μg of the respective mRNAs into *cdx1a/cdx4*-deficient *isl1-GFP* transgenic embryos, and examined the distribution of GFP-positive branchiomotor neurons at 50 hpf (Fig. 7A-C). Previously, it was shown that posterior Hox gene overexpression rescues the loss of the red blood cells observed in *cdx1a/cdx4*-deficient embryos (Davidson and Zon, 2006). Although we were able to observe the rescue of the red blood cell marker *gata1* in our injected embryos (see Fig. S4 in the supplementary material), we never observed the rescue of the CNS defects seen in the *cdx1a/cdx4*-deficient embryos. For example, branchiomotor neurons were still present along the trunk of *cdx1a/cdx4*-deficient embryos overexpressing these posterior Hox genes (Fig. 7A-C). This suggests that Cdx factors have a Hox-independent role in spinal cord specification.



We also examined the effect that posterior Hox gene overexpression had on the mirror-image patterning of the CNS of *cdx1a/cdx4*-deficient embryos (Fig. 7D-I). In the native hindbrain, r3 and r5 *krx20* expression was reduced or absent, whereas the r5/r6 *val* expression domain was mostly unaffected (Fig. 4D-I), consistent with the ability of middle and posterior Hox genes to transform anterior rhombomeres into posterior ones (Bruce et al., 2001). We also found, as previously reported (Shimizu et al., 2006), that posterior Hox gene overexpression prevents ectopic *krx20* expression in the posterior CNS of *cdx1a/cdx4*-deficient embryos (Fig. 7G-I, arrowhead). However, when we additionally examined markers of posterior hindbrain identities, such as *val*, we observed that the expression of these genes in *cdx1a/cdx4*-deficient embryos was still present in both the native hindbrain and posterior CNS after Hox gene overexpression (Fig. 7D-F, asterisks). This result shows that hindbrain patterning genes can be differentially affected by Hox gene overexpression in the absence of Cdx activity.

To test if posterior Hox gene overexpression could rescue spinal cord identities in *cdx1a/cdx4*-deficient embryos, we analyzed the expression of the oligodendrocyte marker *olig2* (Fig. 7G-I). None of the tested Hox genes rescued the loss of *olig2* expression in *cdx1a/cdx4*-deficient embryos (Fig. 7G-I and data not shown). This result, together with our hindbrain marker analysis, suggests that both hindbrain size and spinal cord specification might be independent of Hox activities.

Fig. 6. *Cdx4* overexpression in the zebrafish hindbrain disrupts rhombomere formation and promotes spinal cord development. Wild-type embryos (A,C,E,G,I,K,M,O,Q) and their siblings carrying one copy of a *phsp70:cdx4* transgene (B,D,F,H,J,L,N,P,R) were heat shocked for 1 hour at 37°C at the three-somite stage and then grown at 28°C for a total of 16 (14 somites, A,B), 19 (20 somites, C-H,M-R) or 50 (I-L) hours. (A,B) Confocal images of the hindbrain region of 14-somite stage embryos injected with membrane-anchored *RFP* (*gap43-RFP*) mRNA to reveal rhombomere furrow formation (arrowhead). In all 12 embryos examined, *cdx4* overexpression impaired furrow formation. (C,D) Distinctive rhombomere bulges (arrowheads in C) fail to appear in *cdx4*-overexpressing embryos (D) at the 20-somite stage ($n=15$). (E,F) Embryos overexpressing *cdx4* fail to express the rhombomere boundary marker *rfng* (purple, arrowheads). *krx20* expression was used to visualize r3 and r5 (red). (G,H) Loss of *epha4a* expression in r1 and reduction in r3 and r5 (brackets) in embryos overexpressing *cdx4*. (I,J) *cdx4* overexpression results in the rostral expansion of vagal (nX) motor neurons and the loss of facial (nVII) and trigeminal (nV, not shown) motor neurons in *isl1-GFP* embryos. (K,L) In wild-type embryos, the RMO44-positive *Mid3* reticulospinal neurons are found forming a cluster in r6 (brackets, K). This cluster is replaced by individual r7/8-like, T reticular interneurons in *cdx4*-overexpressing embryos (arrowheads, L). On a few occasions, *Mid2* (r5) and Mauthner (r2) neurons (Ma) were also lost in *cdx4*-overexpressing embryos (data not shown). (M,N) Expression of the r4 marker *hoxb1a* is not affected in embryos overexpressing *cdx4*. (O,P) *hoxb3a*, a gene that is normally transcribed in rhombomeres posterior to r4 (O, arrowhead), is expressed throughout the rostral hindbrain in *cdx4*-overexpressing embryos (bracket in P). (Q,R) *cdx4* overexpression induces ectopic hindbrain expression of the spinal motor neuron and oligodendrocyte marker *olig2* (purple, arrows in R). For the 20-somite stage embryos (C-H,M-R), a minimum of 36 embryos from at least three independent experiments were analyzed. All embryos mounted anterior to the left, lateral views except for A,B,I-L, which are dorsal. o, otic vesicle; r, rhombomere; s, somite. Scale bars: 100 μ m.

DISCUSSION

Our analysis of *cdx1a/cdx4*-deficient zebrafish embryos has shown that Cdx function is required within the neural tissue for spinal cord specification and patterning: loss of Cdx function causes the unsegmented spinal cord to become segmented and take on hindbrain features. In reciprocal gain-of-function experiments we find that *cdx4* overexpression is sufficient to cause the segmented hindbrain to lose aspects of its segmental character and take on features of the spinal cord. We conclude that Cdx factors specify vertebrate spinal cord cell fates and, by regulating posterior Hox gene expression, additionally influence anterior-posterior patterning.

Cdx promotes spinal cord development

In zebrafish, at the beginning of gastrulation, the hindbrain and spinal cord precursor cells are broadly distributed along the margin of the epiblast and are not yet committed to their fate (Woo and Fraser, 1995; Woo and Fraser, 1998). Commitment occurs towards the end of the gastrulation period, when hindbrain and spinal cord cells occupy the anterior and posterior halves of the caudal neural plate, respectively (Woo and Fraser, 1995). This segregation and commitment of prospective hindbrain and spinal cord cells correlates with the restriction of *cdx4* transcripts to the posterior third of the neuroectoderm from an initial broad, ventral-to-dorsal gradient of expression at the margin of the epiblast (see Fig. S1 in the supplementary material) (Davidson et al., 2003;

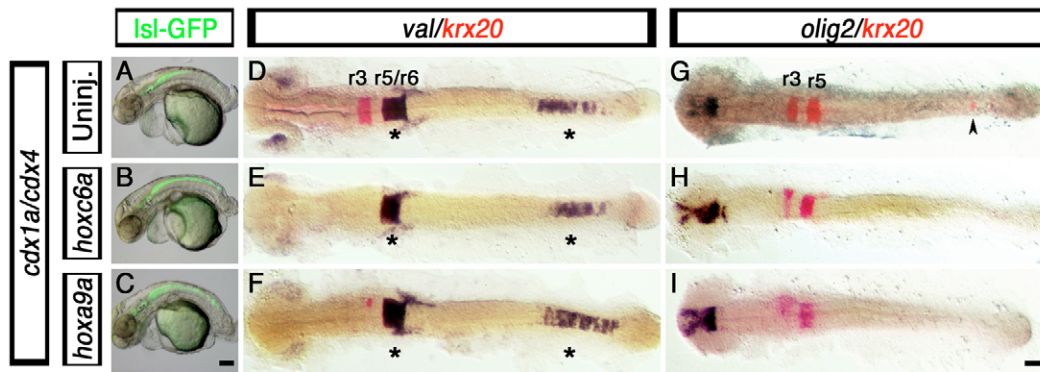


Fig. 7. Hindbrain and spinal cord defects associated with loss of Cdx function are not rescued by posterior Hox gene overexpression.

(A-I) Branchial motor neuron distribution (A-C, GFP-positive cells), hindbrain markers *krx20* (D-I, red) and *val* (D-F, purple), and spinal cord oligodendrocyte marker *olig2* (G-I, purple staining), in *cdx1a/cdx4*-deficient zebrafish embryos injected with 25 pg of *hoxc6a* and *hoxa9a* mRNA. (A-C) At 50 hpf, control and *hoxc6a* and *hoxa9a* mRNA-injected *cdx1a/cdx4*-deficient, *isl1:GFP* transgenic embryos show GFP-positive branchiomotor neurons throughout the posterior CNS. (D-I) At the 20-somite stage (19 hpf), *hoxc6a* and *hoxa9a* overexpression in *cdx1a/cdx4*-deficient embryos results in reduced *krx20* expression in r3 and r5 and its loss in the posterior CNS (E,F,H,I, red; caudal expression indicated with an arrowhead), as compared with uninjected controls (D,G and Fig. 2). (D-F) *val* expression is maintained in r5/6 and posterior CNS of *cdx1a/cdx4*-deficient embryos overexpressing posterior Hox genes (see Fig. 2 for wild-type control). (G,I) Posterior Hox gene overexpression does not rescue spinal cord *olig2* expression in *cdx1a/cdx4*-deficient embryos (see Fig. 1 for wild-type control). Embryos shown in lateral (A-C) or dorsal (D-I) views, anterior to the left. Asterisk indicates *val* expression in hindbrain and posterior CNS. Arrowhead indicates ectopic *krx20* expression in the posterior CNS. A minimum of 15 embryos in three independent experiments were analyzed, with more than 90% of embryos displaying the phenotypes shown. Scale bars: 100 μ m.

Davidson and Zon, 2006; Shimizu et al., 2005). In addition to the posterior CNS expression domain, *cdx4* and *cdx1a* are expressed in a lateral-posterior domain of the tailbud that contains the spinal cord precursor cells, among other lineages (Kanki and Ho, 1997). This nested expression explains the partially redundant function of *cdx1a* and *cdx4* in spinal cord specification and patterning; only the loss of both genes causes severe tail truncations, absence of spinal cord and, as previously shown, lack of hematopoietic stem cells (Davidson and Zon, 2006). This partially redundant function of Cdx1a and Cdx4 in the development of the spinal cord is not unlike the situation described in the paraxial mesoderm of mouse (Chawengsaksophak et al., 2004; van den Akker et al., 2002; van Nes et al., 2006).

Notably, the failure of these Cdx-deficient embryos to develop spinal cord does not appear to be caused simply by a tail truncation, but also involves the posterior expansion of the hindbrain territory. The use of *isl1-GFP* transgenic animals as well as various hindbrain-specific markers has shown that whereas the native anterior hindbrain regions appear unaffected, the native posterior hindbrain region, especially the r7/8 region, has greatly expanded its domain to take up the majority of the former spinal cord region in these embryos. Posterior to the expanded r7/8 territory, the remaining CNS expresses ectopic hindbrain-specific markers, including anterior Hox genes, at the expense of spinal cord-specific markers. In addition, our morphological studies have shown that spinal cord-specific characteristics, such as the formation of spinal nerve roots, are lost from the nervous systems of *cdx1a/cdx4*-deficient embryos. Together, our results suggest that Cdx factors are necessary for the specification and development of the spinal cord region.

The loss of Cdx functions in the developing nervous system leads to the formation of a larger than normal hindbrain region in which both expanded and ectopic rhombomeric identities can be found within the former spinal cord territory. We have also observed that in *cdx1a/cdx4*-deficient embryos, the hindbrain boundary markers *rfg* and *foxb1.2* are ectopically expressed within the CNS in the

tailbud region, and that this incipient segmentation resolves into recognizable rhombomere-like structures upon partial inhibition of RA signaling. The involvement of Cdx factors in not only spinal cord specification and anterior-posterior patterning, but perhaps also inhibition of segment formation, places these factors at an important regulatory crossroad.

Cdx repression of hindbrain development

A formal possibility is that Cdx factors might allow spinal cord specification by repressing hindbrain-specific characteristics within the posterior CNS; such a function would be consistent with the expression pattern of *cdx4* within the spinal cord region of the zebrafish. The creation of a heat-inducible *cdx4* transgenic line has enabled the overexpression of a Cdx factor at the end of the hindbrain determination period. We have shown that *cdx4* overexpression affects correct hindbrain formation. For example, the loss of *rfg* and *foxb1.2* expression within the hindbrain suggests that a relatively late step in the hindbrain segmentation cascade, namely the formation of segmentation boundaries, can be disrupted by *cdx4* overexpression. Therefore, this type of experiment suggests that by interfering with the hindbrain segmentation program, Cdx factors might be able to direct the caudal neural plate cells to a spinal cord fate.

By contrast, the analysis of more-upstream hindbrain segmentation pathway components such as *krx20*, *epha4a* and *hoxb3a*, gave variable results in our overexpression assays, depending on which region of the hindbrain was analyzed. This variation can be attributed to the heterochrony of the region, as different rhombomeres form at different times during development and express different sets of genes (Moens and Prince, 2002). In these experiments, we confined our analyses to cases in which *cdx4* overexpression was accomplished by heat-shock treatment administered at the three-somite stage, the developmental stage at which *cdx4* overexpression caused the most severe hindbrain abnormalities. At this time, genes involved in hindbrain patterning

and rhombomere boundary formation such as *hoxb1a*, *val* and *krx20*, are already expressed in the hindbrain (Moens et al., 1998; Prince et al., 1998b; Waskiewicz et al., 2002). Under these experimental conditions, the hindbrain region expresses what appears to be a mixed hindbrain/spinal cord identity with some aspects of hindbrain fate, such as the formation of vagal motor neurons, now overlapping with aspects of spinal cord fate, such as the ectopic expression of the spinal motor neuron marker *olig2*. It is likely that *cdx4* overexpression at different times of development will give different outcomes, a possibility we are currently testing. Despite this caveat, we have shown that the overexpression of *cdx4* is able to interfere with both the segmentation and specification of individual rhombomeric identities in the zebrafish hindbrain.

Specification and patterning of the spinal cord territory by Cdx

Although our work, like that of many others (reviewed by Deschamps and van Nes, 2005; Lohnes, 2003), shows that Cdx genes have roles in the establishment of Hox gene expression limits, we further propose that the initial function of Cdx in establishing the spinal cord field might be independent of a role in Hox gene regulation. We suggest that Cdx factors initially function to establish the prospective spinal cord territory by preventing the posterior-most region of the caudal neural plate from adopting a segmental developmental program (hindbrain fate) and by inducing or promoting the expression of spinal cord-specific gene expression. Consistent with this hypothesis are our data showing that the overexpression of 5' Hox genes fails to rescue the loss of the spinal cord markers seen in *cdx1a/cdx4*-deficient zebrafish embryos and only causes the posteriorization of the expanded hindbrain. If Cdx functioned solely through the control of Hox gene expression, then the general overexpression of a posterior Hox gene would be predicted to prevent the expansion seen in *cdx1a/cdx4*-deficient embryos. Since this was not the case, we propose that separate hindbrain and spinal cord territories must be established prior to becoming receptive to Hox gene functions. In *cdx1a/cdx4*-deficient embryos, posterior hindbrain identities are still present when 5' Hox genes are overexpressed. Our conclusion differs from that of Shimizu et al. (Shimizu et al., 2006), who interpreted their overexpression studies as showing that Hox genes could prevent the ectopic expression of posterior hindbrain fates in *cdx1a/cdx4*-deficient embryos. We note that Shimizu et al. (Shimizu et al., 2006) only utilized the r5 marker *krx20*; however, when we additionally evaluated the r5/6 marker *val* it was clear that ectopic hindbrain fates were still present in the Hox-overexpressing embryos (Fig. 7). Therefore, our data suggest that in vertebrates, Cdx might have homeotic functions independent of those of Hox factors, similar to the function of the *caudal* gene in the *Drosophila* adult (Moreno and Morata, 1999). This homeotic function may act both prior to and independent of any downstream control of Hox genes, similar to the ability of *Drosophila* Caudal to repress *Abd-B* transcription and induce *Distal-less*, *brachyenteron* and *even skipped* gene expression during analia development (Moreno and Morata, 1999). Further work will be required to characterize the Hox-independent function of Cdx during spinal cord development.

Another function of Cdx factors within the nervous system is to allow the hindbrain and spinal cord regions to differentially respond to gradients of FGF, RA and Wnt signals in the embryo. This is illustrated by the striking mirror image expression of ectopic hindbrain patterning genes in *cdx1a/cdx4*-deficient embryos and by the failure of 5' Hox gene overexpression to prevent this phenotype. As the caudal neural plate fails to be specified as spinal cord in

cdx1a/cdx4-deficient embryos, it retains hindbrain characteristics and remains responsive to surrounding signals, particularly FGF and RA, in a manner similar to the native hindbrain region. For example, in wild-type embryos, r4-derived FGF signals are responsible for inducing and patterning the r5 and r6 regions (Maves et al., 2002). During normal development, the native hindbrain territory is located far removed from the tailbud region, which is another source of FGF signals (Draper et al., 2003; Griffin et al., 1995). However, in *cdx1a/cdx4*-deficient embryos, the expanded hindbrain now comes into close contact with the tailbud. As shown by Shimizu et al. (Shimizu et al., 2006), tailbud-derived FGF signals are able to mimic the FGF-dependent, r5- and r6-inducing activity of r4. This signaling activity, coupled with paraxial mesoderm-derived RA signals, is responsible for the induction of ectopic r4, r5, r6 and r7/8 identities in the trunk region of the embryo (summarized in Fig. 4). Because the gradients of FGF from the tailbud region of the embryo are reversed relative to the FGF gradients found in the native hindbrain region, the pattern of ectopic rhombomeric identities is likewise reversed within the trunk region. Therefore, the mirror-image pattern of rhombomeric identities seen in the trunk region of *cdx1a/cdx4*-deficient embryos could be produced by the normal responses of hindbrain tissues to the same types of signals that they would be exposed to in the hindbrain's native location. This is further supported by transplantation experiments, in which individual *cdx1a/cdx4*-deficient cells in the spinal cord region of a wild-type host responded to FGF and RA factors as if they were located in the hindbrain. Therefore, we propose that the functions of the *cdx1a* and *cdx4* genes in the nervous system of the zebrafish are to inhibit the hindbrain developmental program by preventing the tissue from inappropriately segmenting and taking on inappropriate anterior-posterior identities.

Evolutionary implications of Cdx function in spinal cord development

Based on patterns of expression and functional similarities across species, we propose that the control of hindbrain and spinal cord development by Cdx transcription factors might be common to all vertebrates. Remarkably, the rostral limit of the most anteriorly expressed Cdx gene coincides with the position of the hindbrain-spinal cord transition in zebrafish, *Xenopus*, chick and mouse (Cambronero and Puelles, 2000; Frumkin et al., 1993; Lohnes, 2003; Marom et al., 1997; Meyer and Gruss, 1993; Pillemer et al., 1998; Reece-Hoyes et al., 2002) (this work); Cdx factors thus define the prospective spinal cord territory in the caudal neural plate and, by exclusion, the region that will give rise to hindbrain. Careful re-examination of Cdx function in chordate neural tube patterning might prove useful in addressing the underlying developmental mechanisms and evolutionary origin of the vertebrate spinal cord.

We thank past and present members of the V.E.P. and R.K.H. laboratories for helpful comments and suggestions throughout this work; R. Bielang and J. Chetta for technical assistance and fish care; D.-G. Ahn, A. Bruce, A. Foley, C. Huang, M. Kinkel and R. Muller for critically reading the manuscript; and members of the zebrafish community for probes and reagents, in particular N. Hopkins for the *kgg^{hi2811A}* fish line, E. Amaya for *gap43-RFP* and D. Stanier for the gift of the *cdx4* morpholino. The A4.1025 antibody developed by Helen M. Blau was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. I.S. was supported by The Helen Hay Whitney Foundation and D.T. by an NSF Graduate Research Fellowship. This study was also supported by grants NIH NS043977 to M.H., NIH DK064973-01 and Juvenile Diabetes Research Foundation Grant 1-2003-257 to V.E.P., and NIH GM67714, NIH DK68286 and MOD FY01-623 to R.K.H.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/1134/11/2147/DC1>

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