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Cdx-Hox code controls competence for responding to Fgfs and retinoic acid in zebrafish neural tissue

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Fibroblast growth factor (Fgf) and retinoic acid (RA) signals control the formation and anteroposterior patterning of posterior hindbrain. They are also involved in development processes in other regions of the embryo. Therefore, responsiveness to Fgf and RA signals must be controlled in a context-dependent manner. Inhibiting the *caudal*-related genes *cdx1a* and *cdx4* in zebrafish embryos caused ectopic expression of genes that are normally expressed in the posterior hindbrain and anterior spinal cord, and ectopic formation of the hindbrain motor and commissure neurons in the posteriormost neural tissue. Combinational marker analyses suggest mirror-image duplication in the Cdx1a/4-defective embryos, and cell transplantation analysis further revealed that Cdx1a and Cdx4 repress a posterior hindbrain-specific gene expression cell-autonomously in the posterior neural tissue. Expression of *fgf*s and *retinaldehyde dehydrogenase 2* suggested that in the Cdx1a/4-defective embryos, the Fgf and RA signaling activities overlap in the posterior body and display opposing gradients, compared with those in the hindbrain region. We found that Fgf and RA signals were required for ectopic expression. Expression of the posterior *hox* genes *hoxb7a, hoxa9a* or *hoxb9a*, which function downstream of Cdx1a/4, or activator fusion genes of *hoxa9a* or *hoxb9a* (*VP16-hoxa9a, VP16-hoxb9a*) suppressed this loss-of-function phenotype. These data suggest that Cdx suppresses the posterior hindbrain fate through regulation of the posterior *hox* genes; the posterior Hox proteins function as transcriptional activators and indirectly repress the ectopic expression of the posterior hindbrain genes in the posterior neural tissue. Our results indicate that the Cdx-Hox code modifies tissue competence to respond to Fgfs and RA in neural tissue.

KEY WORDS: *caudal*-related genes, *hox*, Hindbrain, Fibroblast growth factor, Retinoic acid, Zebrafish

INTRODUCTION

The anteroposterior patterning of neural tissue is established through various inductive signals. The neuroectoderm is initially induced by signals from the dorsal organizer and subsequently receives a diffusible signal(s) from the non-axial mesoderm and endoderm (mesendoderm in zebrafish) that is responsible for establishing the anteroposterior axis in the neuroectoderm. Subsequently, the neural tissue receives more defined positional information from the mesodermal tissues and the secondary organizing centers.

The hindbrain is a segmented neural structure that contains seven or eight compartments called rhombomeres (r). The formation and anteroposterior patterning of the posterior hindbrain and anterior spinal cord is regulated by fibroblast growth factor (Fgf) and retinoic acid (RA) signaling. r4 is the first-formed rhombomere, and it functions as a secondary signaling center that expresses *fgf3* and *fgf8*, which are required for r5 and r6 to form (Maves et al., 2002; Walshe et al., 2002; Waskiewicz et al., 2002; Wiellette and Sive, 2004). The *retinaldehyde dehydrogenase 2* gene (*raldh2*) [the *aldehyde dehydrogenase 1 family, member A2* gene (*aldh1a2*) – Zebrafish Information Network] encodes an enzyme that synthesizes RA from retinaldehyde, the intermediate product of vitamin A oxidation (Niederreither et al., 2000); *raldh2* is expressed in early mesendodermal cells and persists in the lateral/paraxial mesoderm in zebrafish (Begemann et al., 2001; Grandel et al., 2002). Mutations in the *raldh2* gene in zebrafish lead to the loss of r7 and the anterior spinal cord (Begemann et al., 2001; Grandel et al., 2002). Inhibition experiments showed that the RA signal is required for the formation of the posterior hindbrain (r5-r7) and anterior spinal cord (Begemann et al., 2001; Grandel et al., 2002); high RA activity is required for the more posterior region (anterior spinal cord) and lower RA activity is required for the more anterior one, as reported for other vertebrate species (Dupe et al., 1999; Dupe and Lumsden, 2001; Gale et al., 1999; Niederreither et al., 2000; Wendling et al., 2001; White et al., 2000). The Fgf and RA signals not only control the formation and patterning of the posterior hindbrain and anterior spinal cord but also regulate other developmental processes. The counter gradients of Fgf and RA signals control neurogenesis in the posterior spinal cord and the segmentation of the paraxial mesoderm (Diez del Corral et al., 2003; Dubrulle et al., 2001; Sawada et al., 2001). It remains to be elucidated how the different tissue responses to Fgf and RA are controlled.

caudal-related homeobox (*cdx*) genes are members of the ParaHox cluster, a cluster of homeobox genes closely related to the Hox cluster that function in the formation of the posterior body in vertebrate and invertebrate species (Deschamps and van Nes, 2005; Lohnes, 2003). Cdx proteins directly regulate the expression of the posterior *hox* genes through direct binding to the *cis*-regulatory elements of the *hox* genes (Charite et al., 1998; Gaunt et al., 2004; Isaacs et al., 1998; Pownall et al., 1996; Subramanian et al., 1995). Zebrafish *cdx4/kugelig* mutant embryos have a reduced posterior body and reduced expression of the posterior *hox* genes (Davidson et al., 2003; Hammerschmidt et al., 1996). Inhibition of both Cdx1a and Cdx4 leads to loss of the *hoxb7a* and *hoxa9a* expression at the early segmentation stage and causes a more severe posterior truncation than does the inhibition of Cdx4 alone (Davidson and Zon, 2006; Shimizu et al., 2005).

Here, we show that the inhibition of Cdx1a and Cdx4 induces ectopic expression of the posterior hindbrain and anterior spinal cord markers in the posteriormost neural tissue in zebrafish. Both Fgf and RA signals are required for this ectopic expression, which can be

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suppressed by expression of the posterior *hox* genes. Our results reveal an essential role for the Cdx-Hox code in modifying tissue responsiveness to Fgf and RA signaling.

MATERIALS AND METHODS

Fish embryos

Wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses of fish with the AB/India genetic background. The islet1-GFP transgenic line *Tg(isl1:GFP)* was used for the analysis of cranial motoneurons (Higashijima et al., 2000). The embryos were incubated at 28.5°C. The morphant embryos and inhibitor-treated embryos showed aberrant development. Developmental stages were determined as hours post fertilization (hpf).

Antisense morpholino oligonucleotides and transcript detection

The antisense morpholino oligonucleotides (MOs) for *cdx1a*, *cdx4*, *raldh2*, *fgf3*, *fgf8*, *wnt3a* and *wnt8* and the preparation of morphant embryos were previously published (Begemann et al., 2001; Davidson et al., 2003; Shimizu et al., 2005; Wiellette and Sive, 2004). The expression patterns of *krox20* (*egr2b* – Zebrafish Information Network), *valentino*, *hoxb1a*, *hoxa2b*, *hoxb4a*, *hoxb5a*, *hoxb6a*, *hoxb7*a, *hoxa9a*, *fgf3*, *fgf8*, *raldh2* and *cyp26a1* have been reported (Begemann et al., 2001; Davidson et al., 2003; Emoto et al., 2005; Furthauer et al., 1997; Grandel et al., 2002; Koshida et al., 2002; Prince et al., 1998a; Prince et al., 1998b; Reifers et al., 1998; Shimizu et al., 2005; Shinya et al., 2001; Walshe and Mason, 2003). BM Purple and FastRed (Roche) were used for whole-mount in situ hybridization. Images were taken using an AxioPlan2 microscope equipped with an AxioCam CCD camera (Zeiss).

Immunostaining and transplantation

Commissure neurons in the hindbrain were stained with the monoclonal antibody zn-5 (Trevarrow et al., 1990; provided by the Zebrafish International Resource Center) and Alexa 488-conjugated antibodies (Invitrogen/Molecular Probes). Transplantation was performed principally as described previously (Ho and Kane, 1990). Briefly, FITC-dextran (Invitrogen/Molecular Probes) was injected with *cdx1a*MO and *cdx4*MO into one-cell-stage embryos. Cells were harvested from the donor embryos and transplanted into the blastoderm of sibling recipient embryos at the sphere stage. After the embryos were fixed, the transcripts were detected by in situ hybridization using BM Purple, and FITC-dextran was detected by immunostaining with an alkaline phosphatase-conjugated anti-FITC antibody (Roche) and FastRed.

Inhibitors for RA and Fgf signaling, and FGF8b treatment

DEAB (Wako) and SU5402 (Calbiochem) were dissolved in DMSO at 100 mmol/l and 20 mmol/l, respectively. Recombinant mouse FGF8b proteins were purchased from R&D Systems. Embryos were treated with 50 μmol/l DEAB and/or 300 μmol/l SU5402, or 100 ng/ml of mFGF8b in the presence of 1 μg/ml heparin in embryonic medium from the shield stage to 22 hpf.

Plasmid construction and synthetic RNAs

To construct expression vectors for *hoxb7a*, *hoxa9a*, *hoxb9a*, *hoxb1a* and *hoxb1b*, the full open reading frames of these genes were amplified by PCR and inserted into pCS2+MT. Plasmids for VP16 fusion proteins VP-Hoxb9a and VP-Hoxb9b were constructed by inserting the PCR fragments containing *hoxb9a* (encoding amino acids 156-251) or *hoxb9b* (amino acids 162-256) into pCS2+NLS VP16AD, which contains the transcriptional activation domain of VP16 (amino acid 412-490) (Shimizu et al., 2002). Plasmids for the Engrailed fusion proteins En-Hoxb9a and En-Hoxb9b were constructed by inserting the *hoxb9a* or *hoxb9b* fragments into pCS2+En, which contains the repressor domain of *Drosophila* Engrailed (amino acids 1-226) (Shimizu et al., 2002). To make synthetic capped RNAs for these genes, the plasmids were linearized with *Not*I and transcribed with SP6 RNA polymerase.

RESULTS

Cdx1a and Cdx4 are required for formation of the posterior spinal cord

We investigated the roles of Cdx1a and Cdx4 in the anteroposterior patterning of neural tissue. We first examined the expression of the *hox* genes *hoxb5a*, *hoxb6a*, *hoxb7a* and *hoxa9a*, which display region-specific expression in the spinal cord (Prince et al., 1998a), in *cdx1a/cdx4*MO-injected embryos (*cdx1a/4* morphant embryos), at 22 hpf (corresponding to the 26-somite stage for the control embryos). In wild-type embryos, the expression domains of these *hox* genes extended anteriorly in the neural tube no further than the level of somite (s) 1 for *hoxb5a*, s2 for *hoxb6a* and s4 for both *hoxb7a* and *hoxa9a* (Fig. 1A,C,E,G), as reported previously (Prince et al., 1998a). In the *cdx1a/4* morphant embryos, no *hoxb7a* or *hoxa9a* was detected (Fig. 1F,H); the expression domains of *hoxb5a* and *hoxb6a* extended noticeably less far posteriorly than they normally do; and a *hoxb5a*- and *hoxb6a*-negative region was observed in the posteriormost domain of the neural tube (Fig. 1B,D). Seen in this light, both Cdx1a and Cdx4 are required for the expression of the posterior *hox* genes, which are normally expressed in the poster spinal cord.

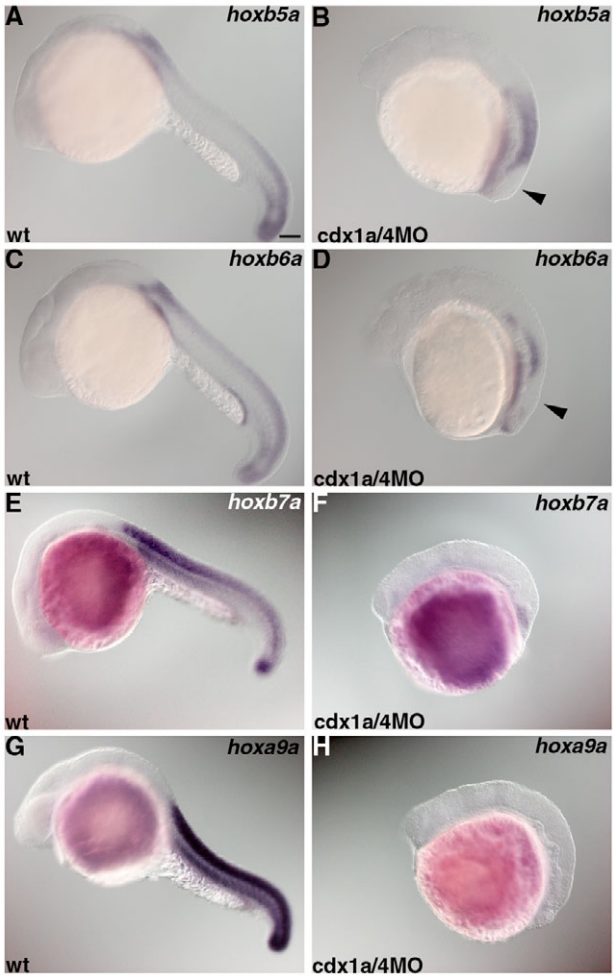

Fig. 1. Loss of posterior *hox* expression in embryos lacking Cdx1a/4. Expression of *hoxb5a* (**A**,**B**), *hoxb6a* (**C**,**D**), *hoxb7a* (**E**,**F**) or *hoxa9a* (**G**,**H**) in wild-type controls (wt) (A,C,E,G) and in embryos that received an injection of 1 ng *cdx1a*MO and 1 ng *cdx4*MO (B,D,F,H) at 22 hpf. *hoxb5a, hoxb6a, hoxb7*a and *hoxa9a* are expressed in the spinal cord. *hoxb5a* is expressed at the level of somite 1 and posterior to it. *hoxb6a* is expressed at the level of somite 2 and posterior to it. *hoxb7a* and *hoxa9a* are expressed at the level of somite 4 and posterior to it. In the *cdx1a/4* morphant embryos, *hoxb5a* or *hoxa6a*-negative domains were detected in the posterior neural tissue (arrowhead in B and D). Scale bar: 100 μm.

Inhibition of Cdx1a and Cdx4 leads to posterior, mirror-image duplication of posterior hindbrain and anterior spinal cord

We next examined the expression of hindbrain markers in the *cdx1a/4* morphant embryos (Fig. 2A) (Prince et al., 1998b). Unexpectedly, we observed the ectopic expression of *krox20* (a marker for r3 and r5, *n*=30/31), *hoxb1a* (r4, *n*=21/22), *hoxa2b* (r2-5, *n*=7/10) and *valentino* (r5, 6, *n*=19/19) in the posteriormost region of the neural tube, in addition to their normal expression domains in the hindbrain region (Fig. 2B-I). The ectopic expression of *valentino*, *krox20* and *hoxb1a* was detected as early as 11, 13 and 15 hpf, respectively, in the *cdx1a/4* morphant embryos (Fig. 2R-W). We did not detect the ectopic expression of *krox20* (*n*=0/17), *hoxb1a* (*n*=0/19), or *valentino* (*n*=0/17) in the embryos that received *cdx1a*MO alone (*cdx1a* morphant embryos, Fig. 2O-Q). Co-staining for *krox20* and *hoxb4a*, which labels r7 and posterior (r7–), *hoxb1a*, or *valentino* revealed that the *cdx1a/4* morphant embryos showed a *hoxb1a*-expressing r4 identity in the posteriormost region (Fig. 2E,L), and, from posterior to anterior, a *krox20*$^{+}$*valentino*$^{+}$ r5 identity (Fig. 2C,I,M; note that the expression domains of *krox20* and *valentino* overlapped in the insets of Fig. 2M), a *krox20*$^{–}$ *valentino*$^{+}$ r6 identity (Fig. 2M; also *hoxb4a*$^{–}$ in Fig. 2K), and a *hoxb4a*-expressing r7 identity (Fig. 2K). The *cdx1a/4* morphant embryos expressed the anterior spinal cord markers *hoxb5a* and *hoxb6a* in the region anterior to the *krox20*-expressing region in the posterior-most region (data not shown). These results suggest that the *cdx1a/4* morphant embryos display ectopic formation of the posterior hindbrain and the anterior spinal cord, and the anteroposterior polarity of the ectopic tissue is opposite to that of the normal one (Fig. 7B).

In addition to its neural expression, *hoxb1a* is normally expressed in the cranial mesoderm (Fig. 2D), while its expression in *cdx1a/4* morphant embryos was expanded posteriorly and reached the posterior end (Fig. 2E). By contrast to ectopic *hoxb1a* expression in the neural tissue, however, we were unable to find any gap between the anterior and posterior mesodermal expression, suggesting that the anterior mesoderm expands instead of posterior mesoderm, rather than being ectopically induced at the posterior end. This is consistent with the posterior expansion of *hoxb5a* expression in the mesoderm of Cdx1a/4-defective embryos (Davidson and Zon, 2006). The data suggest that the ectopic induction of anterior tissues only took place in the neural tissue in the *cdx1a/4* morphant embryos.

In an attempt to reveal whether the *cdx1a/4* morphant embryos contained ectopic hindbrain neurons, we performed immunohistochemistry with zn-5, which stains neurons such as hindbrain commissure neurons and secondary motoneurons (Trevarrow et al., 1990) (Fig. 3A-H). We were able to detect the hindbrain commissure neurons with their axons in the hindbrain of control embryos (Fig. 3A-C), and in the hindbrain and the posteriormost neural tissue of the *cdx1a/4* morphant embryos (Fig. 3E-H). We further examined the formation of cranial motoneurons by injecting the *cdx1a/4*MOs into the islet1-GFP transgenic embryos (Higashijima et al., 2000). Here, we could detect GFP expression in the cranial motoneurons, including trigeminal, facial and vagal neurons in the control embryos (V, VII and X in Fig. 3I). In the *cdx1a/4* morphant embryos, GFP expression was first detected in the hindbrain and the entire posterior neural tissue at the pharyngula period. We also observed a cluster of GFP-positive neurons with their axons in the posteriormost neural tissue at 48 hpf (Fig. 3K, marked by arrowhead). Taken together, the results suggest that inhibition of Cdx1a and Cdx4 leads to posterior, mirror-image duplication of posterior hindbrain and anterior spinal cord.

We also observed the ectopic expression of *krox20*, in the embryos that received *cdx4*MO alone (*cdx4* morphant embryos, Fig. 2N). However, the ectopic transcripts were scattered in the middle trunk region and were not detected in the posteriormost neural tissues (Fig. 2N). These data suggest that: (1) Cdx4 is required for repressing the posterior hindbrain fate at least partly non-redundantly; and (2) the ectopic formation of the posterior hindbrain depends on inductive signals that are affected differently in the *cdx1a/4* morphant and *cdx4* morphant embryos.

As the inhibition of Cdx1a/4 also affects the development of the mesoderm (Davidson et al., 2003; Davidson and Zon, 2006; Shimizu et al., 2005), it was not clear whether *cdx1a* and *cdx4* repressed the formation of the posterior hindbrain and anterior spinal cord cell-autonomously or non-cell-autonomously. To address this issue, we transplanted wild-type or Cdx1a/4-deficient blastomeres into wild-type host embryos (Fig. 4). Although cells from the wild-type donor embryos never expressed *krox20* (*n*=0/18), ectopic expression was occasionally detected in cells from the *cdx1a/4* morphant embryos, when the transplanted cells were incorporated into the neural tissue (*n*=20, 20% of the embryos; Fig. 4), indicating that Cdx1a and Cdx4 suppress the posterior hindbrain fate cell-autonomously. However, this ectopic expression was detected only when the Cdx1a/4-deficient cells were located in the middle trunk region of the neural tissue, and not in the anterior or the posteriormost spinal cord (Fig. 4B,C), further supporting the idea that inductive signals for hindbrain gene expression were localized differently in the wild-type and *cdx1a/4* morphant embryos.

Opposite gradients of Fgf and RA signaling between hindbrain and posterior neural tissues

Since the normal formation and anteroposterior patterning of the hindbrain and anterior spinal cord is regulated by Fgf and RA signals, we considered the possibility that the ectopic expression of the posterior hindbrain and anterior spinal cord markers might also depend on these signals. In an effort to investigate this possibility, we first examined the expression of the *fgf* genes *raldh2* and *cyp26a1*, which codes for an RA-degrading enzyme, in wild-type, *cdx1a* morphant, *cdx4* morphant and *cdx1a/4* morphant embryos, at the early segmentation stage (Fig. 5A-P), as the ectopic expression is initiated at the early segmentation stages (Fig. 2R-W). We found that the expression of these genes did not significantly differ between wild-type and *cdx1a* morphant embryos (Fig. 5). The *fgf3* and *fgf8* expression domains in the anterior neuroectoderm and r4 were not affected in the *cdx4* and *cdx1a/4* morphant embryos, and their expression in the posterior mesoderm was retained at reduced levels (Fig. 5A-H). The expression of *fgf8* in the somitic mesoderm was relatively well maintained in the *cdx4* morphant but was strongly reduced in the *cdx1a/4* morphant embryos (Fig. 5E,G,H). The expression domain of *raldh2* in the trunk region of the paraxial/lateral mesoderm shifted posteriorly in the *cdx4* morphant embryos. The *raldh2* expression domain shifted more posteriorly and was located closer to the posterior end in the *cdx1a/4* morphant embryos, than in the *cdx4* morphant embryos (Fig. 5I-L). The expression of *cyp26a1* in the posteriormost region was retained in the *cdx4* and *cdx1a/4* morphant embryos but that in the anterior spinal cord was strongly increased and shifted posteriorly in the *cdx1a/4* morphant embryos (Fig. 5M-P). The expression of *fgf8*, *raldh2* and *cyp26a1*in the posterior region was initiated at the gastrula period and was affected in the *cdx4* and *cdx1a/4* morphant embryos in a similar way to that observed at the early segmentation stage (see Fig. S1 in the supplementary material).

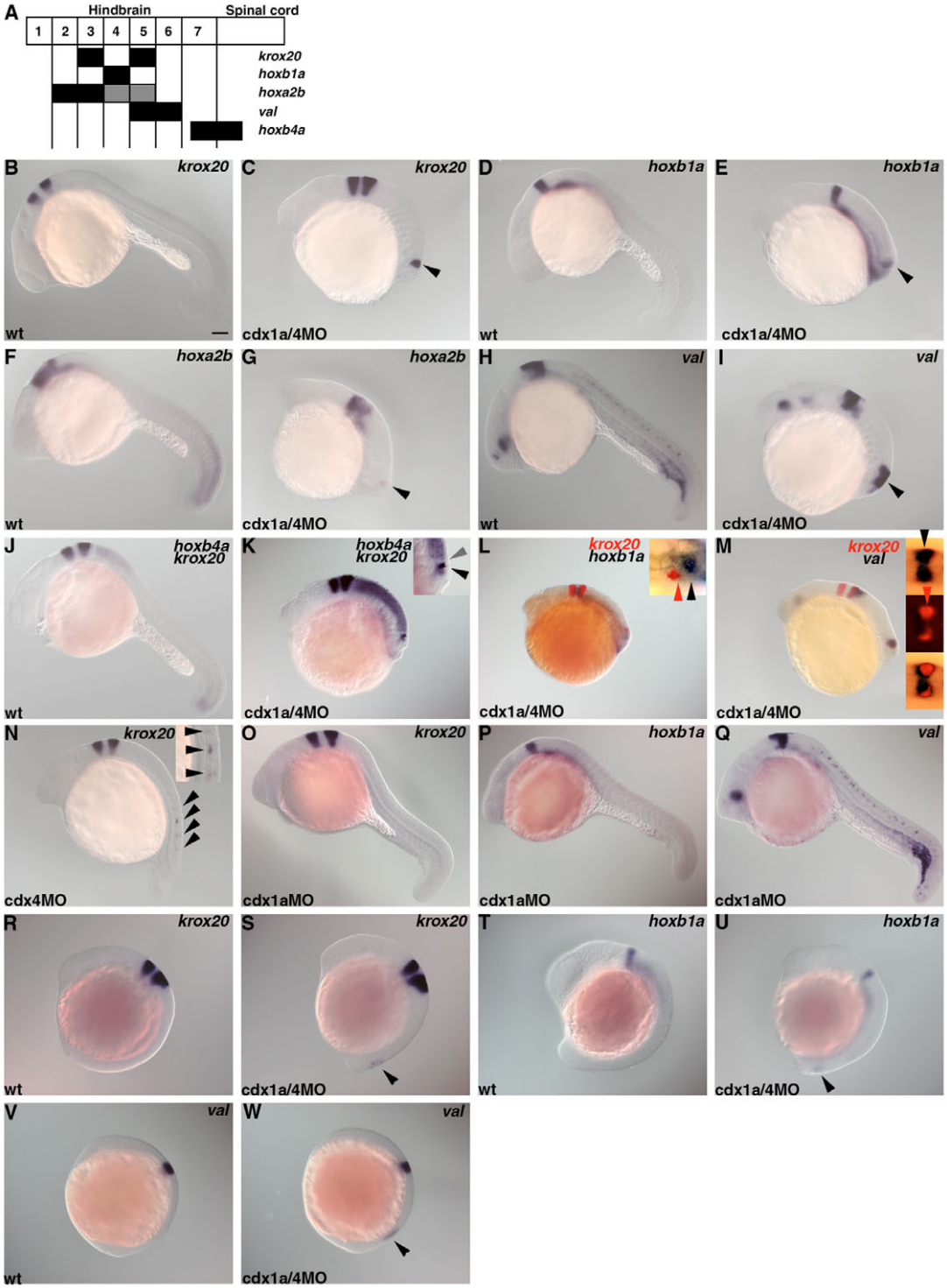

Fig. 2. Ectopic formation of hindbrain in embryos lacking Cdx4 or Cdx1a/Cdx4. (**A**) Schematic presentation of genetic markers for the hindbrain and anterior spinal cord. Numbers indicate rhombomeres. (**B-M**) Expression of *krox20* (B,C), *hoxb1a* (D,E), *hoxa2b* (F,G), *valentino* (*val*, H,I), *hoxb4a* and *krox20* (J and K), *krox20* and *hoxb1a* (L), or *krox20* and *valentino* (M) in wild-type control (B,D,F,H,J) and *cdx1a/4*MO-injected embryos (C,E,G,I,K-M) at 22 hpf. (K-M) Higher magnification dorsal views of the posterior region are in the insets [bright-field images in K,L; bright-field (upper), fluorescent (middle) and superimposed (lower) images in M]. Ectopic expression of the hindbrain markers are indicated by arrowheads (C,E,G,I). In the posterior neural tissue of the *cdx1a/4* morphant embryos, *hoxb1a* expression (r4, black arrowhead in L) was detected just posterior to the *krox20* expression (r5, red arrowhead in L); *valentino* expression (r5, 6, black arrowhead in M) overlapped with *krox20* expression (r5, red arrowhead in M) and extended anteriorly (*krox20*$^{-}$*val*$^{+}$ domain corresponds to r6); the *hoxb4a*$^{-}$ domain (r6, gray arrowhead in K) was anterior to the *krox20* domain (r5, black arrowhead in K). (**N**) Expression of *krox 20* in embryos that received 1 ng *cdx4*MO at 22 hpf. Higher magnification views in the inset. Ectopic expression domains are marked by arrowheads. (**O-Q**) Expression of *krox 20, hoxb1a* and *valentino* in embryos that received 1 ng *cdx1a*MO at 22 hpf. (**R-W**) Expression of *krox20* at 13 hpf, of *hoxb1a* at 15 hpf and of *valentino* at 11 hpf in wild-type control (R,T,V) and *cdx1a/4* morphant embryos (S,U,W). Ectopic expression domains are marked by arrowheads. Scale bar: 100 μm.

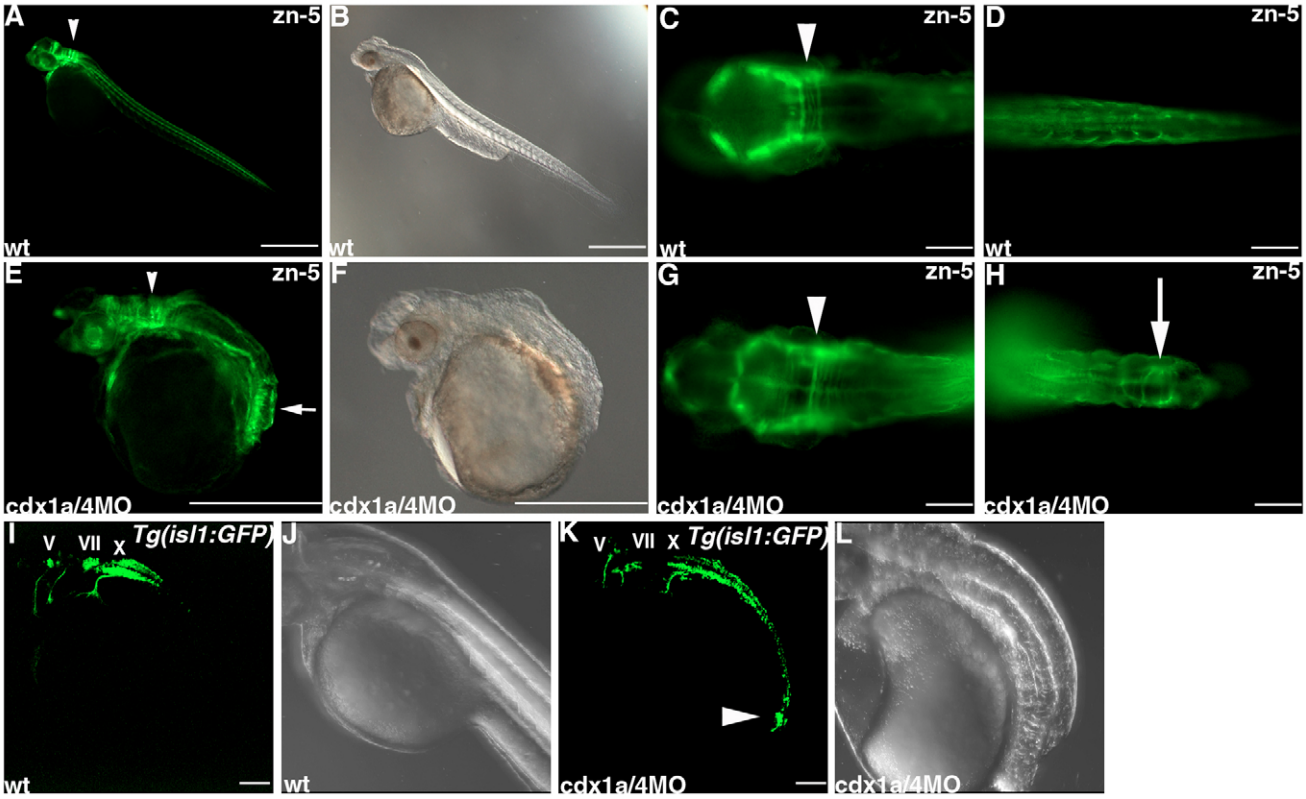

Fig. 3. Ectopic formation of hindbrain neurons in embryos lacking Cdx1a and Cdx4. (**A-H**) Detection of hindbrain commissure neurons by immunostaining with monoclonal antibody zn-5 of wild-type control (A-D) and *cdx1a/4* morphant embryos (E-H) at 3 days post fertilization (dpf). (B,E) Bright field images. Lateral views (A,B,E,F) and high-magnification dorsal views of hindbrain (C,G) and tail regions (D,H) with anterior to the left. zn-5-positive commissure neurons can be recognized by their axonal structures in the hindbrain regions (arrowheads) and ectopically in the posteriormost neural tissue (arrows). (**I-L**) Detection of cranial motoneurons in control *Tg(isl1:GFP)* embryos and *Tg(isl1:GFP)* embryos that received *cdx1a*MO and *cdx4*MO at 48 hpf. Bright field images (J,L). The position of trigeminal (V), facial (VII) and vagal (X) motor nuclei was indicated. A cluster of the GFP+ neurons with their axons were detected in the posteriormost region of *cdx1a/4* morphant embryos (arrowhead, K). Scale bars: 500 μm in A,B,E,F; 100 μm in C,D,G,H,I,K.

Given that Fgf signaling activity was high in the posteriormost region and RA signaling activity was high in the middle trunk region in wild-type animals, the results from the *cdx4* and *cdx1a/4* morphant embryos indicate that the gradients of the Fgf and RA signals in the ectopic posterior neural tissue were opposite to those in the hindbrain and anterior spinal cord (Fig. 5R-T). Our results also show that the region of high activity for Fgf and RA signaling overlapped in the posteriormost region of the *cdx1a/4* morphant embryos (Fig. 5T), but in the *cdx4* morphant embryos these domains overlapped in the middle trunk region, where *raldh2* and *fgf8* are coexpressed in the somitic mesoderm (Fig. 5S). Considering these findings, we hypothesized that the overlapping Fgf and RA signaling recapitulated the signaling conditions for development of the posterior hindbrain and anterior spinal cord, thereby inducing their ectopic development in the posteriormost neural tissue in the *cdx1a/4* morphant embryos and in the posterior-trunk region in the *cdx4* morphant embryos. Consistent with this, we detected ectopic *krox20* transcripts in the vicinity of the *fgf8* expression domain in the somitic mesoderm of the *cdx4* morphant embryos (Fig. 5Q).

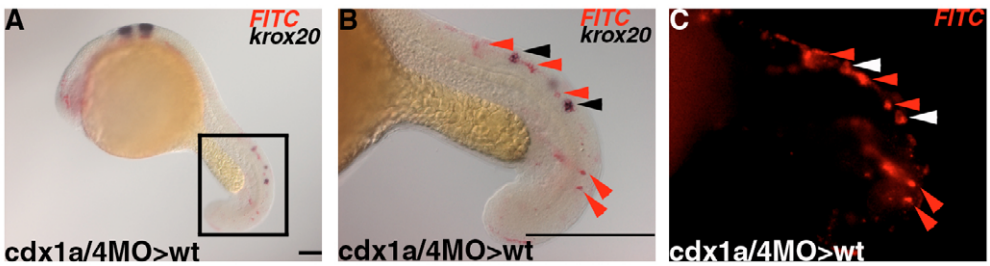

Fig. 4. Cell-autonomous role of Cdx1a/4 in suppressing posterior hindbrain identity. (**A-C**) Transplantation of Cdx1a/4-defective cells into wild-type embryos. Blastomere cells were isolated from embryos that received *cdx1a/4*MOs and FITC-dextran at the sphere stage and transplanted into sibling wild-type embryos. The embryos were fixed at 22 hpf and stained with a *krox20* riboprobe (purple) and an anti-FITC antibody (red). High-magnification bright field (B) and fluorescent images (C) of the posterior neural tissues (encircled by a square in A). *krox20*-expressing and non-expressing transplanted cells are marked with black and red arrowheads, respectively, in B, or with white and red arrowheads in C. Scale bars: 100 μm in A; 500 μm in B.

Fgf and RA signaling are required for the ectopic formation of the posterior hindbrain

To test our hypothesis, we conducted experiments inhibiting Fgf and/or RA signaling in the *cdx1a/4* morphant embryos (Figs 6, 7; see Fig. S4 in the supplementary material). We inhibited Fgf signaling by treating the embryos with 300 μmol/l SU5402, a specific inhibitor of the FGF receptor (Mohammadi et al., 1997) or by co-injecting *fgf3*MO and *fgf8*MO (Wiellette and Sive, 2004). We inhibited RA signaling by treating the embryos with 50 μmol/l 4-(Diethylamino)-benzaldehyde (DEAB), a potent retinaldehyde dehydrogenase inhibitor (Russo, 1997) or by injecting *raldh2*MO (Begemann et al., 2001). Marker expression in embryos treated only with an inhibitor of Fgf or RA signaling is shown in Fig. S2 in the supplementary material (marker expression in wild-type untreated embryos is shown in Fig. 2). Inhibition of either the Fgf or RA signal did not perturb the other signaling gradient in the *cdx1a/4* morphant embryos (Fig. S3 in the supplementary material). Inhibition of the Fgf signal in the *cdx1a/4* morphant embryos by the

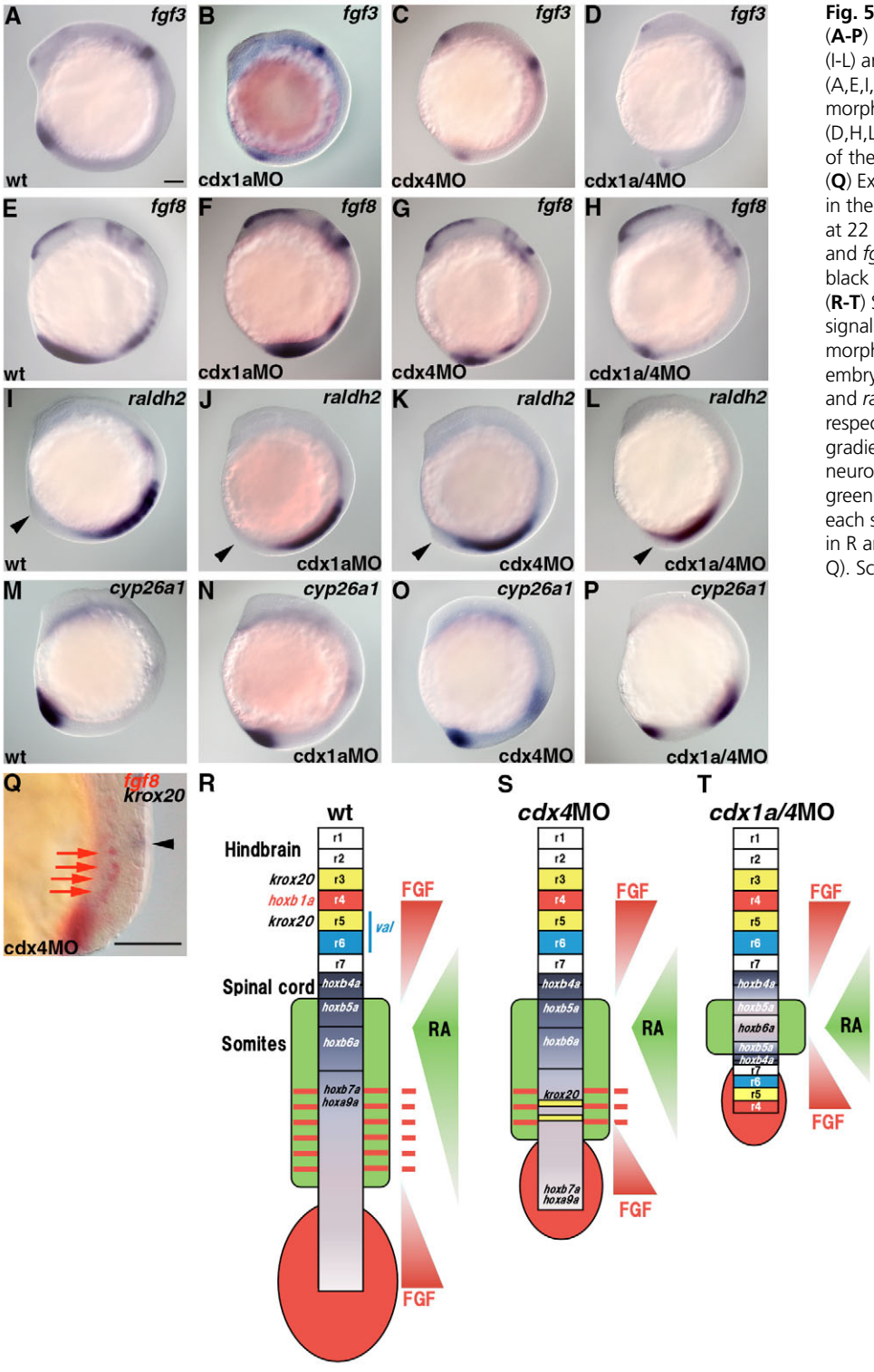

Fig. 5. Gradients of Fgf and RA signals. (**A-P**) Expression of *fgf3* (A-D), *fgf8* (E-H), *raldh2* (I-L) and *cyp26a1* (M-P) in wild-type control (A,E,I,M), *cdx1a* morphant (B,F,J,N), *cdx4* morphant (C,G,K,O) and *cdx1a/4* morphant (D,H,L,P) embryos at 13 hpf. The posterior end of the embryos are marked by arrowheads (I-L). (**Q**) Expression of *fgf8* (red) and *krox20* (purple) in the trunk region of *cdx4* morphant embryos at 22 hpf. The ectopic *krox20* in neural tissue and *fgf8* in somitic mesoderm are marked by a black arrowhead and red arrows, respectively. (**R-T**) Schematic presentation of the Fgf and RA signaling gradients in wild-type (R), *cdx4* morphant (S) and *cdx1a/4* morphant (T) embryos. The expression domains of the *fgf*s and *raldh2* are indicated in red and green, respectively, in the body of each schematic. The gradients of the Fgf and RA signals in the neuroectoderm are also indicated in red and green, respectively, and shown to the right of each schematic. The solid red bars on the right in R and S indicate stripes of Fgf signaling (see Q). Scale bars: 100 μm.

*fgf3/8*MOs abolished the ectopic expression of *hoxb1a* (r4, *n*=26/34), *krox20* (r5, *n*=36/36) and *valentino* (r5, 6, *n*=29/34) and reduced the normal expression of *krox20* in r5, but did not inhibit the expression of *hoxb4a* (r7–) and *hoxb5a* (s1–) (Fig. 6A,E,I,M,Q; Fig. 7C). Inhibition of the Fgf signal by SU5402 abolished the ectopic expression of *hoxb1a* (*n*=14/14) and the ectopic and normal expression of *krox20* (*n*=14/14) and *valentino* (*n*=14/14) but retained the *hoxb4a* and *hoxb5a* expression (Fig. 6B,F,J,N,R; Fig. 7D). The weaker phenotypes with the *fgf3/8*MOs are probably due to incomplete inhibition of the Fgf3 and Fgf8 function under our experimental conditions, as the MOs did not disrupt the normal formation of r5 and r6 (Maves et al., 2002; Walshe et al., 2002; Wiellette and Sive, 2004). The results suggest that the Fgf signal is required for the ectopic expression of the hindbrain r4-r6 markers, but dispensable for both normal and ectopic expression of the r7 and the anterior spinal cord markers. The inhibition of Raldh2 by its MO in the *cdx1a/4* morphant embryos repressed the expression of *hoxb5a* (s1–, *n*=26/26), but did not suppress the normal or ectopic expression of *krox20* (r5), *hoxb1a* (r4), *valentino* (r5, 6) or *hoxb4a* (r7–) (Fig. 7E; see Fig. S4 in the supplementary material). Strong inhibition of RA signaling by DEAB in the *cdx1a/4* morphant embryos completely abolished the expression of *hoxb4a* (r7–; *n*=15/15) and *hoxb5a* (s1–, *n*=11/11) and strongly inhibited the expression of *valentino* (r5, 6), but did not significantly inhibit the expression of *hoxb1a* (r4) (Fig. 5C,G,K,O,S; Fig. 7F). These data indicate that high RA signaling activity is required for the normal and ectopic expression of the anterior spinal cord markers, and lower RA signaling activity is required for both normal and ectopic expression of the posteriormost hindbrain markers (r7 and probably r6).

Inhibition of both the Fgf and RA signals by SU5402 and DEAB in the *cdx1a/4* morphant embryos suppressed the ectopic *hoxb1a* expression (r4, *n*=15/15) and the normal and ectopic expression of *krox20* (r5, *n*=18/18), *valentino* (r5, 6, *n*=9/9), *hoxb4a* (r7–,

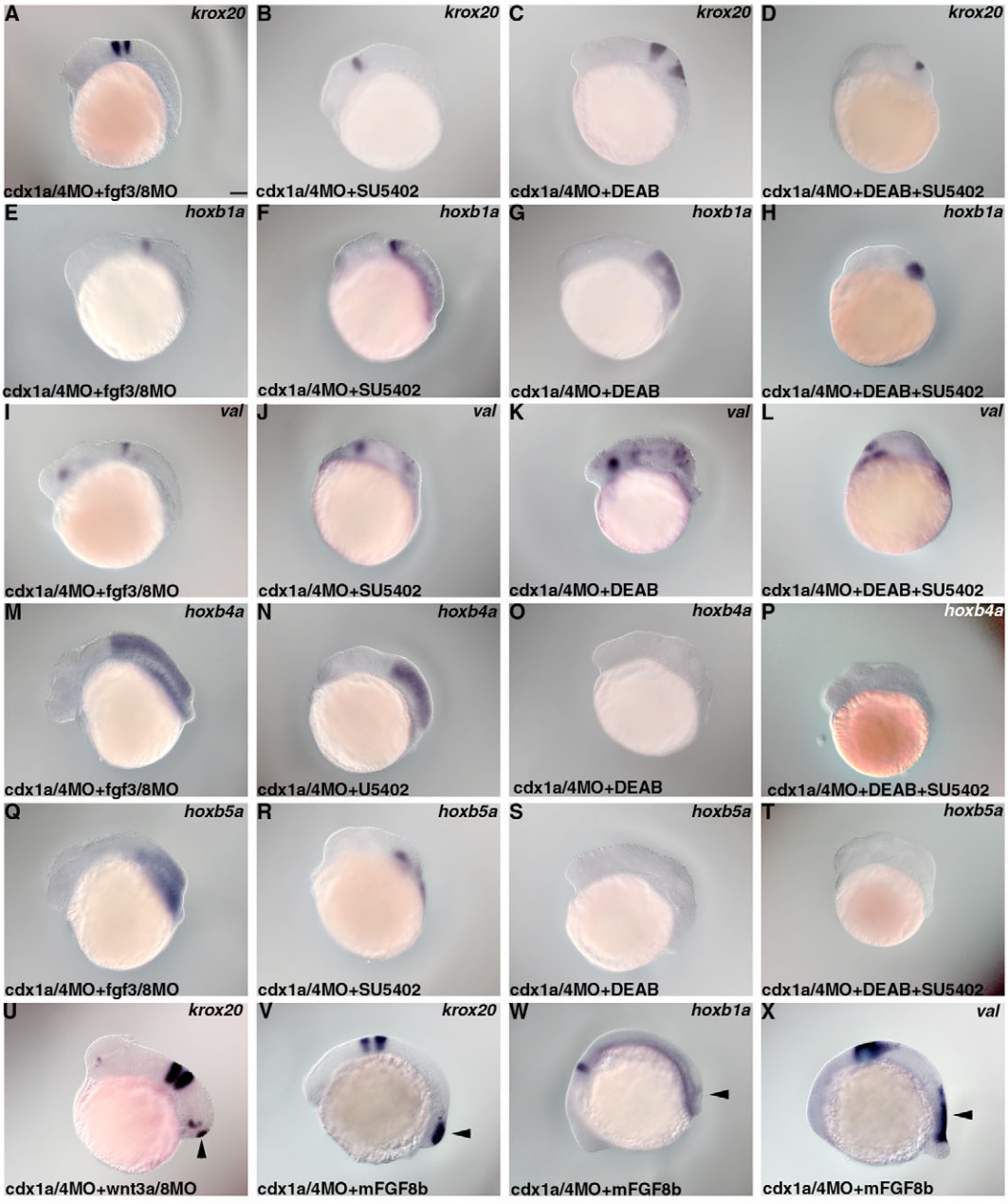

Fig. 6. RA and Fgf signals are involved in the formation of the duplicated neural structure in embryos lacking Cdx1a/4. (**A-U**) Expression of *krox20* (A-D,U), *hoxb1a* (E-H), *valentino* (I-L), *hoxb4a* (M-P) or *hoxb5a* (Q-T) in embryos that received an injection of *cdx1a/4*MOs and 2 ng each of the *fgf3*MO and *fgf8*MOs (A,E,I,M,Q) or injection of *cdx1a/4*MOs and 2 ng each of *wnt3a*MO and *wnt8*MOs (U); *cdx1a/4* morphant embryos that were treated with 300 μmol/l SU5402 (B,F,J,N,R); *cdx1a/4* morphant embryos that were treated with 50 μmol/l DEAB (C,G,K,O,S); *cdx1a/4* morphant embryos that were treated with 300 μmol/l SU5402 and 50 μmol/l DEAB (D,H,L,P,T) at 22 hpf. (**V-X**) Expression of *krox20* (V), *hoxb1a* (W) and *valentino* (X) in *cdx1a/4* morphant embryos that were treated with 100 μg/ml recombinant mouse FGF8b. The ectopic expression is marked by arrowheads. Scale bar: 100 μm.

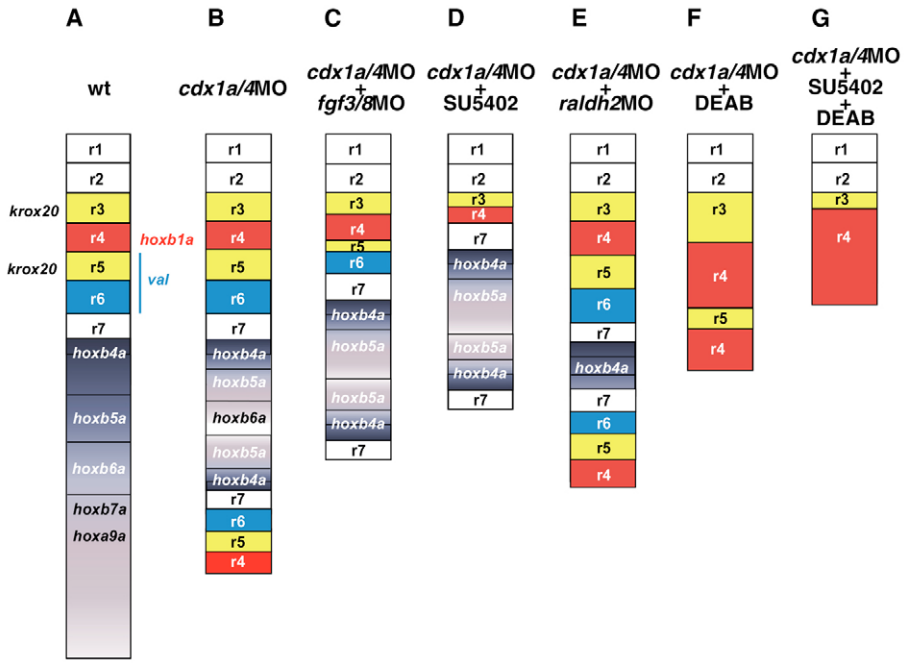

Fig. 7. Schematic presentation of the neural structures of *cdx1a/4* morphant, and Fgf and/or RA signal-defected *cdx1a/4* morphant embryos. (**A**) Wild type (wt). (**B**) *cdx1a/4* morphants. (**C**) *cdx1a/4* morphants that received co-injected *fgf3/8*MOs. (**D**) *cdx1a/4* morphants treated with 300 μmol/l SU5402. (**E**) *cdx1a/4* morphant embryos expressing the *raldh2*MO (see Fig. S4 in the supplementary material). (**F**) *cdx1a/4* morphant embryos treated with 50 μmol/l DEAB. (**G**) *cdx1a/4* morphant embryos treated with SU5402 and DEAB.

n=10/10) and *hoxb5a* (s1–, *n*=10/10) (Fig. 6D,H,L,P,T; Fig. 7G). This inhibition also elicited a slight expansion of the *hoxb1a*-expressing domain (Fig. 6H; Fig. 7G), suggesting that these embryos lost the duplicated posterior hindbrain and anterior spinal cord, but instead showed an expanded r4 domain. Although Wnt signaling is reported to be involved in patterning and neurogenesis in the hindbrain (Amoyel et al., 2005; Riley et al., 2004), inhibition of Wnt3a and Wnt8, which disrupt the posterior body formation in wild-type embryos (Shimizu et al., 2005; Thorpe et al., 2005), did not inhibit the formation of the ectopic neural tissue (Fig. 6U). Our results indicate that the ectopic formation of r4-6 requires high Fgf signaling, whereas that of anterior spinal cord and the posteriormost hindbrain requires high RA signaling, in a similar manner to the normal formation of these tissues.

To gain better insight into this issue, we investigated whether Fgf and RA were sufficient to induce the posterior hindbrain in the absence of Cdx1a and Cdx4. We treated the *cdx1a/4* morphant embryos with mouse FGF8. Compared with untreated *cdx1a/4* morphant embryos (Fig. 2), the FGF8-treated *cdx1a/4* morphant embryos showed anterior expansion of the ectopic expression domains of *krox20* and *valentino*, but not of *hoxb1a* (Fig. 6V,W,X). As the posterior mesoderm expresses *raldh2* in the *cdx1a/4* morphant embryos (Fig. 5L), our results suggest that Fgf and RA signals induced the posterior hindbrain fate in posterior neural tissue lacking Cdx1a/4.

Posterior Hox proteins mediate the repression by Cdx of the posterior hindbrain identity

Cdx proteins are known to regulate the expression of the posterior *hox* genes (Charite et al., 1998; Gaunt et al., 2004; Isaacs et al., 1998; Pownall et al., 1996; Subramanian et al., 1995), and the expression of posterior *hox* genes, such as *hoxb7a* and *hoxa9a*, is absent from the neural tissues of *cdx1a/4* morphant embryos (Shimizu et al., 2005) (Fig. 1F,H), suggesting that the posterior Hox proteins function downstream of Cdx1a/4 to repress the fate of the posterior hindbrain. To address this issue, we injected RNAs for *hoxb7a, hoxa9a* or another posterior *hox* gene, *hoxb9a*, with or without the *cdx1a/cdx4*MOs. The misexpression of these posterior *hox* genes in wild-type embryos suppressed the expression of *krox20* (*n*=15/15 for *hoxa9a*, *n*=19/19 for *hoxb9a* and *n*=11/15 for *hoxb7a*) (Fig. 8A-C, and data not shown for *hoxb7a*). The ectopic expression of *krox20*, which was observed in the *cdx1a/4* morphant embryos (Fig. 2C), was abolished in these embryos (*n*=14/15 for *hoxa9a*, *n*=15/17 for *hoxb9a* and *n*=15/17 for *hoxb7a*) (Fig. 8D,E, and data not shown

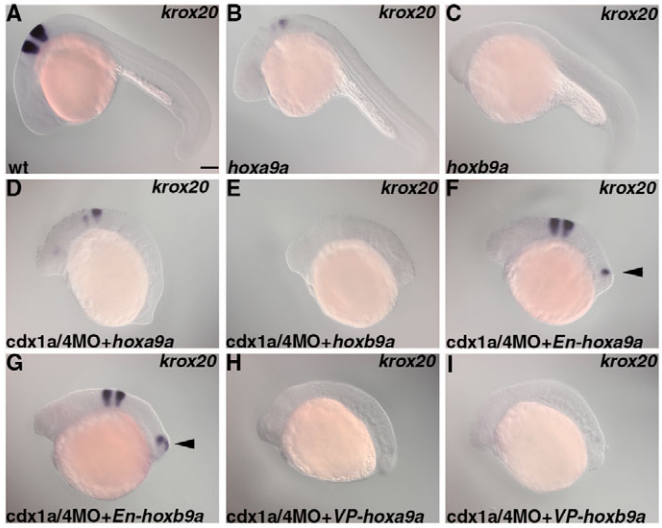

Fig. 8. Posterior Hox proteins function as transcriptional activators leading to the Cdx-mediated inhibition of hindbrain formation. Expression of *krox20* in wild-type control embryos (**A**) and embryos that received an injection of 25 pg of *hoxa9a* RNA (**B**), 25 pg of *hoxb9a* RNA (**C**), *cdx1a/4*MOs and *hoxa9a* RNA (**D**), *cdx1a/4*MOs and *hoxb9a* RNA (**E**), *cdx1a/4*MOs and 25 pg of *En-hoxa9a* RNA (**F**), *cdx1a/4*MOs and 25 pg of *En-hoxb9a* RNA (**G**), *cdx1a/4*MOs and 25 pg of *VP-hoxa9a* RNA (**H**) or *cdx1a/4*MOs and 25 pg of *VP-hoxb9a* RNA (**I**). Overexpression of *hoxa9a, hoxb9a, VP-hoxa9a* or *VP-hoxb9a* suppresses the expression of *krox20* in its normal and ectopic positions. Overexpression of *En-hoxa9a* or *En-hoxb9a* did not suppress the ectopic *krox20* expression (marked by arrowheads). Scale bar: 100 μm.

for *hoxb7a*), indicating that these posterior *hox* genes compensated for the loss of Cdx1a and Cdx4 in repressing the posterior hindbrain fate. Misexpression of the anterior *hox* genes *hoxb1a* or *hoxb1b* did not suppress the ectopic *krox20* expression in the *cdx1a/4* morphant embryos (data not shown). These findings suggest that the posterior *hox* genes function downstream of Cdx1a and Cdx4 to repress the posterior hindbrain fate.

Hox proteins are reported to function as transcriptional activators or repressors (Asahara et al., 1999; Lu et al., 2003; Saleh et al., 2000; Tour et al., 2005). We investigated whether the posterior Hox proteins function as transcriptional repressors of the posterior hindbrain genes or as transcriptional activators that indirectly repress the transcription of these genes. To approach this issue, we constructed activator and repressor versions of Hoxa9a and Hoxb9a, by constructing fusion proteins with either the transcriptional repressor domain of *Drosophila* Engrailed (En-Hoxa9a and En-Hoxb9a) or the activation domain of the Herpes Simplex Virus transcriptional activator VP16 (VP-Hoxa9a and VP-Hoxb9a), respectively. Although the expression of *En-hoxa9a* or *En-hoxb9a* led to a reduction of posterior structures and an expansion of the anterior structure in wild-type (data not shown) and *cdx1a/4* morphant embryos (Fig. 8F,G), neither of these fusion proteins inhibited the ectopic expression of *krox20* (n=17/17 for *En-hoxa9a* and n=25/27 for *En-hoxb9a*) (Fig. 8F,G). By contrast, the expression of *VP-hoxa9a* or *VP-hoxb9a* suppressed the expression of *krox20* in its normal and ectopic positions (n=10/15 for *VP-hoxa9a* and n=11/15 for *VP-hoxb9a*) (Fig. 8H,I), as did the wild-type RNA. Our data show that the posterior Hox proteins function as transcriptional activators that indirectly repress the formation of the posterior hindbrain.

DISCUSSION

Role of the Cdx-Hox code in formation of the hindbrain

In this study, we unexpectedly found that the loss of Cdx1a and Cdx4 functions led to ectopic expression of the hindbrain genes and ectopic formation of the hindbrain neurons (Figs 2, 3). Mutations in, or the inhibition of, *caudal*-related genes is reported to affect the development of the posterior body structure in invertebrate and vertebrate species (Chawengsaksophak et al., 2004; Macdonald and Struhl, 1986; Shinmyo et al., 2005; van den Akker et al., 2002; van Nes et al., 2006), but no role has been reported for them in inhibiting the ectopic formation of an anterior structure in the posterior body. Our data reveal a previously unrecognized role for the *caudal*-related genes in body patterning.

Our data show that expression of the posterior *hox* genes inhibits the ectopic formation of the posterior hindbrain in *cdx1a/4* morphant embryos, suggesting that the posterior *hox* genes mediate the activity of Cdx. The possibility remains that Cdx proteins by themselves or downstream genes other than the posterior *hox* genes suppress the formation of the posterior hindbrain and anterior spinal cord. To address this issue, loss-of-function experiments for all the posterior Hox proteins must be performed. This is not possible currently, because there are too many posterior *hox* genes to be knocked down by MOs. Our findings, however, show that the posterior Hox proteins play at least some part in the Cdx-mediated inhibition of posterior hindbrain formation.

The Hox proteins interact directly – or indirectly through Pbx proteins – with transcriptional co-repressors and histone deacetylases, and they function as transcriptional repressors (Asahara et al., 1999; Saleh et al., 2000), which implies that the posterior Hox proteins might directly repress the expression of the posterior hindbrain genes. Our data show, however, that the posterior Hox proteins function as transcriptional activators to repress the posterior hindbrain genes. Therefore, the Hox proteins may activate the expression of transcriptional repressors that inhibit the expression of the posterior hindbrain genes. Alternatively, posterior Hox proteins might repress the function of transcriptional activators that induce the expression of the posterior hindbrain genes, through a protein-protein interaction or competition for the binding sites. In any case, the posterior Hox proteins indirectly repress the expression of the posterior hindbrain genes.

The Cdx-Hox code modifies tissue response to Fgf and RA

It is well known that the same signaling molecules are often used for different developmental processes. *fgf3* and *fgf8* control the anteroposterior patterning of the hindbrain and spinal cord (Maves et al., 2002; Walshe et al., 2002; Waskiewicz et al., 2002; Wiellette and Sive, 2004), the morphogenesis of the posterior body (Dubrulle et al., 2001; Sawada et al., 2001) and the development of telencephalon (Shinya et al., 2001; Walshe and Mason, 2003). The RA signal controls the anteroposterior patterning of the hindbrain/spinal cord (Begemann et al., 2001; Grandel et al., 2002; Maves et al., 2002; Waskiewicz et al., 2002) in the anterior region and regulates neurogenesis and segmentation in the posterior region (Diez del Corral et al., 2003). The biological activities of the Fgf and RA signals must therefore be controlled differently in the anterior and posterior regions, by other factors. Here, we were able to demonstrate that Cdx1a and Cdx4 function to control the responsiveness of Fgf and RA signals. First, inhibition of Cdx1a/4 led to ectopic expression of the posterior hindbrain and anterior spinal cord genes, and the ectopic expression was suppressed by inhibition of Fgf and/or RA signals (Figs 6, 7). The Fgf signal is known to be required for the formation of posterior body (Griffin et al., 1998), and it is possible that the inhibition of the Fgf signal might secondarily affect ectopic formation through the repression of inductive signals from the posterior body. The incubation of the *cdx1a/4* morphant embryos with the FGF8 protein, however, led to expansion of the ectopic expression but did not significantly affect the posterior body structures (Fig. 6), suggesting that the Fgf signal acts directly on the neural tissue to induce the hindbrain genes, and Cdx1a and Cdx4 repress the Fgf-dependent ectopic expression. This is consistent with the proposed direct role of Fgf and RA signals in the normal formation of the posterior hindbrain and anterior spinal cord. Our transplantation experiment also showed that Cdx1a and Cdx4 function in repressing the hindbrain genes cell-autonomously (Fig. 4). Viewed as a whole, Cdx1a and Cdx4 can be seen as controlling the formation of the posterior neural tissue by modifying the competence of these tissues to respond to the Fgf and RA signals.

It is unlikely, however, that Cdx1a and Cdx4 repress the ectopic formation of the hindbrain and anterior spinal cord through inhibiting the Fgf and RA signaling pathways. Cdx1a requires Fgf signaling to induce the expression of the posterior *hox* genes (Shimizu et al., 2005), and RA signaling is known to be involved in neurogenesis of the spinal cord, where *Cdx* genes are expressed (Diez del Corral et al., 2003). Rather than inhibiting the Fgf and RA signals then, Cdx1a and Cdx4 actually control the responsiveness to the Fgf and RA signaling. Although the molecular mechanism by which the Cdx proteins control the Fgf and RA responsiveness remains unclear, the posterior *hox* genes are suitable candidates to be involved in this mechanism.

Cdx proteins are involved in the special control of Fgf and RA signaling

In addition to the cell-autonomous role of Cdxs in repressing hindbrain gene expression in the neural tissue, Cdxs also control the sources of Fgf and RA signals in the mesodermal tissues. In our study we observed the *raldh2* expression domain in the paraxial/lateral mesoderm to shift posteriorly in the *cdx1a/4* morphant embryos, resulting in overlapping regions of high Fgf and RA signaling in the posteriormost neural tissue (Fig. 5). This is involved in the ectopic formation of the posterior hindbrain and anterior spinal cord. In a previous study we had reported that *cdx1a* and *cdx4* are required for the formation of the posterior mesoderm (Shimizu et al., 2005), suggesting that Cdx1a and Cdx4 function to separate the regions of high Fgf and RA signaling though regulating the posterior body formation, thereby preventing ectopic formation in the posteriormost neural tissue. We also detected upregulation of *cyp26a1* expression in the anterior spinal cord of the *cdx1a/4* morphant embryos (Fig. 5). This is probably due to high RA signaling activity, as *cyp26a1* is strongly responsive to RA signaling (Emoto et al., 2005). It is not yet clear, however, exactly how the RA signaling gradient is generated in the posterior hindbrain in the presence of high Cyp26a1 activity in the *cdx1a/4* morphant embryos. As the RA signal is high in these embryos, it could be the case that some part of the RA may escape from the Cyp26a1-meidated degradation and be sufficient for the formation and patterning of the posterior hindbrain and anterior spinal cord. In the posteriormost neural tissue, the high RA activity probably contributes to the mirror image duplication in the *cdx1a/4* morphant embryos.

Anteroposterior patterning of neural tissue by Fgf, RA and the Cdx-Hox code

How are our present findings integrated with the current model for the anteroposterior patterning of neural tissue? The anteroposterior patterning of neural tissues is initially regulated by inductive signals from the dorsal organizer and the non-axial mesendoderm, in which Wnt and Fgf signaling are believed to be involved. The subsequent regional specification is controlled by inductive signals from the secondary organizing centers and the adjacent mesoderm tissues, in which Fgf and RA signaling are involved (Moens and Prince, 2002). *cdx1a* and *cdx4* are regulated by Wnt and Fgf signals (Shimizu et al., 2005), and they confer on the neural tissues different competences for responding to the local Fgf and RA signals. The region in which *cdx1a* and *cdx4* are not expressed takes on the posterior hindbrain/anterior spinal cord fate in response to the counter gradients of Fgf and RA signaling. In the posterior neural tissue, where *cdx1a* and *cdx4* are expressed, *cdx1a* and *cdx4* not only suppress the posterior hindbrain/anterior spinal cord fate, but also are required for the formation of normal posterior neural tissue (Shimizu et al., 2005).

A previous study (Bel-Vialar et al., 2002) has reported that, in chick embryos, 3′ *HoxB* genes – which correspond to anterior *hox* genes in this study – are responsive to RA signaling, while 5′ *Hox* genes (posterior *hox* genes) are responsive to Fgf signaling. The CDX activity makes the posterior *hox* genes competent to respond to Fgf signaling. We previously reported that Fgf signaling is also required for the Cdx-mediated expression of *hoxa9a* and *hoxb7a* (Shimizu et al., 2005). These reports suggest that Cdx proteins cooperate with Fgf signaling in controlling the patterning and formation of the posterior spinal cord. Consistent with this, our preliminary data show that misexpression of *cdx1a* activates ectopic expression of *hoxb9a* in the hindbrain region in an Fgf-dependent manner (data not shown). From this perspective, *cdx1a* and *cdx4* are key genes for switching the tissue competence to respond to Fgf signaling from the anterior to the posterior mode. As in chick embryos, the anterior *hox* gene *hoxb1b* is shown to be responsive to the RA signaling at the gastrula period in zebrafish (Kudoh et al., 2002). As paralog group1 (PG1) of the anterior *hox* genes have been shown to be involved in the formation of posterior hindbrain (r4-r6) in various species (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Gavalas et al., 1998; Lufkin et al., 1991; Mark et al., 1993; McClintock et al., 2001; Rossel and Capecchi, 1999; Studer et al., 1998), then this suggests that the anterior *hox* genes also cooperate with Fgf and RA signaling in the formation of the posterior hindbrain.

Our findings provide compelling evidence that a Cdx-Hox code controls the tissue competence to respond to the inductive signals that control the anteroposterior patterning of neural tissues. The roles of the Cdx-Hox code in neural patterning illuminate at least one mechanism by which a given inductive signal can control different processes during embryogenesis.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/23/4709/DC1

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