Retinoic acid-metabolizing enzyme Cyp26a1 is essential for determining territories of hindbrain and spinal cord in zebrafish

Yumi Emoto, Hironori Wada, Hitoshi Okamoto, Akira Kudo, Yoshiyuki Imai*

*Department of Biological Information, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan
bLaboratory for Developmental Gene Regulation, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
cCore Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

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Abstract

Retinoic acid (RA) plays a critical role in neural patterning and organogenesis in the vertebrate embryo. Here we characterize a mutant of the zebrafish named giraffe (gir) in which the gene for the RA-degrading enzyme Cyp26a1 is mutated. The gir mutant displayed patterning defects in multiple organs including the common cardinal vein, pectoral fin, tail, hindbrain, and spinal cord. Analyses of molecular markers suggested that the lateral plate mesoderm is posteriorized in the gir mutant, which is likely to cause the defects of the common cardinal vein and pectoral fin. The cyp26a1 expression in the rostral spinal cord was strongly upregulated in the gir mutant, suggesting a strong feedback control of its expression by RA signaling. We also found that the rostral spinal cord territory was expanded at the expense of the hindbrain territory in the gir mutant. Such a phenotype is the opposite of that of the mutant for Raldh2, an enzyme that synthesizes RA. We propose a model in which Cyp26a1 attenuates RA signaling in the prospective rostral spinal cord to limit the expression of hox genes and to determine the hindbrain–spinal cord boundary.

Keywords: giraffe; neckless; cyp26a1; raldh2; hox; Retinoic acid; Common cardinal vein; Hindbrain; Spinal cord; Zebrafish

Introduction

Retinoic acid (RA), a derivative of vitamin A, is an important signaling molecule for the morphogenesis of vertebrate embryos. All-trans RA is the major biologically active retinoid in vivo (Costaridis et al., 1996; Horton and Maden, 1995). This compound is present in embryonic and adult tissues at high levels and binds efficiently to retinoic acid receptors (RARs). All-trans RA is synthesized by way of two oxidation steps (for a review, see Perlmann, 2002). In the first step, vitamin A (retinol) is converted to all-trans retinaldehyde by alcohol dehydrogenases. In the second step, which is thought to be the rate-limiting one, all-trans retinaldehyde is converted to all-trans RA by any of three related aldehyde dehydrogenases, Raldh1, Raldh2, and Raldh3 (also known as Aldh1a1, Aldh1a2, and Aldh1a3, respectively). All-trans RA is in turn metabolized by members of the Cyp26 family of cytochrome P450 enzymes, which include Cyp26a1, Cyp26b1, and Cyp26c1. These enzymes convert all-trans RA to more polar metabolites, including 4-hydroxy-RA, 4-oxo-RA, 18-hydroxy-RA, and 5,8-epoxy-RA (Fujii et al., 1997; Taimi et al., 2004; White et al., 1996, 2000a). Although several assays have shown that these metabolites are biologically active (Idres et al., 2002 and references therein), genetic evidence has suggested that Cyp26a1 metabolites are not bioactive but merely degradation products (Niederreither et al., 2002).

Mutational analyses of the genes involved in the production and degradation of RA have been carried out in mouse and zebrafish. In mice, a loss of the raldh2 function causes impaired body turning and heart looping,
shortening of the trunk region, absence of limb buds, hypoplastic otic vesicles, and lack of externally visible second and third branchial arches (Niederreither et al., 1999). Likewise, mutations in the raldh2 gene in zebrafish named neckless (nls)/no-fin (nof) result in truncation of the anterior–posterior (AP) axis, defects in midline mesendodermal tissues, and absence of pectoral fins and cartilaginous gill arches (Abu-Abed et al., 2001; Grandel et al., 2002). These phenotypes are similar to those of the vitamin A-deficient syndrome, suggesting that Raldh2 is the main source of RA produced during embryogenesis.

Targeted disruption of the mouse cyp26a1 gene causes spina bifida, truncation of the tail, aplasia or hypoplasia of the urogenital system, posterior transformations of cervical vertebrae, and abnormal patterning of the rostral hindbrain (Abu-Abed et al., 2001; Sakai et al., 2001). Most of these phenotypes are similar to those induced by excess RA administration, suggesting that Cyp26a1 is essential for controlling the RA levels during embryogenesis. The early lethal phenotype of the cyp26a1-deficient mouse was rescued by heterozygous disruption of raldh2, suggesting that Cyp26a1 simply functions to protect the embryos from excess all-RA (Niederreither et al., 2002). In addition, a recent study on a cyp26b1-knockout mouse has revealed that the cyp26b1 function is required for proximodistal patterning and outgrowth of the developing limb (Yashiro et al., 2004). In zebrafish, morpholino knockdown of cyp26a1 caused anterior expansion of posterior genes such as hoxb1b and a reduction of the expression domain of the anterior gene otx2 at the late gastrulation stage (Kudoh et al., 2002), suggesting that the Cyp26a1 function is required for the proper AP patterning of the neural ectoderm during gastrulation.

In this study, we isolated and characterized a novel mutant in zebrafish named giraffe (gir) that displays patterning defects in various organs including the common cardinal vein, pectoral fin, tail, hindbrain, and spinal cord. Expression patterns of tbx5.1 and raldh2 suggested that the lateral plate mesoderm is posteriorized in the gir mutant, and such a patterning defect is likely to cause the defective formation of common cardinal vein and pectoral fin. We show evidence that a loss of the cyp26a1 function causes the gir phenotypes. As shown in cyp26a1 knockdown embryos, the anterior neural ectoderm was reduced and the posterior neural ectoderm was expanded at the early stages in the gir mutant. We further show that the expression of the rostral spinal cord markers hoxb5a and hoxb6a were upregulated and expanded rostrally at the mid-segmentation stage in the gir mutant. A strong feedback regulation of the cyp26a1 expression in the prospective rostral spinal cord is likely to be important for defining the rostral expression limit of these hox genes. We propose a model in which Cyp26a1 controls the RA level at the prospective rostral spinal cord and thereby plays a crucial role in territorial determination of the hindbrain and spinal cord.

Materials and methods

Fish and genetic procedures

The gir mutant was found in the background of another mutant rw209 that is defective in jaw development (unpublished). The rw209 mutant was identified in a genetic screen for mutations, in which a RIKEN strain carrying the Islet-1 (Isl1)-GFP transgene (Higashijima et al., 2000) was mutagenized with N-ethyl-N-nitrosourea (ENU) (Masai et al., 2003). For mapping of gir, a gir/+ fish was crossed to a wild-type wik strain (Johnson and Zon, 1999; Nechiporuk et al., 1999); and the F1 progenies were then intercrossed to generate a panel of gir/gir embryos. Isolation of genomic DNA from embryos and mapping relative to SSLP markers were done as described previously (Gates et al., 1999; Knapik et al., 1998). To test directly the linkage between gir and cyp26a1, a cyp26a1 fragment was amplified using primers 5′-CAGGGTTTGAAGCCAGCAATT-3′ and 5′-GCTGCTTTTCATCGCTAAAC-3′, and digested with XbaI, which cleaves the mutant cyp26a1 allele, but not the wild-type allele.

In situ hybridization, histology, immunohistochemistry, and fluorescence microscopy

Whole mount in situ hybridization with digoxigenin-labeled RNA probes was done as described (Thissie et al., 1993). For sectioning, the stained embryos were fixed with 4% paraformaldehyde, embedded using Technovit 8100 (Kulzer) according to the manufacturer’s instructions, and sectioned at 5 μm. Blood cells were stained with o-dianisidine according to Detrich et al. (1995). Alcian blue staining of cartilage tissue was performed as previously reported (Schilling et al., 1996). Acetylated α-tubulin antibody (Sigma, 1:1000 dilution) and a secondary antibody conjugated to Alexa-533 (Santa Cruz Biotechnology, 1:500 dilution) were used for immunofluorescence staining according to the standard protocol (Westerfield, 1995). Observations by fluorescence microscopy were made with a confocal microscope (Zeiss LSM 510).

Morpholino microinjection

A cyp26a1 antisense morpholino (5′-CGCGCAACT-GATGCGAAAAACGAAA-3′), which is complementary to –32 to –8 of the 5′ UTR of cyp26a1 cDNA, a raldh2 antisense morpholino (5′-GTTCACCTCAGTGGGAGGT-CATC-3′; Begemann et al., 2001; Grandel et al., 2002), and a standard control morpholino (5′-CCTCTTACCAGTTCAAATTTATA-3′) were obtained from Gene Tools LLC. The morpholino solution (100 pl) in 5 mg/ml phenol red and 0.2 M KCl was microinjected into one- to eight-cell stage embryos using a pressurized microinjection device (PV820 Pneumatic Picopump, World Precision Instruments).
Results

The gir mutant is defective in the development of common cardinal veins

The phenotype of the giraffe (gir) mutant became apparent at 28 h postfertilization (hpf). At this stage, blood cells begin to circulate in wild-type embryos, whereas no circulation was observed in the gir mutant, although heart beating was observed. In addition, the gir mutant exhibited a shortened and distorted tail (Figs. 1A and B). During embryonic stages, the blood cells are produced in the intermediate cell mass (ICM), a region of the trunk ventral to the notochord, and then these cells enter the heart through the posterior cardinal vein and the common cardinal vein. In gir mutant embryos, blood cells accumulated in the posterior cardinal vein and failed to enter the common cardinal vein (Figs. 1C–F). Consequently, no or few blood cells were found in the dorsal aorta (Fig. 1D) or in the heart (Fig. 1F). By 3 days postfertilization (dpf), the gir mutant embryos exhibited severe edema (Figs. 1G and H) and eventually died within 1 week.

Since the gir mutant was defective in the initiation of blood cell circulation, we examined the expression of flk1, which marks endothelial cells, to ask if patterning of blood vessels was affected. Although most of the flk1 expression was apparently normal, its expression in the common cardinal vein was absent in the gir mutant (Figs. 1I and J), suggesting that the defective formation of the common cardinal vein caused the failure of circulation in the gir mutant.

Patterning defects of pectoral fin and tail in the gir mutant

In addition to the defect in blood cell circulation, the gir mutation caused a patterning defect in the pectoral fins. At 36 hpf, fin bud formation was evident in wild-type embryos, whereas no fin bud was found in gir mutant embryos (Figs. 2A and B). At 3 dpf, no or small fins were observed in the gir mutant (Figs. 2C and D). We examined the expression of tbx5.1, which is expressed in the fin field and is essential for pectoral fin induction (Ahn et al., 2002; Garrity et al., 2002). At 20 hpf (~22-somite stage), the overall expression of tbx5.1 was reduced in the gir mutant, and this reduction was prominent in the posterior region of...
the fin field (Figs. 2E and F). At 28 hpf, tbx5.1 expression in the mutant was found in a region of the lateral plate mesoderm anterior to the normal fin field (Figs. 2G and H). Expression of shh, a marker for zone of polarizing activity, was not detectable in the fin field of the gir mutant, although its expression in the midline was normal (Figs. 2I and J).

Since the gir mutant exhibited a defect in tail development (Figs. 1B and H), we examined the expression of no tail (ntl), the zebrafish ortholog of Brachyury, which is essential for tail development (Schulte-Merker et al., 1994). As a result, we found that although ntl expression in the notochord was not significantly affected, its expression in the tailbud was reduced at segmentation stages in the gir mutant (Figs. 2K and L).

We also noted that the pharyngeal cartilages, in particular Meckel’s cartilage, were malformed in the gir mutant (data not shown). However, at the early larval stages when the cartilages develop, the overall morphology of the gir mutant was distorted because of the severe edema (Fig. 1H). In addition, the gir mutant dies at these stages. Therefore, we did not study the cartilage phenotypes further.

Genetic and molecular analyses of gir

Genetic mapping localized gir to the vicinity of LG12 marker Z27025 (no recombinants among 280 meioses, see Materials and methods). By searching for genes and ESTs that have been mapped to the genomic region around Z27025, we found cyp26a1 and its EST clone fb81e05 on the HS meiotic map (Woods et al., 2000) and T51 radiation hybrid map (Geisler et al., 1999), respectively (Fig. 3A; http://zfin.org). cyp26a1 was considered to be a good candidate for gir, since this gene has been shown to be important for patterning in mouse embryos (Abu-Abed et al., 2001; Sakai et al., 2001). We sequenced the RT-PCR fragment of this gene and found a nonsense mutation at position 814 (Glu272Stop; Fig. 3B). Accordingly, the truncated Cyp26a1 protein produced in the gir mutant lacks the heme-binding motif in the C-terminus, which is conserved among members of the cytochrome P450 superfamily (Fig. 3C; Graham-Lorence and Peterson, 1996), suggesting that the mutant Cyp26a1 protein is nonfunctional. By taking advantage of a restriction site polymorphism generated by the mutation, we found that...
cyp26a1 was tightly linked to gir (no recombinants among 356 meioses; Fig. 3D).

A cyp26a1 morpholino phenocopies the gir mutant

To confirm that gir encodes Cyp26a1, we injected a cyp26a1 antisense morpholino (cyp26a1-MO) into wild-type embryos. Embryos injected with 1 ng of cyp26a1-MO exhibited a shortened tail (Figs. 4A and B), no or small pectoral fins (Figs. 4C and D), and defect in blood cell circulation (Figs. 4E and F), thus phenocopying the gir mutant. We noted that some of the cyp26a1 morphants displayed a milder circulation defect than the gir mutant, such that blood cells entered the common cardinal vein and moved across the yolk to around the inflow tract, but did not circulate further (data not shown). Eighty-three out of 104 embryos injected with cyp26a1-MO phenocopied the gir mutant, whereas none of the 48 embryos injected with the control morpholino exhibited any of these phenotypes. Together with the mutational analysis described above, these results indicate that a loss of cyp26a1 function causes the gir phenotypes.

Genetic interaction between gir/cyp26a1 and raldh2

Since Cyp26a1 is an enzyme that degrades RA, we speculated that an increased RA concentration caused the gir phenotypes. To confirm this hypothesis, we injected a raldh2 antisense morpholino (raldh2-MO) into the gir mutant. Previous studies have shown that injection of 8.5–17 ng (Begemann et al., 2001) or 4 ng (Grandel et al., 2002) of raldh2-MO phenocopies the nls/nof mutant. In our experiments, injection of 4 ng of raldh2-MO partly phenocopied the nls/nof mutant: 59% (17/29) of the injected embryos exhibited complete loss of the pectoral fin and 34% (10/29) exhibited partial loss of the pectoral fin. We found that injection of 4 ng of raldh2-MO rescued the phenotypes of the gir mutant (Figs. 4G–L). Thirty-five out of 36 gir mutants injected with raldh2-MO exhibited normal tail morphology (Figs. 4G and H) and normal blood circulation (Figs. 4K and L). In addition, the pectoral fin defect was also partly rescued: 5 out of 36 mutants had apparently normal pectoral fins (Figs. 4I and J). Injection of 0.5 ng of raldh2-MO had only a mild effect on the wild-type embryos, but this efficiently rescued the circulation defects, but not the pectoral fin or tail defect, of the gir mutant (data not shown). These results suggest that a reduction of the RA level is sufficient to suppress the phenotypes of the gir mutant.

Expression of the cyp26a1 and raldh2 genes is affected in the gir mutant

It has been shown that expression of cyp26a1 is upregulated by RA, whereas that of raldh2 is down-
regulated by RA (Begemann et al., 2001; Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002). Therefore, we expected that expression of these genes might be altered in gir mutant embryos. The cyp26a1 gene is expressed in the presumptive anterior neural ectoderm and around the blastoderm margin during gastrulation, in the tailbud throughout somitogenesis, and in multiple specific tissue types after the mid-segmentation stage (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002). We found that cyp26a1 expression was not significantly affected during gastrulation stage and at bud stage (10 hpf) in the gir mutant (Figs. 5A and B). However, at the early segmentation stage when cyp26a1 began to be expressed in the rostral presumptive spinal cord, its expression was upregulated in the gir mutant (Figs. 5C and D). Interestingly, cyp26a1 expression in the rostral spinal cord was strongly upregulated at 20 hpf in the gir mutant (Figs. 5E and F). A transverse section confirmed that cyp26a1 was expressed in the rostral spinal cord at this stage (Fig. 5G). Its expression was also upregulated and expanded in the tailbud of the gir mutant (Figs. 5E and F). At 28 hpf, cyp26a1 expression in the caudal part of the branchial arch primordium was upregulated, whereas that in the rostral branchial arch primordium was slightly reduced in the gir mutant (Figs. 5H and I).

Expression of the raldh2 gene was also altered in the gir mutant. During gastrulation stages, raldh2 expression in the blastoderm margin was only slightly, if at all, reduced in the gir mutant (data not shown). At bud stage, its expression in the presomitic mesoderm was significantly reduced in the gir mutant (Figs. 5J and K). At 20 hpf, raldh2 expression in the somites was downregulated in the gir mutant (Figs. 5L and M). At 28 hpf, the raldh2 expression domain in the caudal part of the branchial arch primordium was slightly narrowed (Figs. 5N and O). Interestingly, its expression domain in the lateral plate mesoderm including the posterior pectoral fin field was strongly expanded rostrally in the gir mutant (Figs. 5N and O). These results suggest that the gir mutation alters the RA distribution in embryos, which subsequently affects expression patterns of cyp26a1 and raldh2.

**Patterning defects of hindbrain in the gir mutant**

We asked if the gir mutation affects patterning of the hindbrain, since decreased or increased RA signaling affects hindbrain development in zebrafish (Begemann et al., 2001, 2004; Grandel et al., 2002; Hill et al., 1995; Holder and Hill, 1991; Linville et al., 2004; Perz-Edwards et al., 2001). Expressions of krox20, which marks rhombomeres 3 and 5, and that of valentino, which is expressed in rhombomeres 5 and 6, were...
examined together with myoD, a marker for somites. Interestingly, the spacing between rhombomere 5 or 6 and the first somite was significantly expanded at the mid-segmentation stages in the gir mutant (Figs. 6A–D). In addition, we noticed that the expression domain of valentino was reduced in the gir mutant (Figs. 6C and D). By examining expression of pax2.1, a marker for the midbrain–hindbrain boundary, together with krox20, we found that the spacing between the midbrain–hindbrain boundary and rhombomere 3 was reduced in the gir mutant (Figs. 6E and F), indicating that the rostral hindbrain territory was reduced. Likewise, the spacing between rhombomere 1, which is marked by gbx2, and rhombomere 3 was slightly reduced in the gir mutant (Figs. 6G and H). We examined an additional gene ephb2 that has multiple expression domains including rhombomeres 4 and 7 and somites. We found that the spacing between rhombomere 7 and the first somite was expanded in the gir mutant (Figs. 6I and J). These phenotypes were opposite to those of the nls/nof mutant, in which the spacing between rhombomere 5 and the first somite is reduced, the expression domain of valentino is expanded, and the rostral hindbrain territory is expanded (Begemann et al., 2001; Grandel et al., 2002).

To study patterning of more rostral structures, we also examined otx2, which is expressed mainly in the midbrain and in the part of diencephalon beneath the epiphysis at the mid-segmentation stages. The otx2 expression domain in the midbrain was found to be reduced (Figs. 6K and L), suggesting that the midbrain territory was reduced in the gir mutant. Furthermore, the otx2 expression pattern suggested that the forebrain territory of the mutant was also reduced (Figs. 6K and L).

### Patterning defects of spinal cord in the gir mutant

Next, we examined expression of neural tube markers hoxb4a, hoxb5a, and hoxb6a, which have their rostral expression limit at the rhombomere 6/7 boundary, at the level of somite 1 and at the level of somite 2, respectively (Prince et al., 1998a,b). It should be noted that hoxb5, the tetrapod ortholog of hoxb5a, has been often used as a marker for the rostral edge of the spinal cord after approximately 12-somite stage (White et al., 2000b and references therein). We found that expression of these hox genes was strongly upregulated at 20 hpf in the gir mutant (Figs. 7A–F). Furthermore, the spacing between the expression domains of krox20 and hoxb5a or hoxb6a was strongly reduced (Figs. 7C–F). This is in contrast to
the expanded spacing between the expression domains of krox20 and myoD (Figs. 6A and B). These analyses demonstrate that the spinal cord territory is expanded rostrally at the expense of the hindbrain territory in the gir mutant.

**Patterning defects of motor neurons in the gir mutant**

The gir mutant line carried the Isl1-GFP transgene (see Materials and methods), which is expressed in postmitotic motor neurons early in their development (Higashijima et al., 2000). Using this Isl1-GFP transgene, we studied development of motor neurons in the hindbrain and rostral spinal cord. We found that the distance between the trochlear nucleus (nIV) in the midbrain and the trigeminal nucleus (nV) in the rhombomeres 2/3 was reduced in the gir mutant at 48 hpf (Figs. 8A–C), consistent with the reduction in the rostral hindbrain territory. In some cases, the number of branchiomotor neurons in the trigeminal nucleus (nV) was reduced in the gir mutant (Fig. 8C). In addition, bilateral clusters of the vagal nucleus (nX) were found to have broadened (Figs. 8A–C). Despite the abnormal vagal nucleus patterning, projection of the vagal axons was apparently normal in the gir mutant (Figs. 8D and E).

At 3 dpf, Isl1-GFP-positive spinal motor neurons projected their axons dorsally in wild-type embryos (Fig. 8F). However, the axon projection of the rostralmost spinal motor neurons was not seen in the gir mutant (Fig. 8G). To study further the neuronal development in this region, we stained the embryos with acetylated α-tubulin antibody. By this assay, the spinal motor neurons projecting their axons ventrolaterally were detected (Figs. 8H and I). We found that the α-tubulin-positive motor neurons in the rostralmost spinal cord extended their axons even in the absence of the target pectoral fin bud (Figs. 8H and I), in contrast to the absence of axons from Isl1-GFP-positive motor neurons in this region. These results indicate that a subset of motor neurons was more selectively affected than others in the gir mutant.

**Discussion**

In this study, we characterized the gir mutant in zebrafish, which shows pleiotropic phenotypes, including defects in the development of common cardinal vein, pectoral fin, tail, hindbrain, and spinal cord. Genetic analysis and the results of the gene knockdown experiment using morpholino oligonucleotides demonstrate that loss of cyp26a1 gene function causes the gir phenotypes. Cyp26a1 is an enzyme that degrades RA (Fujii et al., 1997; White et al., 1996), suggesting that RA concentration is increased in the gir mutant. Consistent with this hypothesis, injection of raldh2-MO, which is supposed to reduce the RA level (Begemann et al., 2001; Grandel et al., 2002), rescued the phenotypes of the gir mutant (Figs. 4G–L).
Phenotypic similarities and differences between the gir/cyp26a1 mutant and the cyp26a1-knockout mouse

Phenotypes of gir mutant embryos are similar to those of the cyp26a1-knockout mouse, but there are important differences. One of the common phenotypes is the tail truncation. Both the mouse and zebrafish cyp26a1 genes are strongly expressed in the tailbud mesoderm at the segmentation stages, and mutations in both species cause the defect in tail development, concomitant with the reduction in Brachyury/ntl expression. Thus, RA degradation by Cyp26a1 in the tailbud is essential for the normal tail morphogenesis both in mouse and zebrafish.

Some of the phenotypic differences between the mouse and zebrafish cyp26a1 mutants may be partly due to developmental differences between the two species. For example, the mouse mutant exhibits sirenomelia (fusion of the hindlimbs), but such a phenotype cannot be observed in the gir mutant, because the hindlimbs start to develop during metamorphosis, to which stage the gir mutant is unable to survive. Moreover, the gir mutant failed to initiate circulation due to the defective common cardinal vein formation on the surface of the yolk; whereas such a phenotype has not been reported in cyp26a1-knockout mouse. Development and morphology of the blood vessels, especially those of the yolk vessels, are highly divergent between mouse and zebrafish, and such differences are likely to result in phenotypic differences.

In addition, some of the phenotypic differences might be accounted for by gene redundancy and different gene expression patterns. Three members of the cyp26 gene family, cyp26a1, cyp26b1, and cyp26c1, have been identified in mice; and the expression patterns of these genes were found to partly overlap (Abu-Abed et al., 2002; MacLean et al., 2001; Tahayato et al., 2003). Similarly, the zebrafish genome harbors at least three members of this family, including cyp26a1 and cyp26b1 (Nelson, 1999; White et al., 1996; our unpublished data). Although the expression pattern of the zebrafish cyp26b1 gene has not been reported yet, functional redundancy among the members of this family might be different between the mouse and zebrafish. It should be noted that the expression pattern of the cyp26a1 gene is significantly different between mouse and zebrafish. For example, mouse cyp26a1 is expressed transiently in the prospective rhombomere 2, but is not expressed in the prospective rostral spinal cord (Fujii et al., 1997). In contrast, zebrafish cyp26a1 is expressed in the prospective rostral spinal cord but not in the prospective rhombomere 2. Consistently, the mouse and zebrafish cyp26a1 mutants display different phenotypes with respect to patterning in the hindbrain and spinal cord (see below).

Cyp26a1 function is required for the development of the common cardinal vein and pectoral fin

We showed that the cyp26a1 function is required for the development of the common cardinal vein, which is an unusually broad vessel, covering a large portion of the yolk surface. RA signaling has been implicated in the formation of vitelline vessels and the cardiac inflow tract in murine and avian embryos. Vitamin A-deficient quail embryos exhibit the absence of omphalomesenteric veins, as well as the absence of sinus venosa and atria (Heine et al., 1985). In addition, raldh2-knockout mouse embryos lacked an organized network of extraembryonic vessels in their yolk sac membranes (Niederreither et al., 1999). Thus, RA signaling seems to play an important role in the development of blood vessels on the yolk in both zebrafish and higher vertebrates. Although little is known about the molecular mechanisms regulating the development of the common cardinal vein, we speculate that patterning defects in the lateral plate mesoderm (discussed below) might cause the failure of the common cardinal vein development in the gir mutant.

The gir mutant displays a severe defect in the development of its pectoral fin buds, equivalent to
forelimb buds in higher vertebrates (Figs. 2A–D). This result was rather surprising, because RA signaling has been shown to be sufficient and necessary for the induction of a zone of polarizing activity in the developing limb bud (Helms et al., 1996; Stratford et al., 1996; Tickle et al., 1982). In the gir mutant, the expression domain of tbx5.1 was shifted anteriorly and that of raldh2 was expanded anteriorly (Figs. 2E–H and 5N and O), suggesting that the lateral plate mesoderm is posteriorized. Such a patterning defect of the lateral plate mesoderm is likely to cause the defective fin bud development. It has been suggested that the early expression of hox genes in the lateral plate mesoderm along the body axis specifies positions where limbs develop (Cohn et al., 1997). Considering this, the altered RA distribution in the gir mutant might affect the expression of hox genes in the lateral plate mesoderm, which might subsequently cause the defect in specification of the fin field.

RA signaling determines the territories of hindbrain and spinal cord

Because expression of the cyp26al gene is induced by RA (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002), we expected to be able to monitor the RA distribution partly by examining the expression of cyp26al. Surprisingly, the cyp26al expression was highly upregulated in the prospective rostral spinal cord in the gir mutant (Figs. 5C–F), suggesting that a strong feedback control mechanism regulates its expression in this region. Supposing this cyp26al expression to be induced by RA, a considerable amount of RA may be accumulated in the prospective rostral spinal cord. This RA accumulation would likely cause the patterning defects in the hindbrain and spinal cord.

cyp26al is expressed in the prospective forebrain and midbrain with a posterior limit around the future rhombomere 1 during gastrulation and at the early segmentation stage (Dobbs-McAuliffe et al., 2004; Kudoh et al., 2002).
As shown in cyp26a1 knockdown embryos (Kudoh et al., 2002), the expression domain of the anterior gene otx2 was reduced and that of the posterior gene hoxb1b was expanded at the late gastrulation stage in the gir mutant (data not shown). We also found that the territories of the forebrain and midbrain were reduced at the mid-segmentation stage in the gir mutant (Figs. 6K and L). Accordingly, the early expression of cyp26a1 seems to be important for the proper AP patterning of the neural ectoderm, as suggested previously (Kudoh et al., 2002). Although the AP patterning defect of the mutant is likely to be caused by an increased RA concentration, the cyp26a1 expression was not significantly affected by bud stage in the gir mutant (Figs. 5A and B), suggesting that the early cyp26a1 expression is less sensitive to the altered RA concentration. However, its expression was strongly upregulated in the prospective rostral spinal cord at the 5-somite stage (Figs. 5C and D). cyp26a1 expression in this region seems to be important to define the rostral limit of the spinal cord territory.

Several lines of evidence have suggested that diffusion of RA from the paraxial mesoderm to the neural tube is important to activate the hox genes and to pattern the hindbrain (reviewed in Gavalas, 2002). It has been also shown that the murine hoxb5, hoxb6, and hoxb8 genes rapidly respond to exogenous RA to extend their expression domains rostrally (Oosterveen et al., 2003), suggesting that these hox genes are directly regulated by RA signaling. Based on these findings, we propose the following model (Fig. 9): RA is produced in the somitic mesoderm and diffuses into the prospective spinal cord, where it activates the expression of hox genes, including hoxb5a and hoxb6a. Cyp26a1 is expressed in the prospective rostral spinal cord, where it degrades RA, thus attenuating RA signaling (Fig. 9A). In the gir mutant, the RA level cannot be lowered at the prospective rostral spinal cord, causing expanded expression domains of these hox genes. As a result, the territory of the rostral spinal cord is expanded at the expense of that of the hindbrain (Fig. 9B). In contrast, when the RA level is reduced by mutations in the raldh2 gene, the opposite phenotype is observed, that is, the hindbrain territory is expanded at the expense of the rostral spinal cord territory (Begemann et al., 2001; Grandel et al., 2002). Thus, the RA level at the rostral spinal cord is crucial to determine the territories of the hindbrain and spinal cord.

In our model, RA signaling activates hox genes, while Cyp26a1 protects the anterior central nervous system (CNS) from excessive exposure to RA. Interestingly, the inactivation of RA by Cyp26a1 apparently takes place at different positions within the CNS between zebrafish and higher vertebrates, because the mouse and chick cyp26a1 is expressed in the prospective rhombomere 2, where it supposedly controls the RA level (Abu-Abed et al., 2001; Fujii et al., 1997; Sakai et al., 2001; Swindell et al., 1999). Consistently, the targeted disruption of the mouse cyp26a1 gene causes misspecification of the rostral hindbrain, rather than rostral expansion of the spinal cord (Abu-Abed et al., 2001; Sakai et al., 2001). Furthermore, it should be noted that the cyp26b1 and cyp26c1 genes, other members of the cyp26 family, are expressed at unique positions in the CNS: mouse cyp26b1 is expressed throughout rhombomeres 5–6, in the ventral portion of rhombomeres 2–4, and in the upper and lower thoracic regions of the spinal cord (Abu-Abed et al., 2002; MacLean et al., 2001); and mouse cyp26c1 is expressed in rhombomeres 2 and 4 (Tahayato et al., 2003). Considering these expression patterns, members of the Cyp26 family are likely to control the RA level at the multiple discrete areas of the CNS. Further analyses of the expression and function of these cyp26 genes will reveal the overall regulation of the RA level by the members of Cyp26 family in the CNS and in other organs.

The axons from Isl1-GFP-positive rostralmost spinal motor neurons are missing in the gir mutant

Early RA signaling is required for the development of motor neuron progenitors, whereas later RA signaling in postmitotic motor neurons appears to regulate their columnar subtype identity (reviewed in Appel and Eisen, 2003). Blockage of RA signaling in postmitotic brachial (forelimb) motor neurons inhibits lateral motor column differentiation and converts many of these neurons to thoracic columnar subtypes, whereas activation of RA signaling impairs the differentiation of thoracic motor neuron columnar subtypes (Sockanathan et al., 2003). Thus, the level of RA signaling apparently determines motor neuron columnar subtype identity. We found that the Isl1-GFP-positive motor
neurons, but not the acetylated α-tubulin-positive motor neurons, failed to extend their axons in the rostralmost spinal cord of the gir mutant (Figs. 8F–I). Since RA level is considered to be very high in the rostral spinal cord of the gir mutant, these Isl1-GFP-positive motor neurons may be unable to mature under high levels of RA signaling. Alternatively, maturation of these motor neurons might be dependent on some signal(s) other than RA, which is produced in the somitic mesoderm and diffuses into the neural tube. The expanded spacing between the rostralmost spinal cord and somitic mesoderm in the gir mutant can cause a reduction in such signal(s) in the rostralmost spinal cord and impair the signal-dependent maturation of motor neurons. Further study is required to identify the signals that direct maturation of the Isl1-GFP-positive motor neurons in the rostralmost spinal cord.

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