Published in final edited form as:
Dev Biol. 2009 January 1; 325(1): 60–70. doi:10.1016/j.ydbio.2008.09.022.

Combinatorial roles for zebrafish retinoic acid receptors in the hindbrain, limbs and pharyngeal arches

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Abstract

Retinoic acid (RA) signaling regulates multiple aspects of vertebrate embryonic development and tissue patterning, in part through the local availability of nuclear hormone receptors called retinoic acid receptors (RARs) and retinoid receptors (RXRs). RAR/RXR heterodimers transduce the RA signal, and loss-of-function studies in mice have demonstrated requirements for distinct receptor combinations at different stages of embryogenesis. However, the tissue-specific functions of each receptor and their individual contributions to RA signaling in vivo are only partially understood. Here we use morpholino oligonucleotides to deplete the four known zebra fish RARs (*raraa*, *rarab*, *rarga*, and *rargb*). We show that while all four are required for anterior–posterior patterning of rhombomeres in the hindbrain, there are unique requirements for *rarga* in the cranial mesoderm for hindbrain patterning, and *rarab* in lateral plate mesoderm for specification of the pectoral fins. In addition, the alpha subclass (*raraa*, *rarab*) is RA inducible, and of these only *raraa* expression is RA-dependent, suggesting that these receptors establish a region of particularly high RA signaling through positive-feedback. These studies reveal novel tissue-specific roles for RARs in controlling the competence and sensitivity of cells to respond to RA.

Keywords

Danio rerio; Zebrafish; Nuclear hormone receptor; Retinoic acid; Hindbrain; Vitamin A; RAR; Branchial arch; Forelimb; Rhombomere

Introduction

The vitamin A derivative, retinoic acid (RA), is essential for vertebrate development, including the central nervous system (Glover et al., 2006; Maden, 2007), limbs (Lee et al., 2004), and craniofacial skeleton (Mark et al., 2004). Disruption of RA signaling by vitamin A depletion (VAD; Maden et al., 1996; Clagett-Dame and DeLuca, 2002), genetic disruption of RA synthesis (Niederreither et al., 2000, 2002, 2003, Begemann et al., 2001) or

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.09.022.

pharmacological blockage of RA receptors (RARs and RXRs, Dupe and Lumsden, 2001; Linville et al., 2004; Mark et al., 2006) suggests a role in patterning along the anterior–posterior (A–P) axis. An anteriorly-declining gradient of RA is thought to act as a morphogen that promotes posterior development. In the developing hindbrain, RA actively induces expression of homeotic genes (*hox*) in different segments (rhombomeres) in a concentration-dependent manner (Durston et al., 1989; Dupe and Lumsden, 2001;Wendling et al., 2001;White et al., 2007). The output of RA signaling also depends upon the regulated expression and function of RARs and RXRs in cells (Mark et al., 2006), but few studies have investigated their tissue-specific roles during development or in the establishment and maintenance of the RA morphogen gradient.

RARs and RXRs are nuclear hormone receptors that regulate transcription of target genes both in the presence or absence of RA (reviewed in Mark et al., 2006). Receptors heterodimerize and bind a sequence in the regulatory regions of these targets called a retinoic acid response element (RARE). The bound complex recruits co-activators, which enable transcriptional activation. Receptor heterodimers also bind RAREs in the absence of RA, and recruit the co-repressors SMRT and N-CoR to repress gene expression (Koide et al., 2001). Three highly conserved RARs (*alpha — RARa*, *beta — RARb*, *gamma — RARg*) have been studied extensively in mouse, chick, and *Xenopus*, but functional studies have been limited (Mark et al., 2006). Surprisingly, single *RAR* knockouts in mice are viable and display relatively mild phenotypes (Li et al., 1993). Only double *RAR/RAR* or *RAR/RXR* mutants die in utero and resemble VAD embryos, suggesting partial redundancy between receptors (Taneja et al., 1995; Luo et al., 1996; Subbarayan et al., 1997; Dupe et al., 1999;Wendling et al., 2001; Mark et al., 2006). Evidence for redundancy has also come from treating embryos with pharmacological RAR antagonists, which have graded effects on A–P patterning of the hindbrain depending on antagonist concentration, and also disrupt neurogenesis at later stages (Dupe and Lumsden, 2001; Linville et al., 2004).

Zebrafish have two alpha RARs (*raraa* and *rarab*) and two gamma RARs (*rarga* and *rargb*) but appear to lack RARbeta. Expression of these receptors in embryos, as well as four zebrafish RXRs, has been reported (Hale et al., 2006; Tallafuss et al., 2006). However, to date there have been no functional studies other than with non-specific chemical antagonists that block all four receptors. Using antisense morpholino oligonucleotides (MO) we show requirements for individual receptors in A–P patterning of the hindbrain, and for *rarga* in cranial mesoderm and *rarab* in the pectoral fins. We also show that the alpha subclass of receptors is uniquely RA inducible, suggesting that these two receptors establish regions of particularly high RA signaling through positive-feedback.

Materials and methods

RA and DEAB treatments

Wild type embryos (AB) were collected from natural matings and treated in the dark with all-trans RA (1 μM, Sigma) or the aldehyde dehydrogenase (Aldh) inhibitor diethylaminobenzene (DEAB) in embryo medium at 6 hpf (50% epiboly). Embryos were grown at 28 °C and fixed in 4% paraformaldehyde (PFA) at the appropriate stages.

GFP constructs, morpholinos and RNA injections

Antisense morpholino oligonucleotides (MOs; Gene Tools Inc.)were designed as translational blockers, either to the 5′UTR of *raraa* (GGT TCA CAT CCA CAC TCT CAT ACAT) and *rarga* (CCA GAG CCT CCA TAC AGT CGA ACAT), or spanning the translation start site for both *rarab* (CCA CAA CGT CCA CGC TCT CGTACAT) and *rargb* (CGT CTC TCATAC CAAGTG GGC TGTT). Second MOs targeting non-

overlapping sequences in *raraa* and *rarga* caused identical defects, confirming specificity (data not shown). The concentrations used for individual MO injections were: *raraa* (10 ng), *rarab* (5 ng), *rarga* (5 ng) and *rargb* (2.5 ng), although for double *raraa/rarab* morphants, 5 ng of each MO was used. In each case, MOs were injected together with the p53 MO to help suppress MO-induced cell death (2 ng; Plaster et al., 2006). All were injected into wild-type embryos at the 1–2 cell stage and the embryos were either fixed in 4% paraformaldehyde for in situ hybridization or alcian blue staining. To test for MO efficiency and specificity, each MO target sequence was fused to GFP, which was used to synthesize capped mRNA (mMessage mMachine Kit, Ambion). This was co-injected with the corresponding MO to detect loss of GFP expression (Suppl. Fig. 2).

Whole-mount in situ hybridization and histology

Whole-mount in situ hybridization was performed as previously described (Thisse et al., 1993). Fragments of *raraa*, *rarab*, *rarga* and *rargb* were amplified from cDNA (SuperScript III) synthesized from pooled total RNA extracted from embryos of multiple stages (Trizol, Invitrogen) and directionally cloned into pCS2+ to synthesize riboprobes: *raraa* (ClaI/T7), *rarab* (EcorI/T7), *rarga* (BamHI/T7) and *rargb* (Waxman and Yelon, 2007). Additional riboprobes used were *hoxb5a* (Prince et al., 1998), *valentino* (Moens et al., 1998), *krox20* (Oxtoby and Jowett, 1993), *myoD* (Weinberg et al., 1996), and *tbx5* (Begemann and Ingham, 2000). Embryos were sectioned after in situ hybridization at 12 μm using a Leica CM3050S Cryostat. Alcian staining was performed at 4 dpf and stained cartilages were dissected and flat-mounted for photography (Javidan and Schilling, 2004).

Quantitative reverse transcription-PCR

Total RNA was extracted from embryos (Trizol, Invitrogen) either injected with all four rar MOs (*raraa*, 5 ng; *rarab*, 5 ng; *rarya*, 5 ng; *raryb*, 2.5 ng), or treated with 1 μM DEAB along with controls at approximately 7–8 hpf. cDNA was synthesized from these pools of total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen), and qRT-PCR was performed using these cDNAs with SYBR Green RT-PCR Master Mix (Roche) on an Opticon DNA Engine (MJ Research).

Confocal microscopy and transgenic embryos

RARE-YFP homozygous transgenic embryos (Perz-Edwards et al., 2001) were obtained through natural matings and injected at the 1–2 cell stage with either *raraa*, *rarab*, *rarga* or *rargb* MOs. Morphants were raised to 26–30 hpf in embryo medium containing phenylthiourea (PTU) to inhibit pigmentation, anesthetized with tricaine (MS-222; Sigma), and mounted in 0.7% agarose prepared in embryo medium on bridged coverslips and imaged with a Zeiss LSM 510 confocal microscope at 2.95 μm intervals.

Mosaic analysis

Wild-type donor embryos were injected at the 1–2 cell stage with 3% biotinylated dextran/3% tetramethylrhodamine (Molecular Probes). Sibling embryos to be used as hosts were simultaneously injected with 5 ng of the *rarab* MO. At 4 hpf, cells from the labeled donors were transplanted to the margins of the unlabeled, morphant hosts, into a fate map position that gives rise to mesendoderm (Begemann et al., 2001). These mosaic embryos were grown to 32–36 hpf, during which time the cells were tracked by rhodamine fluorescence, and then fixed with 4% paraformaldehyde. Biotin was visualized using the Vectastain ABC kit as previously described before proceeding with in situ hybridization (Westerfield, 1998).

Results

Tissue-specific expression patterns of zebrafish rars

Several previous studies described the expression of zebrafish *rar*s (Joore et al., 1994; Stafford et al., 2006; Hale et al., 2006;Waxman and Yelon, 2007). However, these did not examine germ-layer specific expression of each receptor during gastrulation, when RA signaling is required for A–P patterning. To investigate this issue, we first examined expression of all four known zebrafish *rar* receptors (*raraa*, *rarab*, *rarga*, and *rargb*) by whole-mount in situ hybridization and in sectioned material during gastrula stages (Fig. 1). Distinct expression patterns were detected for three out of the four RARs at 7–9 h post-fertilization [hpf] (Figs. 1A, E, I). Expression of the fourth and most recently discovered receptor, *rargb*, was ubiquitous at gastrula stages, with stronger expression on the dorsal side (data not shown; Waxman and Yelon, 2007). Parasagittal sections of whole mounts revealed that expression of *raraa* at these stages was restricted to the posterior epiblast (future ectoderm; including weak expression in the enveloping layer, EVL) and excluded from the hypoblast (future mesoderm and endoderm, Figs. 1B, C). Expression of *rarab* was detected posteriorly in both layers, but much stronger in the epiblast (Figs. 1F, G). In contrast, *rarga* expression was detected anteriorly in the hypoblast, in a pattern complementary to *raraa* and *rarab* (Fig. 1I) as well as at the dorsal margin (Fig.1M) and in non-neural ectoderm (Fig. 1N). Hypoblast expression was visible in sections as a thin layer of cells next to the yolk (Figs. 1J, K). These expression patterns are summarized in Suppl. Fig. 1K.

Complementary patterns of expression of alpha and gamma subclasses persisted at later stages (Hale et al., 2006). In whole mounts, both *raraa* and *rarab* expression were restricted along the A–P axis of the neural plate at 11–15 hpf, with *raraa* expressed up to the rhombomere 6/7 (r6/7) boundary, and *rarab* up to the r1/2 boundary (Suppl. Figs. 1A–F). In contrast, *rarga* expression was limited to the cranial mesoderm and tailbud (Suppl. Figs. 1G, H). These distinct patterns of expression were confirmed in sections of whole mounts at 18 hpf (Fig. 1) – *raraa* and *rarab* retained expression in the neural tube (Figs. 1D, H) while *rarab*, *rarga* and *rargb* were expressed in the pharyngeal arches (Figs. 1L, O; Waxman and Yelon, 2007) – and persisted at 48 hpf (Suppl. Figs. 1C, F, I, J). Expression domains of alpha and gamma subclasses of RARs overlap in a few places, including the pectoral fin buds, where *rarab* is expressed proximally, *rarga* distally, and *rargb* throughout the fin mesenchyme (insets in Suppl. Figs. 1C, F, I, J). These expression patterns are summarized in Suppl. Fig. 1K. From these results we infer that RA signaling in the neural ectoderm in zebrafish is mediated predominantly by the alpha subclass of RARs, while signaling in other tissues requires both alphas and gammas.

Functional requirements for rars in the hindbrain

Expression of *raraa* and *rarab* in the neural plate suggests that these two receptors mediate the A–P patterning functions of RA in the hindbrain. To test this hypothesis, we designed antisense morpholino oligonucleotides (MOs) targeted to the translation start sites or 5′UTR of each of the four *rar*s. We confirmed the binding efficiency of each receptor MO by generating fusion constructs containing the MO target sequence upstream of eGFP and co-injected these with the corresponding MOs, all of which efficiently inhibited GFP expression (Suppl. Fig. 2). We also confirmed specificity of the *raraa* and *rarga* MO-induced defects with second MOs targeting non-overlapping sequences (data not shown).

A–P patterning of hindbrain rhombomeres in RAR morphants was assessed by expression of *hoxb5a* in the anterior spinal cord (Prince et al., 1998; Bruce et al., 2001), *krox20* in r3 and r5 (Oxtoby and Jowett, 1993), and *valentino* in r5 and r6 (*val*; Moens et al., 1998) (Fig. 2).

Embryos treated with the pan-RAR antagonist, AGN 193109, which are severely deficient in RA signaling, lacked *hoxb5a* and *val* expression as well as the r5 stripe of *krox20* (Figs. 2N, R). In contrast, embryos injected with *raraa* or *rarab* MOs alone showed a reduction of *hoxb5a* expression (Figs. 2A–C), but little or no effect on expression of *krox20* or *val* (Figs. 2F–H). Double morphants for *raraa* and *rarab* also continued to express *hoxb5a* (22/22; Fig. 2K) and *val* (35/38; Fig. 2O) and their hindbrains were not strongly anteriorized, suggesting that these are not the only essential receptors for RA in the hindbrain.

Similarly, injections of *rarga* and *rargb* MOs, either alone or in combination, reduced *hoxb5a* (Figs. 2D, E, L) but not *val* expression (Figs. 2I, J, P) and when combined with *raraa* and *rarab* MOs these only slightly enhanced the reduction in *hoxb5a* (Figs. 2M, Q). This suggests that all four receptors are required for hindbrain patterning. Unfortunately, embryos injected with all four receptor MOs had widespread cell death and did not survive long enough to assay gene expression in the hindbrain, even when co-injected with a p53-targeted MO to block apoptosis (data not shown; Plaster et al., 2006). Therefore, we examined expression of RA target genes in these embryos during gastrulation by in situ and qRT-PCR (Fig. 3). Quadruple *rar* morphants (injected with all four MOs and hereafter referred to as *rar*-MO) were compared to embryos treated with the Aldh inhibitor, DEAB, for changes in *hoxb1a* expression in presumptive hindbrain (which requires RA), and *cyp26a1* in presumptive forebrain–midbrain (which is largely RA independent; White et al., 2007). *hoxb1a* expression was strongly reduced in DEAB-treated and even more so in *rar*-MO embryos, compared to controls (Figs. 3D–F), while *cyp26a1* expression appeared unchanged (Figs. 3A–C). Expression of another RA target, *hoxd4a*, was absent in both DEAB-treated and *rar*-MO embryos (Figs. 3G–I), suggesting that it is more sensitive to RA levels than *hoxb1a*. These results were further supported by qRT-PCR analyses with RNA isolated from siblings of the embryos used for in situ hybridization (Fig. 3J). Depletion of all four *rars* eliminates *hoxb1a* and *hoxd4a* expression, and reduces expression of *cyp26a1* (White et al., 2007). These results suggest partial functional redundancy among the four RARs in hindbrain development.

rarga functions in pharyngeal arch development

RA signaling is also required for development of the craniofacial skeleton, including the pharyngeal arches. Posterior arches are reduced or lost in *aldh1a2* mutant zebrafish (Begemann et al., 2001) and mice (Niederreither et al., 2000). All four zebrafish rars are expressed in the arches — *raraa*, *rarab* and *rargb* in NC cells that form the pharyngeal skeleton, and *rarga* in mesodermal cells that form the pharyngeal muscles and endothelia (Figs. 1, 2; Schilling and Kimmel, 1994). Injection of an *rarga* MO caused reductions in the arch skeleton, similar to *aldh1a2* mutants, but *raraa* and *rarab* MOs had no affect on the arches (data not shown). *dlx2* expression was reduced in migrating NC precursors of the skeleton in arches 3–5 in *rarga* morphants (28/30; Figs. 4A, B). Co-injection of the *rarga* MO with the *rargb* MO did not substantially increase the severity of the arch defects (35/35; Fig. 4C). *rarga* morphant embryos also lacked the 5th endodermal pouch, as revealed by immunostaining with the zn5 antibody that recognizes the cell surface adhesion molecule, DM-GRASP (Figs. 4D–F; Trevarrow et al., 1990). These results indicate a unique role for *rarga* in development of the pharyngeal arches. Based on its expression in the cranial mesoderm, these data suggest that the requirements for *rarga* in neural crest and endoderm within the arches may be, at least in part, indirect.

rarab functions in pectoral fin budding

RA is also a key player in vertebrate limb development. Zebrafish embryos treated with RAR-antagonists (Gibert et al., 2006; data not shown) or mutant for *aldh1a2* (Begemann et al., 2001) lack pectoral fin buds and fail to express *tbx5*, a T-box containing protein that

specifies forelimb identity (Begemann and Ingham, 2000, Ahn et al., 2002). *rarab*, *rarga* and *rargb* are all expressed in the pectoral fin buds (Fig. 2). Therefore, we tested their functions with MOs and stained the MO-injected larvae for *tbx5* in the fin buds at 32 hpf (Figs. 5D–F) as well as cartilage at 5 dpf (Figs. 5A–C). In wild type controls, cartilaginous endoskeletal discs protrude into the fins on both sides of the larvae, and these are lost in *aldh1a2*$^{-/-}$ mutants (*n*=12; Figs. 5A, B). Similarly, injection of *rarab*-MO caused a loss of fin cartilage, while the scapulocoracoid cartilage of the pectoral girdle, to which the fin is normally attached, was unaffected (24/24; Fig. 5C). Like *aldh1a2*$^{-/-}$ mutants, *rarab* morphant embryos lacked *tbx5* expression in the fin bud mesenchyme at 32 hpf, suggesting a defect in budding (7/7; Figs. 5E, F). *rarab* morphants were otherwise healthy when compared to uninjected larvae at 4 dpf, lacking only their fin blades (12/14; Figs. 5G, H). In some cases, small buds formed unilaterally (1/14; Fig. 5H, arrow). In contrast, *raraa*, *rarga* or *rargb* MOs caused no fin defects (data not shown). This suggests a central role for the *rarab* receptor in pectoral fin development.

rarab is expressed in the lateral plate mesoderm (LPM), which contributes to the pectoral fins, but not in paraxial and intermediate mesoderm (IM; Fig. 5I). To determine if the pectoral fin defects in *rarab* morphants are due to cell autonomous requirements for this receptor in LPM, we performed mosaic rescue studies. We transplanted wild-type cells from labeled donors into *rarab*-morphant hosts and confined them to mesoderm by targeting the transplants to the margin of the blastoderm at 4–5 hpf (Fig. 6A). These were typically confined to one side of the animal and extended across many segments along the A–P axis (Begemann et al., 2001). Wild-type cells transplanted into the LPM rescued *tbx5* expression (Figs. 6B–D) and fin outgrowth unilaterally in *rarab* morphants, either partially (15/50) or fully (2/50). These results indicate cell autonomous requirements for *rarab* in the LPM for pectoral fin development.

Regulation of rar expression by RA

RA directly regulates RAR transcription in other species (De The et al., 1990; Hoffman et al., 1990; Leroy et al., 1991a,b). To determine if RA signaling activates expression of the four zebrafish *rar*s, we treated embryos with either a pan-RAR antagonist, AGN 193109 (Allergan, Inc.), or with RA during gastrulation (Fig. 7). *raraa* expression was dramatically reduced in the posterior neural plate during gastrulation and at later stages in the hindbrain in embryos treated with 10 μMAGN(Figs. 7A, B; 32/32 treated embryos) and in *aldh1a2*$^{-/-}$ mutants (Figs. 7J, K; 24/24 mutants). These results suggest that RA initiates and maintains *raraa* expression. Conversely, high RA concentrations (1 μM) caused *raraa* expression to shift anteriorly (Fig. 7C, arrowheads; 23/23).

In contrast, expression of the other three rars was unaffected by similar AGN and RA treatments (*rarab* — Figs. 7D, E, L, M; *rarga* — Figs. 7G, H, N, O [*N*=25 in each case]). 1 μM RA upregulated *rarab* expression in the anterior neuroectoderm (Fig. 7F, arrowheads; 23/25), but appeared to inhibit *rarga* expression (Fig. 7I; 19/22). Given its unique sensitivity to RA, *raraa* may provide a mechanism for amplifying RA signaling in this region of the embryo through positive feedback.

RARs and tissue sensitivity to RA

To examine RA sensitivity in more detail, we depleted each *rar* with MOs and simultaneously treated the embryos with DEAB, to inhibit RA synthesis (Fig. 8). These experiments were performed in transgenic zebrafish embryos expressing yellow fluorescent protein (YFP) driven by RA response elements (RARE-YFP; Perz-Edwards et al., 2001), as a direct reporter for RA signaling. At 24 hpf this labels the posterior hindbrain and spinal cord up to the r6/r7 boundary, adjacent to the first somite (s1; Fig. 8A), and expression was

completely eliminated with treatments of 1 μM DEAB (Figs. 8B, F, J, N; N>10 in each case). Lower concentrations of DEAB (0.1 μM) caused a posterior shift in the domain of RARE-YFP expression, relative to the first somite (Figs. 8C, D; 9/10), and similar shifts were seen in *rarab* morphants (Fig. 8I; 10/11). Injection of the *raraa* MO caused little to no change in the severity of the defect in RARE-YFP expression caused by treatments with 0.1 μM or 0.01 μM DEAB (Figs. 8G, H; 5/5). In contrast, depletion of *rarab* dramatically enhanced the severity at both concentrations (Figs. 8 K, L; 6/6). *raraa*/*rarab* double morphants were similar to *rarab* morphants alone (Figs. 8O, P; 10/10). Thus while *raraa* is more strongly induced by RA, *rarab* is more essential in the neural tube for direct activation of an RARE. This correlates with the distinct expression patterns of the two receptors, with *rarab* throughout the hindbrain and *raraa* restricted to r7 and the anterior spinal cord where the highest levels of RA signaling are achieved.

Discussion

RARs show remarkable structural and functional conservation across vertebrates. We have shown requirements for all four zebrafish RARs in the hindbrain, as well as distinct roles for *rarab* and *rarga* in the pectoral fins and pharyngeal arches, respectively. These defects resemble loss of function phenotypes in the RA signaling pathway in a variety of vertebrate model systems. In addition, we show: 1) previously unrecognized functions for RARs in the mesoderm, 2) differences between alpha and gamma RAR subtypes in their regulation by RA, and 3) divergent functions for duplicate RARs within each subtype. We propose a model (Fig. 9) in which the early expression of different RARs establishes anterior boundaries of RA responsiveness during gastrulation, both in the hindbrain and cranial mesendoderm, and later establishes a second domain of high RA signaling at the brain/spinal cord junction. This region of higher signaling also induces budding of the forelimbs.

Unique and redundant functions for RARs

RARs and RXRs transduce RA signals as heterodimers, which are developmentally regulated (reviewed in Mark et al., 2006). Mechanisms by which different heterodimers combine to control distinct target genes could explain many of the pleiotropic effects of RA. Our studies suggest partial redundancy both within and between subtypes of RARs. Similarly, single *RAR* null mutant mice are viable, while compound loss-of-function mutations in *RARα*/*RARβ* (Dupe et al., 1999) and *RARα*/*RARγ* (Wendling et al., 2001), more closely resemble an RA-deficient phenotype.

Compound mutants or morphants, however, may be incorrectly interpreted as reflecting redundancy if genes function in different cell types that contribute to the same tissues. For example, alpha and gamma subclasses of RARs in zebrafish are expressed in complementary domains in the embryo – alphas predominantly in the CNS and endoderm, gammas predominantly in mesoderm – yet both are required to pattern the hindbrain (Fig. 9). Similarly, RA signaling-deficient zebrafish (Begemann et al., 2001;Grandel et al., 2002;Kopinke et al., 2006) and mice (Lohnes et al., 1993;1994;Mendelsohn et al., 1994a,b;Matt et al., 2003;Niederreither et al., 2003;Mark et al., 2004) lack pharyngeal arches 3–6. Expression of an *RARE-lacZ* reporter in mice suggests that RA signals directly to both endoderm and mesoderm that contribute to the arches (Niederreither et al., 2003). RA also signals directly to the endoderm in zebrafish (Stafford et al., 2006) and is essential for formation of segmental pouches in the pharyngeal endoderm, which may secondarily cause many of the NC defects seen in RA-deficient embryos (Wendling et al., 2000;Kopinke et al., 2006).

Embryos depleted of all four RARs with MOs have more severe defects than even the most severe vitamin A deficiencies. One explanation for this may be that RARs both activate and

repress their targets, depending on cellular context. Activation is ligand-dependent, and lack of ligand can lead to excess target gene repression. Embryos treated with RAR antagonists (such as AGN used here) lose the activation of RA target-genes because the antagonist increases the affinity of co-repressors (N-CoR/Smrt) for the RAR/RXR heterodimer complex (Koide et al., 2001). These antagonists are sometimes referred to as “inverse agonists”. In contrast, MOs block RAR expression, eliminating both activating and repressing forms. Our results suggest that both functions of RARs are essential for early development.

Functions of RARs in forelimb development

RA is primarily synthesized in the paraxial (somitic) and lateral plate mesoderm (Begemann et al., 2001; Cui et al., 2003; Gibert et al., 2006) and plays important roles in somite formation (Rancourt et al., 1995). *Rarab*, *rarga* and *rargb* are all expressed in the LPM of the pectoral fin bud, and we have shown a central role for *rarab* in limb budding. To test which tissues require *rarab* we transplanted morphant cells into the LPM of the fin bud of wild type hosts and found cell autonomous requirements for *rarab* in this mesoderm. Similarly, selective depletion of *raraa* and *rarab* expression in endodermal cells in the zebrafish disrupts the specification of pancreatic beta cells (Stafford et al., 2006).

Pectoral fin development in zebrafish requires RA produced in the paraxial mesoderm (Gibert et al., 2006; Linville et al., 2004). Previous studies have suggested that this acts on adjacent mesodermal populations to induce *wnt2b* expression in intermediate mesoderm (IM) (Mercader et al., 2006) and *tbx5* expression in the LPM. *Tbx5* is required for the migration of future fin LPM into the fin bud (Ahn et al., 2002; Garrity et al., 2002; Agarwal et al., 2003; Rallis et al., 2003). We show *rarab* expression in the LPM but not in IM, suggesting that the LPM responds directly to RA (Fig. 6). Injection of an *rarab* MO eliminated fin budding and *tbx5* expression, similar to *aldh1a2*$^{-/-}$ mutants (Begemann et al., 2001) and *tbx5* morphants (Ahn et al., 2002). Thus, despite co-expression of several receptors in the fin bud, only *rarab* is required. Other receptors may act later in fin outgrowth or differentiation.

The LPM gives rise to the limb skeleton, while mesodermal cells of the hypaxial myotome form limb muscles (Ahn et al., 2002). The position of the forelimb field along the A–P axis is controlled by *hox* genes in the LPM, particularly *hoxb5*, *hoxc6* and *hoxc8*, all of which are regulated by RA signaling. *Hoxb5*$^{-/-}$ mutant mice have defects in the shoulder girdle (Rancourt et al., 1995). In contrast, the pectoral girdle is unaffected in both *tbx5* and *rarab* morphant zebrafish; only the fin blade fails to form (Ahn et al., 2002). This suggests that *rarab* is not required to specify the forelimb field, but rather maintains expression of *tbx5* and promotes budding. Other RARs may mediate an earlier cascade of *hox* expression in the LPM that positions the fin field (Waxman and Yelon, in preparation).

Autoregulation through the rar alpha subclass and sensitivity to RA

In zebrafish, *raraa* requires RA for expression, but other *rars* are either completely RA independent or much less sensitive. In mammals, RA upregulates all three RARs, in some cases directly (De The et al., 1990; Hoffman et al., 1990; Sucov et al., 1990; Leroy et al., 1991a,b; Kamei et al., 1993; Serpente et al., 2005). Autoregulation within the pathway can impart a differential sensitivity to RA in regions where *raraa* is expressed (i.e. in r7 of the hindbrain and in the anterior spinal cord; Fig. 9). This domain correlates precisely with expression of the RARE-YFP transgenic reporter, which is thought to reflect sustained, high levels of RA signaling (Perz-Edwards et al., 2001).

Differential regulation of RARs may contribute to the robustness of the RA signal (White et al., 2007). By treating *rar* morphant zebrafish with DEAB we found that *rarab* has more influence on the sensitivity of the embryo to RA levels. These data also reveal functional differences between two close duplicates within the alpha subclass in zebrafish (Hale et al., 2006). In contrast, several *RARS* in the chick require RA for their expression (Cui et al., 2003). All three mammalian RARs have alternative isoforms generated by two different promoters (P1 and P2) and alternative splicing, but only the P2 promoter in RARα and RARβ contains an RARE and is RA-regulated (Mollard et al., 2000; Mark et al., 2006).

Evolution of the RAR gene family

Recent comparative studies of the genomic organization of the RAR and RXR families in zebrafish and other vertebrates suggest that these animals lack a true RARβ receptor (Joore et al., 1994; Hale et al., 2006; Tallafuss et al., 2006). Instead, zebrafish have duplicate alpha and gamma genes. MOs targeting *raraa* cause hindbrain and pharyngeal defects, similar to mouse *RAR*β mutants. In addition, *raraa* expression appears relatively late during gastrulation at 8 hpf while both *rarab* and *rarga* are maternally provided (Hale et al., 2006 and unpublished results). Similarly, mouse *RAR*α and *RAR*γ are widely expressed at primitive streak stages (E6.5) prior to the onset of RA synthesis in both embryonic and extra-embryonic tissues, while *RAR*β expression is not detected until E7.5 in the neuroectoderm and adjacent mesenchyme (Ang and Duester, 1997). *raraa* is also the only zebrafish *rar* that is not expressed in the forelimbs, similar to mouse *RAR*β. Thus, based on its expression and function in zebrafish, *raraa* appears to have arisen as a recent duplicate in teleosts that has subsequently acquired a beta-like function.

RARs and their roles in A–P patterning appear to have arisen in the deuterostome lineage and are absent in protostomes (e.g. nematodes and arthropods) (Marletaz et al., 2006). Studies of comparative ligand-binding as well as expression in non-vertebrate chordates, such as amphioxus, suggests that the ancestral RAR was most closely related to the RARbeta subclass (Escriva et al., 2006). Its expression defines a region of competence that extends throughout the presumptive hindbrain region. This combined with localized degradation of RA anteriorly by the Cyp26 enzymes may have led to the emergence of the ancestral A–P patterning system in which RA signaling is graded from posterior to anterior (White et al., 2007). The incorporation of additional RARs into the A–P patterning system in vertebrates with different boundaries of expression and RA sensitivities, may have been crucial in the evolution of the sharp boundaries of gene expression that define the segmental organization of the hindbrain and pharyngeal arches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank members of the Schilling lab for comments on the manuscript. This work was supported by NIH grant R01NS41353.

References

Agarwal P, Wylie JN, Galceran J, Arkhitko O, Li C, Deng C, Grosschedl R, Bruneau BG. Tbx5 is essential for forelimb bud initiation following patterning of the limb field in the mouse embryo. Development 2003;130:623–633. [PubMed: 12490567]

Ahn DG, Kourakis MJ, Rohde LA, Silver LM, Ho RK. T-box gene tbx5 is essential for formation of the pectoral limb bud. Nature 2002;417:754–758. [PubMed: 12066188]

Ang HL, Duester G. Initiation of retinoid signaling in primitive streak mouse embryos: spatiotemporal expression patterns of receptors and metabolic enzymes for ligand synthesis. Dev. Dyn 1997;208:536–543. [PubMed: 9097025]

Begemann G, Ingham PW. Developmental regulation of Tbx5 in zebrafish embryogenesis. Mech. Dev 2000;90:299–304. [PubMed: 10640716]

Begemann G, Schilling TF, Rauch GJ, Geisler R, Ingham PW. The zebrafish neckless mutation reveals a requirement for raldh2 in mesodermal signals that pattern the hindbrain. Development 2001;128:3081–3094. [PubMed: 11688558]

Bruce AE, Oates AC, Prince VE, Ho RK. Additional hox clusters in the zebrafish: divergent expression patterns belie equivalent activities of duplicate hoxB5 genes. Evol. Dev 2001;3:127–144. [PubMed: 11440248]

Clagett-Dame M, DeLuca HF. The role of vitamin A in mammalian reproduction and embryonic development. Ann. Rev. Nutr 2002;22:347–381. [PubMed: 12055350]

Cui J, Michaille JJ, Jiang W, Zile MH. Retinoid receptors and vitamin A deficiency: differential patterns of transcription during early avian development and the rapid induction of RARs by retinoic acid. Dev. Biol 2003;260:496–511. [PubMed: 12921748]

De The H, Vivanco-Ruiz MM, Tiollais P, Stunnenberg H, Dejean A. Identification of a retinoic acid response element in the retinoic acid receptor beta gene. Nature 1990;343:177–180. [PubMed: 2153268]

Dupe V, Lumsden A. Hindbrain patterning involves graded responses to retinoic acid signalling. Development 2001;128:2199–2208. [PubMed: 11493540]

Dupe V, Ghyselinck NB, Wendling O, Chambon P, Mark M. Key roles of retinoic acid receptors alpha and beta in the patterning of the caudal hindbrain, pharyngeal arches and otocyst in the mouse. Development 1999;126:5051–5059. [PubMed: 10529422]

Durston AJ, Timmermans JPM, Hage WJ, Hendriks HFJ, De Vries NJ, Heideveld M, Nieuwkoop P. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 1989;340:140–144. [PubMed: 2739735]

Escriva H, Bertrand S, Germain P, Robinson-Rechavi M, Umbhauer M, Cartry J, Duffraisse M, Holland L, Gronemeyer H, Laudet V. PLoS Genet 2006;2:e102. [PubMed: 16839186]

Garrity DM, Childs S, Fishman MC. The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. Development 2002;129:4635–4645. [PubMed: 12223419]

Gibert Y, Gajewski A, Meyer A, Begemann G. Induction and prepatterning of the zebrafish pectoral fin bud requires axial retinoic acid signaling. Development 2006;133:2649. [PubMed: 16774994]

Glover JC, Renaud JS, Rijli FM. Retinoic acid and hindbrain patterning. J. Neurobiol 2006;66:705–725. [PubMed: 16688767]

Grandel H, Lun K, Rauch GJ, Rhinn M, Piotrowski T, Houart C, Sordino P, Kuchler AM, Schulte-Merker S, Geisler R, Holder N, Wilson SW, Brand M. Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. Development 2002;129:2851–2865. [PubMed: 12050134]

Hale LA, Tallafuss A, Yan YL, Dudley L, Eisen JS, Postlethwait JH. Characterization of the retinoic acid receptor genes raraa, rarab and rarg during zebrafish development. Gene. Expr. Patterns 2006;6:546–555. [PubMed: 16455309]

Hoffmann B, Lehmann JM, Zhang XK, Hermann T, Husmann M, Graupner G, Pfahl M. The retinoic acid receptor-specific element controls the retinoic acid receptor-beta promoter. Mol. Endocrinol 1990;4:1727–1736. [PubMed: 2177841]

Javidan Y, Schilling TF. Development of cartilage and bone. Meth. Cell. Biol 2004;76:415–436.

Joore J, van der Lans GB, Lanser PH, Vervaart JM, Zivkovic D, Speksnijder JE, Kruijer W. Effects of retinoic acid on the expression of retinoic acid receptors during zebrafish embryogenesis. Mech. Dev 1994;46:137–150. [PubMed: 7918098]

Kamei Y, Kawada T, Kazuki R, Sugimoto E. Retinoic acid receptor gamma 2 gene expression is upregulated by retinoic acid in 3T3-L1 preadipocytes. Biochem. J 1993;293:807–812. [PubMed: 8394693]

Koide T, Downes M, Chandraratna RA, Blumberg B, Umesono K. Active repression of RAR signaling is required for head formation. Genes Dev 2001;15:2111–2121. [PubMed: 11511542]

Kopinke D, Sasine J, Swift J, Stephens WZ, Piotrowski T. Retinoic acid is required for endodermal pouch morphogenesis and not for pharyngeal endoderm specification. Dev. Dyn 2006;235:2695–2709. [PubMed: 16871626]

Lee GS, Kochhar DM, Collins MD. Retinoid-induced limb malformations. Curr. Pharm. Des 2004;10:2657–2699. [PubMed: 15320736]

Leroy P, Krust A, Zelent A, Mendelsohn C, Garnier JM, Kastner P, Dierich A, Chambon P. Multiple isoforms of the mouse retinoic acid receptor alpha are generated by alternative splicing and differential induction by retinoic acid. EMBO J 1991a;10:59–69. [PubMed: 1846598]

Leroy P, Nakshatri H, Chambon P. Mouse retinoic acid receptor alpha 2 isoform is transcribed from a promoter that contains a retinoic acid response element. Proc. Natl. Acad. Sci. U.S.A 1991b; 88:1110138–1110142.

Li E, Sucov HM, Lee KF, Evans RM, Jaenisch R. Normal development and growth of mice carrying a targeted disruption of the alpha 1 retinoic acid receptor gene. Proc. Natl. Acad. Sci. U. S. A 1993;90:1590–1594. [PubMed: 7679509]

Linville A, Gumusaneli E, Chandraratna RA, Schilling TF. Independent roles for retinoic acid in segmentation and neuronal differentiation in the zebrafish hindbrain. Dev. Biol 2004;270:186–199. [PubMed: 15136149]

Lohnes D, Kastner P, Dierich A, Mark M, LeMeur M, Chambon P. Function of retinoic acid receptor gamma in the mouse. Cell 1993;73:643–658. [PubMed: 8388780]

Lohnes D, Mark M, Mendelsohn C, Dolle P, Dierich A, Gorry P, Gansmuller A, Chambon P. Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. Development 1994;120:2723–2748. [PubMed: 7607067]

Luo J, Sucov HM, Bader JA, Evans RM, Giguere V. Compound mutants for retinoic acid receptor (RAR) beta and RAR alpha 1 reveal developmental functions for multiple RAR beta isoforms. Mech. Dev 1996;55:33–44. [PubMed: 8734497]

Maden M. Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat. Rev. Neurosci 2007;8:755–765. [PubMed: 17882253]

Maden M, Gale E, Kostetskii I, Zile M. Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. Curr. Biol 1996;6:417–426. [PubMed: 8723346]

Mark M, Ghyselinck NB, Chambon P. Retinoic acid signalling in the development of branchial arches. Curr. Opin. Genet. Dev 2004;14:591–598. [PubMed: 15380252]

Mark M, Ghyselinck NB, Chambon P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu. Rev. Pharmacol. Toxicol 2006;46:451–480. [PubMed: 16402912]

Marletaz F, Holland LZ, Laudet V, Schubert M. Retinoic acid signaling and the evolution of chordates. Int. J. Biol. Sci 2006;2:38–47. [PubMed: 16733532]

Matt N, Ghyselinck NB, Wendling O, Chambon P, Mark M. Retinoic acid-induced developmental defects are mediated by RARbeta/RXR heterodimers in the pharyngeal endoderm. Development 2003;130:2083–2093. [PubMed: 12668623]

Mendelsohn C, Mark M, Dolle P, Dierich A, Gaub MP, Krust A, Lampron C, Chambon P. Retinoic acid receptor beta 2 (RAR beta 2) null mutant mice appear normal. Dev. Biol 1994a;166:246–258. [PubMed: 7958449]

Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P, Mark M. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 1994b;120:2749–2771. [PubMed: 7607068]

Mercader N, Fischer S, Neumann CJ. Prdm1 acts downstream of a sequential RA, Wnt and Fgf signaling cascade during zebrafish forelimb induction. Development 2006;133:2805–2815. [PubMed: 16790478]

Moens CB, Cordes SP, Giorgianni MW, Barsh GS, Kimmel CB. Equivalence in the genetic control of hindbrain segmentation in fish and mouse. Development 1998;125:381–391. [PubMed: 9425134]

Mollard R, Viville S, Ward SJ, Decimo D, Chambon P, Dolle P. Tissue-specific expression of retinoic acid receptor isoform transcripts in the mouse embryo. Mech. Dev 2000;94:223. [PubMed: 10842077]

Niederreither K, Vermot J, Schuhbaur B, Chambon P, Dolle P. Retinoic acid synthesis and hindbrain patterning in the mouse embryo. Development 2000;127:75–85. [PubMed: 10654602]

Niederreither K, Vermot J, Schuhbaur B, Chambon P, Dolle P. Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse. Development 2002;129:3563–3574. [PubMed: 12117807]

Niederreither K, Vermot J, Le Roux I, Schuhbaur B, Chambon P, Dolle P. The regional pattern of retinoic acid synthesis by RALDH2 is essential for the development of posterior pharyngeal arches and the enteric nervous system. Development 2003;130:2525–2534. [PubMed: 12702665]

Oxtoby E, Jowett T. Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. Nucleic Acids Res 1993;21:1087–1095. [PubMed: 8464695]

Perz-Edwards A, Hardison NL, Linney E. Retinoic acid-mediated gene expression in transgenic reporter zebrafish. Dev. Biol 2001;229:89–101. [PubMed: 11133156]

Plaster N, Sonntag C, Busse CE, Hammerschmidt M. p53 deficiency rescues apoptosis and differentiation of multiple cell types in zebrafish flathead mutants deficient for zygotic DNA polymerase delta1. Cell Death Differ 2006;13:223–235. [PubMed: 16096653]

Prince VE, Moens CB, Kimmel CB, Ho RK. Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, valentino. Development 1998;125:393–406. [PubMed: 9425135]

Rallis C, Bruneau BG, Del Buono J, Seidman CE, Seidman JG, Nissim S, Tabin CJ, Logan MP. Tbx5 is required for forelimb bud formation and continued outgrowth. Development 2003;130:2741–2751. [PubMed: 12736217]

Rancourt DE, Tsuzuki T, Capecchi MR. Genetic interaction between hoxb-5 and hoxb-6 is revealed by nonallelic noncomplementation. Genes Dev 1995;9:108–122. [PubMed: 7828847]

Schilling TF, Kimmel CB. Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. Development 1994;120:483–494. [PubMed: 8162849]

Serpente P, Tumpel S, Ghyselinck NB, Niederreither K, Wiedemann LM, Dolle P, Chambon P, Krumlauf R, Gould AP. Direct crossregulation between retinoic acid receptor beta and Hox genes during hindbrain segmentation. Development 2005;132:503–513. [PubMed: 15634700]

Stafford D, White RJ, Kinkel MD, Linville A, Schilling TF, Prince VE. Retinoids signal directly to zebrafish endoderm to specify insulin-expressing beta-cells. Development 2006;133:949–956. [PubMed: 16452093]

Sucov HM, Murakami KK, Evans RM. Characterization of an autoregulated response element in the mouse retinoic acid receptor type beta gene. Proc. Natl. Acad. Sci. U.S.A 1990;87:5392–5396. [PubMed: 2164682]

Subbarayan V, Kastner P, Mark M, Dierich A, Gorry P, Chambon P. Limited specificity and large overlap of the functions of the mouse RAR gamma 1 and RAR gamma 2 isoforms. Mech. Dev 1997;66:131–142. [PubMed: 9376317]

Tallafuss A, Hale LA, Yan YL, Dudley L, Eisen JS, Postlethwait JH. Characterization of retinoid-X receptor genes rxra, rxrba, rxrbb, and rxrg during zebrafish development. Gene. Expr. Patterns 2006;6:556–565. [PubMed: 16448862]

Taneja R, Bouillet P, Boylan JF, Gaub MP, Roy B, Gudas LJ, Chambon P. Reexpression of retinoic acid receptor (RAR) gamma or overexpression of RAR alpha or RAR beta in RAR gamma-null F9 cells reveals a partial functional redundancy between the three RAR types. Proc. Natl. Acad. Sci. U. S. A 1995;92:7854–7858. [PubMed: 7644503]

Thisse C, Thisse B, Schilling TF, Postlethwait JH. Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 1993;119:1203. [PubMed: 8306883]

Trevarrow B, Marks DL, Kimmel CB. Organization of hindbrain segments in the zebrafish embryo. Neuron 1990;4:669–679. [PubMed: 2344406]

Waxman JS, Yelon D. Comparison of the expression patterns of newly identified zebrafish retinoic acid and retinoid X receptors. Dev. Dyn 2007;236:587–595. [PubMed: 17195188]

Wendling O, Dennefeld C, Chambon P, Mark M. Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches. Development 2000;127:1553–1562. [PubMed: 10725232]

Wendling O, Ghyselinck NB, Chambon P, Mark M. Roles of retinoic acid receptors in early embryonic morphogenesis and hindbrain patterning. Development 2001;128:2031–2038. [PubMed: 11493525]

Westerfield, M. The Zebrafish Book. U Oregon Press; 1998.

White R, Nie Q, Lander AD, Schilling TF. Complex regulation of Cyp26a1 creates a robust retinoic acid signaling gradient in the zebrafish embryo. PLoS. Biol 2007;5(11):e304. [PubMed: 18031199]

Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, Andermann P, Doerre OG, Grunwald DJ, Riggleman B. Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. Development 1996;122:271–280. [PubMed: 8565839]

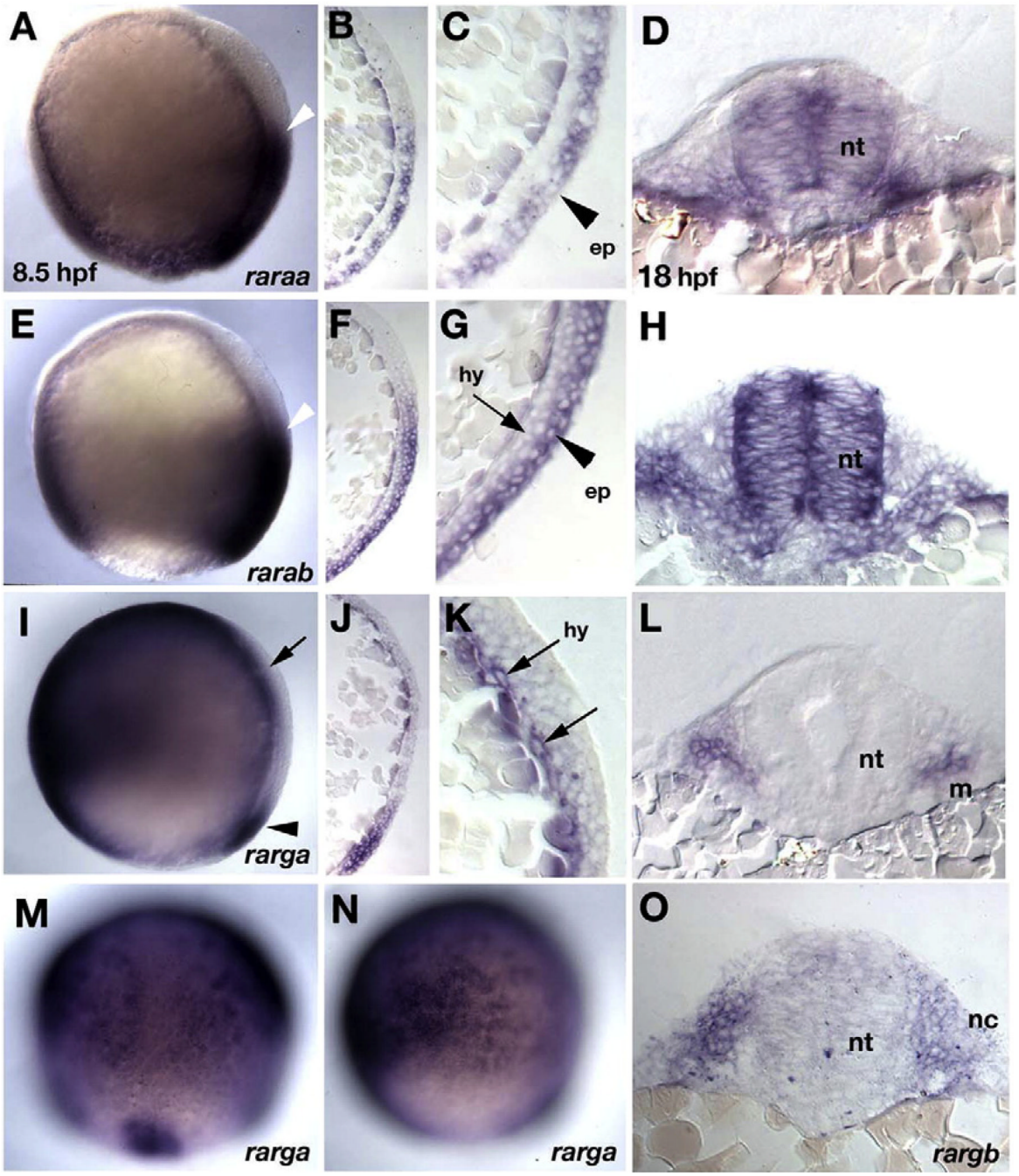

Fig. 1.

Germ layer-specific expression of zebrafish *rars*. In situ hybridization in whole mounts (A, E, I, M, N) and parasagittal sections (B, C, F, G, J, K) at 8.5 hpf (90% epiboly), and transverse sections at 18 hpf (18 somites) at hindbrain levels (D, H, L, O). (A–D) *raraa*. (A) Expression is in posterior ectoderm in the gastrula, with an anterior border in the presumptive hindbrain (white arrowhead). (B–D) Sections reveal that expression is restricted to epiblast, shown at higher magnification in panel C (arrowhead), and persists in the neural tube (D). (E–H) *rarab*. (E) Expression is in posterior ectoderm in the gastrula, with an anterior border in the presumptive hindbrain (white arrowhead), similar to *raraa*. (F–H) Sections reveal strong expression in epiblast (arrowhead in panel G), weaker in hypoblast,

which persists in the neural tube and pharyngeal arches (H). (I–N) *rarga*. (I, M, N) Expressed anteriorly and ventrally during gastrulation and at the dorsal margin (arrowheads in panels I and M). Panel M shows a dorsal view and panel N is focused laterally to show expression in ventral epiblast (presumptive epidermis). (J–L) Sections near the dorsal side reveal expression restricted to the hypoblast (arrows in panel K) and later in cranial mesoderm (L). (O) *rargb*. Expression is ubiquitous at early stages (not shown), and by 18 hpf sections reveal expression in pharyngeal arch mesenchyme. Abbreviations: ep, epiblast; hy, hypoblast; m, mesoderm; nc, neural crest; nt, neural tube.

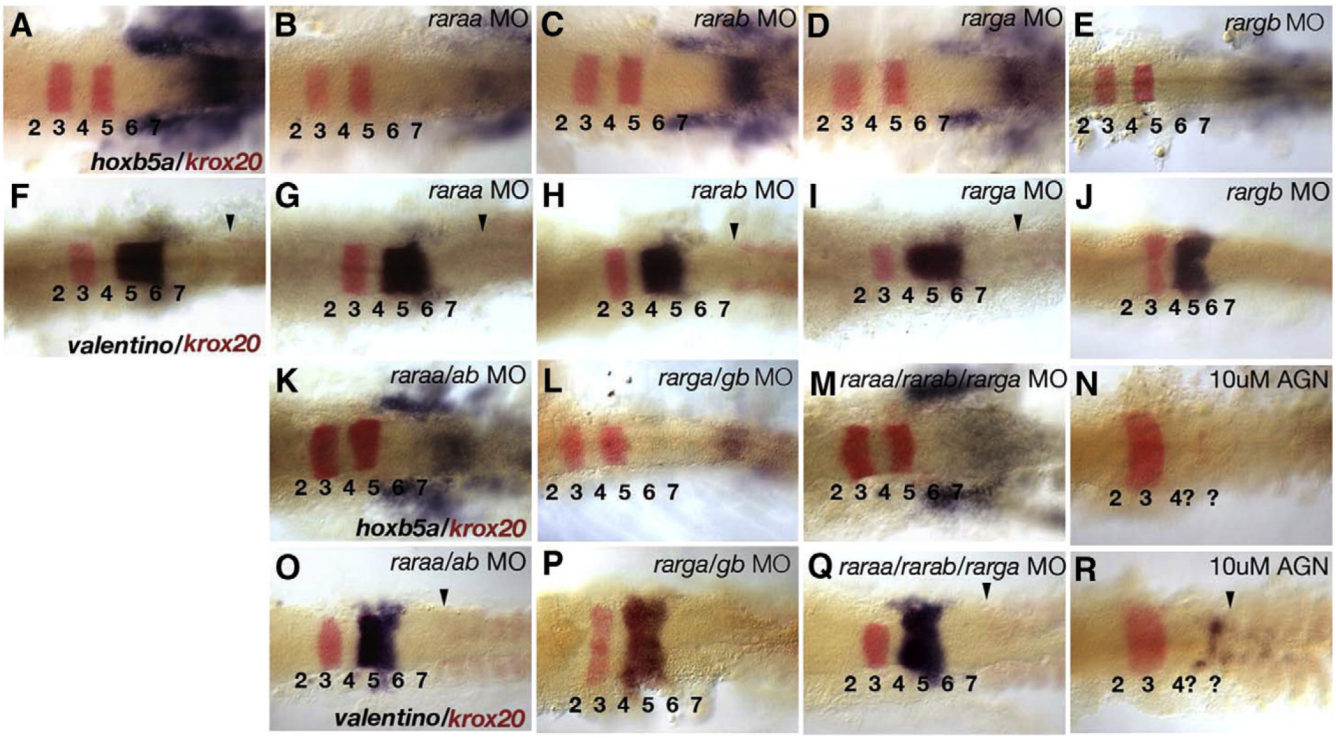

Fig. 2.

Redundant requirements for *rars* in hindbrain patterning. Dorsal views of flat-mounted hindbrains at 15 hpf (12-somite), anterior to the left, labeled by two-color in situ hybridization for either *hoxb5a*/*krox20* (A–E, K–N) or *valentino*/*krox20* (F–J, O–R). Hindbrain rhombomeres are numbered (1–7), arrowheads indicate the location of the first somite. (A, F) Controls. (B–E) Embryos injected with MOs targeting *raraa* (B), *rarab* (C) *rarga* (D) or *rargb* (E) show reduced *hoxb5a* expression in posterior neural tube and mesoderm (blue), but no defects in *krox20* expression in r3 or r5 (red). (G–J) Embryos injected with each of the four MOs alone show no defects in *valentino* (*val*) expression, or in the position of somite 1 (arrowheads). (K, L) Combined injection of *raraa* and *rarab* MOs (K; *N*=22), or *rarga* and *rargb* MOs (L; *N*=25) causes a reduction in *hoxb5a* expression. (M) Combined injection of the *rarga* MO with both *raraa* and *rarab* MOs (*N*=25) causes severe reduction in *hoxb5a* expression in the neural tube, but mesodermal expression persists. These triple morphants retain the r5 stripe of *krox20* expression unlike embryos treated with a pan-RAR antagonist, AGN 193109 (N). (O–Q) *raraa/rarab* double morphants (O; 35/38), *rarga*/*rargb* double morphants (P; *N*=20), and *raraa/rarab/rarga* triple morphants (Q; *N*=22) all show slight reductions in *val* expression, while its expression is almost completely eliminated by AGN treatments (R; *N*=25).

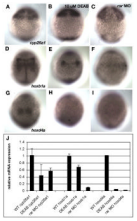

Fig. 3.

Depleting all four *rars* eliminates *hox* expression in the hindbrain. (A–I) Dorsal views of late gastrula stage embryos (8.5 hpf), labeled by whole mount in situ hybridization for *cyp26a1* (A–C), *hoxb1a* (D–F), and *hoxd4a* (G–I). Anterior is to the top. Controls (left column), DEAB-treated (middle column) or quadruple morphants (*rar*-MO) injected with MOs targeting *raraa*, *rarab*, *rarga* and *rargb*. (J) Histogram summarizing results of quantitative PCR (qPCR) to detect relative amounts of the same three gene products, using RNA isolated from the siblings of embryos used for panels A–I. (A–C) No changes were detected in the pattern of *cyp26a1* expression in presumptive forebrain and midbrain in DEAB-treated (B) or *rar*-MO quadruple morphants (C), but total levels were significantly reduced in both cases (J). (D–F) *hoxb1a* expression in presumptive hindbrain was dramatically reduced by DEAB treatments (E) and eliminated by *rar*-MO (F), and this was confirmed by qPCR (J). (G–I) *hoxd4a* expression was virtually eliminated by both DEAB treatments (H) and *rar*-MO (I), which was confirmed by qPCR (J).

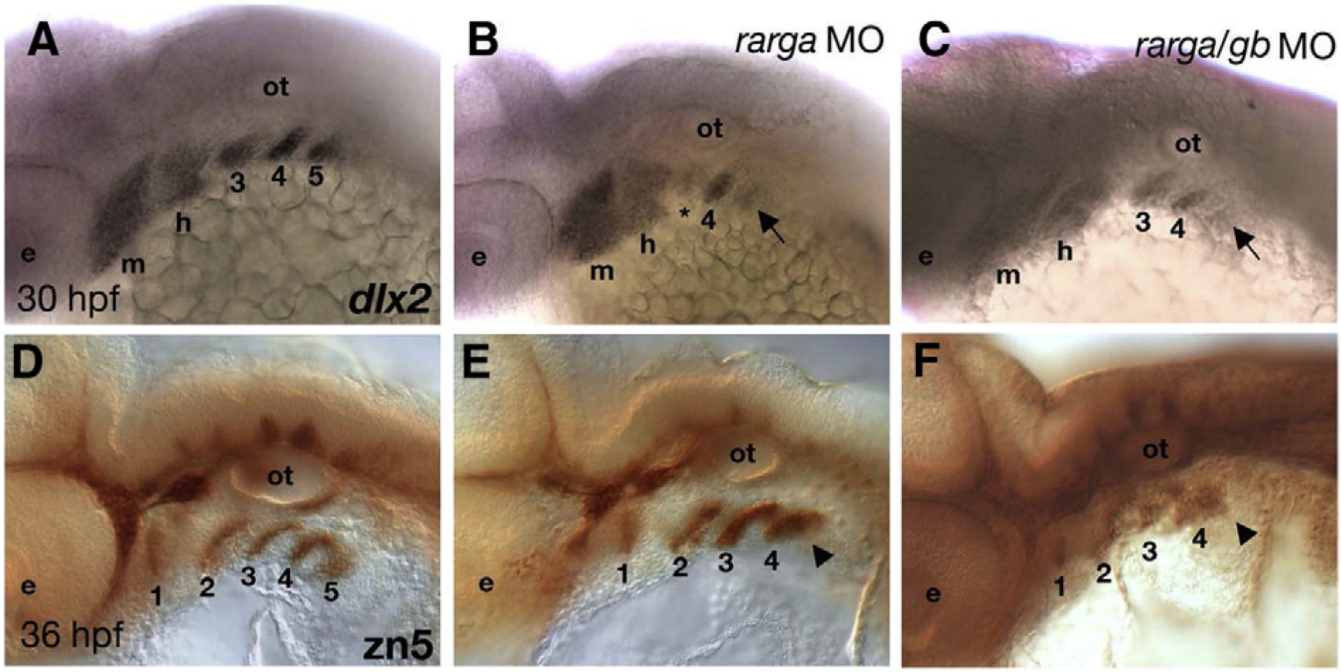

Fig. 4.

Depletion of *rarga* alone disrupts posterior pharyngeal arches. Lateral views, anterior to the left. (A, D) Controls. (B, E) *rarga* morphants (C, F) *rarga/rargb* double morphants. (A–C) Whole mount in situ hybridization to detect *dlx2* mRNA reveals reductions in migrating neural crest cells in arches 3–5 (arrows) in both single (B; 28/30) and double (C; 35/35) morphants. (D–F) Whole mount, immunostaining with the zn-5 antibody reveals reductions in the pharyngeal pouch endoderm (particularly pouch #5, arrowheads) in these arches, but no defects in zn5+ cells in the hindbrain or cranial ganglia in single (E) and double (F) morphants. Abbreviations: e, eye; m, mandibular; h, hyoid; ot, otic vesicle.

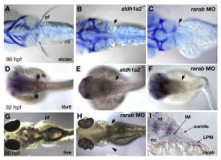

Fig. 5.

Depletion of *rarab* alone disrupts pectoral fin budding. (A–C) Alcian blue stained cartilage in larvae (4 dpf). Ventral views, anterior to the left. (A) In controls the pectoral fins protrude laterally just posterior to the skull. (B, C) Lack of fins and fin cartilages in both *aldh1a2* mutants (B, arrow; *N*=12) and *rarab* morphants (C, arrow; *N*=24). (D–F) *tbx5* expression at 32 hpf, detected by whole mount in situ hybridization. Dorsal views, anterior to the left. (D) In controls, *tbx5* labels the bilateral pair of early pectoral fin buds, which are absent in *aldh1a2* mutants (E, arrow) and *rarab* morphants (F, arrow; *N*=7). (G, H) Live 96 hpf larvae, dorsal views, anterior to the left. *rarab* morphants occasionally form a small pectoral fin on one side (H, arrowhead). (K) Transverse sections at forelimb levels at 24 hpf reveal *rarab* expression in the neural tube and lateral plate mesoderm, but absent from the adjacent somite or intermediate mesoderm. Abbreviations: fb, fin bud; IM, intermediate mesoderm; LPM, lateral plate mesoderm; pf, pectoral fin; nc, notochord; nt, neural tube.

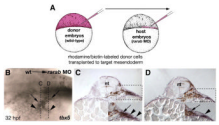

Fig. 6.

Grafts of wild-type mesoderm rescue *tbx5* expression in *rarab* morphants. (A) Cell transplantation technique. Donor embryos were injected at the one-cell stage with both a fixable biotinylated dextran and a fluorescent rhodamine-dextran (red). Hosts were simultaneously injected with the *rarab* MO. At 4 hpf small groups of cells were transferred from donors to the margin (which forms the mesendoderm) of hosts, which were raised to 32 hpf, and stained for biotin (brown, grafted cells) and *tbx5* (blue, pectoral fin). (B) Whole mount, anterior to the left, showing partial unilateral rescue of *tbx5* expression — arrowheads indicate *tbx5*-expressing cells, and arrows indicate locations of wild-type, donor cells. Rescue was either partial (15/50) or completely restored the fin (2/50). Dotted lines indicate locations of transverse sections in panels C and D. (C, D) Transverse sections through the fin fields of mosaic embryos, showing the absence of *tbx5* expression on the untransplanted, left side and *tbx5+* cells in the LPM (arrowheads) on the right side in close proximity to biotin-labeled, donor cells (arrows). Abbreviations: nt, neural tube; wt, wild type.

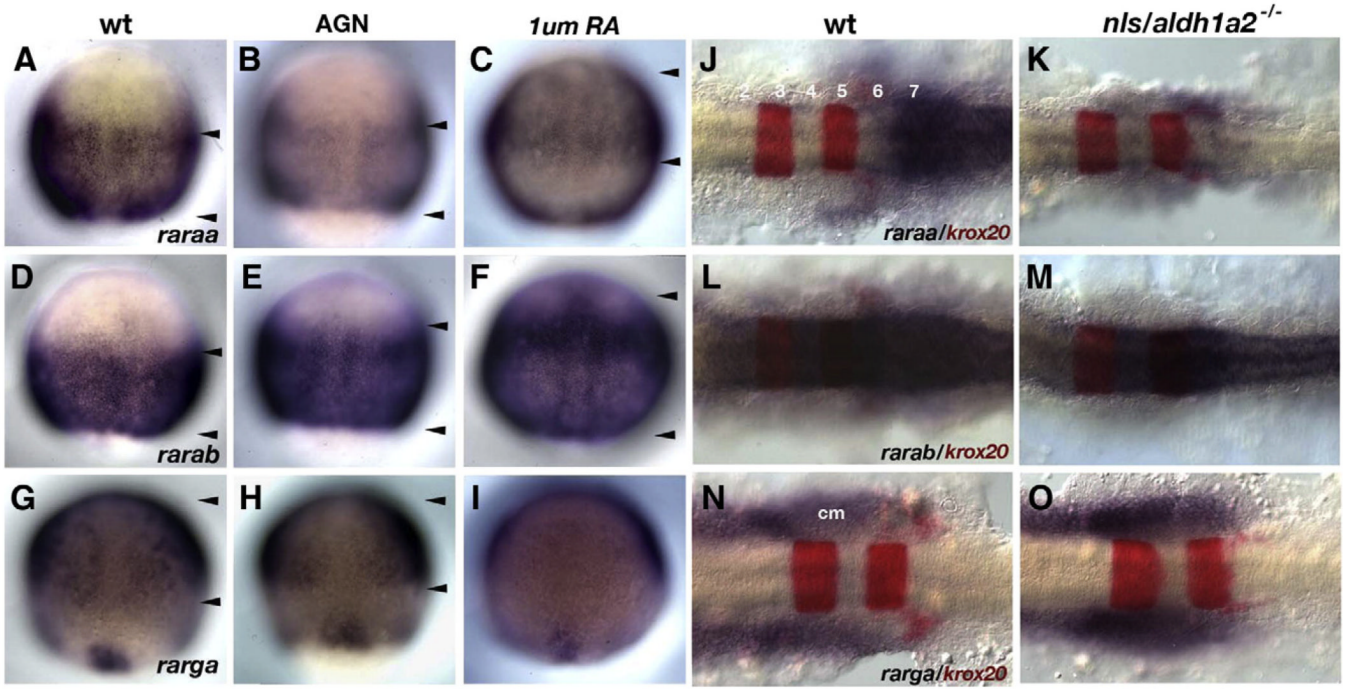

Fig. 7.

rars are differentially regulated by RA. (A–I) Whole mount in situ hybridization for three of the four *rars* during gastrulation (8.5 hpf). Dorsal views, anterior to the top, of wild-types (A, D, G), AGN-treated (B, E, H), and RA-treated (C, F, I) embryos. Arrowheads indicate approximate borders of receptor expression along the anterior–posterior axis. (A–C) Only *raraa* expression is reduced by AGN treatment (B) and induced by RA (C). (D–F) AGN has no effect on *rarab* expression (E) though expression is upregulated anteriorly by RA (F). (G–I) AGN treatments also have little effect on *rarga* expression (H), but surprisingly RA treatments downregulate expression (I). (J–O) Expression of *rars* in the hindbrain at 18 hpf. Dorsal views, anterior to the left, of flat-mounted hindbrains showing rar expression (blue) and *krox20* in r3 and r5 (red). Similar to the gastrula studies, only *raraa* expression is reduced in *aldh1a2* mutants (K) while other receptors appear unaffected (M, O). Abbreviations: cm, cranial mesoderm.

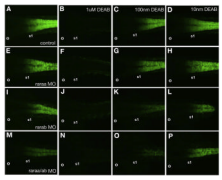

Fig. 8.

Depletion of *raraa* or *rarab* alters sensitivity to exogenous RA. Confocal images of RARE-YFP transgenics at 20 hpf. Dorsal views, anterior to the left. (A) Controls express YFP up to an anterior border between r6 and r7 in the hindbrain (adjacent to somite 1), and in the anterior spinal cord. (B) YFP expression is lost in embryos treated with 1 μM DEAB, and reduced/shifted posteriorly at lower DEAB concentrations (C, D). (E–H) *raraa* morphants. Untreated morphants express YFP similar to controls (E), and lack expression when treated with 1 μM DEAB (F), but show little change in expression at lower DEAB concentrations (G, H). (I–L) *rarab* morphants show reduced YFP expression that shifts posteriorly (I), lack expression when treated with 1 μM DEAB (J) and show stronger reduction/shifts in expression when treated with lower DEAB concentrations than controls (K, L). (M–P) *raraa/rarab* double morphants also show reduced/shifted YFP expression (M), and almost completely lose expression when treated with 0.1 μMDEAB (O). Abbreviations: o, otic vesicle; s1, somite #1.

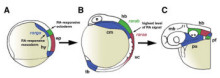

Fig. 9.

Model for roles of zebrafish RARs. Expression of *raraa* (red), *rarab* (green) and *rarga* (blue) at gastrula (A), 12 hpf (B) and 36 hpf (C). Lateral views, anterior to the left. (A) From the onset of their expression, *raraa* and *rarab* define anterior limits of responsiveness to RA in the ectoderm, while *rarga* mediates responses in cranial mesoderm that have secondary consequences for hindbrain patterning. (B) By neurula stages, *raraa* expression defines a second domain of stronger RA signal, with an anterior border near the head-trunk boundary. (C) *rarga* plays a crucial role in pharyngeal arch development, while *rarab*, which is expressed in the proximal pectoral fin bud, is crucial for fin budding. Abbreviations: cm, cranial mesoderm; e, eye; ep, epiblast; hb, hindbrain; hy, hypoblast; mb, midbrain; np, neural plate; pa, pharyngeal arches; pf, pectoral fin; sc, spinal cord; tb, tailbud.