# Multiple points of interaction between retinoic acid and FGF signaling during embryonic axis formation

**Jason Shiotsugu[1,*], Yu Katsuyama[1,*,†], Kayo Arima[1], Allison Baxter[1], Tetsuya Koide[1], Jihwan Song[2], Roshantha A. S. Chandraratna[3] and Bruce Blumberg[1,‡]**

[1]Department of Developmental and Cell Biology, University of California, Irvine, CA 92697-2300, USA
[2]Laboratory of Stem Cell Biology, Cell & Gene Therapy Research Institute, Pochon CHA University College of Medicine, Seoul 135-081, Korea
[3]Retinoid Research, Departments of Chemistry and Biology, Allergan, Irvine, CA 92623, USA
*These authors contributed equally to this work
†Present address: Department of Neuroscience, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
‡Author for correspondence (e-mail: blumberg@uci.edu)

*Accepted 17 February 2004*

Development 131, 2653-2667
Published by The Company of Biologists 2004
doi:10.1242/dev.01129

## Summary

**Anteroposterior (AP) patterning of the developing CNS is crucial for both regional specification and the timing of neurogenesis. Several important factors are involved in AP patterning, including members of the WNT and FGF growth factor families, retinoic acid receptors, and HOX genes. We have examined the interactions between FGF and retinoic signaling pathways. Blockade of FGF signaling downregulates the expression of members of the RAR signaling pathway, *RARα*, *RALDH2* and *CYP26*. Overexpression of a constitutively active *RARα2* rescues the effects of FGF blockade on the expression of *XCAD3* and *HOXB9*. This suggests that RARα2 is required as a downstream target of FGF signaling for the posterior expression of *XCAD3* and *HOXB9*. Surprisingly, we found that posterior expression of *FGFR1* and *FGFR4* was dependent on the expression of *RARα2*. Anterior expression was also altered with *FGFR1* expression being lost, whereas *FGFR4* expression was expanded beyond its normal expression domain. RARα2 is required for the expression of *XCAD3* and *HOXB9*, and for the ability of XCAD3 to induce *HOXB9* expression. We conclude that RARα2 is required at multiple points in the posteriorization pathway, suggesting that correct AP neural patterning depends on a series of mutually interactive feedback loops among FGFs, RARs and HOX genes.**

Key words: Retinoic acid, FGF, *Xenopus*, XCAD3

## Introduction

Anteroposterior (AP) patterning of the developing neural tube is a crucial early step in the generation of the vertebrate nervous system. The isolated animal pole (animal cap) of a blastula stage *Xenopus* embryo forms epidermal tissue when cultured, although dissociated animal cap cells will express neural markers (Sato, 1989). Animal caps cultured in the presence of molecules normally expressed in Spemann's Organizer such as noggin, follistatin or chordin become neuralized in the absence of mesoderm induction (Hawley et al., 1995; Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994; Holley et al., 1995; Lamb et al., 1993). These neural inducers are required in the ectoderm to block activity of BMP-4, a secreted TGFβ growth factor superfamily member (Hawley et al., 1995; Holley et al., 1995; Xu et al., 1995) that normally acts to repress neural fate. Organizer-expressed neural inducing genes neutralize BMP activity by directly binding to them, thereby blocking BMP inhibition of neural fate (Fainsod et al., 1997; Piccolo et al., 1996; Zimmerman et al., 1996).

The direct neural inducers described above generate neural tissue of anterior character (Hawley et al., 1995; Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994; Holley et al., 1995; Lamb et al., 1993). These findings support the activation-transformation model of neural patterning wherein the initial basal state of the neural ectoderm is anterior with additional factors being required to generate the posterior parts of the nervous system (Eyal-Giladi, 1954; Nieuwkoop, 1952). The major components of the activation signal are FGF and WNT signals that act before gastrulation to induce the organizer to secrete inhibitors of BMP and WNT signaling such as noggin, chordin, cerberus, follistatin and dickkopf during gastrulation (reviewed by Harland, 2000). In turn, these induce the neuroectoderm to adopt an anterior fate.

The transformation signal has been more elusive and is only recently becoming better understood. It has previously been shown that basic fibroblast growth factor (bFGF, FGF2) could posteriorize anterior neuroectoderm in vitro, suggesting an endogenous role for FGFs in neural induction and patterning (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995) (reviewed by Doniach, 1995). *eFGF* (*FGF4*) overexpression posteriorizes the axis via induction of downstream genes *Xcad3* and *Hoxa7* in vivo (Pownall et al., 1996); and inhibition of FGF signaling via overexpression of the dominant-negative FGF receptor 1, *XFD*, reduced the expression of the posterior markers *HOXA7* and *XCAD3* (Pownall et al., 1996). Papalopulu and colleagues have

shown that FGF8 acting through FGFR4 (rather than eFGF acting through FGFR1) is likely to be the major FGF pathway in neural posteriorization (Hardcastle et al., 2000).

We and others have shown that signaling through retinoic acid receptors (RARs) is necessary for correct AP patterning. Hindbrain and posterior patterning is abnormal in vitamin A-deficient quail (Maden et al., 1996) and rats (Dickman et al., 1997; White et al., 1998), and these defects can be reversed by appropriate temporal administration of retinoic acid (RA). We used overexpression of a dominant-negative *RAR* to show that signaling through RARs is required for the expression of the posterior markers *HOXB9*, *N-tubulin* and *XLIM1* (Blumberg et al., 1997). Positional changes were observed in the hindbrain along with posterior coordinate shifts in the expression of anterior markers. By contrast, locally increasing RAR signaling yielded the opposite result (Blumberg et al., 1997). Others showed that retinoid signaling was required to specify positional identity in the hindbrain (Kolm et al., 1997; van der Wees et al., 1998). Overexpression of the *Xenopus* retinoic acid hydroxylase (*CYP26*), which targets RA for degradation, leads to expansion of anterior structures (de Roos et al., 1999; Hollemann et al., 1998), whereas inhibition of *CYP26* expression led to expansion of posterior structures (Kudoh et al., 2002). Overexpression of the RA biosynthetic enzyme *RALDH2* led to reduction of anterior structures (Chen et al., 2001). *RALDH2* loss of function led to a variety of axial defects in mice, including axial shortening, loss of posterior rhombomere identity, limb buds and a variety of retinoic acid inducible molecular markers (Niederreither et al., 1999). Lumsden and colleagues recently showed that RA is the endogenous transforming factor active during hindbrain patterning and that it acts in a concentration-dependent fashion to specify the identity of rhombomeres 5-8 (Dupe and Lumsden, 2001). Together, these results indicate that retinoid signaling through RARs is essential for correctly restricting the expression of anterior genes, and to enable the expression of posterior marker genes. It should also be noted that RA could posteriorize anterior neuroectoderm injected with XFD, whereas FGF could not (Bang et al., 1997). Therefore, both retinoid and FGF signaling can posteriorize anterior neural tissue in vitro, perhaps acting synergistically, as was suggested previously based on transplantation experiments (Cho and De Robertis, 1990).

A role for WNT signaling in posteriorizing the embryonic axis has been suggested by studies showing that overexpression of *XWNT3A* posteriorized anterior neuroectoderm (McGrew et al., 1997; McGrew et al., 1995). Blockade of XWNT8 signaling caused loss of posterior fates (Bang et al., 1999; Fekany-Lee et al., 2000; McGrew et al., 1997), whereas inappropriate activation of WNT target genes caused by loss of the headless/*Tcf3* gene resulted in severe anterior defects in zebrafish (Kim et al., 2000). The combination of ectopic FGF or WNT signaling and suppression of RA by overexpression of *CYP26* has been shown to leave the presumptive neuroectoderm without any AP identity (Kudoh et al., 2002). Loss-of-function and genetic analysis has shown that WNT8 is an important transforming factor in zebrafish and *Xenopus*, and that either WNT8, or a factor crucially dependent on WNT8 for its expression is an endogenous neural transforming factor (Erter et al., 2001; Lekven et al., 2001). It has recently been shown that WNT8 signaling is required, together with BMP and nodal, for formation of the tail organizer in zebrafish (Agathon et al., 2003). Krumlauf and colleagues recently showed that the WNT/β-catenin pathway posteriorizes *Xenopus* neural tissue via an indirect mechanism requiring FGF signaling, suggesting that the posteriorization pathway might be WNT→FGF→XCAD3→posterior HOX genes (Domingos et al., 2001). This model does not account for the observation that inhibiting RAR signaling blocks the expression of posterior neural markers, while FGF and WNT signaling are presumably normal (Blumberg, 1997; Blumberg et al., 1997). Therefore, we aimed to determine where RAR signaling fits into the scheme of neural posteriorization.

We hypothesized that as both FGF (Isaacs et al., 1998; Pownall et al., 1996) and retinoid signaling (Blumberg et al., 1997) are required for the expression of posterior markers, these pathways might converge on one or more common target genes. *XCAD3* is a key downstream gene in the FGF-mediated posteriorization pathway (Isaacs et al., 1998; Pownall et al., 1996) and retinoids have been shown to influence the expression of caudal family genes in other systems (Allan et al., 2001; Houle et al., 2000; Prinos et al., 2001). Therefore, we tested the effects of modulating retinoid signaling on the expression of XCAD genes. *XCAD3* is upregulated by increasing RAR signaling and downregulated by inhibiting RAR signaling or the expression of *RARα*. Morpholino antisense oligonucleotide (MO) mediated inhibition of *RARα2* expression led to loss of *XCAD3* and *HOXB9* expression, confirming that RARs are required for posterior gene expression. Epistasis experiments showed that *FGF8* overexpression could not rescue the effects of *RAR* loss of function on *XCAD3* or *HOXB9* expression. However, overexpression of a constitutively active *RAR* (but not RA treatment) rescued the effects of FGF gene loss of function on *XCAD3* and *HOXB9*. This suggests that FGF signaling is not downstream of RAR signaling but that RAR might be downstream of FGF. FGF receptor function was required for the expression *RARα*, *RALDH2* and *CYP26* in whole embryos, and FGF8 microinjection induced expression of *RARα*, *CYP26* and *RALDH2* in the animal cap assay. Taken together, these results suggest that RAR is downstream of FGF signaling. However, we also found that RAR is required for the correct expression of *FGF8*, *FGFR4* and *FGFR1*, and that RA induces expression of *FGF8*, *FGFR1* and *FGFR4* in animal caps, arguing against a simple linear pathway. Co-injection of *XCAD3* mRNA and an MO directed against RARα2.2 (RAR-MO) showed that regulation of HOXB9 expression by XCAD3 requires RARα2 function, thus placing *RARα2* both upstream and downstream of XCAD3. Last, we show that *XCAD3* expression requires RAR function for its correct expression at stage 16 but not at stage 26. These data suggest the existence of a mutually reinforcing feedback loop among FGF8/FGFR4, XRARα and XCAD3. Thus, it appears that RAR signaling is required at multiple steps in the embryonic posteriorization pathway, suggesting that RAR and FGF signaling have multiple points of interaction rather than being in a simple linear pathway.

## Materials and methods

### Embryos

*Xenopus* eggs were fertilized in vitro as described (Koide et al., 2001)

and embryos staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Treatments with RAR agonists and antagonists were performed as described (Blumberg et al., 1996; Blumberg et al., 1997; Koide et al., 2001).

### Microinjection

Morpholino antisense oligonucleotides (MO) used in this study were the following: *XRARα2.1*, AAC TGA CCA TAG AGT GGA ACC GAG C; *XRARα2.2*, ATC CAA AGG AAG GTG AGT GTG TGT G. In all experiments using MO, control embryos were injected with 20 ng of standard control MO CCT CTT ACC TCA GTT ACA ATT TAT A (GeneTools). The following plasmids were constructed by PCR amplification of the protein-coding regions of the indicated genes and cloning into the expression vector pCDG1 or pCDG1-VP16: *XRARα2.2* (Sharpe, 1992) and *XCAD3* (Northrop and Kimelman, 1994). mRNA was prepared from these plasmids as well as pSP36T-XFD (Amaya et al., 1993) and pCS2-FGF8 (Hardcastle et al., 2000) using mMessage Machine (Ambion).

### Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Koide et al., 2001). Probes used in this study were the following: *HOXB9* (Sharpe et al., 1987), *XCAD1*, *XCAD2* (Blumberg et al., 1991) *XCAD3* (Northrop and Kimelman, 1994), *RARα* (Blumberg et al., 1992), *FGF8* (Hardcastle et al., 2000), *FGFR4* (Hongo et al., 1999), FGFR1 (Amaya et al., 1993), *XRALDH2* (Chen et al., 2001) and *CYP26* (de Roos et al., 1999). Lineage analysis using 100 pg/embryo of β-galactosidase was performed as described (Blumberg et al., 1997), except that the chromogenic substrate was 5-bromo-6-chloro-3-indolyl β-D-galactopyranoside (magenta-gal, Biosynth AG), which produces an insoluble red precipitate after cleavage by β-galactosidase (Sambrook and Russell, 2001).

### QRT-PCR

Embryo RNA was isolated using Trizol reagent (InVitrogen Life Technologies), DNAse treated and LiCl precipitated, then reverse transcribed using Superscript II reverse transcriptase according to the manufacturer-supplied protocol (InVitrogen Life Technologies). QRT-PCR was performed as described (Tabb et al., 2003) using the following primer sets: *FGF8*, 5′ AATCCTGGCGAACAAGAAGA and 3′ TAACCAGTCTCCGCACCTTT; *FGFR4*, 5′ GCCAGCTG-GTAACACAGTCA and 3′ TGATGGAACCACACTCTCCA; eFGF, 5′ GTTTTACCGGACGGAAGGAT and 3′ TCCATACAGCTT-CCCCTTTG; *FGFR1*, 5′ GGTGTCCAGCAAATGGAACT and 3′ ATGGGACAACGGAATCCATA; *XCAD1*, 5′ CAGCCTTGTGTT-GGGGTATT and 3′ GGTTTCCTGAGCCATTCGTA; *XCAD2*, 5′ ACCAGCGCCTTGAATTAGAA and 3′ GAGTGGTTGTTG-AGGCCTGT; *XCAD3*, 5′ AAGGGCAGCCTATGGAGTTT and 3′ GTCCCAGATGGATGAGGAGA; *XRARα*, 5′ ATCAAGACGGTG-GAATTTGC and 3′ CAGTCCGTCAGAGAACGTCA; *RALDH2*, 5′ GCCCTTTTGATCCCACTACA and 3′ TCTTCCCAATGCT-TTTCCAC; *CYP26*, 5′ TGTTCGTGGTGGAATTGTGT and 3′ TTAGCGGGTAGGTTGTCCAC; Histone H4, 5′ AACATCCAGG-GCATCACCAA and 3′ AGAGCGTACACCACATCCAT.

Each primer set was found to amplify only a single band as determined by gel electrophoresis and melting curve analysis.

## Results

### Expression patterns of *RARα* in early Xenopus development

*RARα* is expressed as two major isoforms during early development, *XRARα1* and *XRARα2* (Sharpe, 1992). These isoforms are the result of alternative promoter usage in most vertebrates (reviewed by Chambon, 1996). Both *RARα1* and *RARα2* are expressed maternally; however, only *RARα2* is expressed zygotically in *Xenopus* (Blumberg et al., 1992; Koide et al., 2001; Sharpe, 1992). *XRARα2* is expressed as two different forms, *XRARα2.1* and *XRARα2.2* that are presumably the product of the pseudotetraploid nature of the *Xenopus laevis* genome (Sharpe, 1992). We performed a detailed analysis of the temporal and spatial expression of *XRARα* using whole-mount in situ hybridization. Although, the probe used for in situ hybridization can detect all isoforms of *RARα*, its temporal expression pattern indicates that the detected signal is derived from *XRARα2* (Koide et al., 2001; Sharpe, 1992). Zygotic expression of *RARα2* was detected at as early as stage 9 and by stage 10 as a faint signal in the involuting surface layer surrounding the blastopore (Fig. 1E), and became stronger as gastrulation proceeded (Fig. 1G,I) (Koide et al., 2001). During neurula stages (stage 14-18), *RARα* expression was detected predominantly in the posterior neural tube with weaker staining throughout the embryo (Fig. 1B,D,F,H). As previously reported (Sharpe, 1992) the strong staining of *XRARα2* has a sharp anterior border (Fig. 1J). Lower level expression continues anteriorly with prominent later expression in the developing eyes (Fig. 1J).

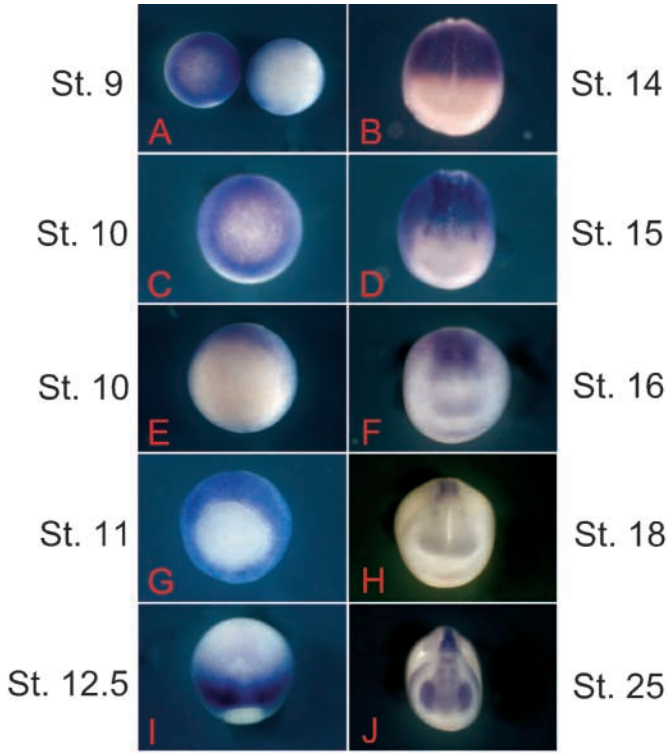


**Fig. 1.** Developmental expression of *XRARα*. Whole-mount in situ hybridization was performed on embryos from stage 9 to stage 25 using a probe that recognizes all isoforms of *XRARα*. (A) Dorsal (left) and ventral (right) view of a stage 9 embryo. (B,D,F,H,J) Frontal views. (C) A dorsal view of the stage 10 embryo. (E) A vegetal view of the stage 10 embryo. Note the sharp anterior border of strong staining.

### *RARα* loss-of-function causes anterior and posterior truncations

To investigate the function of RARα2, we used morpholino antisense oligonucleotide-mediated loss-of-function analysis

(Heasman et al., 2000; Koide et al., 2001). MO were prepared to specifically inhibit the expression of either *XRARα2.1* or *XRARα2.2*. Microinjection of the XRARα2.1 MO was nontoxic and did not elicit a phenotype at doses up to 20 ng/embryo (data not shown). By contrast, microinjection of the XRARα2.2 MO (hereafter RAR-MO) affected both anterior and posterior development in the *Xenopus* tadpole (Fig. 2) (Koide et al., 2001). Injection of the RAR-MO into both blastomeres of the two-cell embryo produced a dose-dependent spectrum of phenotypes; microinjection of 10 ng RAR-MO/embryo consistently gave rise to observable phenotypes with varying severity (Fig. 2A-C). We used co-injection of β-galactosidase lineage tracer to correlate the observed phenotypes with RAR-MO distribution and found that the phenotypic variation was related to the distribution of the RAR-MO in the affected embryos. The most severe phenotypes were observed when the RAR-MO was distributed dorsally (*n*=41/50) (Fig. 2A). Mild phenotypes resulted when the RAR-MO was widely distributed (*n*=6/50) (Fig. 2b) and no abnormalities were observed when the lineage tracer was observed in the ventral or lateral parts of the embryo (*n*=3/50) (Fig. 2c). This lack of a phenotype from lateral or ventral distribution of the lineage tracer was confirmed by targeted injection into the marginal zone of the two-cell stage embryos (*n*=19/21) (not shown). These observations suggest that *XRARα2.2* function is predominantly required for development of the dorsal parts of the *Xenopus* embryo, consistent with the zygotic expression pattern of this gene (Fig. 1) (Koide et al., 2001; Sharpe, 1992). Co-injection of 1 ng RARα2 mRNA with RAR-MO consistently rescued the morphological abnormalities (Fig. 2D) (Koide et al., 2001). Doses higher than 20 ng of RAR-MO/embryo were lethal and injection of more than 10 ng per embryo could not be rescued by co-injection of the rescue construct. Therefore, we used 10 ng of RAR-MO and 1 ng of rescue construct per whole embryo (or 5 ng of RAR-MO for unilateral injections) for the experiments described below.

*XRARα* is expressed in the region surrounding the blastopore in gastrulating embryos (Fig. 1E,G), overlapping the reported expression patterns of *XWNT8* and *XBRA* (Christian and Moon, 1993; Smith et al., 1991). Therefore, we examined *XWNT8* and *XBRA* expression in RAR-MO-injected embryos because embryos lacking either of these genes showed posterior truncations similar to those we found (Conlon et al., 1996; Hoppler et al., 1996). Embryos were co-injected with 5 ng RAR-MO and 100 pg β-galactosidase mRNA lineage tracer unilaterally at the two-cell stage, allowed to develop until stage 11 then fixed and processed for in situ hybridization. Expression of *XWNT8* and *XBRA* were not altered in the β-galactosidase positive regions of the embryo (Fig. 2E,F) indicating that XRARα2.2 is not required for their expression. Therefore, it is unlikely that the posterior truncations elicited by downregulating *XRARα2.2* expression with the RAR-MO resulted from effects on *XWNT8* or *XBRA* expression.

We have previously shown that overexpression of a dominant negative *RARα1* suppressed expression of the spinal cord marker, *HOXB9* and the posterior markers XLIM1 and N-tubulin (Blumberg et al., 1997). As the dominant-negative RARα used in those experiments can also inhibit expression from RARβ and RARγ target genes (Blumberg, 1997; Damm et al., 1993), we tested whether RARα was required for *HOXB9* expression using RAR-MO injected embryos. Five or 10 ng of RAR-MO were injected bilaterally into the animal pole of two-cell embryos that were allowed to develop until stage 18 then fixed for whole-mount in situ hybridization. *HOXB9* expression was inhibited in a dose-dependent manner (Fig. 2H-J) and showed a range of phenotypes that could be classified into three groups. Class I embryos (*n*=5/23 for 5 ng MO, *n*=0/30 for 10 ng MO) showed weaker *HOXB9* staining than controls (Fig. 2G) and the presumptive posterior neural tube region (marked by *HOXB9* expression) was wider than that of control embryos (Fig. 2H). *HOXB9* expression in class II embryos (10/23 for 5 ng MO, 5/30 for 10 ng MO) was weaker yet and the expression boundary was shifted posteriorly (Fig. 2I). Class III embryos expressed *HOXB9* only in the posterior terminus of the embryo (5/23 for 5 ng MO,

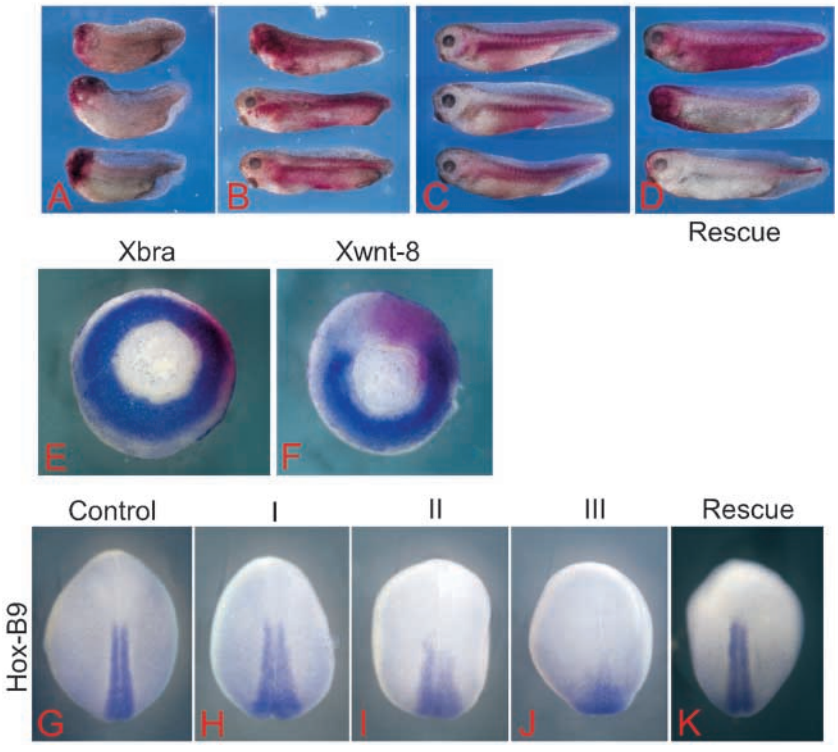


**Fig. 2.** XRARα2.2 loss-of-function leads to axial truncations and reduction of *HOXB9* expression. (A-D) Microinjection of RAR-MO causes anterior and posterior truncations at highest frequency when expressed in the head region (A) or dorsally (B). (C) Phenotypes are mild to undetectable when the lineage tracer is distributed laterally or ventrally. (D) Phenotypes are rescued by co-injection of *XRARα2* mRNA, irrespective of where the lineage tracer is located. (E,F) Neither *XBRA* (E) nor *XWNT8* (F) expression is affected by RAR-MO injection. (G-K) Effects of RAR-MO on the expression of *HOXB9*. (H-J) The types of phenotypes obtained. (K) *HOXB9* expression was restored by co-injecting *XRAR* mRNA and RAR-MO.

25/30 for 10 ng MO) (Fig. 2I). Complete suppression of *HOXB9* expression by injection of 10 ng RAR-MO was not obtained ($n$>100). Co-injection of 1 ng *XRARα2* mRNA rescued *HOXB9* expression (26/32 embryos were normal, 6/32 were class I) (Fig. 2J). Based on these observations, we conclude that signaling through XRARα2.2 is indispensable for the expression of *HOXB9*, in accord with previous studies using a dominant-negative *Xenopus* RAR (Blumberg et al., 1997).

## RA signaling is required for *XCAD3* expression

The homeobox gene *XCAD3* has been implicated in the posteriorization pathway as a downstream target of eFGF (FGF4) signaling (Isaacs et al., 1998; Pownall et al., 1998; Pownall et al., 1996). The embryonic expression pattern of *XCAD3* is strikingly similar to that of *HOXB9* (Northrop and Kimelman, 1994). We identified *XCAD3* in a screen to identify RAR target genes (R. Niu and B. Blumberg, unpublished), which led us to hypothesize that *XCAD3* might be a common target for retinoid and FGF signaling upstream of *HOXB9*. Two approaches were taken to investigate this possibility. First, embryos were treated with either the synthetic retinoid agonist TTNPB, which specifically activates all three subtypes of RAR or the antagonist AGN193109, which specifically blocks the ability of RARs to activate transcription (Koide et al., 2001). These reagents were chosen because they affect RARs but not RXRs (Boehm et al., 1994; Johnson et al., 1995). TTNPB treatment enhanced the expression of *XCAD3* in the posterior neural tube (Fig. 3M), whereas significant differences were not observed for *XCAD1* (Fig. 3C) or *XCAD2* (Fig. 3H) compared with control embryos. Conversely, AGN193109 treatment suppressed *XCAD3* expression (Fig. 3K), but did not alter expression of *XCAD1* (Fig. 3A) or *XCAD2* (Fig. 3F). QRT-PCR analysis showed that *XCAD3* was slightly upregulated by TTNPB (1.3-fold) and strongly downregulated by antagonist (2.5-fold). *XCAD1* was downregulated by both TTNPB and 193109 (indicating a non-specific effect on gene expression) and *XCAD2* was slightly downregulated by TTNPB (1.5 fold) but not affected by AGN193109 (1.07 fold up) (data not shown).

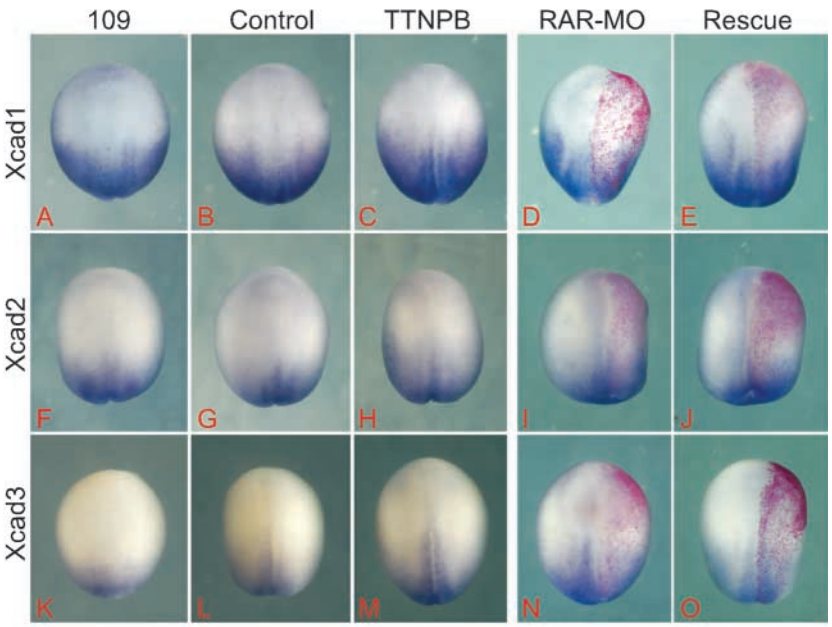


**Fig. 3.** Modulating retinoid signaling affects the expression of *XCAD3* but not *XCAD1* or *XCAD2*. (A-C,F-H,K-M) Embryos were treated with the indicated compound or ethanol solvent controls from the early blastula stage (stage 7) until harvesting when control embryos reached stage 18. Embryos were fixed and processed for whole-mount in situ hybridization with the indicated probes. (A,F,K) $10^{-6}$ M AGN193109 (RAR-selective antagonist), (B,G,L) ethanol solvent control, (C,H,M) $10^{-6}$ M TTNPB (RAR-selective agonist). RAR-MO was injected unilaterally at the two-cell stage with β-galactosidase lineage tracer alone (D,I,N) or together with 1 ng XRARα2 mRNA (E,J,O). Embryos were fixed when controls reached stage 18, stained for β-galactosidase activity and processed for whole-mount in situ hybridization with the indicated probes. Some embryos were used for RNA extraction and QRT-PCR analysis as described in the text.

We next tested the requirements for XRARα2.2 signaling on XCAD gene expression using RAR-MO mediated loss-of-function. Two-cell embryos were unilaterally injected with 5 ng RAR-MO and 100 pg β-galactosidase mRNA, fixed when controls reached the late neurula stage (stage 16-18), and stained for β-galactosidase activity. Embryos exhibiting appropriate β-galactosidase staining were selected for in situ hybridization. *XCAD3* expression was suppressed in 10/12 RAR-MO injected embryos (Fig. 3N) and was rescued by co-injection of *XRARα2* mRNA (Fig. 3O). In agreement with the retinoid treatments, expression of *XCAD1* ($n$=12) and *XCAD2* ($n$=12) showed no significant differences between the injected side and the uninjected contralateral control (Fig. 3D,E,I,J). These results indicate that only *XCAD3* is regulated by RA in early *Xenopus* embryos.

## RA and FGF signaling converge on *XCAD3*

Slack, Isaacs and colleagues have shown that *XCAD3* is a direct target for FGF signaling and that an important embryonic posteriorization pathway begins with eFGF (FGF4) activation of *XCAD3*, which induces expression of *HOXA7* and other posterior HOX genes (Isaacs et al., 1998; Pownall et al., 1996). They showed that XCAD3 was necessary and sufficient to activate posterior HOX genes (Isaacs et al., 1998). Our previous results (Blumberg et al., 1997) and those described above show that RAR signaling is also required for the expression of posterior markers such as *HOXB9* and *XCAD3*. As both retinoid and FGF signaling appear to be important for the expression of posterior genes, we carried out epistasis experiments designed to reveal the relationship between RAR and FGF signaling.

*bFGF* (*FGF2*) treatment of neuralized *Xenopus* animal cap explants induces posterior gene expression (Bang et al., 1997; Papalopulu and Kintner, 1996). Recent publications suggested that FGF8 is likely to be the bona fide posteriorizing FGF acting in the early embryo (Christen and Slack, 1997; Hardcastle et al., 2000; Hongo et al., 1999); hence, we next tested the effects of microinjecting *FGF8* mRNA, RAR-MO or both together. Bilateral microinjection of 25 pg *FGF8* mRNA

into two-cell embryos led to ectopic and enhanced expression of *XCAD3* and *HOXB9* in anterior neural tissues (*n*=16/19; Fig. 4C,H). The observed anterior expansion of *XCAD3* and *HOXB9* was similar to that observed for *eFGF* overexpression (Pownall et al., 1996). We next asked whether FGF signaling is downstream of RAR signaling by testing whether *FGF8* overexpression could rescue the effects of the RAR-MO on posterior gene expression. Injection of RAR-MO (10 ng) suppressed expression of *XCAD3* (*n*=8/11) and *HOXB9* (*n*=10/11) (Fig. 4B,G). Co-injection of as much as 50 pg of *FGF8* mRNA could not rescue expression of either gene (*n*=10/11 for each gene; Fig. 4D,I). Lineage traced, unilateral microinjections confirmed this observation (Fig. 4E,J). Therefore, we infer that FGF8 signaling is not downstream of RAR signaling.

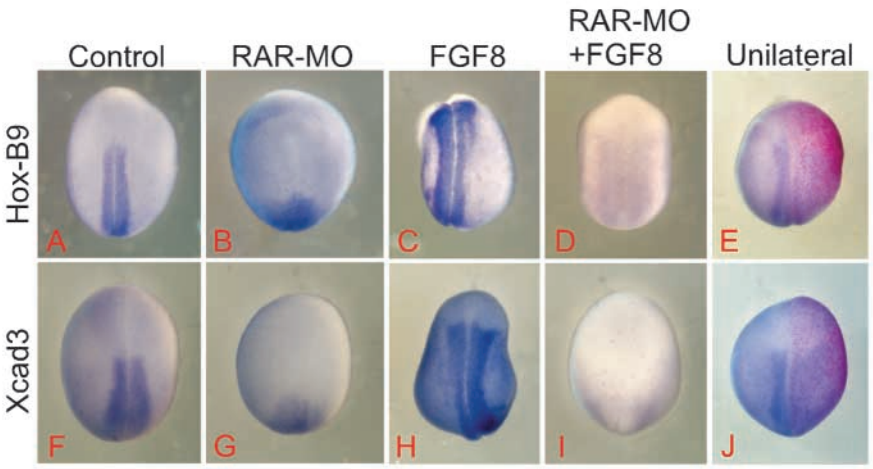


**Fig. 4.** FGF8 cannot rescue the effects of XRARα2.2 loss-of-function on posterior marker genes. Embryos were microinjected at the two-cell stage with the indicated reagents, allowed to develop until controls reached stage 18 and processed for whole-mount in situ hybridization with either HOXB9 (A-E) or XCAD3 (F-J) probes.

It has previously been shown that expression of a dominant-negative FGF receptor 1 (*FGFR1*) mutant (*XFD*) in early *Xenopus* embryos suppressed expression of *HOXB9* and *XCAD3* (Pownall et al., 1998). We confirmed this observation by microinjecting 1 ng XFD mRNA together with 100 pg of β-galactosidase mRNA into one blastomere of two- or four-cell embryos. Embryos were fixed and stained for β-galactosidase activity when untreated siblings reached stage 18. *XFD* injection typically delays morphogenesis during gastrulation and neurulation leaving the dorsal side of the embryos open, showing endodermal cells that would otherwise be covered by the mesodermal and ectodermal layers during gastrula and neurula stages (Amaya et al., 1991) (Fig. 5). As the closing edge of the ectodermal layer of *XFD*-overexpressing embryos is the presumptive posterior neural tube region, we selected embryos showing β-galactosidase staining in this region for in situ analysis. *HOXB9* and *XCAD3* expression were unaffected in the uninjected side whereas the neural tube region of the injected side did not express either *HOXB9* or *XCAD3* (*n*=40/40) (Fig. 5A,D). Treatment of *XFD*-overexpressing embryos with $10^{-6}$ M all-*trans*-RA did not rescue expression of either *HOXB9* or *XCAD3* in the injected side (*n*=12/12) (Fig. 5B,E). Co-injection of 1 ng *XRARα2.2* mRNA with *XFD* partially rescued *HOXB9* (*n*=4/10) and *XCAD3* (*n*=2/10) expression (data not shown). Co-injection of the constitutively active VP16-XRARα2 (Blumberg, 1997; Koide et al., 2001) yielded nearly complete rescue of both *HOXB9* (*n*=14/16) and *XCAD3* (*n*=16/16) (Fig. 5C,F). Complete rescue by the constitutively active (ligand-independent) RAR and partial rescue by the wild-type RAR suggests that both the expression of *XRARα2.2* and the synthesis of RA are deficient in *XFD* injected embryos. These results suggest that retinoid signaling through XRARα2.2 is required for FGF signaling to induce posterior genes in the neural ectoderm, which may place RAR downstream of FGF signaling in neural patterning.

## FGF signaling is required for the expression of RA signaling pathway components

One possible explanation for the results described above is that FGF signaling regulates the RAR signaling pathway. Therefore, we examined the effects of XFD-mediated FGF loss-of-function on the expression of mRNAs encoding *XRARα*, the RA-synthesizing enzyme *RALDH2* and the RA-degrading enzyme *CYP26*. Embryos were fixed at stage 11 and those showing β-gal expression in the region of the blastopore were selected for in situ analysis. As previously reported, XFD blocks *XCAD3* expression at this stage (Pownall et al., 1998; Pownall et al., 1996) (Fig. 6B). *XRARα* and *XCAD3* are expressed in similar patterns at stage 11 and we found that XFD also inhibits the expression of *XRARα* (Fig. 6d). Strikingly, XFD also led to the downregulation of *RALDH2*

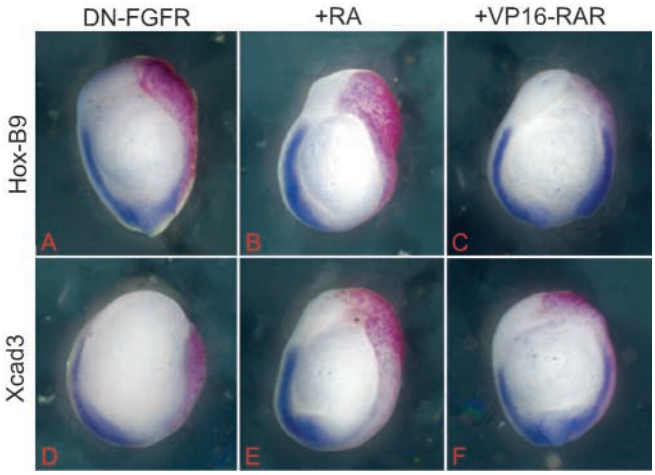


**Fig. 5.** A constitutively active RAR, but not RA, can rescue the effects of FGF gene loss-of-function on posterior markers. Embryos were microinjected unilaterally at the two cell stage with β-galactosidase mRNA as lineage tracer and (A,D) 1 ng of XFD mRNA, (B,E) 1 ng of *XFD* mRNA then treated with $10^{-6}$ M atRA, or (C,F) 1 ng of *XFD* and 1 ng VP16-XRARα2 mRNA. When control embryos reached stage 18, the embryos were fixed, stained for β-galactosidase activity and processed for whole-mount in situ hybridization with either *HOXB9* (A-C) or *XCAD3* (D-F) probes.

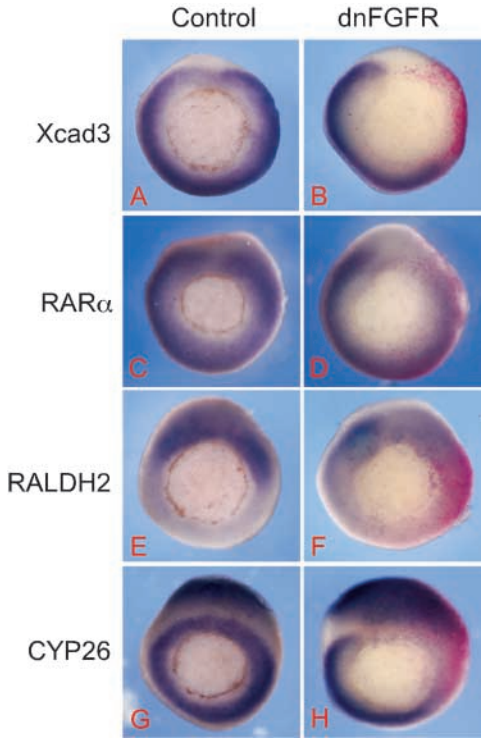


**Fig. 6.** FGF gene loss of function alters the expression of RAR signaling pathway components in microinjected embryos. Embryos were microinjected unilaterally into one blastomere at the two- or four-cell stage with 1 ng of *XFD* mRNA and β-galactosidase mRNA as lineage tracer. Embryos were allowed to develop until controls reached stage 11 then fixed and processed for whole-mount in situ hybridization with the probes (A,B) *XCAD3*, (C,D) *XRARα*, (E,F) *RALDH2* or (G,H) *CYP26*.

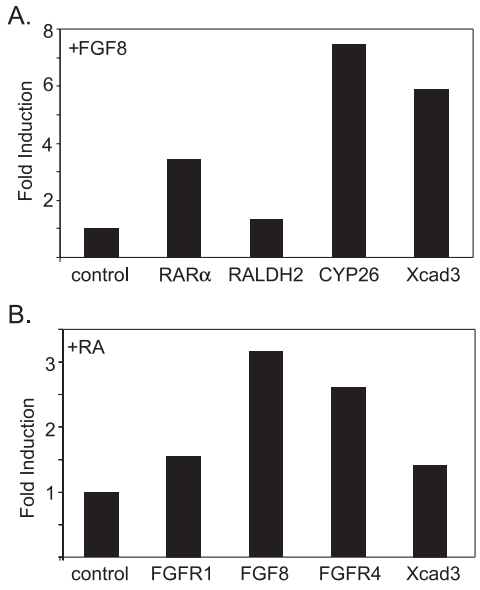


**Fig. 7.** FGF8 and RA induce members of the other signaling pathways in animal caps. Embryos were microinjected with mRNA encoding FGF8 mRNA. Caps were cut from FGF8 or control embryos at or before stage 9 and allowed to develop until untreated sibling embryos reached stage 20 in the presence or absence of $10^{-6}$ M all-trans RA. RNA was prepared from the caps and control embryos and QRT-PCR analysis performed with the indicated primer sets. Experiments were performed in triplicate and reproduced in independent experiments (Student's paired *t*-test). $P<0.03$ for all data sets.

(Fig. 6F) in the injected cells. These results are consistent with the rescue experiments shown in Fig. 5. *CYP26* is normally expressed dorsally and in the lateral mesoderm of gastrula embryos (de Roos et al., 1999; Hollemann et al., 1998). XFD did not inhibit the dorsal expression of CYP26 but strongly inhibited it in the lateral and ventral regions of the embryo (Fig. 6H).

Another approach to show the dependence of RAR on FGF relies on the ability of FGF8 to modulate the expression of RAR pathway components in animal cap explants. Embryos were injected bilaterally at the two-cell stage with 50 pg of mRNA encoding *FGF8* and animal caps were cut at or before stage 9 (Sive et al., 2000). The isolated animal caps were either cultured in MBS or MBS containing $10^{-6}$ M all-trans RA and allowed to develop until untreated siblings reached stage 18 when RNA was prepared from the caps. The expression of *XCAD3*, *XRARα2*, *RALDH2*, *CYP26* and the control histone H4 were evaluated using QRT-PCR in untreated caps whereas *XCAD3*, *eFGF*, *FGF8*, *FGFR1* and histone H4 were evaluated in RA-treated caps (Fig. 7). FGF8 upregulated the expression of *XCAD3*, *XRARα2*, *RALDH2* and *CYP26* (Fig. 7A). Taken together with the loss-of-function experiments described above, this suggests that the expression of components in the RAR signaling pathway, in vivo, depends on FGF signaling.

## RARα regulates FGF signaling

Although it is clear from the results shown above that the expression of RAR signaling pathway components requires FGF function, the effects of removing FGF and RAR function were not identical (compare Fig. 2 with Fig. 5), which cannot be explained by a simple linear pathway. RA treatment led to upregulation of *FGFR1*, *FGFR4* and *FGF8* compared with controls in animal caps (Fig. 7B). We detected small amount of *eFGF* in whole stage 18 embryos but were unable to detect it in animal caps (data not shown). It is possible that RAR signaling is required for the initiation or maintenance of the components in the FGF pathway; therefore, we decided to test the effects of the RAR-MO on FGF signaling pathway components.

RAR-MO was unilaterally microinjected into two-cell embryos together with β-galactosidase lineage tracer. Embryos were fixed when controls reached stage 18-20, stained for β-galactosidase activity then processed for whole-mount in situ hybridization. The RAR-MO elicited an increase in the size and staining intensity of the anterior lateral epidermal crescent expression domain of *FGF8* (Christen and Slack, 1997) (Fig. 8B), which was restored by microinjection of *XRARα2* mRNA (Fig. 8C). Posterior expression near the blastopore was slightly enhanced and extended anteriorly in RAR-MO injected embryos (Fig. 8E) and rescued by co-injection of *XRARα2* mRNA (Fig. 8F). Overall, we observed relatively minor, but reproducible changes in the expression of *FGF8* in RAR-MO injected embryos.

In contrast to the modest effects on *FGF8* mRNA

expression, RAR-MO injection led to strong effects on *FGFR4* and *FGFR1* expression. In the anterior region, RAR-MO led to a lateral expansion of *FGFR4* with a concomitant loss of regional boundaries seen in control embryos (Fig. 8G) or in the uninjected contralateral side (Fig. 8H). Strikingly, posterior expression of *FGFR4* in the spinal cord was strongly inhibited by RAR-MO injection (Fig. 8K). *FGFR1* was strongly downregulated throughout the embryo by RAR-MO injection (Fig. 8N,Q). The effects of microinjecting the RAR-MO could be rescued by co-injection of *XRARα2* mRNA, demonstrating its specificity (Fig. 8I,L,O,R). These results suggest that signaling through XRARα2 is required for the correct expression of these FGF signaling pathway components.

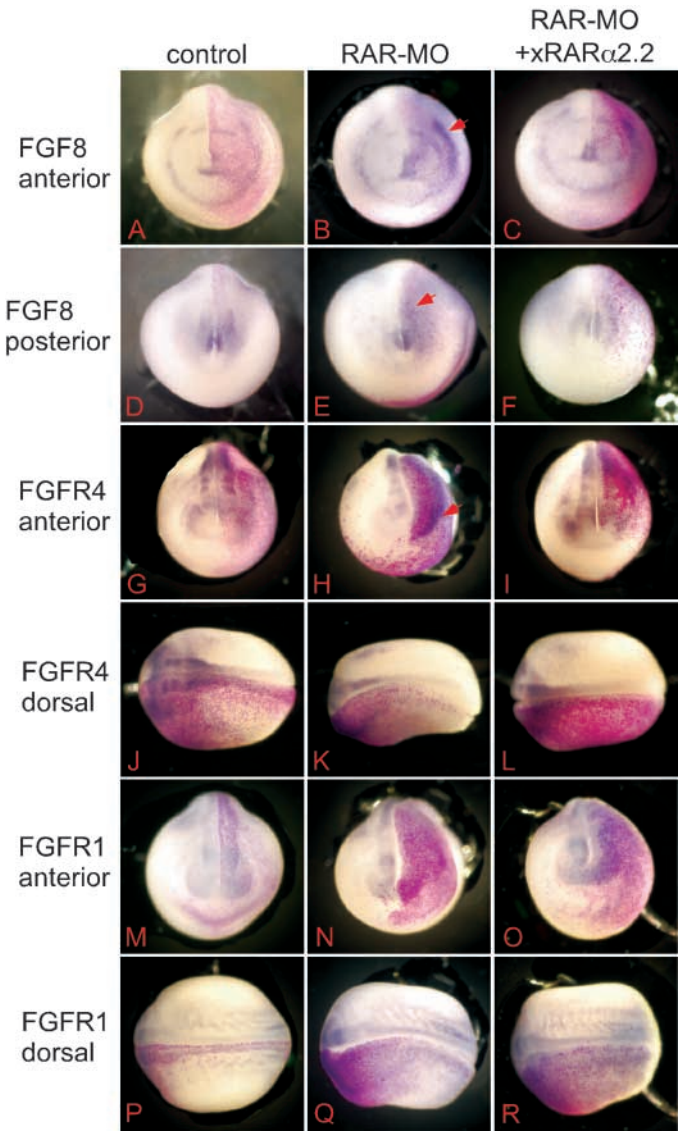


**Fig. 8.** *XRARα2.2* loss-of-function alters the expression of *FGF8*, *FGFR4* and *FGFR1*. Embryos were microinjected unilaterally with β-galactosidase mRNA plus 10 ng RAR-MO or 10 ng RAR-MO plus 1 ng *XRARα2* mRNA. Embryos were fixed when control uninjected embryos reached stage 18, stained for β-galactosidase activity and then processed for in situ hybridization with *FGF8* (A-F), *FGFR4* (G-L) or *FGR1* (M-R) probes.

## RA signaling is required both upstream and downstream of XCAD3

It has been suggested that posterior HOX genes are regulated by caudal family genes based on the effects of *XCAD3* loss of function (Isaacs et al., 1998) and the identification of CDX-binding motifs in HOX gene promoters (Subramanian et al., 1995). Knockout and transgenic mouse studies with caudal family genes showed alterations in HOX gene expression (Charite et al., 1998; Subramanian et al., 1995). Posterior HOX genes are also known to be sensitive to RA in cell culture (Simeone et al., 1991; Simeone et al., 1990; Simeone et al., 1995), and in mouse (Gavalas and Krumlauf, 2000; Kessel, 1992; Kessel and Gruss, 1991; Ogura and Evans, 1995a; Ogura and Evans, 1995b) and *Xenopus* embryos (Durston et al., 1998; Godsave et al., 1998; van der Wees et al., 1998). One possible inference that can be drawn from the experiments described above is that RAR regulates *HOXB9* through the function of XCAD3, because *XCAD3* expression requires XRARα (Figs 3, 4). However, those experiments do not rule out the possibility that RAR signaling directly regulates the expression of *HOXB9* and perhaps other HOX genes. Several known and putative retinoic acid response elements have been identified in HOX genes (Dupe et al., 1997; Huang et al., 2002; Huang et al., 1998; Marshall et al., 1994; Ogura and Evans, 1995a; Zhang et al., 1997) suggesting that RAR may act in concert with XCAD3 and perhaps other factors, instead of acting upstream of XCAD3 within a strict hierarchy. Therefore, we examined this possibility by co-injecting the RAR-MO together with *XCAD3* mRNA. If XCAD3 is strictly downstream of XRARα2 in the regulation of *HOXB9*, microinjection of *XCAD3* mRNA should rescue the effects of RAR-MO on *HOXB9*. *XCAD3* overexpression induced ectopic anterior neural expression of *HOXB9* (*n*=8/8; Fig. 9C) as previously reported (Isaacs et al., 1998; Pownall et al., 1998). Injection of the RAR-MO alone led to strong downregulation

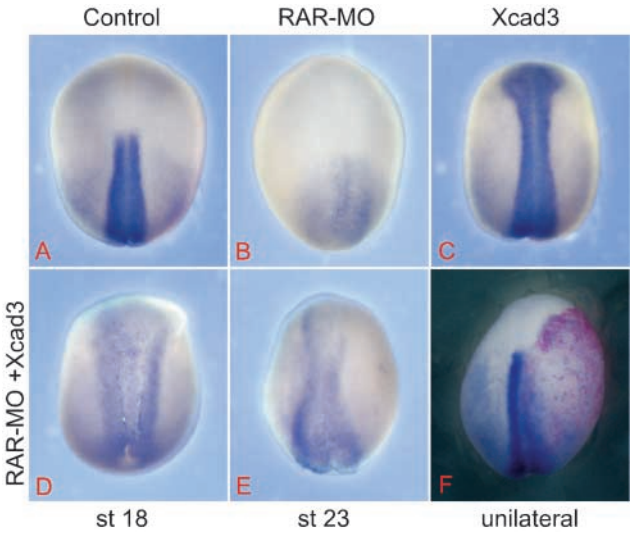


**Fig. 9.** XRARα2.2 is required for XCAD3-mediated upregulation of *HOXB9*. Embryos were microinjected with 10 ng RAR-MO (B), 1 ng *XCAD3* mRNA (C) or 10 ng RAR-MO plus 1 ng *XCAD3* mRNA (D-F), then allowed to develop until untreated embryos reached stages 18 (A-D,F) or 23 (E), fixed and processed for whole mount in situ hybridization with *HOXB9*.

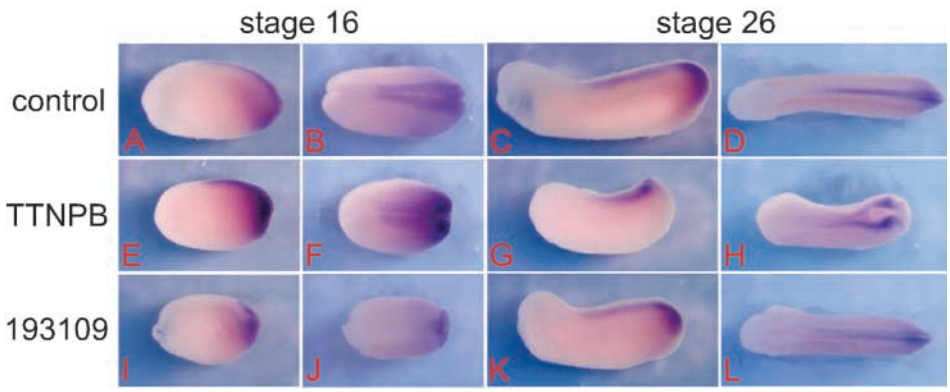


**Fig. 10.** XCAD3 expression requires RAR at early but not late stages. Embryos were treated with either TTNPB (E-H) or AGN193109 (I-L) at the blastula stage and cultured until controls (A-D) reached the indicated stages, fixed and processed for in situ hybridization with XCAD3. (A,C,E,G,I,K) Lateral views; (B,D,F,H,J,L) dorsal views.

of HOXB9 together with a posterior shift in its expression boundary (Fig. 9B). Co-injection of the RAR-MO and *XCAD3* mRNA led to a substantial reduction in the intensity of *HOXB9* staining compared with *XCAD3* or control embryos ($n$=10/10) (Fig. 9D,E) although *HOXB9* expression was never completely eliminated. Unilateral injections confirmed that *HOXB9* expression was reduced by the RAR-MO, even in the presence of overexpressed *XCAD3* (Fig. 9F). These results suggest that XRARα2 is required both upstream and downstream of XCAD3.

### Temporal requirement for RAR signaling in posterior gene expression

Although the results presented above clearly demonstrate that RAR signaling is required for the expression of XCAD3 and HOXB9, there are published data from other laboratories suggesting that retinoid signaling is not required for posterior gene expression (see Discussion). One possible explanation for these disparate results is that posterior gene expression occurs in two phases. The first would require early retinoid signaling for the establishment of posterior gene expression, whereas the second phase would be retinoid independent. In this scenario, the stage at which embryos are analyzed would play an important role in determining whether or not posterior markers were expressed. To test this possibility, we treated embryos with either TTNPB or AGN193109 continuously from the blastula stage (stage 9) and then evaluated *XCAD3* expression at early (stage 16) and late (stage 26) stages. In accordance with our model, antagonist treatment led to a substantial downregulation of *XCAD3* at stage 16 (Fig. 10I,J) whereas the expression of *XCAD3* appeared essentially normal at stage 26 (Fig. 10K,L). TTNPB treatment led to an increase in *XCAD3* expression at stage 16 (Fig. 10E,F), whereas the expression had normalized by stage 26, save for the apparent loss of *XCAD3* expression in the extreme posterior of the embryo (Fig. 10G,H). Interestingly, this is the same region where *CYP26* is normally expressed (Hollemann et al., 1998). We infer from these results that the early expression of *XCAD3* requires retinoid signaling whereas later expression does not.

## Discussion

### RA and FGF signaling in neural posteriorization

Activities of three distinct types of intercellular signaling pathways (WNTs, FGFs and retinoids) contribute to the transforming (posteriorizing) signal in Nieuwkoop's activation-transformation model. Loss-of-function analyses indicate that these factors are all required for posterior patterning but the interrelationships and dependencies among the pathways were unclear. We focused on the relationship between RA and FGF signaling in specifying posterior neural structures. The observations that both FGF (Isaacs et al., 1998; Pownall et al., 1996) and retinoid signaling (Blumberg et al., 1997) are required for the expression of posterior HOX genes led us to hypothesize that these pathways converge on one or more common target genes. *XCAD3* is a key downstream gene in the FGF-mediated posteriorization pathway and retinoids have been shown to influence the expression of caudal family genes in other systems (Allan et al., 2001; Houle et al., 2000; Prinos et al., 2001), making XCAD genes likely targets for both retinoid and FGF signaling. Indeed, we found that modulating retinoid signaling with RAR agonists or antagonists predictably altered the expression of *XCAD3* (Fig. 3) and that RARα2.2 is required for the expression of *XCAD3* (Figs 3, 4) and *HOXB9* (Figs 2, 4).

Overexpression of the dominant-negative FGF receptor 1 (*XFD*) mRNA suppresses mesoderm formation (Amaya et al., 1993) and the expression of posterior neural genes (Fig. 5) (Pownall et al., 1998; Pownall et al., 1996). Microinjection of the constitutively active VP16-*XRARα2* completely rescued *XCAD3* and *HOXB9* expression in *XFD*-injected embryos, whereas *XRARα2* led to partial rescue and RA treatment did not rescue at all (Fig. 5). Therefore, we infer that XFD is downregulating a crucial component of retinoid signaling. The failure of RA to rescue the effects of *XFD* overexpression suggests that expression of the receptor itself is the key missing component, although the incomplete rescue elicited by the wild-type receptor also implicates retinoid synthesis. The constitutively active receptor does not require endogenous RARs or RA, and therefore would be expected to rescue if retinoid is downstream of FGF signaling.

Inhibition of posterior gene expression by RAR-MO-mediated *XRARα2.2* loss of function could not be overcome by overexpressing *FGF8* (Fig. 4) or *XCAD3* (Fig. 9). The suppression of genes involved in RA signaling such as *XRARα2*, *RALDH2* and *CYP26* by *XFD* injection (Fig. 6) is consistent with a model wherein FGF signaling modulates RAR signaling by regulating the availability of components in the RAR signaling pathway. Zygotic expression of *XRARα*, *RALDH2* and *CYP26* is detectable from the onset of gastrulation in the periblastoporal region (Fig. 6) where various FGF signaling pathway components are also expressed (Golub

et al., 2000; Hongo et al., 1999; Isaacs et al., 1995; Lombardo et al., 1998; Song and Slack, 1994; Song and Slack, 1996). We note that XRARα, XRARγ and bioactive retinoids are all present in the unfertilized egg (Blumberg et al., 1992). As RAR signaling is required for the expression of FGF receptors in neural tissue (Fig. 9), it is possible that the maternally expressed RAR genes are permissive for FGF signaling which is, in turn, instructive for the zygotic expression of RAR pathway components.

FGF gene and *XFD* overexpression experiments using *Xenopus* embryos suggested that FGF signaling is essential for neural posteriorization (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995). Analysis of transgenic frogs overexpressing *XFD* yielded somewhat conflicting results with one group suggesting that FGF signaling is involved in gastrulation, but not in posteriorization (Kroll and Amaya, 1996), and another demonstrating an absolute requirement for FGF signaling in neural posteriorization (Pownall et al., 1998). The discrepancy between mRNA injections and the two transgenic studies could be due to the different timing and levels of XFD protein produced from the transgenic promoters, as opposed to the relatively earlier expression of protein from the microinjected mRNA relative to the transgenic promoters. XFD protein might not be produced at sufficient levels early enough in the transgenic *Xenopus* embryo to completely block the zygotic expression of the genes required for RAR signaling. Our observations that inhibition of FGF signaling affected the expression of mRNAs encoding RA pathway components in the early gastrula (Fig. 6) and that RAR-MO injection alters the expression of FGF8, FGFR1 and FGFR4 (Fig. 8) suggests that RAR and FGF signaling crossregulate each other. The observation that RA upregulates *FGF8*, *FGFR1* and *FGFR4* while FGF8 upregulates *RARα*, *RALDH2* and *CYP26* in animal cap experiments (Fig. 7) supports the existence of a feedback loop that allows these posteriorizing factors to maintain each other's expression. The loss of *FGF8* and *FGF3* expression in *Raldh2*$^{-/-}$ mice (Niederreither et al., 1999) is also consistent with our findings.

## RA signaling is involved in multiple steps of neural posteriorization

The alteration in the expression of FGF signaling components by the RAR-MO (Fig. 8) together with the requirement for FGF signaling to express RAR pathway components (Fig. 6) supports the existence of such a mutual feedback loop. Isaacs and colleagues showed that XCAD3 upregulates *HOXB9* expression in *Xenopus* embryos using gain- and loss-of-function experiments (Isaacs et al., 1998; Pownall et al., 1998; Pownall et al., 1996). Their model was further supported by the identification of caudal/Cdx homeodomain-binding sites in the promoters of region of mouse and chick HOX genes (Charite et al., 1998; Subramanian et al., 1995). We noted that ectopic expression of *HOXB9* induced by *XCAD3* overexpression is restricted to the neural tube (Fig. 9C) and next examined the role of *XRARα* in *HOXB9* expression. Injection of *XCAD3* mRNA alone induced ectopic expression of *HOXB9* anterior to where it is normally expressed (Fig. 9C). Co-injection of the RAR-MO led to severely reduced expression of *HOXB9* throughout the embryo (Fig. 9D-F), suggesting that RAR function is required for XCAD3 to induce expression of *HOXB9*. Expression of *HOXB9* (Fig. 9C) is limited to neural regions after *XCAD3* overexpression, suggesting that other factors are responsible for permitting or restricting *HOXB9* expression to the developing neural tube.

## RAR signaling and regional boundaries within the developing CNS

The expression of *XCAD3* and *HOXB9* is reduced by RAR antagonists and receptor loss of function. Loss of *XCAD3* and *HOXB9* expression resulting from XFD-mediated blockade of FGF signaling can be rescued by co-injection of the constitutively active VP16-XRARα2 (Fig. 5). Therefore, we infer that RAR functions in the spinal cord region as a transcriptional activator, downstream of FGF signaling. This function for RAR is consistent with the restricted expression of the RA-synthesizing enzyme, *RALDH2*, in the spinal cord and lateral mesoderm of early frog embryos (Chen et al., 2001) and with RA rescue of posterior gene expression in XFD treated zebrafish embryos (Kudoh et al., 2002) or *Xenopus* animal cap explants (Bang et al., 1997). Increasing RAR signaling by microinjecting VP16-XRARα1 (Blumberg, 1997), treating embryos with RA or microinjecting *XRALDH2* (Chen et al., 2001) induces an anterior shift in the expression boundaries of midbrain and hindbrain markers. However, the position of the anterior border of *HOXB9* and *XCAD3* expression is unaffected by increases in RAR signaling (Fig. 5) (Blumberg, 1997; Chen et al., 2001) suggesting that RAR signaling is not responsible for setting this boundary. When expression of *HOXB9* (Fig. 5C) or *XCAD3* (Fig. 5F) is rescued by *XRARα2* or VP16-*XRARα2* in XFD injected embryos, the anterior border of the rescued expression is similar to that in the uninjected control side of the embryo. This argues that FGF signaling is probably not involved in regulating the anterior boundary of posterior marker expression. *FGF8* and *XCAD3* overexpression can elicit ectopic *HOXB9* expression in the anterior (Fig. 4). Both are required for XRARα expression but also need *XRARα2.2* to exert their effects on downstream genes such as *HOXB9*. The insufficiency of VP16-*XRARα2* (which activates transcription of RAR target genes strongly in the absence of retinoid ligands) to ectopically induce HOXB9 or *XCAD3*, argues against *CYP26* (which degrades RA) being the major factor blocking the expression of posterior genes in the head, as has been suggested for the zebrafish (Kudoh et al., 2002).

Inhibition of RAR signaling using an RAR antagonist or dominant-negative RAR led to the upregulation of anterior markers (Koide et al., 2001) and a caudal shift in the expression boundaries of anterior and hindbrain markers in frog embryos (Blumberg et al., 1997). Similar results were obtained by overexpressing the RA catabolizing enzyme *CYP26* (de Roos et al., 1999; Hollemann et al., 1998; Maden, 1999) in frog embryos or treating chick embryos with RAR antagonists (Dupe and Lumsden, 2001). Kudoh and colleagues recently showed that knockdown of *CYP26* expression led to downregulation of the anterior marker *OTX2* and anterior expansion of *HOXB1B*, *MEIS3* and *IRO3* (Kudoh et al., 2002). We previously reported that unlike the hindbrain and spinal cord, which require RAR as a transcriptional activator, RARα is required as a transcriptional repressor to allow anterior patterning (Koide et al., 2001). This aspect of RAR function coincides with the expression of the RA degrading enzyme

*CYP26* in regions anterior to the hindbrain (de Roos et al., 1999; Hollemann et al., 1998). Taken together, these results suggest that a delicate balance exists between RAR-mediated repression of target genes in the head that is required for anterior patterning and the RAR-mediated activation of target genes that is indispensable for the expression of posterior neural markers. The expression of region-specific markers in the hindbrain is exquisitely sensitive to alterations in RA signaling (Godsave et al., 1998; Kolm et al., 1997; van der Wees et al., 1998). Lumsden and colleagues showed convincingly that RA acts as a classic morphogen in the hindbrain but that it appeared to be generated locally at precise levels rather than forming a long-range gradient (Dupe and Lumsden, 2001). In the absence of retinoid signaling the hindbrain develops as a default R4, whereas increasing RA-signaling yields posterior rhombomeres in a concentration-dependent fashion (Dupe and Lumsden, 2001).

### Is there a role for retinoids and retinoid receptors other than in the hindbrain?

Although it is now well established that position in the hindbrain is set by precisely delivered levels of RA (Bel-Vialar et al., 2002; Chen et al., 2001; Dupe and Lumsden, 2001; Godsave et al., 1998; Hollemann et al., 1998; Kolm et al., 1997; van der Wees et al., 1998) the role of retinoids and retinoid receptors anterior and posterior to the hindbrain remains controversial. For example, we showed that inhibition of RAR function by overexpressing a dominant negative *XRARα1* led to posterior expansion of the forebrain marker *OTX2*, a posterior shift in the expression of the midbrain marker *EN2*, a posterior shift in the rhombomere 3 expression and loss of the rhombomere 5 expression of the hindbrain marker *KROX20* and downregulation of the posterior markers *HOXB9*, *XLIM1* and *N-tubulin* (Blumberg et al., 1997). By contrast, increasing RAR function by microinjecting the constitutively active VP16-*XRARα1* led to the opposite effect: a decreased and anterior shift in the expression border of *OTX2* and rostral shifts in *EN2* and *KROX20* expression although the anterior boundary of *HOXB9* expression was unaffected (Blumberg et al., 1997). Thus, we concluded that RARs were required for correct positional specification along the entire AP axis (Blumberg, 1997; Blumberg et al., 1997).

In contrast to our results, Sive and colleagues used a dominant-negative *Xenopus* RARα2.2 to show that interfering with retinoid signaling altered hindbrain pattering in *Xenopus* but had no observable effects on anterior (*XCG*, *OTX2*) or posterior (*HOXB9*) gene expression (Kolm et al., 1997). Durston and colleagues used a dominant-negative chicken RARα2 and came to a similar conclusion: that inhibition of RAR function substantially altered the expression patterns of certain genes expressed in the hindbrain (*KROX20*, *HOXB3*) but not others (*EN2*, *HOXB1*) although their results differed in some details from the previously noted studies (van der Wees et al., 1998). No alterations were noted in the expression patterns of *OTX2*, *HOXB7* and *HOXB9* (van der Wees et al., 1998).

These latter studies have led to the model that retinoids and retinoid receptors are only required for patterning of the hindbrain (Godsave et al., 1998; Niederreither et al., 2000; van der Wees et al., 1998). However, it is also plausible that the hindbrain is extremely sensitive to levels of retinoid signalling, whereas more anterior and posterior regions might be less sensitive. In this scenario, the efficacy of the reagents used could play an important role in the type of outcome observed. Less potent antagonists or dominant-negative receptors would be expected to show effects in the hindbrain but not in the forebrain or spinal cord. In accordance with this model and consistent with our results using a dominant-negative RAR (Blumberg et al., 1997), decreasing the amount of RA by microinjecting *XCYP26* mRNA into *Xenopus* embryos led to posterior expansion of *OTX2* expression, caudal shifts in the r3 band of *KROX20* and the loss of *KROX20* in r5 (or fusion with r3) (Hollemann et al., 1998). Similarly, increasing RA levels by microinjecting *RALDH2* mRNA into *Xenopus* embryos led to anterior shifts in the expression borders of the midbrain band of *OTX2* expression and of both r3 and r5 expression of *KROX20* (Chen et al., 2001) in accordance with our results using the constitutively active VP16-XRARα1 (Blumberg et al., 1997). No alteration in the expression boundary of *HOXB9* was noted by microinjection of *RALDH2*, *CYP26*, DN-RAR or VP16-RAR mRNAs (Blumberg et al., 1997; Chen et al., 2001; Hollemann et al., 1998). Taken together, these results show that RARs are required in the head as well as the hindbrain, confirming a role for RARs in the anterior regions of the embryo.

As noted above, no changes in *HOXB9* expression were noted in *Xenopus* embryos treated with RA, injected with dominant-negative *Xenopus RARα2.2* or chicken *RARα2* mRNAs (Godsave et al., 1998; Kolm et al., 1997; van der Wees et al., 1998). Consistent with these observations, VAD quail embryos (Maden et al., 1996) and chick embryos electroporated with a dominant-negative *Xenopus RARα1* (Bel-Vialar et al., 2002) show alterations in anterior (3′) HOX genes (*HOXB1*, *HOXB3*, *HOXB4*, *HOXB5*) but not posterior (5′) HOX gene (*HOXB6*, *HOXB9*) expression. These results stand in stark contrast to the results shown above and our previously published data showing inhibition by posterior marker genes after downregulation of RAR function by three different methods: microinjection of a dominant negative RARα1 (Blumberg et al., 1997), morpholino oligonucleotide-mediated knockdown of *RARα2.2* (Figs 2, 3, 4, 9) and treatment with the RAR antagonist AGN193109 (Figs 3, 10). One possible resolution for these discrepancies would be for the early and late expression of posterior markers to have different regulatory mechanisms. We propose that the early expression of posterior markers such as *XCAD3* depends on signaling through XRARα2 as a downstream target of FGFs (Figs 5, 10). This early retinoid dependent phase is followed by a later retinoid-independent, FGF-independent stage wherein the expression of these genes recovers, even in the apparent absence of retinoid (Fig. 10I,J) or FGF signaling (Pownall et al., 1998). This model has the advantage of explaining all of the observed data while simultaneously providing a potential function for the posterior expression of genes such as *XRARα2* (Fig. 1) (Sharpe, 1992), *XRARα2* (Ellinger-Ziegelbauer and Dreyer, 1993; Pfeffer and De Robertis, 1994) and *RALDH2* (Chen et al., 2001). Consistent with this proposal, the murine caudal gene, *CDX1* is induced by retinoic acid and downregulated in $Rara^{-/-}/Rarg^{-/-}$ embryos (Houle et al., 2000). Subsequent experiments showed that *CDX1* expression requires RA for expression only in early stages, later becoming dependent on WNT3A for its continued expression (Prinos et al., 2001). Posterior expression of

*HOXA1* is lost in *Raldh2*$^{-/-}$ embryos at day 8.75 (Niederreither et al., 1999) but has normalized by day 9.5 (Niederreither et al., 2000). *HOXB5A* and *HOXB6B* expression were also shown to be downregulated in no-fin mutant zebrafish, which are deficient in RALDH2 signaling (Grandel et al., 2002).

Experiments in *Xenopus* showed that RAR and FGF signaling patterned largely non-overlapping regions in the embryonic hindbrain and posterior (Kolm et al., 1997), and that XCAD3 is required for the expression of posterior HOX genes as a downstream target of FGF signaling (Isaacs et al., 1998; Pownall et al., 1998; Pownall et al., 1996). 3′ HOX genes that are expressed in the hindbrain and midaxial regions such as *HOXB1*, *HOXB3* and *HOXB4* were shown to be extremely sensitive to retinoid signaling whereas they are insensitive to changes in FGF signaling in both chicken (Bel-Vialar et al., 2002) and *Xenopus* (Epstein et al., 1997; Isaacs et al., 1998). Our data are consistent with an instructive role for RAR signaling in the hindbrain and midaxial regions. By contrast, posterior genes such as *XCAD3* and *HOXB9* require RAR signaling in a permissive role downstream of FGF signaling but are also required for the expression of FGF signaling pathway components. Therefore, we propose that the developing CNS can be divided into three regions that differ with regard to the expression of genes responsive to RAR signaling. In the first region, most anterior neural tissue requires RAR as a transcriptional repressor, reinforced by the action of CYP26 to degrade any RA that might be present outside the restricted areas that require RA, such as the developing eye. The hindbrain represents the limits of the second region, and expresses neither *CYP26* nor *RALDH2*. Hindbrain specification requires localized activation of RAR to express region specific genes specifying rhombomere identity (Dupe and Lumsden, 2001; Maden, 1999; Maden, 2002). The third region, the spinal cord, which is represented by *XCAD3* and *HOXB9* expression, requires RAR as a transcriptional activator in frogs (Figs 5, 10) and zebrafish (Kudoh et al., 2002), although RAR may be playing a permissive, rather than an instructive, role in this process.

Left unexplained is the observation that knockout of multiple murine RARs (e.g. *Rara*$^{-/-}$/*Rarb*$^{-/-}$) is required to obtain unambiguous developmental phenotypes (Mark et al., 1999), whereas our results using morpholino oligonucleotide-mediated gene knockdown presented here (Figs 2-4, 8, 9) and elsewhere (Koide et al., 2001) show that one need only inhibit expression of a single RAR isoform to generate consistent phenotypes. This observation becomes rather more remarkable when once considers that retinoids can activate other signaling proteins such as NURR1 (Perlmann and Jansson, 1995; Zetterstrom et al., 1996) RORβ (Stehlin-Gaon et al., 2003) and PPARβ/δ (Shaw et al., 2003). We previously speculated that the maternal expression of *Xenopus RARα* and *RARγ* mRNAs (Blumberg et al., 1992; Sharpe, 1992), coupled with the relatively late initiation of transcription in *Xenopus* (mid-blastula, ~4000-cell stage) (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b) compared with mouse (two-cell stage) made *Xenopus* more sensitive to perturbations in RAR levels than mouse. The requirement for RAR function relatively early in developmental patterning (Fig. 10) (Koide et al., 2001) is consistent with the possibility that RAR protein might be required too early for loss of expression to be compensated for by upregulation of another RAR gene.

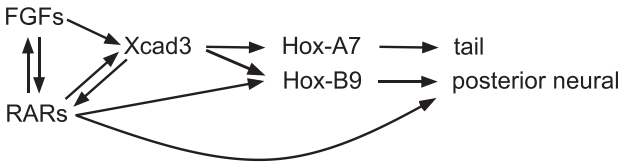


**Fig. 11.** Schematic model of the interactions among FGF, RAR and XCAD3 signaling pathways.

The experiments described above reveal complex interactions among FGF, XCAD3 and retinoid signaling (Fig. 11). Our results demonstrate that RARs act both upstream and downstream of FGF signaling to pattern the AP axis. *Xenopus* RARα2.2 is required for the correct expression of *FGF8*, *FGFR1* and *FGFR4* (Fig. 8), while FGF signaling is required for the zygotic expression of RAR signaling pathway components (Fig. 6). Embryos deficient in *RARα* do not form heads (Koide et al., 2001) and primary neurons do not differentiate (Blumberg et al., 1997; Sharpe and Goldstone, 2000; Sharpe and Goldstone, 1997). This suggests that retinoid signaling is an essential component of the positional patterning system operational at the very earliest stages of embryonic development. The observations relating retinoid signaling and the expression of neurogenic markers all clearly indicate a subsequent role for retinoids in neuronal differentiation. Activity of the RARs is required for the correct expression of proneural and prepatterning genes operating at the earliest steps of neural development (Franco et al., 1999; Paganelli et al., 2001). Considering that the timing of neuronal differentiation is coupled with that of AP patterning (Papalopulu and Kintner, 1996), it is plausible that retinoid signaling may play a key role in linking these two crucial developmental processes.

We thank K. Cho, I. Blitz, C. Zilinski and members of the Blumberg laboratory for comments on the manuscript, and N. Papalopulu, H. Isaacs and H. Okamoto for contributions of plasmids and probes. Supported by grants from the NIH (GM-60572) and NSF (IBN-9904764) to B.B. J.S. was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (01-PJ1-PG1-01CH11-0003).

## References

**Agathon, A., Thisse, C. and Thisse, B.** (2003). The molecular nature of the zebrafish tail organizer. *Nature* **434**, 448-452.

**Allan, D., Houle, M., Bouchard, N., Meyer, B. I., Gruss, P. and Lohnes, D.** (2001). RARgamma and Cdx1 interactions in vertebral patterning. *Dev. Biol.* **240**, 46-60.

**Amaya, E., Musci, T. J. and Kirschner, M. W.** (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell* **66**, 257-270.

**Amaya, E., Stein, P. A., Musci, T. J. and Kirschner, M. W.** (1993). FGF signalling in the early specification of mesoderm in Xenopus. *Development* **118**, 477-487.

**Bang, A. G., Papalopulu, N., Kintner, C. and Goulding, M. D.** (1997). Expression of Pax-3 is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non- axial mesoderm. *Development* **124**, 2075-2085.

**Bang, A. G., Papalopulu, N., Goulding, M. D. and Kintner, C.** (1999). Expression of Pax-3 in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. *Dev. Biol.* **212**, 366-380.

**Bel-Vialar, S., Itasaki, N. and Krumlauf, R.** (2002). Initiating Hox gene

expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. *Development* **129**, 5103-5115.

**Blumberg, B.** (1997). An essential role for retinoid signaling in anteroposterior neural specification and neuronal differentiation. *Semin. Cell Dev. Biol.* **8**, 417-428.

**Blumberg, B., Wright, C. V. E., de Robertis, E. M. and Cho, K. W. Y.** (1991). Organizer-specific homeobox genes in Xenopus laevis embryos. *Science* **253**, 194-196.

**Blumberg, B., Mangelsdorf, D. J., Dyck, J., Bittner, D. A., Evans, R. M. and de Robertis, E. M.** (1992). Multiple retinoid-responsive receptors in a single cell: families of RXRs and RARs in the Xenopus egg. *Proc. Natl. Acad. Sci. USA* **89**, 2321-2325.

**Blumberg, B., Bolado, J., Jr, Derguini, F., Craig, A. G., Moreno, T. A., Charkravarti, D., Heyman, R. A., Buck, J. and Evans, R. M.** (1996). Novel RAR ligands in Xenopus embryos. *Proc. Natl. Acad. Sci. USA* **93**, 4873-4878.

**Blumberg, B., Bolado, J., Moreno, T. A., Kintner, C., Evans, R. M. and Papalopulu, N.** (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

**Boehm, M. F., Zhang, L., Badea, B. A., White, S. K., Mais, D. E., Berger, E., Suto, C. M., Goldman, M. E. and Heyman, R. A.** (1994). Synthesis and structure-activity relationships of novel retinoid X receptor-selective retinoids. *J. Med. Chem.* **37**, 2930-2941.

**Chambon, P.** (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940-954.

**Charite, J., de Graaff, W., Consten, D., Reijnen, M. J., Korving, J. and Deschamps, J.** (1998). Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. *Development* **125**, 4349-4358.

**Chen, Y., Pollet, N., Niehrs, C. and Pieler, T.** (2001). Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of Xenopus embryos. *Mech. Dev.* **101**, 91-103.

**Cho, K. W. Y. and de Robertis, E. M.** (1990). Differential activation of Xenopus homeobox genes by mesoderm inducing growth factors and retinoic acid. *Genes Dev.* **4**, 1910-1916.

**Christen, B. and Slack, J. M.** (1997). FGF-8 is associated with anteroposterior patterning and limb regeneration in Xenopus. *Dev. Biol.* **192**, 455-466.

**Christian, J. L. and Moon, R. T.** (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus. *Genes Dev.* **7**, 13-28.

**Conlon, F. L., Sedgwick, S. G., Weston, K. M. and Smith, J. C.** (1996). Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. *Development* **122**, 2427-2435.

**Cox, W. G. and Hemmati-Brivanlou, A.** (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development* **121**, 4349-4358.

**Damm, K., Heyman, R. A., Umesono, K. and Evans, R. M.** (1993). Functional inhibition of retinoic acid response by dominant negative retinoic acid receptor mutants. *Proc. Natl. Acad. Sci. USA* **90**, 2989-2993.

**de Roos, K., Sonneveld, E., Compaan, B., ten Berge, D., Durston, A. J. and van der Saag, P. T.** (1999). Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and Xenopus laevis embryogenesis. *Mech. Dev.* **82**, 205-211.

**Dickman, E. D., Thaller, C. and Smith, S. M.** (1997). Temporally-regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* **124**, 3111-3121.

**Domingos, P. M., Itasaki, N., Jones, S. A., Mercurio, S., Sargent, M. G., Smith, J. C. and Krumlauf, R.** (2001). The Wnt/b-catenin pathway posteriorizes neural tissue in Xenopus by an indirect mechanism requiring FGF signaling. *Dev. Biol.* **239**, 148-160.

**Doniach, T.** (1995). Basic FGF as an inducer of anteroposterior neural pattern. *Cell* **83**, 1067-1070.

**Dupe, V., Davenne, M., Brocard, J., Dolle, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. M.** (1997). In vivo functional analysis of the Hoxa-1 3′ retinoic acid response element (3′RARE). *Development* **124**, 399-410.

**Dupe, V. and Lumsden, A.** (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. *Development* **128**, 2199-2208.

**Durston, A. J., van der Wees, J., Pijnappel, W. W. and Godsave, S. F.** (1998). Retinoids and related signals in early development of the vertebrate central nervous system. *Curr. Top. Dev. Biol.* **40**, 111-175.

**Ellinger-Ziegelbauer, H. and Dreyer, C.** (1993). The pattern of retinoic acid receptor g (RARg) expression in normal development of Xenopus laevis and after manipulation of the main body axis. *Mech. Dev.* **41**, 33-46.

**Epstein, M., Pillemer, G., Yelin, R., Yisraeli, J. K. and Fainsod, A.** (1997). Patterning of the embryo along the anterior-posterior axis: the role of the caudal genes. *Development* **124**, 3805-3814.

**Erter, C. E., Wilm, T. P., Basler, N., Wright, C. V. and Solnica-Krezel, L.** (2001). Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. *Development* **128**, 3571-3583.

**Eyal-Giladi, H.** (1954). Dynamic aspects of neural induction. *Arch. Biol.* **65**, 180-259.

**Fainsod, A., Deissler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H. and Blum, M.** (1997). The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech. Dev.* **63**, 39-50.

**Fekany-Lee, K., Gonzalez, E., Miller-Bertoglio, V. and Solnica-Krezel, L.** (2000). The homeobox gene bozozok promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways. *Development* **127**, 2333-2345.

**Franco, P. G., Paganelli, A. R., Lopez, S. L. and Carrasco, A. E.** (1999). Functional association of retinoic acid and hedgehog signaling in Xenopus primary neurogenesis. *Development* **126**, 4257-4265.

**Gavalas, A. and Krumlauf, R.** (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev.* **10**, 380-386.

**Godsave, S. F., Koster, C. H., Getahun, A., Mathu, M., Hooiveld, M., van der Wees, J., Hendriks, J. and Durston, A. J.** (1998). Graded retinoid responses in the developing hindbrain. *Dev. Dyn.* **213**, 39-49.

**Golub, R., Adelman, Z., Clementi, J., Weiss, R., Bonasera, J. and Servetnick, M.** (2000). Evolutionarily conserved and divergent expression of members of the FGF receptor family among vertebrate embryos, as revealed by FGFR expression patterns in Xenopus. *Dev. Genes Evol.* **210**, 345-357.

**Grandel, H., Lun, K., Rauch, G. J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Kuchler, A. M., Schulte-Merker, S., Geisler, R. et al.** (2002). 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* **129**, 2851-2865.

**Hardcastle, Z., Chalmers, A. D. and Papalopulu, N.** (2000). FGF-8 stimulates neuronal differentiation through FGFR-4a and interferes with mesoderm induction in Xenopus embryos. *Curr. Biol.* **10**, 1511-1514.

**Harland, R.** (2000). Neural induction. *Curr. Opin. Genet. Dev.* **10**, 357-362.

**Hawley, S. H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. and Cho, K. W. Y.** (1995). Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-2935.

**Heasman, J., Kofron, M. and Wylie, C.** (2000). Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

**Hemmati-Brivanlou, A. and Melton, D. A.** (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77**, 273-281.

**Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A.** (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.

**Hollemann, T., Chen, Y., Grunz, H. and Pieler, T.** (1998). Regionalized metabolic activity establishes boundaries of retinoic acid signalling. *EMBO J.* **17**, 7361-7372.

**Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., de Robertis, E. M., Hoffmann, F. M. and Ferguson, E. L.** (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. *Nature* **376**, 249-253.

**Hongo, I., Kengaku, M. and Okamoto, H.** (1999). FGF signaling and the anterior neural induction in Xenopus. *Dev. Biol.* **216**, 561-581.

**Hoppler, S., Brown, J. D. and Moon, R. T.** (1996). Expression of a dominant-negative Wnt blocks induction of MyoD in Xenopus embryos. *Genes Dev.* **10**, 2805-2817.

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

**Huang, D., Chen, S. W. and Gudas, L. J.** (2002). Analysis of two distinct retinoic acid response elements in the homeobox gene Hoxb1 in transgenic mice. *Dev. Dyn.* **223**, 353-370.

**Huang, D., Chen, S. W., Langston, A. W. and Gudas, L. J.** (1998). A conserved retinoic acid responsive element in the murine Hoxb-1 gene is required for expression in the developing gut. *Development* **125**, 3235-3246.

**Isaacs, H. V., Pownall, M. E. and Slack, J. M.** (1995). eFGF is expressed in the dorsal midline of Xenopus laevis. *Int. J. Dev. Biol.* **39**, 575-579.

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

**Johnson, A. T., Klein, E. S., Gillett, S. J., Wang, L., Song, T. K., Pino, M. E. and Chandraratna, R. A.** (1995). Synthesis and characterization of a highly potent and effective antagonist of retinoic acid receptors. *J. Med. Chem.* **38**, 4764-4767.

**Kengaku, M. and Okamoto, H.** (1995). bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in Xenopus. *Development* **121**, 3121-3130.

**Kessel, M.** (1992). Respecification of vertebral identities by retinoic acid. *Development* **115**, 487-501.

**Kessel, M. and Gruss, P.** (1991). Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell* **67**, 89-104.

**Kim, C. H., Oda, T., Itoh, M., Jiang, D., Artinger, K. B., Chandrasekharappa, S. C., Driever, W. and Chitnis, A. B.** (2000). Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature* **407**, 913-916.

**Koide, T., Downes, M., Chandraratna, R. A., Blumberg, B. and Umesono, K.** (2001). Active repression of RAR signaling is required for head formation. *Genes Dev.* **15**, 2111-2121.

**Kolm, P. J., Apekin, V. and Sive, H.** (1997). Xenopus hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1-16.

**Kroll, K. L. and Amaya, E.** (1996). Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122**, 3173-3183.

**Kudoh, T., Wilson, S. W. and Dawid, I. B.** (2002). Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* **129**, 4335-4346.

**Lamb, T. M. and Harland, R. M.** (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3627-3636.

**Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M.** (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713-718.

**Lekven, A. C., Thorpe, C. P., Waxman, J. S. and Moon, R. T.** (2001). Zebrafish Wnt8 encodes two Wnt8 proteins on a bicistronic transcript and is required for mesoderm and neuroectoderm patterning. *Dev. Cell* **1**, 103-114.

**Lombardo, A., Isaacs, H. V. and Slack, J. M.** (1998). Expression and functions of FGF-3 in Xenopus development. *Int. J. Dev. Biol.* **42**, 1101-1107.

**Maden, M.** (1999). Heads or tails? Retinoic acid will decide. *BioEssays* **21**, 809-812.

**Maden, M.** (2002). Retinoid signalling in the development of the central nervous system. *Nat. Rev. Neurosci.* **3**, 843-853.

**Maden, M., Gale, E., Kostetskii, I. and Zile, M.** (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6**, 417-426.

**Mark, M., Ghyselinck, N. B., Wendling, O., Dupe, V., Mascrez, B., Kastner, P. and Chambon, P.** (1999). A genetic dissection of the retinoid signalling pathway in the mouse. *Proc. Nutr. Soc.* **58**, 609-613.

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

**McGrew, L. L., Hoppler, S. and Moon, R. T.** (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in Xenopus. *Mech. Dev.* **69**, 105-114.

**McGrew, L. L., Lai, C. J. and Moon, R. T.** (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. *Dev. Biol.* **172**, 337-342.

**Newport, J. and Kirschner, M.** (1982a). A major developmental transition in early Xenopus embryos: II. Control of the onset of transcription. *Cell* **30**, 687-696.

**Newport, J. and Kirschner, M.** (1982b). A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* **30**, 675-686.

**Niederreither, K., Subbarayan, V., Dolle, P. and Chambon, P.** (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.

**Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dolle, P.** (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.

**Nieuwkoop, P. D.** (1952). Activation and organization of the central nervous system in amphibians. Part III. synthesis of a new working hypothesis. *J. Exp. Zool.* **120**, 83-108.

**Nieuwkoop, P. O. and Faber, J.** (1967). *A Normal Table of* Xenopus laevis *(Daudin)*. Amsterdam: North Holland.

**Northrop, J. L. and Kimelman, D.** (1994). Dorsal-ventral differences in Xcad-3 expression in response to FGF- mediated induction in Xenopus. *Dev. Biol.* **161**, 490-503.

**Ogura, T. and Evans, R. M.** (1995a). Evidence for two distinct retinoic acid response pathways for HOXB1 gene regulation. *Proc. Natl. Acad. Sci. USA* **92**, 392-396.

**Ogura, T. and Evans, R. M.** (1995b). A retinoic acid-triggered cascade of HOXB1 gene activation. *Proc. Natl. Acad. Sci. USA* **92**, 387-391.

**Paganelli, A. R., Ocana, O. H., Prat, M. I., Franco, P. G., Lopez, S. L., Morelli, L., Adamo, A. M., Riccomagno, M. M., Matsubara, E., Shoji, M. et al.** (2001). The Alzheimer-related gene presenilin-1 facilitates sonic hedgehog expression in Xenopus primary neurogenesis. *Mech. Dev.* **107**, 119-131.

**Papalopulu, N. and Kintner, C.** (1996). A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in Xenopus neuroectoderm. *Development* **122**, 3409-3418.

**Perlmann, T. and Jansson, L.** (1995). A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes Dev.* **9**, 769-782.

**Pfeffer, P. L. and de Robertis, E. M.** (1994). Regional specificity of RAR gamma isoforms in Xenopus development. *Mech. Dev.* **45**, 147-153.

**Piccolo, S., Sasai, Y., Lu, B. and de Robertis, E. M.** (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.

**Pownall, M. E., Tucker, A. S., Slack, J. M. W. and Isaacs, H. V.** (1996). eFGF, Xcad3, and Hox genes form a molecular pathway that establishes the anteroposterior axis in Xenopus. *Development* **122**, 3881-3892.

**Pownall, M. E., Isaacs, H. V. and Slack, J. M.** (1998). Two phases of Hox gene regulation during early Xenopus development. *Curr. Biol.* **8**, 673-676.

**Prinos, P., Joseph, S., Oh, K., Meyer, B. I., Gruss, P. and Lohnes, D.** (2001). Multiple pathways governing Cdx1 expression during murine development. *Dev. Biol.* **239**, 257-269.

**Sambrook, J. and Russell, D. W.** (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

**Sato, S. M. and Sargent, T. D.** (1989). Development of neural inducing capacity in dissociated Xenopus embryos. *Dev. Biol.* **134**, 263-266.

**Sharpe, C. and Goldstone, K.** (2000). The control of Xenopus embryonic primary neurogenesis is mediated by retinoid signalling in the neurectoderm. *Mech. Dev.* **91**, 69-80.

**Sharpe, C. R.** (1992). Two isoforms of retinoic acid receptor alpha expressed during Xenopus development respond to retinoic acid. *Mech. Dev.* **39**, 81-93.

**Sharpe, C. R. and Goldstone, K.** (1997). Retinoid receptors promote primary neurogenesis in Xenopus. *Development* **124**, 515-523.

**Sharpe, C. R., Fritz, A., de Robertis, E. M. and Gurdon, J. B.** (1987). A homeobox-containing marker of posterior neural differentiation shows the importance of predetermination in neural induction. *Cell* **50**, 749-758.

**Shaw, N., Elholm, M. and Noy, N.** (2003). Retinoic acid is a high affinity selective ligand for the peroxisome proliferator-activated receptor beta/delta. *J. Biol. Chem.* **278**, 41589-41592.

**Simeone, A., Acampora, P., Arcioni, Andrews, P. W., Boncinelli, E. and Mavilio, F.** (1990). Sequential activation of Hox 2 homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature* **346**, 763-766.

**Simeone, A., Acampora, D., Nigro, V., Faiella, A., D'Esposito, M., Stornainolo, A., Mavillio, F. and Boncinelli, E.** (1991). Differential regulation by retinoic acid of the homeobox genes of the four HOX loci in human embryonal carcinoma cells. *Mech. Dev.* **33**, 215-228.

**Simeone, A., Avantaggiato, V., Moroni, M. C., Mavilio, F., Arra, C., Cotelli, F., Nigro, V. and Acampora, D.** (1995). Retinoic acid induces stage-specific antero-posterior transformation of rostral central nervous system. *Mech. Dev.* **51**, 83-98.

**Sive, H. L., Grainger, R. M. and Harland, R. M.** (2000). *Early Development of* Xenopus laevis*: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

**Smith, J. C., Price, B. M., Green, J. B., Weigel, D. and Herrmann, B. G.** (1991). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

**Song, J. and Slack, J. M.** (1994). Spatial and temporal expression of basic fibroblast growth factor (FGF-2) mRNA and protein in early Xenopus development. *Mech Dev.* **48**, 141-151.

**Song, J. and Slack, J. M.** (1996). XFGF-9: a new fibroblast growth factor from Xenopus embryos. *Dev. Dyn.* **206**, 427-436.

**Stehlin-Gaon, C., Willmann, D., Zeyer, D., Sanglier, S., van Dorsselaer, A., Renaud, J. P., Moras, D. and Schule, R.** (2003). All-trans retinoic acid is a ligand for the orphan nuclear receptor ROR beta. *Nat. Struct. Biol.* **10**, 820-825.

**Subramanian, V., Meyer, B. I. and Gruss, P.** (1995). Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell* **83**, 641-653.

**Tabb, M. M., Sun, A., Errandi, J. L., Zhou, C., Grün, F., Romero, K., Inoue, S., Mallick, S., Lin, M., Forman, B. M. et al.** (2003). Vitamin K2 regulation of bone homeostasis is mediated by the orphan receptor, SXR. *J. Biol. Chem.* **278**, 43919-43927.

**van der Wees, J., Schilthuis, J. G., Koster, C. H., Diesveld-Schipper, H., Folkers, G. E., van der Saag, P. T., Dawson, M. I., Shudo, K., van der Burg, B. and Durston, A. J.** (1998). Inhibition of retinoic acid receptor-mediated signalling alters positional identity in the developing hindbrain. *Development* **125**, 545-556.

**White, J. C., Shankar, V. N., Highland, M., Epstein, M. L., DeLuca, H. F. and Clagett-Dame, M.** (1998). Defects in embryonic hindbrain development and fetal resorption resulting from vitamin A deficiency in the rat are prevented by feeding pharmacological levels of all-trans-retinoic acid. *Proc. Natl. Acad. Sci. USA* **95**, 13459-13464.

**Xu, R. H., Kim, J., Taira, M., Zhan, S., Sredni, D. and Kung, H. F.** (1995). A dominant negative bone morphogenetic protein 4 receptor causes neuralization in Xenopus ectoderm. *Biochem. Biophys. Res. Commun.* **212**, 212-219.

**Zetterstrom, R. H., Solomin, L., Mitsiadis, T., Olson, L. and Perlmann, T.** (1996). Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. *Mol. Endocrinol.* **10**, 1656-1666.

**Zhang, F., Popperl, H., Morrison, A., Kovacs, E. N., Prideaux, V., Schwarz, L., Krumlauf, R., Rossant, J. and Featherstone, M. S.** (1997). Elements both 5′ and 3′ to the murine Hoxd4 gene establish anterior borders of expression in mesoderm and neurectoderm. *Mech. Dev.* **67**, 49-58.

**Zimmerman, L. B., de Jesus-Escobar, J. M. and Harland, R. M.** (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.