Dorsoventral patterning of the *Xenopus* eye: a collaboration of Retinoid, Hedgehog and FGF receptor signaling

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Summary

In the developing spinal cord and telencephalon, ventral patterning involves the interplay of Hedgehog (Hh), Retinoic Acid (RA) and Fibroblast Growth Factor (FGF) signaling. In the eye, ventral specification involves Hh signaling, but the roles of RA and FGF signaling are less clear. By overexpression assays in *Xenopus* embryos, we found that both RA and FGF receptor (FGFR) signaling ventralize the eye, by expanding optic stalk and ventral retina, and repressing dorsal retina character. Co-overexpression experiments show that RA and FGFR can collaborate with Hh signaling and reinforce its ventralizing activity. In loss-of-function experiments, a strong eye dorsalization was observed after triple inhibition of Hh, RA and FGFR signaling, while weaker effects were obtained by inhibiting only one or two of these pathways. These results suggest that the ventral regionalization of the eye is specified by interactions of Hh, RA and FGFR signaling. We argue that similar mechanisms might control ventral neural patterning throughout the central nervous system.

Key words: Ventral retina, Dorsal retina, Optic stalk, Retinoic acid, Hedgehog, FGF receptor, *Xenopus*

Introduction

Dorsoventral (DV) patterning of the vertebrate eye underlies important properties of the visual system. First, it controls subdivision of the eye into optic stalk (OS) and retina, which form from the ventromedial and the dorsolateral parts of the optic vesicle, respectively (Chow and Lang, 2001). Second, the retina itself is patterned along the DV axis. A landmark of retina DV polarity is the choroid fissure at the ventral pole of the retina. Retinal neurogenesis is initiated in this region and then progresses to the DR (Peters, 2002; Russell, 2003). DV asymmetries also exist in the distribution of differentiated cell types within the retina (Peters, 2002). Finally, ganglion cells located in the dorsal retina (DR) and the ventral retina (VR) send their axons to the lateral and medial optic tectum, respectively, creating an inverted topographic map of retinotectal projections (McLaughlin et al., 2003).

Before these morphogenetic events of DV specification, asymmetrically expressed transcription factors regionalize the optic vesicle into three main DV compartments. From ventral to dorsal, these are: the OS, which expresses *Pax2*, *Vax1* and *Vax2*, but not *Pax6* and *Tbx5*; the VR, which expresses *Vax2* and *Pax6*, but not *Pax2*, *Vax1* or *Tbx5*; the DR, which expresses *Pax6* and *Tbx5*, but not *Pax2*, *Vax1* and *Vax2* (Barbieri et al., 2002; Bertuzzi et al., 1999; Hallonet et al., 1999; Koshiba-Takeuchi et al., 2000; Liu et al., 2001; Mui et al., 2002; Schwarz et al., 2000; Torres et al., 1996).

How is this transcriptional code established? In the developing spinal cord, opposing ventralizing and dorsalizing activities of Hedgehog (Hh) and Bone Morphogenetic Protein (BMP) signaling pathways have key roles in the specification of DV polarity upstream of transcription factors (Ruiz i Altaba et al., 2003). Nonetheless, recent studies have indicated that ventral patterning cannot be ascribed to Hh signaling alone, and Retinoic Acid (RA) and Fibroblast Growth Factor (FGF) signaling are also crucial players in this process (Appel and Eisen, 2003; Diez del Corral and Storey, 2004; Harris, 2003). Recent work has also shown that RA and FGF are required, in addition to Hh signaling, to elicit full specification of the ventral telencephalon (Lupo et al., 2002; Marklund et al., 2004; Shinya et al., 2001).

By analogy to the neural tube, it has been suggested that eye DV polarity may also be established by Hh and BMP antagonistic activities (Russell, 2003; Wilson and Houart, 2004; Yang, 2004). BMP overexpression in the retina has a strong dorsalizing effect (Koshiba-Takeuchi et al., 2000; Sasagawa et al., 2002), while BMP inhibition ventralizes the eye (Murali et al., 2005; Sakuta et al., 2001; Sasagawa et al., 2002). As to Hh signaling, although its role in OS specification

is well known on the basis of both loss- and gain-of-function assays (Russell, 2003; Yang, 2004), its role in DV patterning of the retina is less clear (Perron et al., 2003; Zhang and Yang, 2001). RA and FGF signaling pathways may also play a role in the ventralization of the eye. The VR is richer in RA than the DR (Drager et al., 2001), suggesting that high RA levels may specify ventral character in the eye. RA treatments upregulate the OS marker *Pax2* in zebrafish embryos (Hyatt et al., 1996). By contrast, reduction of embryonic RA signaling by means of vitamin A deficiency, knock out of RA receptors (RAR) or exposure to citral caused morphological deficits in the VR (Kastner et al., 1994; Marsh-Armstrong et al., 1994; Ross et al., 2000). However, a lack of appropriate molecular markers meant that it was not possible to determine whether the observed morphological defects were due to impaired DV specification rather than abnormal morphogenesis, growth or survival of the VR. As to FGF signaling, although it may be responsible for maintaining some ventral eye gene expression in the absence of all Nodal and Hh signaling in zebrafish (Take-uchi et al., 2003), in the studies reported so far, inhibition of FGF signals produced only weak effects on the expression of ventral eye genes (Shanmugalingam et al., 2000; Walshe and Mason, 2003), thus leaving the role of FGF signaling during ventral eye specification not fully resolved.

Here, we study the role of Hh, RA and FGF Receptor (FGFR) signaling in DV patterning of the *Xenopus* eye. Our results suggest that specification of the OS and VR depends on the levels of Hh, RA and FGFR signaling interacting on these territories during development. We argue that similar mechanisms may control ventral patterning throughout the central nervous system (CNS).

Materials and methods

Cloning of full-length Raldh3 cDNA

A 1.3 kb fragment was amplified by RT-PCR from stage 40 head cDNA using the following primers: RAL-3F 5′-AA(AG) AT(ACT) TT(CT) AT(ACT) AA(CT) AA(CT) GAI TGG-3′; RAL-3R 5′-GAC AT(CT) TT(AG) AAI CCI CC(AG) AAI GG-3′. After gel purification, the PCR product was cloned into pGEM-T vector (Promega). Plasmid DNA was recovered from 18 independent clones: 11 corresponded to *Xenopus Raldh1*; 2 to *Xenopus Raldh2*, while five clones showed the highest homology to chick and mouse *Raldh3* and corresponded to *Xenopus Raldh3* (*Xraldh3*). Full-length *Xraldh3* cDNA was obtained by RACE-PCR using a SMART RACE cDNA amplification kit (Clontech).

Xenopus embryos and in situ hybridization

Embryos were obtained and staged as previously described (Nieuwkoop and Faber, 1967). Whole-mount in situ hybridization was performed as described by Harland (Harland, 1991). Whole-mount in situ hybridization on dissected retinas, double in situ hybridizations, and sectioning of whole-mount hybridized embryos were carried out as previously described (Liu et al., 2001).

RNA methods and microinjections

Capped mRNAs were synthesized from linearized plasmid templates using mMESSAGE mMACHINE kits (Ambion). Embryos were injected as previously described (Vignali et al., 2000). Injections were performed at the eight-cell stage in one or both dorsal-animal blastomeres. The following template plasmids were used: *banded hedgehog*, pT7TS-Xbhh (Ekker et al., 1995); *iFGFR1*, pCS2+-iFGFR1 (Pownall et al., 2003); and *Raldh3*, pCS2+-Xraldh3. iFGFR1 activity was induced by immersion of embryos in 0.1×MBS supplemented with 1.25 μM AP20187 (ARIAD Pharmaceuticals; http://www.ariad.com), from 1 mM stock in 100% ethanol. When β-galactosidase (β-gal) was used as a lineage tracer, embryos were co-injected with 100-500 pg of β-gal mRNA and stained as previously described (Andreazzoli et al., 1999).

Treatments with retinoids and receptor antagonists

For retinoid treatments, embryos were treated in the dark with all-trans-Retinoic Acid (Sigma) or all-trans-Retinal (Sigma), diluted in 0.1×MBS from 10-100 mM stocks in DMSO. Loss-of-function experiments were performed with the following compounds: AGN194310 (Allergan; http://www.allergan.com), dissolved in 25 mM stock in DMSO; cyclopamine (a gift from W. Gaffield, and Toronto Research Chemicals), dissolved in 20 mM stock in 95% ethanol; SU5402 (Calbiochem), dissolved in 25 mM stock in DMSO. Embryos were exposed in the dark to appropriate concentrations of these inhibitors diluted in 0.1×MBS. Control embryos were treated with identical concentrations of DMSO and/or ethanol.

Results

Overexpression of Hh, RA and FGFR signaling pathways causes ventralization of the eye with dose-dependent effects

The *Xenopus* eye at stage 33 comprises three main regions (Fig. 1): the most ventral OS, which is *Pax2*, *Vax1b*, *Raldh3* and *Vax2* positive but *Pax6* negative; the VR, which is *Vax2* and *Pax6* positive but negative for both OS markers and *ET*; and the DR, which is *ET* and *Pax6* positive but negative for all the ventral eye markers. This DV organization was clearly affected in embryos unilaterally injected with high doses (250 pg) of *bhh* mRNA (Fig. 1B). On the injected sides, both *Vax2* and the OS markers *Pax2*, *Vax1b* and *Raldh3* were upregulated, while *Pax6* and *ET* were downregulated, suggesting that high levels of Hh signaling transform the retina into OS. To address whether Hh overexpression could also modify the DV character of the retina, without transforming it into OS, we injected low doses (1 pg) of *bhh* mRNA. As shown in Fig.1A, these doses caused a partial expansion of OS markers and slightly reduced *Pax6* domain. However, expression of the VR marker *Vax2* spread throughout the retina, while expression of the DR marker *ET* was reduced, suggesting that the DR has, at least in part, acquired VR, rather than OS, character. Similar results were obtained after overexpression of *shh* mRNA (data not shown).

RA treatments during gastrulation cause caudalization and loss of head structures (Durston et al., 1989). Therefore, we started exposure to RA at early neurula stages (stage 12.5/13), and performed dose-response experiments with concentrations of RA varying from 0.01 μM to 10 μM. As shown in Fig. 2B and see Fig. S1 in the supplementary material, treatments of *Xenopus* embryos with high doses of RA (10 μM) from stage 12.5/13 to stage 33 expanded *Vax2* expression throughout the eye, while both DR markers (*Vent2* and *ET*) and OS markers (*Pax2*, *Vax1b* and *Raldh3*) were repressed. In these embryos, *Pax6* was normally expressed in the retina. Although doses of 10 μM repressed the OS, lower RA doses (0.1-0.2 μM) caused a different effect. In these cases, the expression of *Vax2* and *ET* was only slightly affected, while the expression domains of the OS markers *Pax2*, *Vax1b* and *Raldh3* were all significantly

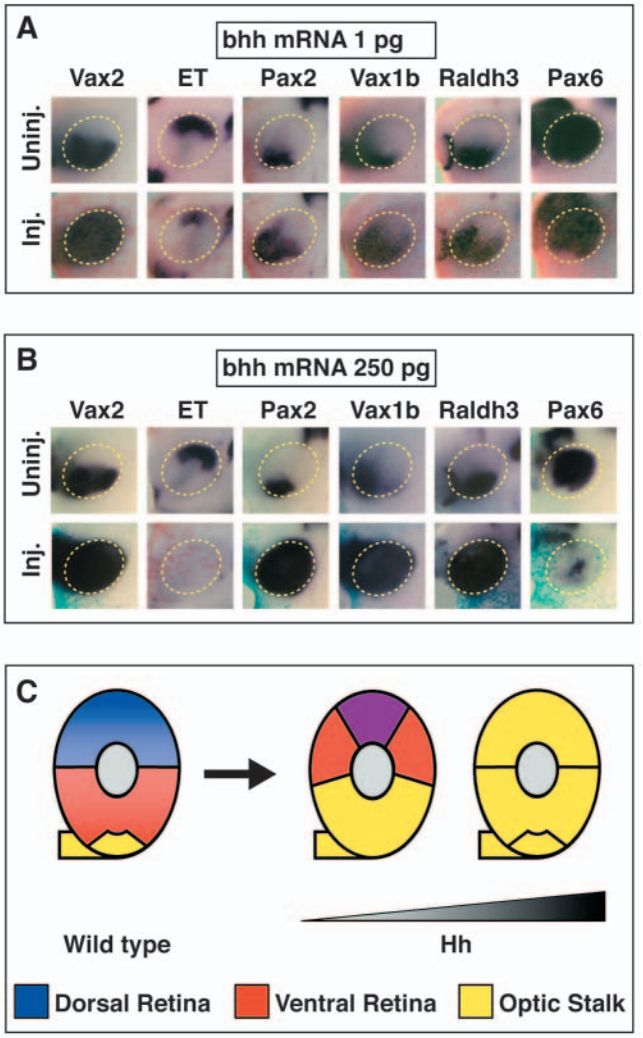

Fig. 1. Effects of Hh signaling overexpression on eye DV polarity. (A,B) The red or light-blue β-gal staining identifies the injected side. The broken yellow circles highlight the eye region. (A) Unilateral injection of low doses of *bhh* mRNA (1 pg) upregulates *Vax2* in the DR and reduces *ET* in stage 33 *Xenopus* embryos. *Pax2*, *Vax1b* and *Raldh3* are partially upregulated, but not in the DR, and the *Pax6* domain is slightly reduced in these embryos. On the uninjected side, *Vax2* is normally expressed in the OS and the VR; *ET* is expressed in the DR; *Pax2*, *Vax1b* and *Raldh3* are expressed in the OS region and *Pax6* is expressed in the retina region. (B) High doses of *bhh* mRNA (250 pg) upregulate *Vax2*, *Pax2*, *Vax1b* and *Raldh3* throughout the eye, and repress *ET* and *Pax6* expression. (C) Schematic representation of the results shown in A and B. Low Hh levels partially expand the OS and ventralize in part the DR. Purple indicates the overlap of ventral and dorsal character in the most dorsal retina. High Hh levels transform the entire retina into OS.

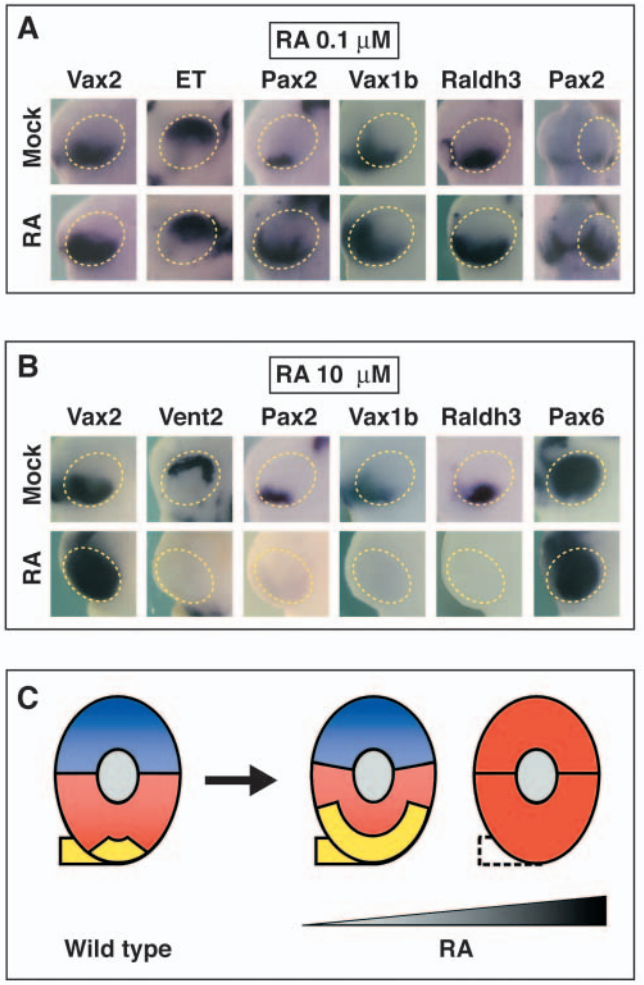

Fig. 2. Effects of RA signaling overexpression on eye DV polarity. (A) Low RA doses (0.1 μM) expand *Pax2*, *Vax1b* and *Raldh3* expression domains, but do not significantly affect *Vax2*- and *ET*-positive regions in stage 33 *Xenopus* embryos. *Pax2*-hybridized embryos are also shown in frontal view (right column). (B) High RA doses (10 μM) upregulate *Vax2* in the DR and repress *Vent2*, *Pax2*, *Vax1b* and *Raldh3*, but do not change *Pax6* expression. (C) Schematic representation of the results shown in A and B. Low RA levels enlarge the OS. High RA levels ventralize the retina and repress OS formation.

expanded (Fig. 2A), though expression of these genes never extended into the dorsal retina but was always confined to ventral eye regions.

To minimize the known effects of early FGF signaling on anteroposterior patterning along the body axis, we took advantage of an inducible form of FGF receptor 1 (iFGFR1) (Pownall et al., 2003). *iFGFR1* mRNA was unilaterally injected into early *Xenopus* embryos, and receptor activity was induced from stage 12.5/13. As shown in Fig. 3B, injection of 4 pg *iFGFR1* mRNA caused ventralization of the eye. In these eyes, the expression of the OS markers *Pax2*, *Vax1b* and *Raldh3*, as well as that of *Vax2*, was expanded dorsally, while expression of the DR marker *ET* was reduced. The *Pax6* domain was also clearly reduced on the injected side, suggesting that these doses of *iFGFR1* mRNA expand the OS at the expense of the retina (Fig. 3C). As this effect is similar to that induced by high Hh levels, we tested whether FGFR signaling could also produce similar dose-dependent effects on OS and VR specification by performing dose-response experiments with iFGFR-1. As shown in Fig. 3A, 2 pg *iFGFR1* mRNA caused only a partial enlargement of *Pax2*, *Vax1b* and *Raldh3* domains, while *Pax6* expression was only slightly reduced in the ventralmost part of the eye. At this same dose, only weak effects were detected on the VR and DR markers *Vax2* and *ET*, respectively. These results suggest that, unlike Hh, FGFR signaling cannot ventralize the

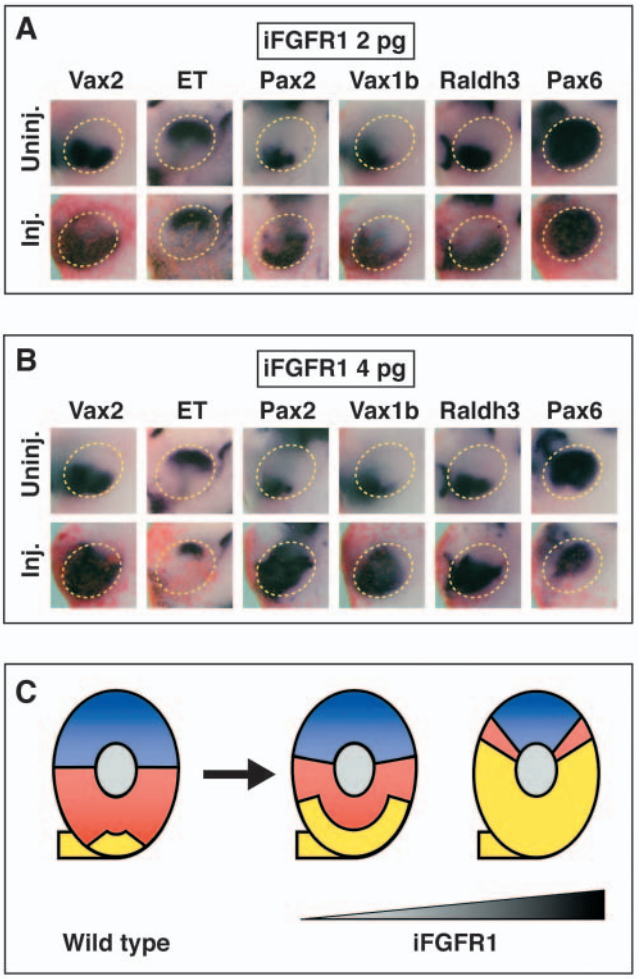

Fig. 3. Effects of FGFR signaling overexpression on eye DV polarity. (A) Unilateral injection of 2 pg *iFGFR1* mRNA, followed by induction with AP20187 at stage 12.5/13, partially expands the expression domains of *Vax2*, *Pax2*, *Vax1b* and *Raldh3* in the ventral eye, and weakly represses *ET* and *Pax6* in stage 33 *Xenopus* embryos. (B) Injection of 4 pg *iFGFR1* mRNA upregulates *Vax2*, *Pax2*, *Vax1b* and *Raldh3* in the dorsal eye and strongly repress *ET* and *Pax6*. (C) Schematic representation of the results shown in A and B. Increasing FGFR levels progressively expands the OS at the expense of the retina.

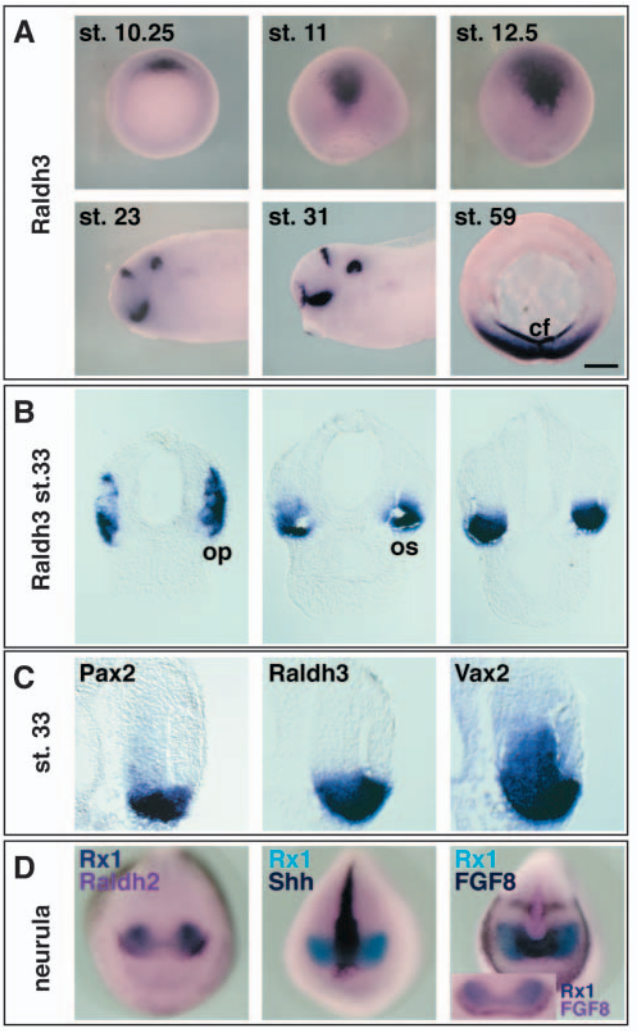

Fig. 4. Expression pattern of *Raldh3* during *Xenopus* development. (A) *Raldh3* expression as detected by whole-mount in situ hybridization of whole embryos (stage 10.25-31) or dissected neural retinas, without lens and pigmented epithelium (stage 59). cf, choroid fissure. Scale bar: 350 μm. (B) *Raldh3* expression in transverse sections of stage 33 embryos after whole-mount in situ hybridization. From left to right, sections show expression at progressively posterior levels. op, olfactory placode; os, optic stalk. (C) Comparison of *Pax2*, *Raldh3* and *Vax2* expression in the optic cup in transversal sections of stage 33 embryos after whole-mount in situ hybridization. (D) Double in situ hybridizations of *Rx1* with *Raldh2*, *Shh* or *FGF8* on mid- to late neurula embryos shown from anterior view. The inset shows a double in situ hybridization of *Rx1* with *FGF8* on an early neurula embryo.

DR at conditions where the OS is moderately expanded (Fig. 3C).

In conclusion, Hh and RA signaling can affect DV specification in the eye with dose-dependent effects. In particular, high Hh and low RA levels preferentially induce OS character, high RA levels preferentially induce VR character, while low Hh levels locally induce OS character ventrally and VR character dorsally. FGFR signaling can induce OS character, but is not efficient at inducing VR character.

Localization of Hh, FGF and RA signaling components supports a role in patterning the *Xenopus* eye field

In all model systems examined so far, *Shh* is expressed in the anterior midline at gastrula and neurula stages, then in the ventral forebrain adjacent to the developing eye during later development (Wilson and Houart, 2004). By double in situ hybridization with the eye field marker *Rx1* (Casarosa et al., 1997), we confirmed that the most anterior domain of *Shh* expression overlaps with the medial part of the eye field at neurula stages (Fig. 4D). *FGF8* is expressed in the anterior neural ridge (ANR) adjacent to the eye field from neurula stages onwards. At later stages, *FGF8* expression is maintained in ventral forebrain regions close to the ventral eye and in the OS (Wilson and Houart, 2004). Double in situ hybridization with *Rx1* shows that, at early neurula stages, *FGF8* expression

in the ANR is adjacent to the eye field. However, during mid-late neurula stages, this *FGF8* domain becomes more medially restricted and overlaps with the medial part of the eye field (Fig. 4D). Hh and FGF receptors are also expressed in the prospective anterior neuroectoderm from early stages of *Xenopus* development (Hongo et al., 1999; Koebernick et al., 2001).

Less is known about RA signaling in the eye field region, although RAR receptors are widely distributed in early *Xenopus* embryos (Shiotsugu et al., 2004), RA synthesis has been detected in neurula stage *Xenopus* embryos (Ang and Duester, 1999b) and high levels of RA synthesis are present in the ventral retina at later stages of development (Drager et al., 2001). To investigate the regulation of RA synthesis during eye development, we screened for *Raldh* homologs, coding for retinaldehyde dehydrogenases, in *Xenopus*. Some of the isolated clones corresponded to *Raldh1*, which is not expressed during early eye development in *Xenopus* (Ang and Duester, 1999a), or *Raldh2*, which is expressed in the ANR adjacent to eye field during neurula stages, and subsequently in the ectoderm flanking the ventrolateral regions of the evaginating optic vesicle (Chen et al., 2001). Double in situ hybridization with *Rx1* showed that *Raldh2* expression in the ANR is stronger next to the anterolateral part of the eye field, and weaker next to the medial part of the eye field (Fig. 4D). Finally, a third group of clones showed the highest homology to chick, mouse and human *Raldh3*, thus corresponding to the *Xenopus Raldh3* ortholog, *Xraldh3* (Accession Number, AY692028). Isolation of full-length *Xraldh3* cDNA indicated that *Xraldh3* codes for a predicted protein of 512 amino acids, showing 79.9%, 78.7% and 79.9% homology with chick, mouse and human Raldh3, respectively (see Fig. S2 in the supplementary material).

Raldh3 is transcribed in the dorsal blastopore lip by early gastrula stages, and, later in gastrulation, in the involuting anterior mesendoderm underlying the anterior neural plate (Fig. 4A). This expression is quickly downregulated, but a new expression domain becomes evident by late neurula stages in the ventral part of the evaginating optic vesicle (data not shown). At early tailbud stages (stage 22/23), *Raldh3* is expressed in the ventral optic vesicle, the midbrain-hindbrain boundary and the dorsal part of the otic vesicle; at later stages (stage 33) it is also expressed in the olfactory placode (Fig. 4A). Transverse sections of *Raldh3*-hybridized embryos at this stage confirmed these expression domains (Fig. 4B and data not shown). Comparative in situ hybridization analysis revealed that *Pax2*, *Raldh3* and *Vax2* show nested expression domains within the ventral optic cup (Fig. 4C). *Raldh3* transcripts persist in the ventral part of the eye throughout development, including metamorphosis (stage 59/60), in a domain still contained within that of *Vax2* (Fig. 4A; data not shown).

Raldh2 can mediate RA synthesis from all-trans Retinal (ATR) in *Xenopus* embryos (Chen et al., 2001). In order to determine whether Raldh3 could also efficiently promote RA synthesis in vivo, we performed overexpression experiments by bilateral microinjection of *Raldh3* mRNA, and checked whether it could mimic the ventralizing activity of RA on the developing eye. No significant effects on DV specification in the eye were detected after injection of up to 5 ng *Raldh3* mRNA (data not shown), possibly because of the complex regulation of substrate availability in vivo (Chen et al., 2001). To test this, we provided low doses of exogenous ATR. Injection of 1 ng *Raldh3* mRNA in combination with 0.5 μM ATR treatment from stage 12.5/13 induced a similar expansion of *Pax2* expression to that observed after treatments with low doses of RA (Fig. 5A; compare with Fig. 2A). By increasing the doses of ATR to 2.5 μM ATR and

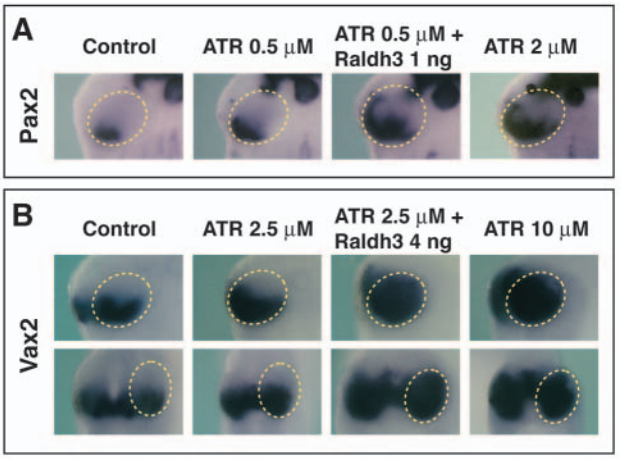

Fig. 5. Raldh3 overexpression in the presence of ATR reproduces the effects of RA treatments on eye DV polarity. (A) Embryos were bilaterally injected with a total of 1 ng *Raldh3* mRNA and treated with 0.5 μM ATR from stage 12.5/13, followed by molecular marker analysis at stage 33. The combination of Raldh3 and ATR expands *Pax2* expression, while ATR alone has only a weak effect. A comparable expansion of *Pax2* is obtained by a dose of 2 μM ATR in the absence of exogenous Raldh3. (B) The combination of 4 ng *Raldh3* mRNA and 2.5 μM ATR can upregulate *Vax2* in the DR, while ATR alone has only a weak effect. Strong *Vax2* upregulation is also caused by a dose of 10 μM ATR in the absence of exogenous Raldh3, as shown in both lateral (upper row) and anterior view (lower row).

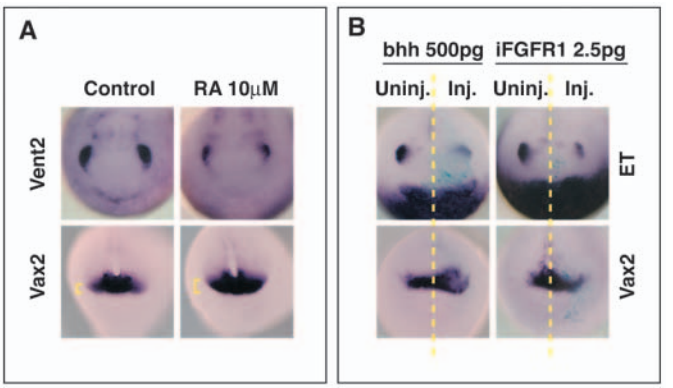

Fig. 6. RA, Hh and FGFR signaling pathways can partially ventralize the eye field at neurula stages. (A) In embryos treated with 10 μM RA from stage 12.5/13 (right column), the lateral region of *Vax2* domain (indicated by the yellow brackets) is expanded at late neurula stages with respect to control embryos, while *Vent2* domain is reduced. (B) Mid-late neurula embryos unilaterally injected either with 500 pg *bhh* mRNA or with 2.5 pg *iFGFR1* mRNA, followed by induction with AP20187 from stage 12.5/13. Compared with the uninjected side, *Vax2* expression is expanded, while *ET* is repressed. The broken yellow lines indicate the embryo midline.

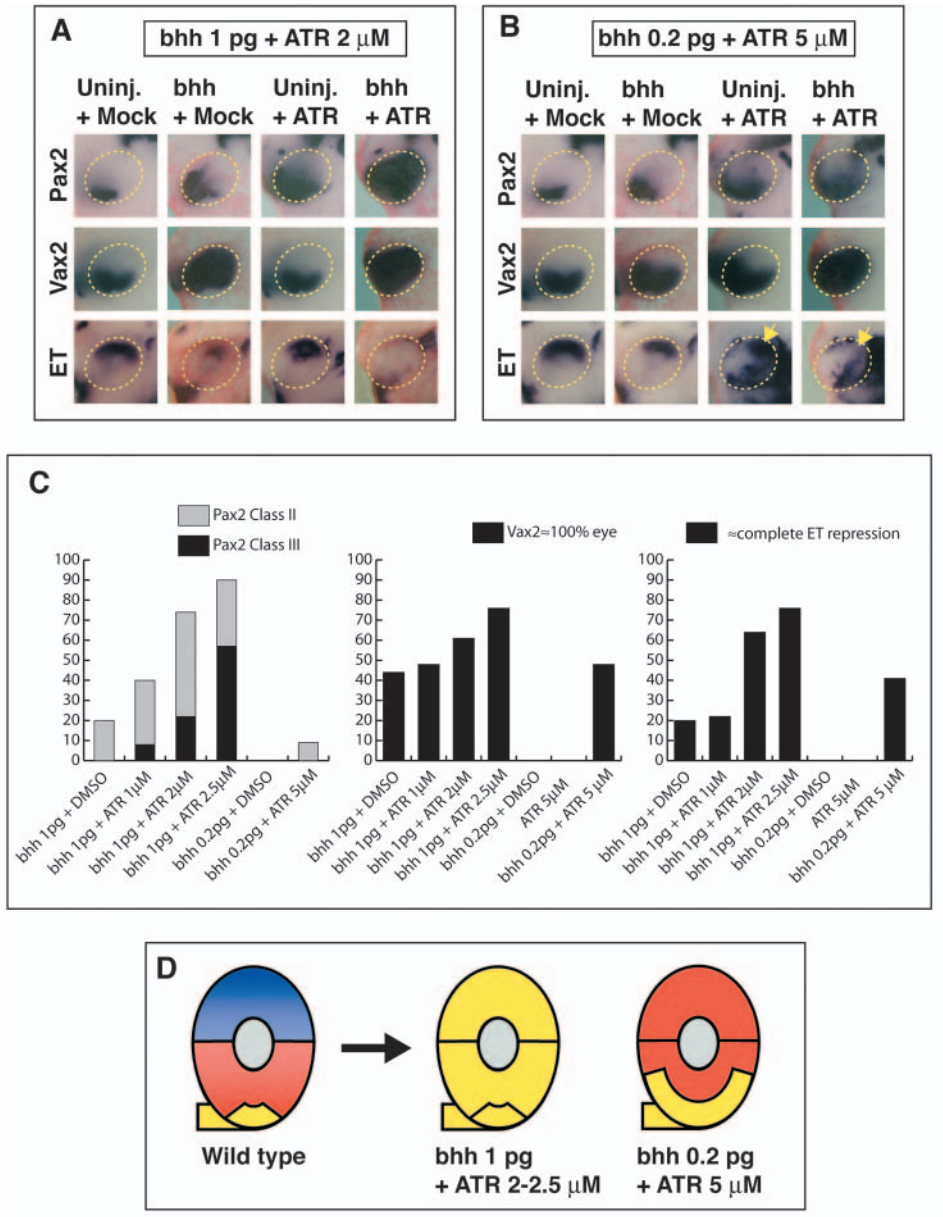

Fig. 7. RA and Hh signaling can collaborate in eye ventralization. (A) Embryos were unilaterally injected with 1 pg *bhh* mRNA and treated with 2 μM ATR from stage 12.5/13. *Pax2* is strongly upregulated in the eye by the combination of *bhh* and ATR, but weakly by *bhh* or ATR alone in stage 33 *Xenopus* embryos. *Vax2* expression can be activated in the DR by these doses of *bhh* in the absence of ATR. *ET* is reduced by *bhh* or ATR alone, but is strongly repressed by the combination of *bhh* and ATR. (B) Embryos were unilaterally injected with 0.2 pg *bhh* mRNA and treated with 5 μM ATR from stage 12.5/13. *Vax2* is upregulated in the DR by the combination of *bhh* and ATR, but not by *bhh* or ATR alone. These doses of *bhh* and ATR weakly activate *Pax2* expression. *ET* expression in the DR is partially reduced by *bhh* or ATR alone, but is strongly repressed by the combination of *bhh* and ATR. The yellow arrow indicates *ET* expression domain in the DR. (C) Quantification of the effects of *bhh* and ATR on *Pax2*, *Vax2* and *ET* expression. Between 21 and 25 embryos were analyzed for each sample in these experiments. (D) Schematic representation of the results shown in A and B. The combination of 1 pg *bhh* mRNA with 2-2.5 μM ATR strongly expands the OS. The combination of 0.2 pg *bhh* with 5 μM ATR ventralizes the DR.

Raldh3 mRNA to 4 ng, we could phenocopy the strong *Vax2* upregulation produced by treatments with high RA doses (Fig. 5B; compare with Fig. 2B). At the same doses of ATR alone, *Pax2* and *Vax2* expression was only slightly increased. Thus, Raldh3 can efficiently convert ATR to RA in vivo. We also observed similar ventralizing effects after treatments with higher doses of ATR in the absence of injected *Raldh3* mRNA (Fig. 5A,B), which were prevented by the RAR antagonist AGN194310 (Hammond et al., 2001) (see Fig. S3 in the supplementary material).

In conclusion, components of the Hh, FGFR and RA signaling pathways are already localized in the eye field at neurula stages, and they remain expressed in or close to the ventral eye at later stages (Figs 4, 9). To test whether these signaling systems play an early role in patterning of the eye field, we performed overexpression experiments in *Xenopus* embryos (as above), but examined the relative expression of eye field markers at neurula stages, and found expansion of ventral eye markers and repression of dorsal eye markers in the eye field (Fig. 6).

RA and FGFR signaling can collaborate with Hh signaling in ventral eye specification

We then performed co-overexpression experiments, in which we simultaneously activated two pathways at a time, at doses that were sub-optimal for each factor alone.

We first unilaterally injected doses of 1 pg *bhh* mRNA, which partially expanded the expression domain of OS markers (Fig. 1A), and exposed injected embryos to different doses of ATR. In these experiments, we scored activation of the OS marker *Pax2* by classifying *Pax2*-hybridized embryos in three classes: (1) class I embryos, where *Pax2* expression was approximately confined to the ventral half of the eye; (2) class II embryos, where *Pax2*-positive domain spread to the dorsal half of the eye, without covering it completely; (3) class III embryos, in which *Pax2* expression covered all or nearly all the eye region. Examples of class I, II and III eyes are shown in Fig. 8A. Although doses of ATR in the range of 2 μM can expand the expression domain of OS markers in the ventral eye, they never upregulate OS markers in dorsal eye regions (Fig. 5A). However, as shown in Fig. 7A, ATR treatments clearly reinforced induction of the OS marker *Pax2* by low

doses of Hh signaling. At doses of 2-2.5 μM ATR, the majority of the embryos had upregulated *Pax2* in the dorsal eye, while only a minority of mock-treated *bhh*-injected embryos showed upregulation of *Pax2* in the dorsal eye (Fig. 7C). On the uninjected side of ATR-treated embryos, *Pax2* was upregulated only within the ventral eye region (Fig. 7A). Therefore, RA and Hh signaling can collaborate in OS specification. As described before, low doses of *bhh* mRNA in the range used for these experiments can ventralize the DR as shown by the upregulation of the VR marker *Vax2* in nearly the whole of the eye region, and the downregulation of the DR marker *ET* (Fig. 7A). We found that the percentages of embryos showing *Vax2* upregulation throughout the eye region, and nearly complete *ET* repression in the DR, were increased by ATR treatments, suggesting that RA and Hh signaling can also collaborate in VR specification (Fig. 7C). In order to clarify this issue, we used lower doses of *bhh* mRNA, which do not significantly affect DV specification in the eye, in combination with ATR treatments. As shown in Fig. 7B, doses of 0.2 pg *bhh* mRNA caused only a slight upregulation of *Pax2* and *Vax2* in the eye, whereas doses of 5 μM ATR locally expanded *Pax2* in the ventral part of the eye, and only slightly increased *Vax2* expression in the VR. Both *bhh* and ATR partially reduced ET expression at these doses. When embryos unilaterally injected with 0.2 pg *bhh* mRNA were also treated with 5 μM ATR, no strong increase was detected in the expression of *Pax2* on the injected side with respect to the uninjected side and few class II and no class III embryos were detected. By contrast, the expression of *Vax2* was upregulated throughout the eye region and the expression of *ET* was almost completely repressed in the DR in substantial fractions of injected eyes (Fig. 7B,C), indicating ventralization of the DR. Similar results were obtained with the DR marker *Tbx5* (data not shown). Therefore, RA and Hh signaling can also cooperate in ventralizing the DR, in conditions where OS specification is weakly affected.

To address whether Hh and FGFR signaling can also collaborate in ventral eye specification, we co-injected suboptimal doses of *bhh* and *iFGFR1* mRNAs, followed by induction of iFGFR1 activity from stage 12.5/13 as before. As already reported (Fig. 1A, Fig. 7A), low doses of 1 pg *bhh* mRNA only partially upregulated the OS marker *Pax2*, and ventralized the DR as shown by *Vax2* upregulation over nearly the whole of the eye region in a proportion of the embryos. Low doses of 0.5-1 pg *iFGFR1* mRNA did not cause significant effects on the expression of *Pax2* and *Vax2*. However, co-injection of 1 pg *bhh* and 0.5-1 pg *iFGFR1* mRNAs caused

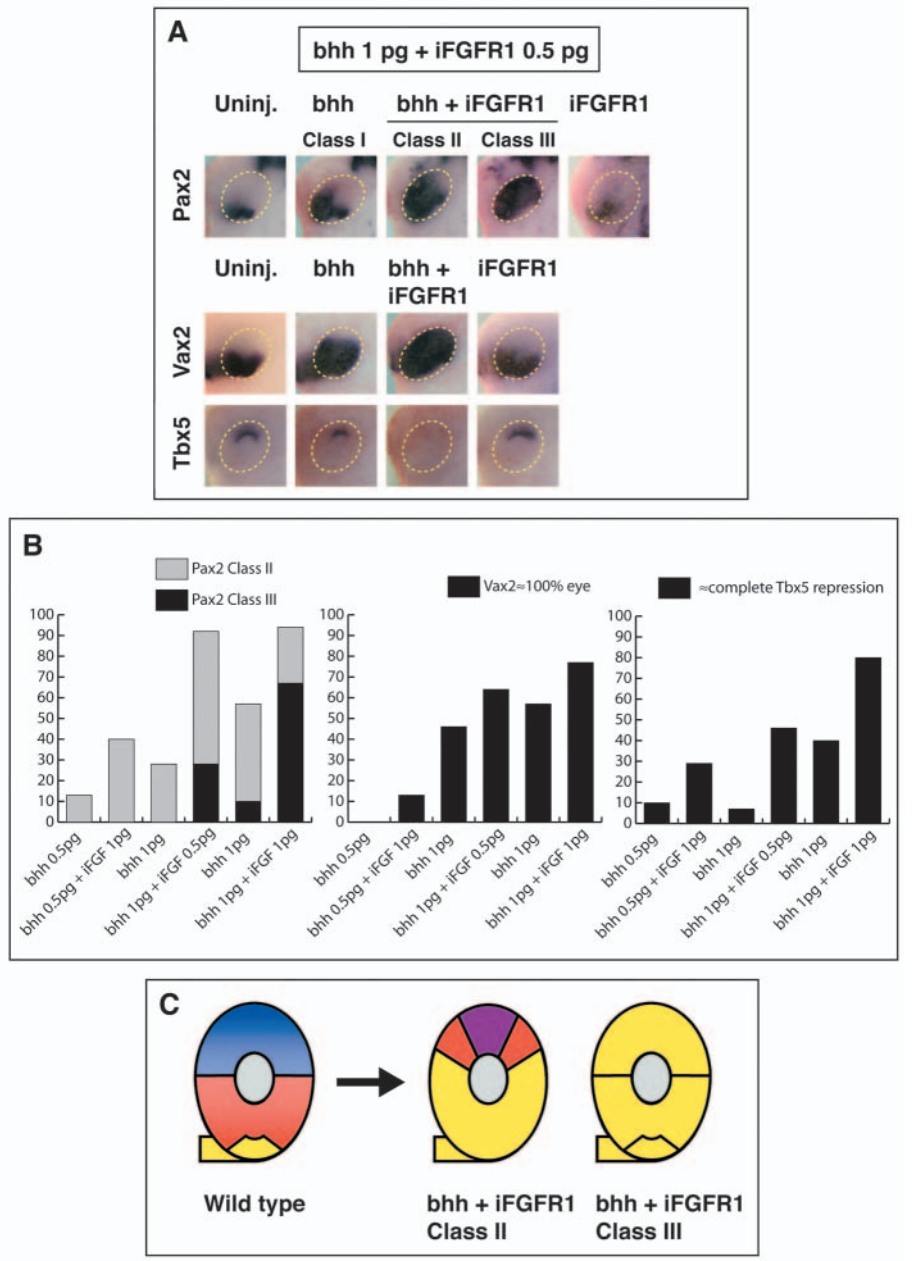

Fig. 8. FGFR and Hh signaling can collaborate in eye ventralization. (A) Embryos were unilaterally co-injected with 1 pg *bhh* mRNA and 0.5 pg *iFGFR1* mRNA, and induced with AP20187 from stage 12.5/13, followed by molecular marker analysis at stage 33. *Pax2* is mainly activated within the ventral half of the eye (class I eyes) by *bhh* alone, while in the presence of *iFGFR1* and *bhh Pax2* expression extends into the dorsal eye (class II and class III eyes). Co-injection of *bhh* and *iFGFR1* mRNAs also slightly increases the frequency of eyes with ubiquitous *Vax2* expression and more strongly represses *Tbx5* when compared with *bhh* alone. Under these conditions, *iFGFR1* mRNA alone has no obvious effect on *Pax2*, *Vax2* and *Tbx5* expression. (B) Quantification of the effects of *bhh* and *iFGFR1* on *Pax2*, *Vax2* and *Tbx5* expression. Between 24 and 30 embryos were analyzed for each sample in these experiments. (C) Schematic representation of the results shown in A. In class II eyes, the expanded OS does not reach the most dorsal retina, which can acquire in part ventral identity. In class III eyes, the retina is mostly or completely transformed into OS.

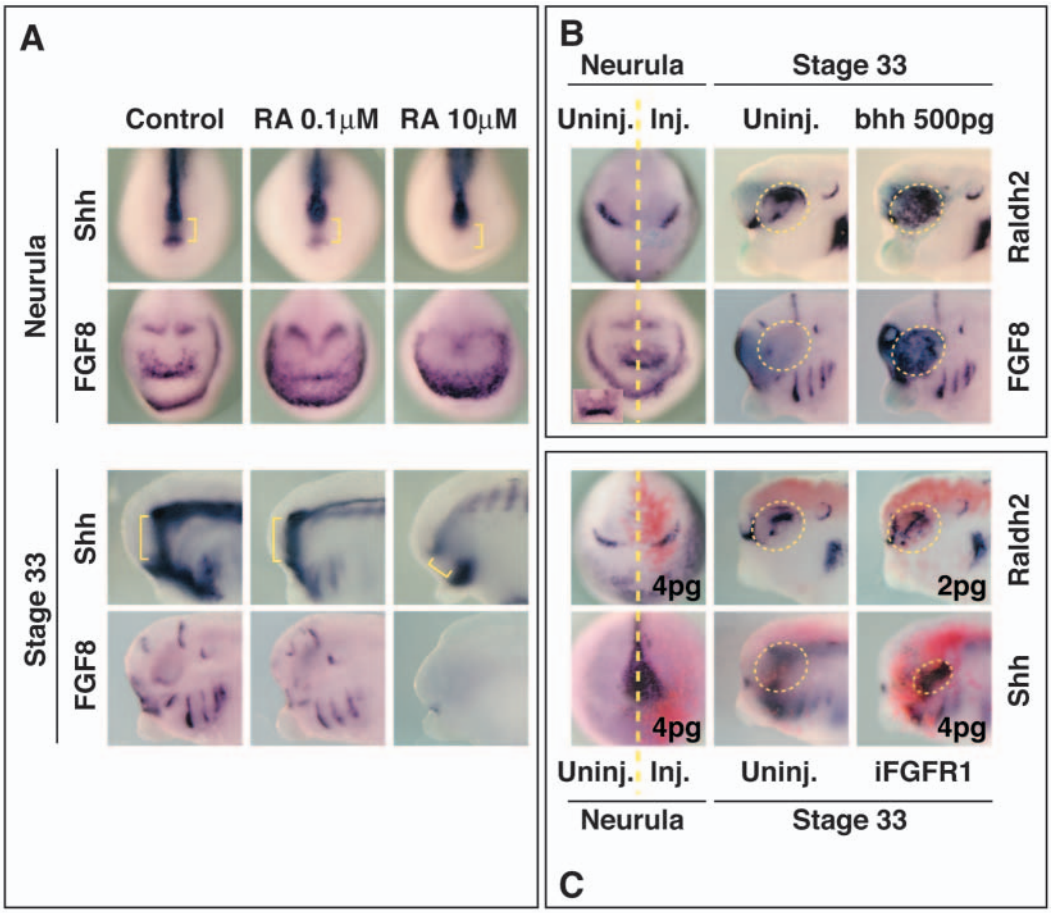

Fig. 9. RA, Hh and FGFR signaling pathways can crossregulate each other at the transcriptional level. (A) High dose RA treatments (10 μM) strongly repress *Shh* expression in the anterior midline (indicated by the yellow bracket) both at neurula and stage 33. The same RA doses cause fusion of the two stripes of *FGF8* expression in the ANR and the anteroventral ectoderm in neurula stage embryos, while at stage 33 *FGF8* expression is strongly downregulated in the whole head region. Low dose RA treatments (0.1 μM) have weaker effects on both *Shh* and *FGF8*. (B) Unilateral injection of 500 pg *bhh* mRNA laterally expands *FGF8* expression in the anterior neural ridge but does not affect *Raldh2* expression at neurula stages. The inset shows *FGF8* expression in the ANR of an uninjected embryo at the same stage. The same dose of *bhh* upregulates both *FGF8* and *Raldh2* in the eye region at stage 33. (C) Unilateral injection of 4 pg *iFGFR1* mRNA, followed by induction with AP20187 at stage 12.5/13, expands *Shh* expression in the prospective hypothalamic region at neurula, and activates *Shh* in the eye regions at stage 33. At doses of 4 and 2 pg, *iFGFR-1* mRNA has no significant effect on *Raldh2* expression at neurula stages and stage 33, respectively.

a clear increase in the percentage of class II and class III *Pax2*-hybridized eyes compared with single *bhh* overexpression (Fig. 8). Co-overexpression of *bhh* and *iFGFR1* also slightly increased the proportion of embryos showing roughly complete upregulation of *Vax2* in the eye region, compared with single *bhh* overexpression, and the percentage of these embryos was slightly higher than the percentage of class III *Pax2*-hybridized embryos (Fig. 8A,B). In addition, the percentage of eyes with nearly complete repression of the DR marker *Tbx5* was increased in embryos co-injected with *bhh* and *iFGFR1* compared with *bhh* alone (Fig. 8A,B). Expression of the DR marker *ET* was also decreased after co-injection of *bhh* and *iFGFR1*, compared with single *bhh* injection, although some *ET* expression in the dorsalmost eye was still retained in the majority of the embryos (data not shown). These data suggest that, when the eye does not completely acquire OS character, *bhh* and *iFGFR1* may also ventralize, at least in part, the remaining retina tissue. Similar results were obtained after co-overexpression of 1 pg *iFGFR1* mRNA with lower doses of 0.5 pg *bhh* mRNA (Fig. 8B). Therefore, FGFR1 and Hh signaling can collaborate in OS specification and they may also weakly interact in VR specification (Fig. 8C).

In conclusion, Hh, RA and FGFR signaling can collaborate in ventral eye specification. In particular, OS character is preferentially specified at higher Hh and FGFR signaling levels, and lower RA signaling levels, whereas VR character is preferentially specified at lower Hh and higher RA levels.

RA, Hh and FGF signaling pathways can cross-regulate each other at the transcriptional level

Eye ventralization by RA, Hh and FGF signals may involve crossregulatory interactions among these three signaling pathways. We found that both *bhh* and *iFGFR1* overexpression upregulate *Raldh3* expression in the eye at stage 33 (Fig. 1B, Fig. 3B). Unilateral injection of 500 pg *bhh* mRNA did not have appreciable effects on *Raldh2* expression in the ANR at neurula stages (Fig. 9B), but upregulated it in stage 33 eyes (Fig. 9B); it also caused the *FGF8*-positive domain in the ANR to expand laterally and upregulated *FGF8* in the eye region at stage 33 (Fig. 9B).

We also found that RA treatments downregulated *Shh* expression in the anterior midline (Fig. 9A). Treatments with high RA doses (10 μM) from stage 12.5/13 repressed the most anterior domain of *Shh* transcription at neurula stages, which overlaps with the medial part of the eye field (Fig. 4D). This effect was also evident at stage 33. Low RA doses (0.1 μM) partially downregulated *Shh* expression in the anterior midline at neurula stages, while no clear effect was evident at stage 33.

FGF8 expression was also affected in RA-treated embryos

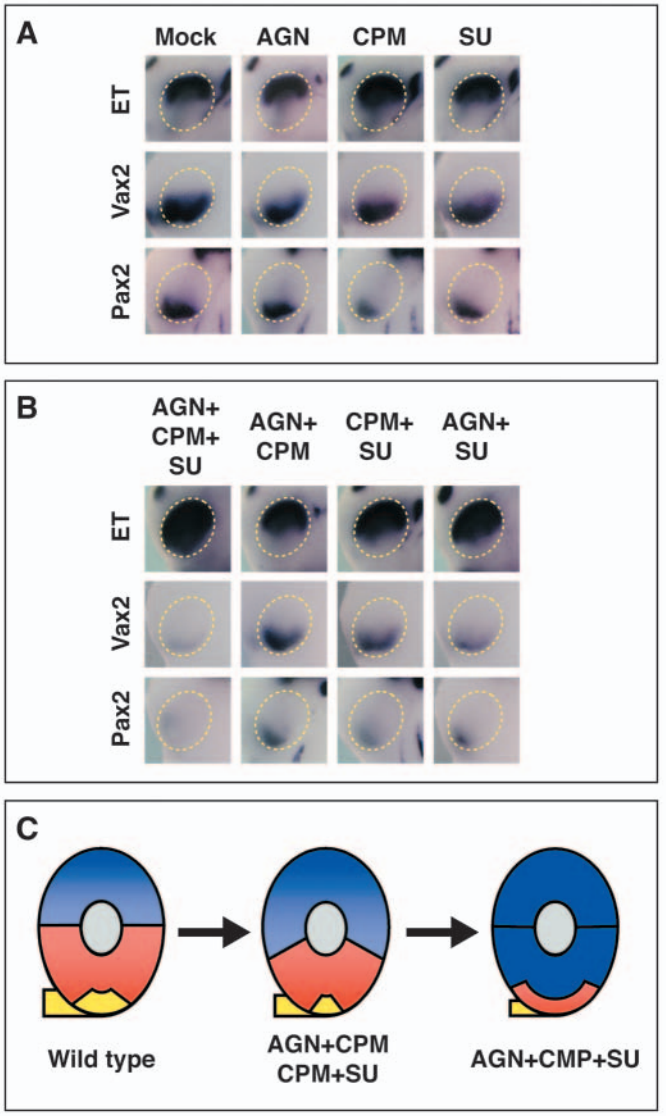

Fig. 10. Loss-of-function effects of RA, Hh and FGFR signaling pathways on eye DV polarity. Embryos were treated from stage 10.5 with 10 μM AGN194310 (AGN), 100 μM cyclopamine (CPM) and 25 μM SU5402 (SU) in different combinations, and analyzed for molecular marker expression at stage 30/31. (A) Effects of the single inhibition of any of the RA, Hh and FGFR signaling pathways, when compared with mock-treated embryos. (B) Effects of double and triple inhibition of RA, Hh and FGFR pathways. (C) Schematic representation of the results shown in A and B. Strong eye dorsalization is caused by triple inhibition of RA, Hh and FGFR signaling, while double inhibitions produce weaker effects.

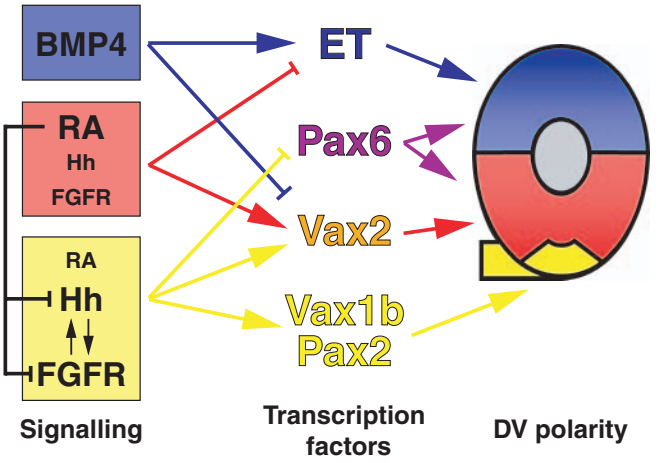

Fig. 11. A proposed model for signaling events controlling DV patterning of the eye. Interaction of high levels of Hh and FGFR with low levels of RA signaling repress *Pax6* and induce the expression of *Vax1*, *Pax2* and *Vax2*, leading to OS specification. High levels of RA in collaboration with low levels of Hh and FGFR signaling repress *ET* and induce the expression of *Vax2* in the absence of *Vax1* and *Pax2*, but in the presence of *Pax6*, thus specifying VR identity. BMP signaling inhibits *Vax2* and induces *ET* expression, causing DR specification. In addition, high levels of RA repress the expression of Hh and FGFR signals, while Hh and FGFR signaling crossactivate each other at the transcriptional level.

(Fig. 9A). At neurula stages, *FGF8* mRNA is transcribed in two anterior stripes, one in the ANR, and the other in the anteroventral ectoderm outside the neural plate. These two stripes were closer to each other in low dose RA-treated embryos, and appeared to be merged in one broader stripe of expression in high dose RA-treated embryos. At tadpole stages, a strong general repression of *FGF8* transcription was caused by high RA doses.

As shown in Fig. 9C, 4 pg *iFGFR1* mRNA injections did not affect *Raldh2* expression in the ANR at neurula after induction at stage 12.5/13, while *Shh* expression was expanded on the injected side at the level of the prospective hypothalamic region. Ectopic upregulation of *Shh* in the eye region at stage 33 was also detectable in these conditions. At lower doses (2 pg), only weak effects on *Shh* and *Raldh2* expression were detected. Analysis of *Pax2* and *Vax2* expression showed that, in this experiment, 2 pg *iFGFR1* mRNA induced eye ventralization similar to that reported in Fig. 3, while a 4 pg dose caused eye reductions (Fig. 9C and data not shown).

In conclusion, RA, Hh and FGF signals are able to crossregulate the expression of one another, although these effects are mainly mediated by doses of signal higher than those required to ventralize the eye. *Shh* downregulation in the anterior midline of RA-treated embryos suggested that the loss of the OS seen with high RA may be a secondary consequence of this effect. To address this issue, we analyzed whether Hh signaling could rescue OS formation in RA-treated embryos. Indeed, we found that expression of OS markers was recovered in embryos injected with 25 pg *bhh* mRNA followed by incubation in 10 μM RA from stage 12.5/13 (see Fig. S4 in the supplementary material). Therefore, RA treatments may repress OS formation indirectly by downregulating *Shh* expression in the anterior midline, although a direct inhibitory effect on OS gene expression cannot be ruled out.

Loss-of-function experiments suggest that ventral eye specification involves interactions among Hh, RA and FGFR signaling

To address whether interactions between Hh, RA and FGFR signaling pathways play an important role in ventral eye specification, we inhibited them in all possible combinations. Hh signaling was blocked with cyclopamine (Incardona et al., 1998). RA signaling was inhibited with the pan-RAR

antagonist AGN194310, which specifically antagonizes activity of all RAR receptors, but not RXR receptors (Hammond et al., 2001). FGFR signaling was inhibited with SU5402 (Mohammadi et al., 1997). *Xenopus* embryos were treated with these antagonists from stage 10.5, at conditions in which each inhibitor effectively reduced the levels of its target signaling pathway, without significantly affecting the other two (see Figs S5, S6 in the supplementary material). As shown in Fig. 10, significant DV organization was retained after any of the single inhibitions of Hh, RA or FGFR signaling. Double inhibition of Hh, RA and FGFR signaling in different combinations increased reduction of ventral eye territories when compared with single inhibitions, especially after Hh and FGFR or RA and FGFR inhibitions. The strongest effects were obtained with the simultaneous inhibition of all three (Hh, RA and FGFR) signaling pathways, which caused a dorsalized eye phenotype, with strong repression of ventral eye markers and upregulation of the DR markers *ET* within the ventral eye. In conclusion, the results of loss-of-function experiments support a model where ventral eye specification involves interactions among Hh, RA and FGFR signaling pathways.

Discussion

Roles of Hh, RA and FGFR signaling pathways in OS and VR specification

Hh signaling has a crucial role in OS specification. In zebrafish, *Xenopus* and chick embryos, Hh overexpression can upregulate OS markers in the retina region, while repressing the expression of retinal markers. By contrast, downregulation of Hh signaling by *Shh* knock out in mouse, *smoothened* knock out in zebrafish or cyclopamine treatments in *Xenopus* all cause suppression of OS fate, thus indicating that Hh signaling is both necessary and sufficient for OS specification (Russell, 2003; Yang, 2004). Within the spinal cord, *Shh* released from the floor plate has been shown to work as a morphogen, inducing the expression of ventral genes at high doses, and more dorsal genes at lower doses (Ruiz i Altaba et al., 2003). By analogy to the neural tube, it has been suggested that Hh signals may also act in a graded manner in the eye, specifying OS, VR and DR fates at different threshold concentrations (Wilson and Houart, 2004). We show that, in the DR, low doses of Hh signaling can upregulate *Vax2*, but not OS markers, and decrease the expression of *ET*, but not *Pax6* (Fig. 1A), suggesting that low Hh levels can at least partially ventralize the DR without concomitant expansion of the OS.

RA has been suggested as an alternative signaling molecule that may control DV specification within the retina (Drager et al., 2001). RA treatments upregulated the OS marker *Pax2* in zebrafish (Hyatt et al., 1996), while reduced RA signaling caused abnormal development of the VR in different animal models (Marsh-Armstrong et al., 1994; Ross et al., 2000), at least at the morphological level. In this paper, we confirm in a different model system that increasing RA levels can induce OS character in the eye (Fig. 2A). More importantly, we provide evidence that RA can act as a ventralizing factor within the retina. This ventralizing effect requires higher levels of RA than those required to induce OS expansion, and it is accompanied by a strong repression of OS genes, probably owing to the downregulation of *Shh* in the anterior midline (Fig. 9A). Upregulation of *Vax2* in the presence of *Pax6*, but in the absence of OS markers, together with the strong downregulation of DR markers, indicates that high RA levels cause the DR to acquire VR character (Fig. 2B, see Fig. S1 in the supplementary material). These ventralizing activities of RA can be mimicked, albeit less efficiently, by the intermediate metabolic precursor ATR, suggesting that correct localization of endogenous RA-generating enzymes is important for DV patterning of the eye (Fig. 5). These effects are strongly inhibited by a RAR antagonist, indicating that they are specifically mediated by activation of RAR receptors (see Fig. S3 in the supplementary material).

Loss-of-function analyses of FGF signals in zebrafish have suggested that this pathway may play a role in OS specification, although decreased FGF signaling seems to have much more profound effects on the adjacent ventral forebrain (Take-uchi et al., 2003; Walshe and Mason, 2003). There are no reports that we are aware of suggesting that FGF signaling may have a role in DV patterning within the retina. We show that overexpression of FGFR signaling has a strong ventralizing effect on the developing *Xenopus* eye. At all doses analyzed, FGFR signaling expands the expression of *Vax2* and OS markers to a similar extent, while the expression domain of *Pax6* is proportionally reduced, suggesting that FGFR signaling on its own can enhance specification of OS character, but it cannot efficiently modify the DV character of the retina (Fig. 3). FGFR and Hh signaling can collaborate in the specification of OS character, and they may weakly interact in specifying VR character (Fig. 8). Finally, strong effects on VR specification were observed when FGFR signaling was inhibited together with RA and Hh signaling (Fig.10). Although further work is needed to determine the precise role of FGFR signaling in DV patterning of the retina, our results suggest that it may be involved in controlling patterning throughout the eye DV axis; higher levels of FGFR activation may promote OS fates, while lower levels of FGFR activation may collaborate with other signals in the specification of VR fates.

RA, Hh and FGFR signaling interact in Xenopus ventral eye specification

The fact that RA, Hh and FGFR overexpression cause similar ventralizing effects in the eye, and the observation that these signaling components are expressed in adjacent or overlapping domains at early stages of eye development, suggested that these pathways may interact during DV patterning of the eye in *Xenopus*. Two lines of evidence in this work support this idea. First, in co-overexpression experiments, Hh, RA and FGFR signaling can collaborate in ventral eye specification (Figs 7, 8). Second, in loss-of-function experiments, stronger effects on eye DV patterning were observed by inhibiting more than one pathway compared with single inhibitions (Fig. 10).

We propose a model of ventral eye specification that involves interactions among RA, Hh and FGFR signaling pathways (Fig. 11). According to this model, high levels of Hh and FGFR signaling interact with low levels of RA signaling to specify the OS by repressing retina-determination genes such as *Pax6*, and promoting the expression of *Vax1* and *Pax2*. By contrast, high levels of RA act in concert with lower levels of Hh and FGFR signaling to specify the VR by repressing DR-specific genes such as *ET* and by inducing the expression of *Vax2* in the presence of *Pax6*, but not *Vax1* and *Pax2*. As

previously proposed (Koshiba-Takeuchi et al., 2000; Sasagawa et al., 2002), BMP signaling specifies DR regions by repressing *Vax2* and inducing *ET* and other members of the *Tbx* gene family, such as *Tbx5* (Koshiba-Takeuchi et al., 2000). In vivo, ventrodorsal (ventral high) gradients of Hh and FGFR signaling may be created by diffusion of Hh and FGF signals from their sources in the anteromedial neural plate (Fig.4D). The regulation of RA gradients is more complex and the localization of different anabolic and catabolic enzymes needs to be considered. However, early expression of *Raldh2* and *Raldh3* appears to be localized close to the presumptive ventral eye (Fig. 4A,D), and the mediolateral gradient (lateral high) of *Raldh2* expression in the ANR (Fig. 4D) may contribute to create higher RA levels in the VR compared with the OS region.

DV patterning in the eye and the neural tube

Recent studies have shown that ventral patterning in the spinal cord and the telencephalon involves interactions between Hh, RA and FGF signaling pathways. In the spinal cord, motoneurons and V3, V2 ventral interneurons originate from the ventral neural tube, while V1 and V0 ventral interneurons originate from more intermediate regions. Hh signals from the notochord and floorplate are thought to specify the progenitor domain of motoneurons, V3 and V2 interneurons (Ruiz i Altaba et al., 2003), while RA signaling is crucial for the specification of V1 and V0 interneurons (Pierani et al., 1999). In addition, although FGF signaling appears to function as a general repressor of ventral neural patterning, RA and FGF in combination can efficiently induce motoneuron progenitors both in explants and in vivo (Novitch et al., 2003).

In the telencephalon, the medial ganglionic eminence (MGE) originates from the ventral part of the telencephalic vesicle, while the lateral ganglionic eminence (LGE) originates from a more intermediate region. Hh signaling is involved in the specification of the MGE, while RA signaling appears to play a crucial role in the specification of the LGE (Gunhaga et al., 2000; Marklund et al., 2004). In addition, FGF signaling is involved in the specification of ventral, but not intermediate, telencephalic fates (Marklund et al., 2004; Shinya et al., 2001).

In the developing eye, cells located more ventrally in the anlage give rise to the OS, while the VR originates from a more intermediate region. As shown in Fig. 11, Hh and FGFR signaling play a crucial role in OS specification, although low levels of RA signaling may also be involved. Moreover, RA signaling could control specification of the VR in collaboration with low levels of Hh and possibly FGFR signaling.

In conclusion, similar mechanisms of ventral specification involving Hh, RA and FGFR signaling pathways appear to be at least partially conserved in different CNS regions. Several questions remain to be addressed concerning the precise role and the mechanism of action of these signaling systems. Clearly, DV patterning of the vertebrate CNS is a complex process, and the eye, because of its distinct regional composition, its finely graded topography and its experimental accessibility, is an exciting model with which to study how different signaling pathways interact to execute specific developmental programs.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/7/1737/DC1

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