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Hedgehog signalling maintains the optic stalk-retinal interface through the regulation of Vax gene activity

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SUMMARY

During early formation of the eye, the optic vesicle becomes partitioned into a proximal domain that forms the optic nerve and a distal domain that forms the retina. In this study, we investigate the activity of Nodal, Hedgehog (Hh) and Fgf signals and Vax family homeodomain proteins in this patterning event. We show that zebrafish *vax1* and *vax2* are expressed in overlapping domains encompassing the ventral retina, optic stalks and preoptic area. Abrogation of Vax1 and Vax2 activity leads to a failure to close the choroid fissure and progressive expansion of retinal tissue into the optic nerve, finally resulting in a fusion of retinal neurons and pigment epithelium with forebrain tissue.

We show that Hh signals acting through Smoothened act downstream of the Nodal pathway to promote Vax gene expression. However, in the absence of both Nodal and Hh signals, Vax genes are expressed revealing that other signals, which we show include Fgfs, contribute to Vax gene regulation. Finally, we show that Pax2.1 and Vax1/Vax2 are likely to act in parallel downstream of Hh activity and that the *bel* locus (yet to be cloned) mediates the ability of Hh-, and perhaps Fgf-, signals to induce Vax expression in the preoptic area. Taking all these results together, we present a model of the partitioning of the optic vesicle along its proximo-distal axis.

Key words: Zebrafish, Vax, Eye, Optic stalk, Coloboma, *fgf*, *hedgehog*

INTRODUCTION

The vertebrate eye originates from cells of the eye field located within the anterior neural plate. Following neural tube formation, prospective eye tissue evaginates from the brain to form the optic vesicles at the interface between telencephalon and diencephalon. Cells within proximal regions of the optic vesicles give rise to the optic stalks (which later differentiate as glial cells of the optic nerves), while cells more distally form the optic cup. The optic cup give rises to the pigmented epithelium and neural layers of the retina.

During the formation of the optic vesicles, signals derived from axial structures promote proximal optic stalk fates while inhibiting distal retinal fates. Hedgehog signalling proteins (Hh) are among the signals that promote proximal optic vesicle fates. For instance, overexpression of Hh induces optic stalk marker genes such as *pax2* and *vax1*, while inhibiting retinal marker genes such as *pax6* and *Rx* (Ekker et al., 1995; Hallonet et al., 1999; Macdonald et al., 1995). Furthermore, loss of function mutations in the Sonic hedgehog gene in mouse (*Shh*) lead to a loss of *pax2* expression, absence of optic stalks, and cyclopia (Chiang et al., 1996). Although *shh* mutations in fish do not exhibit this phenotype (Schaurte et al., 1998), probably because of redundancy with other Hh proteins (Nasevicius and Ekker, 2000), absence of *pax2.1/noi* expression is observed in the optic vesicles of fish lacking the activity of Smoothened (Chen et al., 2001; Varga et al., 2001), a transmembrane protein essential for the transduction of all Hh signals (Ingham and McMahon, 2001).

Nodal signals are also implicated in early proximo-distal patterning of the optic vesicles, as evident from the cyclopic defects in fish carrying mutations in a variety of genes functioning in the Nodal signalling pathway (e.g. Gritsman et al., 1999; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). Some of the forebrain and eye defects in Nodal pathway mutants are indirectly because of loss of Hh activity in anterior regions of the mutant embryos (Macdonald et al., 1995; Masai et al., 2000; Rohr et al., 2001; Mathieu et al., 2002). However, the extent of cyclopia in Nodal pathway mutants is often more severe than in zygotic Hh pathway mutants, suggesting that signals in addition to Hhs contribute to the proximo-distal patterning of the eyes. Indeed, it is probable that Fgfs contribute to the patterning of proximal optic vesicle tissue. Several Fgfs are expressed in and around the optic stalks (Reifers et al., 1998; Reifers et al., 2000; Shinya et al., 2001), and abrogation of Fgf activity leads to disrupted patterning of midline tissue between the eyes, including the optic chiasm (Shanmugalingam et al., 2000).

Our understanding of genes that function downstream of the signals that promote proximal optic vesicle fates is fragmentary. In this context, the best-studied protein is the transcription factor Pax2, which mediates optic stalk

differentiation and chiasm formation in both mice and fish (Torres et al., 1996; Macdonald et al., 1997). Pax2.1/Noi-expressing optic stalk cells in fish differentiate as reticular astrocytes of the optic nerve, and it seems probable that these glial cells play key roles in the guidance of retinal and other axons across the midline of the brain (Macdonald et al., 1997). A second phenotype observed in humans, mice and fish with disrupted Pax2 function is coloboma, a failure to close the choroid fissure (Sanyanusin et al., 1995; Favor et al., 1996; Torres et al., 1996; Macdonald et al., 1997). The choroid fissure is the opening from the ventral side of the retina into the optic stalk that allows exit of axons from, and entrance of blood vessels into, the eye. During normal development, the nasal and temporal retina on either side of the choroid fissure fuses around the axons and blood vessels. The conserved nature of the coloboma phenotype indicates that Pax2 is likely to regulate as yet unknown genes that directly mediate the fusion event.

A second family of genes implicated in optic stalk development and proximal retinal patterning are the homeodomain protein-encoding Vax genes. Vax genes are *emx*-like homeobox-containing genes which have now been identified in mice, human, *Xenopus*, rat, chicken and medaka fish (Barbieri et al., 1999; Hallonet et al., 1998; Ohsaki et al., 1999; Schulte et al., 1999; Winkler et al., 2000). Vax genes are classified into *vax1* and *vax2* subfamilies according to their gene structure and expression pattern. *vax1* genes are expressed in the optic stalk and preoptic area, whereas *vax2* genes are expressed in the optic stalk, preoptic area and ventral retina. The developmental roles of Vax genes have been studied through overexpression experiments in *Xenopus* and chick (Barbieri et al., 1999; Schulte et al., 1999), and gene targetting in mice (Barbieri et al., 2002; Hallonet et al., 1999; Bertuzzi et al., 1999; Mui et al., 2002). These studies have implicated *Vax* gene activity in the dorso-ventral patterning of retinal cells (and hence in the retinotopic projection of retinal ganglion cell axons), and similar to Pax2, in optic nerve differentiation, the guidance of retinal axons and closure of the choroid fissure. Although *vax1* mutant mice do not exhibit cyclopia, the expression of at least some retinal markers is maintained in the optic nerves, long after such genes are normally downregulated (Bertuzzi et al., 1999; Hallonet et al., 1999). This suggests that proximal optic vesicle tissue maintains some distal (retinal) character in the absence of Vax1 function.

Here we describe the isolation and characterization of zebrafish *vax1* and *vax2*. *vax1* is expressed in the anterior neural keel and later in the preoptic area and optic stalk. *vax2* is expressed in the anterior neural keel and later in the preoptic area, optic stalk and ventral retina. Concurrent abrogation of Vax1 and Vax2 function using morpholino oligonucleotides (MOs) results in a coloboma phenotype in which the choroid fissure fails to fuse. Subsequent to this, a medial expansion of retinal tissue along the optic nerve and into the forebrain occurs in Vax activity-depleted embryos. Thus, Vax1 and Vax2 proteins act synergistically to restrict retinal tissue to the optic cup.

To assess the upstream regulators of Vax gene expression, we examined the involvement of Hh, Nodal and Fgf signaling pathways. Overexpression of Hh induces *vax1* and *vax2* whereas conversely, expression of *vax1* and *vax2* is lost in Hh pathway mutants. Thus the expression of Vax genes in the preoptic area, optic stalk and ventral retina is dependent on the Hh pathway. We further investigate the nature of genes that might act downstream of Hh signalling to regulate Vax gene expression. We show that Noi/Pax2.1 is not essential for Vax gene expression, whereas Bel function is required within the preoptic area for Hh signals to induce Vax gene expression.

We also show that induction of Vax gene expression is lost in embryos with reduced Nodal activity and that this loss is at least in part because of reduced or absent Hh signalling in anterior regions of the Nodal mutants. However, complete loss of Nodal signalling activity restores expression of Vax genes, despite the apparent lack of Hh activity in such embryos. We provide evidence suggesting that this induction is mediated by Fgf signals.

MATERIALS AND METHODS

Fish lines

Zebrafish (*Danio rerio*) were maintained on a 14-hour light/10-hour dark cycle and bred as described in Westerfield (Westerfield, 1993). Developmental age is given as somite stage (somites) or hours post-fertilization (hpf) at 28.5°C. Prior to morphological phenotypes becoming apparent, $noi^{-/-}$ embryos were recognized by reduced expression of *eng3* in the midbrain and $bel^{-/-}$ embryos were recognized by loss of expression of *vax1* and *vax2* in the preoptic area (both phenotypes were apparent in 25% of embryos of clutches from heterozygous parents).

Cloning of *vax1* and *vax2* cDNAs

To isolate zebrafish homologues of the Vax genes, the following oligo nucleotide primers were used for degenerate reverse-transcription PCR:

MTv502D=GARACICARGTGAARGTITGGTTYCARAA;
MTv301D=GTAICGGGCIGAIAGYTCITGIARRTT;
MTv521D=ATHMGIGARATHGTIYTICCIAAAGG;
MTv321D=GGIACISWIARIARICKICCYTGYTC.

Mixed bases are indicated based on IUB codes and I=deoxyinosine. mRNA was purified from total-RNA extracted from 24 hpf zebrafish embryos. Superscript-II (Gibco) was used for the reverse-transcription and two types of cDNA fragments were obtained by subsequent PCR. 5′ and 3′ regions of the cDNAs were obtained by 5′RACE and 3′RACE. Full-length coding sequence of *vax1* and *vax2* are available at GenBank (Accession Numbers AY185348 and AY183363, respectively).

MO antisense injections

MO antisense oligo nucleotides (GeneTools) were designed against 25 bases including the AUG of the *vax1* and the 5′ UTR of the *vax2* mRNAs (sequences available on request). Injections of MOs were done at concentration 25 μg/μl. To confirm the specificity of the Vax MOs, four constructs were made. Two of the constructs have the MO target sequence of *vax1* or *vax2* cloned 5′ to the coding sequence for green fluorescent protein (*vax1*GFP and *vax2*GFP, respectively). The *vax1*GFP is an in-frame fusion of the target sequence of *vax1* gene to GFP coding sequence, and the *vax2*GFP is a fusion of 5′ UTR of the *vax2* gene to 5′ upstream of GFP coding sequence. The injected transcripts of these constructs generated fluorescent proteins in the embryos. Co-injection of the *vax1*MO or *vax2*MO removed the fluorescence following *vax1*GFP or *vax2*GFP injection respectively, indicating that the MOs can prevent translation of the fusion protein. The other two constructs have three base changes in the MO target sequence of *vax1* or *vax2* in front of the GFP-coding sequence. The injected transcripts of these constructs generated fluorescent protein in the embryo both with and without injected MO. We conclude that

the MOs against *vax1* and *vax2* are able to inhibit translation from RNA carrying the target sequence.

Plastic sectioning and toluidine blue staining

Embryos to be sectioned were dehydrated in methanol and embedded in JB4 resin (Agar Scientific Ltd). The polymerized block was cut at 10-μm sections on a Jung 20555 Microtome with Leica blade, and the sections were mounted onto glass slides. Toluidine blue staining was performed on the sections. The slides were mounted with DPX and analysed with DIC optics on a Nikon optiphot-2 compound microscope.

Confocal microscopy of DiI-labelled optic nerves

Embryos were fixed with 4% paraformaldehyde (PFA) for eight hours at 4°C. The lipophilic fluorescent dye, DiI, was injected intraocularly and incubated at 4°C for 20 hours to allow dye to diffuse to the tectum. Melanin pigment was bleached by hydrogen peroxide. DiI-injected embryos were mounted in 1% agarose in PBS for confocal microscopy. Confocal analysis was performed on a Leica TCS SP Confocal Microscope using 20X water immersion objective. Three-dimensional series of images were acquired at 2 μm intervals and imported into NIH Image to merge.

RNA injection

Capped mRNAs were synthesized in vitro using the MEGAscript kit (Ambion). Injections were performed as described (Barth and Wilson, 1995).

In situ hybridization

Digoxigenin-labelled antisense RNA probes were synthesized from cDNA clones. Staged embryos were fixed in 4% PFA and stored in methanol at –20ºC. In situ hybridizations were performed using standard procedures (Macdonald et al., 1994). Sections of embryos were performed after whole-mount stainings.

Fate mapping of optic stalk

Embryos were injected with caged fluorescein (Molecular Probes) at the one-cell stage. Photoactivation of fluorescent dye was performed as described (Rohr and Concha, 2000) at around the 14-somite stage. Following photoactivation, DIC and fluorescent images were acquired using Openlab software (Improvision) with a cooled CCD camera (Hammamatsu) attached to an Axioplan microscope (Zeiss). At later stages, live embryos were mounted in 1% agarose and fluorescent images were acquired with Leica TCS SP Confocal Microscope using a 20X water immersion objective. Three-dimensional series of images were acquired at 2 μm intervals. As an alternative approach, rhodamine-dextran injection was performed using iontophoresis. Embryos were treated with pronase and mounted in 1.2% low-melting agarose. Iontophoric injection of one or two medial optic vesicle cells was performed using 1 μm tip micropipettes filled with 4% rhodamine-dextran. Fates of fluorescent cells were followed and DIC and fluorescent images were acquired using Openlab software.

SU5402 treatment

To block Fgf receptor activity, embryos were treated with 21 μM SU5402 from 70% epiboly to the 24 hpf stage as previously described (Shanmugalingam et al., 2000).

RESULTS

Cloning zebrafish *vax1* and *vax2* genes and phylogenic analysis of their gene products

Degenerate RT-PCR was used to isolate two different zebrafish Vax gene fragments from 24 hpf zebrafish mRNA. Subsequent 5′RACE and 3′RACE of these fragments revealed the full-length sequences of the open reading frames (accession numbers AY185348 and AY183363). In other species, there are *vax1* and *vax2* subdivisions of the Vax gene family. Vax1 was originally cloned as a homeodomain transcription factor similar to Not1, and Vax2 was cloned by similarity to *Drosophila Ems* (Barbieri et al., 1999; Hallonet et al., 1998). Phylogenic analysis using *emx1* as an ‘out group gene’ in the phylogenic tree, showed one of the novel genes fell within the *vax1* subgroup and the other within the *vax2* subgroup (Fig. 1). Therefore, we termed these genes zebrafish *vax1* and *vax2*.

Zebrafish *vax1* encodes 317 amino acids and is most closely related to medaka *vax1* (Winkler et al., 2000) with which it shares 70% identical amino acids. Zebrafish *vax2* encodes 307 amino acids and has 46% identity to *Xenopus vax2* (Barbieri et al., 1999) over the full sequence and 100% within the homeodomain.

Zebrafish Vax genes are expressed in the optic stalk and the preoptic area

In situ hybridization analysis showed that similar to previously described Vax genes, zebrafish *vax1* and *vax2* are expressed in the anterior forebrain, including the optic stalks, ventral retina and preoptic area (Fig. 2), territories that probably all originate from a similar region of the anterior medial neural plate (Varga et al., 1999). A low level of *vax1* mRNA is first detected in the anterior neural keel at 7 somites (Fig. 2A). By 12 somites, expression is more robust in the medial forebrain and forming eyes. By this stage, optic vesicles have evaginated from the brain and the optic recess of the third ventricle is visible as a

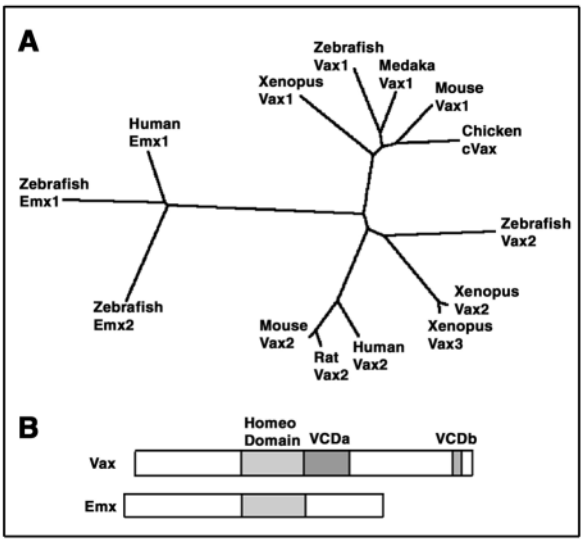


Fig. 1. Phylogenic relationship of the Vax gene family. (A) Lineage tree of vertebrate Vax genes using Emx genes as an ‘out group’. The out group branch separates Vax1 and Vax2 subgroups, both of which contain putative orthologues from different Classes of vertebrates, suggesting that the ancestral Vax gene was duplicated prior to the separation of the vertebrate Classes. Amino acid sequences from GenBank database entries were used. (B) Vax protein structure. Emx and Vax proteins share more than 60% amino acid sequence in the homeodomain (Hallonet et al., 1999), and additionally Vax proteins share nearly invariant homeodomains and have two Vax gene-specific conserved domains, VCDa and VCDb.

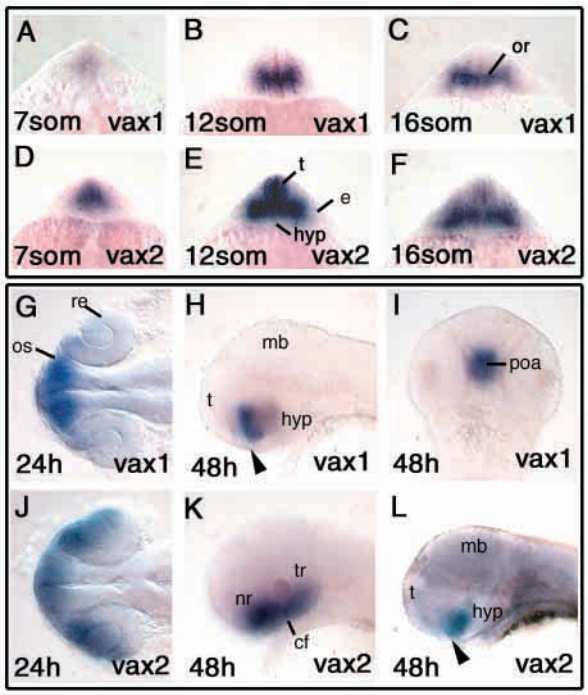


Fig. 2. Zebrafish Vax genes are expressed in the optic stalk and the preoptic area. Expression patterns of *vax1* and *vax2* genes (indicated bottom right) in the anterior neural keels (A,D) and brains (B,C,E-L) of wild-type embryos at various stages (indicated bottom left). Dorsal is to the top in the frontal views (A-F,I). Anterior is to the left in the dorsal views (G,J) and in the lateral views (H,K,L). Eyes are removed to observe the preoptic area in L. Arrowheads indicate optic recess (H,L). Abbreviations: cf, choroid fissure; e, eye; hyp, hypothalamus; mb, midbrain; nr, nasal retina; poa, preoptic area; or, optic recess; os, optic stalk; re, retina; t, telencephalon; tr, temporal retina.

prominent cleft within the rostral brain and optic vesicles (Fig. 2B,C). The region of the brain around the optic recess is termed the pre-optic area and is considered to be the boundary between telencephalon and diencephalon. Laterally, the preoptic area is in direct continuity with the optic stalks that in turn connect to the prospective retinae at the site of future choroid fissure formation.

By 16 somites, *vax1* expression remains within the optic stalk, but like *pax2.1* (see Macdonald et al., 1997) is predominantly restricted to tissue ventral to the optic recess in the preoptic area (Fig. 2C). In addition to the optic stalk and ventral preoptic area, *vax1* is weakly expressed in ventral retina near the choroid fissure region by 24 hpf (Fig. 2G). At 48 hpf, *vax1* expression remains prominent in the ventral preoptic area but is much reduced in the choroid fissure and optic stalk (Fig. 2H,I).

The expression domain of *vax2* is wider than that of *vax1* throughout all developmental stages analysed. By 7 somites, *vax2* mRNA is detected in the anterior brain (Fig. 2D) and expression is strong within optic stalks, preoptic area and telencephalon by 12 somites (Fig. 2E). By 16 somites, expression in the telencephalon is reduced but that in the preoptic area, optic stalk and ventral retina remains (Fig. 2F), and this pattern of expression is similar at 24 hpf (Fig. 2J). By 48 hpf, *vax2* expression is restricted to the ventral preoptic area, anterior dorsal hypothalamus and ventral retina (Fig. 2K,L).

The expression domain of *vax1* is nested within that of *vax2* at all stages observed, and because of their highly conserved homeodomain sequences, these two transcription factors probably have many of the same target genes. To address the function of these genes, we therefore considered it probable that removal of activity of both Vax1 and Vax2 might reveal phenotypes not detected following loss of function of either gene alone.

Abrogation of Vax1 and Vax2 function results in ectopic ipsilateral optic nerve projections and severe coloboma

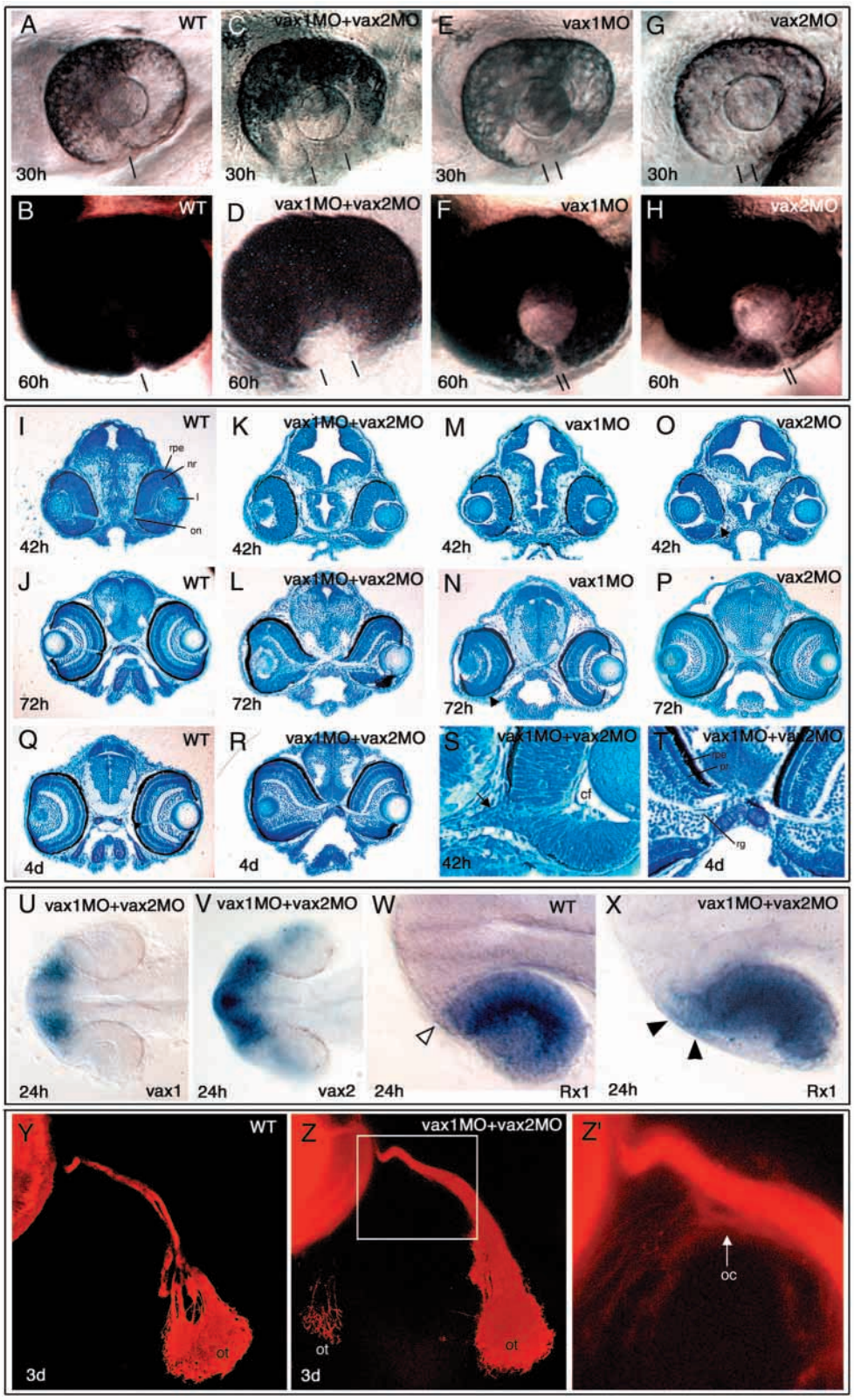
To assess the consequences of reducing the activity of the Vax genes, we injected MOs against 5′UTR sequence of the Vax gene mRNAs (see Materials and Methods for controls). Injection of either *vax1*MO or *vax2*MO led to defects in the ventral eye. Early morphogenesis of the optic stalks and eye cups preceded apparently normally in the injected embryos. However, by 30 hpf it was apparent that the choroid fissure failed to close (Fig. 3E,G), a condition called coloboma that is also observed following loss of Pax2.1/Noi activity (Macdonald et al., 1997), and in mice lacking Vax1 or Vax2 activity (Bertuzzi et al., 1999; Hallonet et al., 1999; Barbieri et al., 2002; Mui et al., 2002). In injected embryos, the retinal pigment epithelium around the choroid fissure appeared reduced and some cell disorganization was evident around the exit point of the optic nerve from the back of the retina (Fig. 3F,H,M-P).

To assess changes in gene expression that might accompany the eye defects in the *vax*MO-injected embryos, we assessed expression of markers of optic stalk, retina and pre-optic area. Although Vax2 is involved in dorso-ventral patterning of the retina in mouse, chicken and frog (Barbieri et al., 2002; Barbieri et al., 1999; Mui et al., 2002; Schulte et al., 1999), we were unable to detect any significant changes in the expression of region-specific markers, *tbx5.1*, *ephrinB2a* and *EphB2* in *vax2*MO embryos (not shown). The only change we detected was in *vax2* itself, which showed slightly weaker expression in the ventral retina of *vax2*MO embryos. Similarly, the optic stalk or choroid fissure markers *fgf8/ace*, *pax2.1/noi* and *netrin2* all appeared largely normal in *vax2* morphants. In *vax1*-MO-injected embryos, expression of *vax1* was slightly reduced in the preoptic area but no other significant changes in patterns of gene expression were detected (not shown).

Co-injection of both *vax1*MO and *vax2*MO resulted in severe disruptions to eye development. *vax1*/*vax2* morphants showed a severe coloboma and pigmentation defects in the ventral retina (Fig. 3C,D). Consistent with the very localized expression of *vax1* and *vax2*, all other aspects of development appear normal and *vax1/vax2* morphants remain viable at 5 dpf. In the *vax1*-MO/*vax2*-MO-injected embryos, expression of *vax1* is reduced in the preoptic area and *vax2* expression is weaker than in wild type in the ventral retina (Fig. 3U,V), suggesting that Vax1 and Vax2 function contributes to the maintenance of *vax1* and *vax2* expression at these sites.

Defects in the medial optic stalk tissue or preoptic area frequently lead to defects in commissural axon guidance (e.g.

Macdonald et al., 1997), and studies in other species have indicated roles for Vax genes in retinal axon guidance (Schulte et al., 1999; Bertuzzi et al., 1999; Hallonet et al., 1999; Barbieri et al., 2002; Mui et al., 2002). We therefore examined optic nerve projections by injecting the lipophilic fluorescent dye, DiI, into the eye. In *vax1*/*vax2* morphants, a minority of axons showed aberrant trajectories at the optic chiasm and ectopically projected to the ipsilateral tectum (Fig. 3Y,Z). No ipsilateral projections were observed in embryos in which *vax1*MO or *vax2*MO were injected alone.



Abrogation of Vax1 and Vax2 function results in a medial expansion of retinal tissue into the forebrain

To assess retinal defects in Vax abrogated embryos, sections through the eyes of the *vax1*/*vax2* morphants were examined. Although the lamination of the retina was normal, there was a progressive expansion of retinal tissue to the midline of the brain (Fig. 3K,L,R-T). At 42 hpf, disruption to the retinal neuroepithelium and the pigmented epithelium is evident at the junction between the eye and optic nerve (Fig. 3K,S). By 72 hpf, retinal lamination has occurred and retinal neuron differentiation appears superficially unaffected in the MO-injected embryos. However, in contrast to wild type, the

Fig. 3. Abrogation of Vax1 and Vax2 function disrupts eye development. Stages indicated bottom left. The morpholino oligonucleotides (MOs) injected are indicated top right. (A-H) Lateral views of left eyes of living embryos with anterior to the left. Bars indicate the choroid fissure, which fails to close in the MO-injected embryos. (I-T) Toluidine blue-stained transverse sections at the level of the choroid fissure or optic nerve head. S and T show higher magnifications of K and R, respectively. Arrows indicate disorganization of cells at the choroid fissure (O,S) and the arrowhead indicates reduced retinal pigment epithelium in the ventral retina (N). (U-X) Dorsal views of Vax (U,V) and *rx1* (W,X) expression with anterior to the left (compare U and V with Fig. 2G,J). Arrowheads indicate that the retinal marker *rx1* is extended into the optic stalk region in the MO-injected embryos. (Y,Z) Confocal projection of DiI-labeled optic nerve with Z′ showing a higher magnification of the midline of Z. Abbreviations: cf, choroid fissure; l, lens; nr, neural retina; oc, optic chiasm; on, optic nerve; ot, optic tectum; pr, photoreceptor layer; rg, retinal ganglion cells; rpe, retinal pigment epithelium.

differentiating neurons extend out of the back of the eye and along the optic nerve towards the brain (Fig. 3L). By four days, retinal neurons are observed all the way to the optic chiasm and pigment epithelium appears in direct contact with the forebrain neuroepithelium (Fig. 3R,T).

The expansion of retina to the brain could either be because of degeneration of optic stalk or nerve tissue coupled with over-proliferation of retinal neurons, or alternatively optic stalk or nerve cells may change fate and differentiate as retina. We performed several sets of experiments to determine which of these possibilities is the more probable. First, in 24 hpf *vax1/vax2* morphants, expression of the retinal marker *rx1* extended into the optic stalks (Fig. 3W,X), suggesting that optic nerve territory does adopt retinal character when Vax activity is abrogated.

To more directly assess whether optic nerve territory forms retina in Vax morphants, we attempted to follow the fate of these cells over time. To do this, cells in the medial region of the optic vesicles of wild-type and *vax1/vax2* morphants were labelled by photoactivating caged fluorescein at the 14 somite stage (Fig. 4A). The position and viability of the fluorescently labelled cells was subsequently assessed at 2, 3 and 4 dpf (n=15 morphants; Fig. 4B-G). In both wild-type and *vax1/vax2* morphants, cells labelled at the junction of optic stalk and brain gave descendents in the preoptic area and optic stalks (Fig. 4B,E). In contrast to wild type, however, the labelled territory remained very broad and did not constrict to form a nerve in the *vax1/vax2* morphants. There was no obvious disappearance of the fluorescent cells in the *vax1/vax2* morphants as might be expected if the optic stalks cells were dying and being replaced by retinal neurons. Indeed, labelling of individual medial optic vesicle cells through iontophoresis of rhodamine dextran confirmed the viability of these cells in *vax1/vax2* morphants (data not shown). By four days, the labelled cells in *vax1/vax2* morphants occupied a broad domain at the interface of the eye with the brain. Given the faintness of the fluorescence at this stage, it was not possible to definitively show that individual fluorescent cells formed retinal neurons. However, by this stage, plastic sectioning suggests that most or all optic vesicle cells of *vax1/vax2* morphants are retinal in character (Fig. 3T). These data therefore favour the possibility that cells that should form optic nerve eventually differentiate as retina in embryos lacking Vax1/Vax2 activity.

Altogether, our data shows that Vax gene activity is required to limit retinal development to the optic cup. When Vax function is compromised retinal tissue differentiates in a territory that should only normally contain reticular astrocytes of the optic nerve (Macdonald et al., 1997). Given the importance of Vax genes in regional patterning of eye structures, it is crucial that the spatial regulation of their expression is tightly controlled. We therefore next assessed the pathways involved in regulation of Vax gene expression.

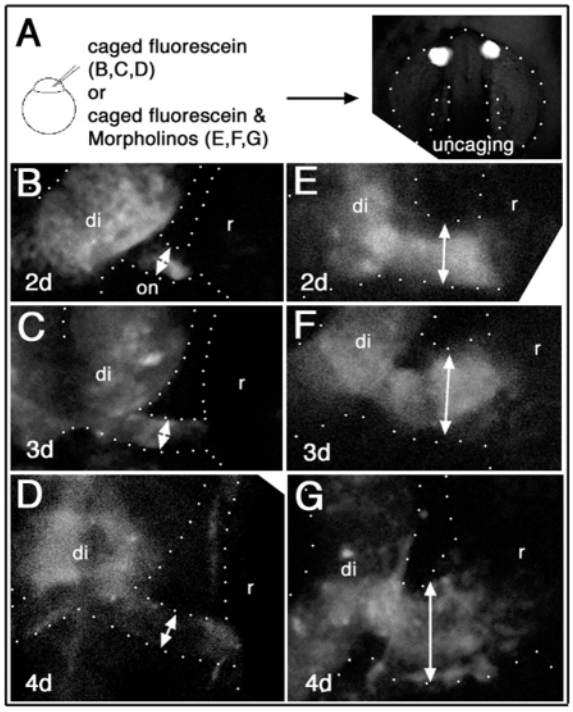


Fig. 4. Medial optic vesicle cells are viable but fail to constrict to form an optic nerve in *vax1/vax2* morphants. (A) Schematic representation of the experiment (left) and dorsal view of an embryo with fluorescein uncaged in both left and right optic stalks (right). (B-G) Confocal images of living eyes, optic nerves and preoptic area of wild-type (B-D) and *vax1*-morpholino oligonucleotide (MO)/*vax2*-MO (E-G) embryos at the stages indicated bottom left. Arrowbars indicate the width of the optic stalk or nerve territory. Abbreviations: di, diencephalon; on, optic nerve; r, retina.

Hedgehog signals acting through Smoothened induce Vax gene expression

The Hedgehog (Hh) pathway is an upstream regulator of *pax2.1* within the optic stalks (Ekker et al., 1995; Macdonald et al., 1995; Varga et al., 2001). To determine whether Hh activity also regulates Vax gene expression, we initially assessed the consequences of overexpression of *shh*. In *shh*-injected embryos, the Hh target gene *ptc2* is strongly induced throughout the eye (Fig. 5I,L), suggesting that Hh activity is no longer spatially localized to medial regions of the optic vesicle. In such embryos, the optic stalk expands at the expense of retinal tissue (Ekker et al., 1995; Macdonald et al., 1995), and *vax1* and *vax2* expression domains are expanded within the mispatterned optic vesicle (Fig. 5D,E). *vax2* expression expands to the dorsal retina (Fig. 5E), and complimentary to this, expression of the dorsal retinal markers *tbx5.1* and *msxC* are reduced or absent in *shh*-injected embryos (Fig. 5C,F) (Macdonald et al., 1995). Thus, Hh signals expand not only optic stalk markers but also the ventral retinal marker *vax2*. These experiments suggest that Hh signals promote Vax gene expression and change the fate of eye and optic stalk tissue from distal to proximal fates. Overexpression of *shh* does not induce Vax gene expression elsewhere within the CNS, indicating that a spatially restricted domain of the CNS is competent to express Vax genes in response to Hh signals.

To study the requirement of Hh signals to induce Vax genes, we analysed expression in *smu* mutant embryos that lack zygotic activity of the Smoothened (Smu) membrane protein (Varga et al., 2001), an essential mediator of Hh signalling (Ingham and McMahon, 2001). In $smu^{-/-}$ embryos, expression of both Vax genes is severely reduced or absent (Fig. 5G,H), revealing an essential requirement for Hh signalling in patterning of the optic stalks, ventral retina and pre-optic area. The weak Vax expression retained in some $smu^{-/-}$ embryos

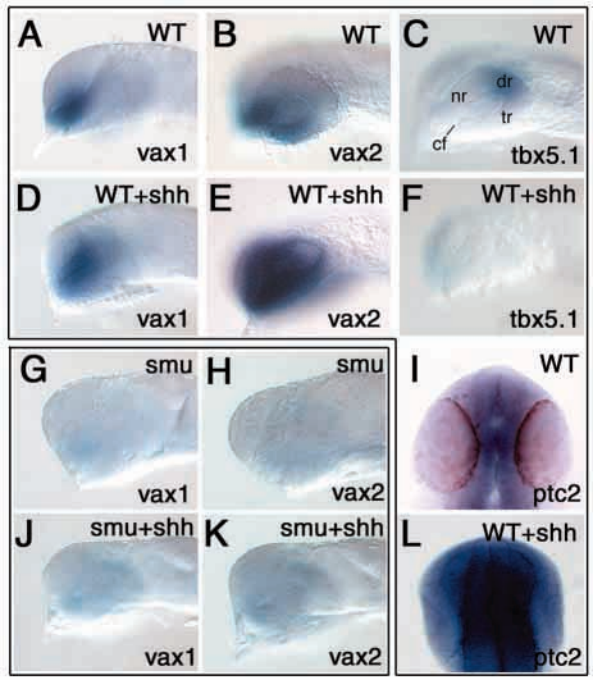


Fig. 5. Hedgehog signals acting through Smoothened induce Vax gene expression. Anterior is to the left in the lateral views (A-H,J,K) and anterior is to the top in the dorsal views (I,L) of 24 hpf brains showing expression of genes (indicated bottom right) in wild-type (WT) (A-C,I), *shh*-injected wild-type (WT+shh) (D-F,L) embryos, s*moothened*$^{-/-}$ (smu) (G,H), and *shh*-injected *smoothened*$^{-/-}$ (smu+shh) (J,K) mutant embryos. In *shh*-injected wild-type embryos, expression of the ventral retinal gene, *vax2*, expands dorsally (E), whereas expression of the dorsal retinal gene, *tbx5*, is abolished (F). Abbreviations: cf, choroid fissure; dr, dorsal retina; nr, nasal retina; tr, temporal retina.

may be because of the Hh signalling through residual maternal Smu protein as has been suggested for *pax2.1* and *nk2.2* within the brain (Varga et al., 2001; Chen et al., 2001).

To assess whether the induction of the Vax genes by overexpression of Shh is mediated through the Smu pathway, *shh*-injected *smu*$^{-/-}$ mutants were examined. Vax (Fig. 5J,K) and *pax2.1* (data not shown) gene expression did not recover in the *shh*-injected *smu*$^{-/-}$ embryos, suggesting that, as expected, exogenous Shh acts entirely through a Smu-dependent pathway.

Hedgehog signals act downstream of the Nodal signalling pathway to induce Vax genes

In addition to the Hh pathway, Nodal signalling is crucial for various aspects of midline patterning, including the establishment of ventral territories in the brain. In some cases, Nodal signalling influences patterning of the brain through regulation of the Hh pathway, whereas in others it functions independent of Hh activity (Masai et al., 2000; Mathieu et al., 2002; Rohr et al., 2001). *cyc*$^{-/-}$ embryos lack activity of the Nodal ligand Cyclops (Rebagliati et al., 1998; Sampath et al., 1998) and *oep*$^{-/-}$ embryos lack zygotic activity of Oep, an EGF-CFC family membrane protein essential for the reception of Nodal signals (Zhang et al., 1998; Gritsman et al., 1999). Vax gene expression was always either severely reduced or absent in *cyc*$^{-/-}$ and zygotic *oep*$^{-/-}$ (Z*oep*) embryos, indicating that as for the Hh pathway, Nodal signalling is required for patterning the preoptic area, optic stalks and ventral retina (Fig. 6A-D).

In Nodal pathway mutants, expression of *shh* is reduced or lost in anterior regions of the embryo (e.g. Rohr et al., 2001), indicating that activity of both Nodal and Hh signalling pathways is compromised. To study the genetic epistasis of the Nodal and Hedgehog pathways on the induction of Vax genes, we injected *shh* into Z*oep*$^{-/-}$ embryos. The presence of exogenous Shh in anterior regions of Z*oep*$^{-/-}$ embryos restored Vax gene expression in the rostral forebrain (Fig. 6E,F). In most cases, expression was induced in medial tissue at the interface between the ventral telencephalon and the fused eye, a position one would expect preoptic area and optic stalks to differentiate in wild-type embryos. Thus tissue capable of expressing Vax genes is present in Nodal mutants, but appears to fail to receive the signals necessary for induction of expression. These observations suggest that Hh signalling acts downstream of the Nodal pathway to induce Vax genes.

Lack of Nodal signalling activity induces Vax genes

Although Z*oep*$^{-/-}$ embryos lack zygotic expression of *oep*, they retain maternally derived *oep* mRNA (Zhang et al., 1998) and hence possess residual Nodal signalling at early developmental stages. In contrast to Z*oep*$^{-/-}$, embryos lacking both maternal and zygotic *oep* mRNA (MZ*oep*$^{-/-}$) showed *vax1* and *vax2* expression in the brain adjacent to the fused eye (Fig. 7A,B). Expression was broader than that of the optic stalk-specific marker *pax2.1* (Masai et al., 2000) (Fig. 7C), suggesting that forebrain tissue with at least some features of preoptic area identity is present in MZ*oep*$^{-/-}$ embryos but not in Z*oep*$^{-/-}$ embryos. The recovery of Vax gene expression in MZ*oep*$^{-/-}$ embryos indicates that early Nodal activity indirectly suppresses the induction of Vax gene expression.

To complement these studies, we also inhibited Nodal

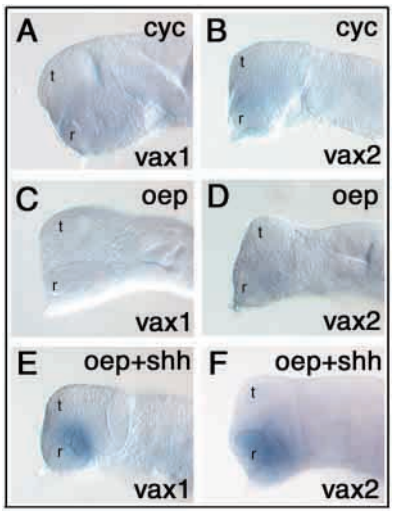


Fig. 6. Hedgehog signals act downstream of the Nodal signalling pathway to induce Vax gene expression. Lateral views of gene expression (indicated bottom right) in 24-hour stage *cyc*$^{-/-}$ (cyc) (A,B), *oep*$^{-/-}$ (oep) (C,D), shh-injected *oep*$^{-/-}$ (oep+shh) (E,F) embryos. Abbreviations: r, retina; t, telencephalon.

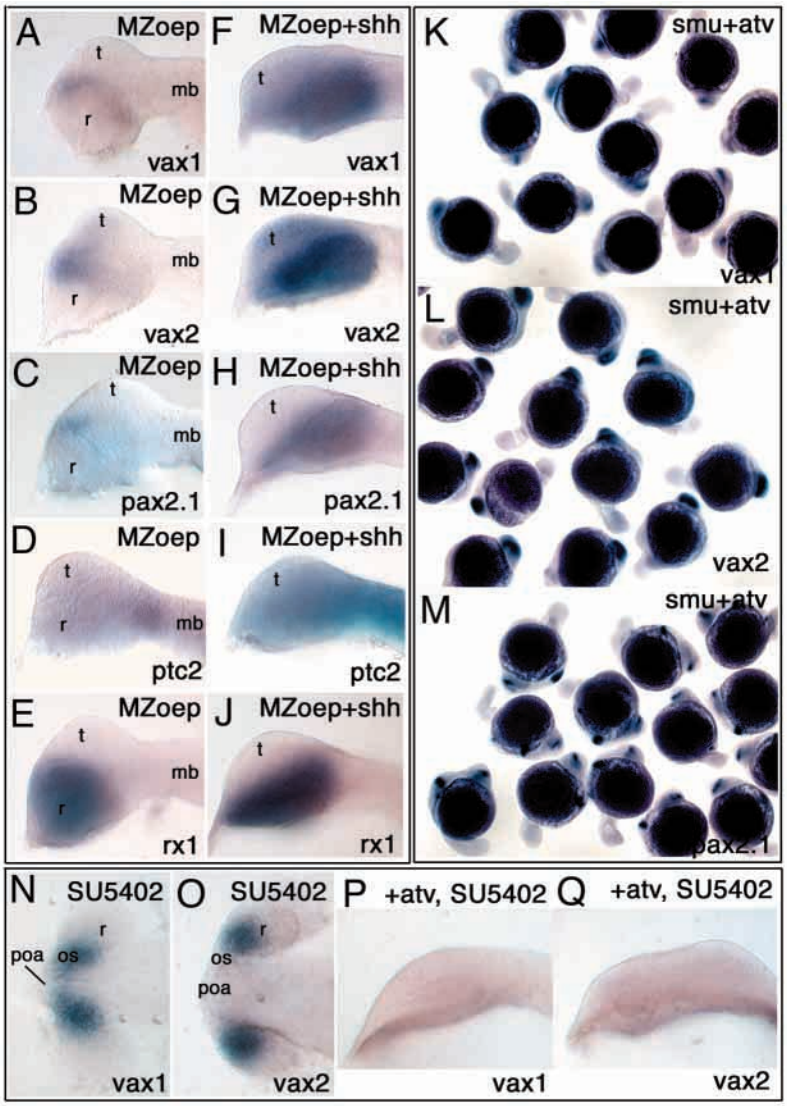


Fig. 7. Lack of Nodal signaling activity leads to Fgf receptor-dependent induction of Vax gene expression. (A-J) Lateral views of brains of maternal and zygotic *oep*$^{-/-}$ (MZoep) (A-E), and *shh*-injected maternal and zygotic *oep*$^{-/-}$ (MZoep+shh) (F-J) mutant embryos analysed for expression of various genes (indicated bottom right). (K-M) *atv*-injected sibling embryos from *smu*$^{+/-}$ × *smu*$^{+/-}$ crosses showing expression of Vax genes and *pax2.1* expression. (N-O) Dorsal views of Vax expression in embryos treated with the Fgf receptor inhibitor SU5402. (P-Q) Lateral views showing absence of Vax expression in *atv*-injected embryos treated with SU5402. Abbreviations: mb, midbrain; os, optic stalk; poa, preoptic area; r, retina; t, telencephalon.

activity through overexpression of Antivin/Lefty1 (Atv/Lft1), an extracellular inhibitor of Nodal signalling (Bisgrove et al., 1999; Meno et al., 1999; Thisse et al., 2000; Thisse and Thisse, 1999). Injection of a high concentration of *atv* inhibits all Nodal signalling and results in a phenotype similar to MZ*oep*$^{-/-}$ embryos or *cyc/sqt* double mutants. As expected, overexpression of high levels of *atv/lft1* led to restoration of Vax expression in a pattern similar to that in MZ*oep*$^{-/-}$ embryos (data not shown). Altogether these experiments reveal a complex requirement for Nodal activity in the regulation of Vax gene expression in that early Nodal activity inhibits Vax gene expression (through an unknown mechanism), whereas later Nodal signalling promotes Vax gene expression (at least in part through promoting Hh activity).

Induction of Vax and *pax2.1* gene expression in the absence of Nodal activity is Smu-independent

The restoration of Vax gene expression in the absence of Nodal activity could either be because of a restoration of Hh activity in the vicinity of the Vax expression, or it could be that the Vax genes are induced by other signals independent of Hh activity. To assess which of these possibilities is more probable, we assayed whether Hh activity is present in the rostral brain of MZ*oep*$^{-/-}$ embryos and sought to simultaneously inhibit both Nodal and Hh signalling to ask whether this suppressed Vax expression.

Neither *twhh* nor *shh* appears to be expressed at early stages in anterior regions of MZ*oep* embryos and later *shh* expression is restricted to posterior forebrain tissue far from the site of *pax2.1* induction (see Rohr et al., 2001). Indeed, induction of the probable Hh target genes *patched2* and *nk2.1a* is restricted to the posterior forebrain in the vicinity of the late *shh* expression (Fig. 7D) (Rohr et al., 2001). These results suggest that Hh signalling is not occurring in the vicinity of the *pax2.1* and Vax expression domains of MZ*oep*$^{-/-}$ embryos.

Analysis of Vax and *pax2.1* expression in embryos compromised in both Hh and Nodal signalling confirmed that Smu activity is unlikely to be required to induce Vax or *pax2.1* expression in Nodal activity compromised embryos. By using Atv to inhibit Nodal signalling in *smu*$^{-/-}$ embryos, we found that there was no apparent difference in the expression of Vax genes or Pax2.1 among all injected siblings from smu$^{+/-}$ × *smu*$^{+/-}$ crosses (Fig. 7K-M). Thus, Vax genes are induced both in smu$^{-/-}$ and in wild-type embryos when Nodal activity is inhibited and therefore this induction is probably independent of Smu activity. We cannot completely exclude the possibility that maternal Smu promotes Vax expression following inhibition of Nodal activity, but given that maternal Smu has very little ability to promote Vax expression in otherwise wild-type embryos, we think that this is a remote possibility.

MZ*oep* and *atv*-injected embryos are sensitized to induction of Vax/*pax2.1* expression by Hh signals

Given that repression of Vax gene expression appears to be relieved in MZ*oep*$^{-/-}$ embryos as compared to Z*oep*$^{-/-}$ embryos, we hypothesized that MZ*oep*$^{-/-}$ forebrain tissue may be sensitive to factors that promote Vax gene expression. We therefore assessed the consequences of overexpressing *shh* in MZ*oep*$^{-/-}$ embryos. This led to extensive induction of *vax1*, *vax2* and *pax2.1* in the rostral CNS, to a much greater extent than in Z*oep*$^{-/-}$ embryos (Fig. 7F-H). *shh* injection alters morphogenesis of the eye in MZ*oep*$^{-/-}$ embryos such that there

is no obvious optic cup or eye structure, and instead eye tissue (as assayed by *rx1* expression, Fig. 7J) remains in continuity with the rest of the diencephalon. Vax gene expression is induced throughout this eye or diencephalon territory. Similar results were obtained in embryos injected with both *atv* and *shh*. These results suggest that a complete lack of Nodal signals renders the diencephalon sensitive to induction of Vax and *pax2.1* genes in response to Hh signals.

Fgf signalling promotes Vax expression in the absence of Nodal signalling

Fgf activity is required for development of midline tissue between the eyes, raising the possibility that this pathway may contribute to the regulation of Vax gene activity. We therefore attempted to assess whether Fgf signals contribute to the regulation of Vax expression in wild type and in Nodal signalling-depleted embryos.

Vax gene expression is not obviously affected in *ace* mutant embryos that lack Fgf8 activity (data not shown), but other Fgfs, such as Fgf3, are still active in the anterior CNS of these embryos (see Shinya et al., 2001). Therefore to more comprehensively inhibit Fgf signalling, embryos were treated with SU5402, a chemical inhibitor of Fgf receptor function (Mohammadi et al., 1997). Embryos treated with SU5402 from 70% epiboly to 24-hour stage lacked expression of Vax genes in the preoptic area (Fig. 7N,O), consistent with previous data implicating Fgfs in midline patterning of the anterior forebrain (Shanmugalingam et al., 2000).

Given the likely requirement of Fgf signals in the regulation of Vax gene expression, we next assessed whether the recovery of Vax expression in Nodal-signalling depleted embryos is dependent upon Fgf receptor activity. In support of this possibility, *antivin*-injected embryos that were treated with SU5402 lacked Vax gene expression (Fig. 7P,Q).

Altogether, these experiments suggest that Fgf signalling promotes Vax gene expression and Fgf activity may be responsible for the recovery of Vax gene expression following severe abrogation of Nodal activity.

Belladonna regulates Vax gene expression in the preoptic area

Next, we attempted to investigate the nature of the genes that might act downstream of Hh or Fgf signals to regulate Vax gene expression. As *pax2.1* expression is promoted by Hh and Fgf activity (Ekker et al., 1995; Macdonald et al., 1995; Shanmugalingam et al., 2000; Varga et al., 2001) and precedes Vax gene expression in the optic stalks, then Pax2.1 is a candidate for inducing the Vax genes in this region. However, induction and maintenance of Vax gene expression proceeds normally within the optic stalks of *noi* (*pax2.1*) mutant embryos (Fig. 8A,B). Furthermore, exogenous *shh* efficiently induces Vax gene expression in *noi*$^{-/-}$ mutants as in wild type (Fig. 8C,D).

Defects in patterning of medial optic stalk tissue or preoptic area frequently lead to defects in commissural axon guidance (e.g. Macdonald et al., 1997; Shanmugalingam et al., 2000; Varga et al., 2001). We therefore screened other lines of fish known to have defects in commissure formation for alterations to Vax gene expression.

belladonna (*bel*) is a mutation that leads to ipsilateral projection of the optic nerves and consequent reversal of visual behavioural responses (Karlstrom et al., 1996; Neuhauss et al., 1999; Rick et al., 2000). In *bel*$^{-/-}$ embryos there is a reduction of Vax gene expression in the preoptic area, whereas expression in the optic stalks and ventral retina appears normal (Fig. 8E,F).

bel has not previously been suggested to be a Hh pathway mutation and normal expression of hh pathway genes in *bel*$^{-/-}$ embryos suggests that Bel is not an upstream regulator of Hh signalling. To test whether Bel might act downstream or in parallel to Hh signalling, we injected *shh* into *bel*$^{-/-}$ embryos. In these embryos, Vax gene expression remained absent from the midline preoptic area, even though expression is expanded in the optic stalks and retina (Fig. 8G,H). Thus Bel function is required within the preoptic area downstream of, or parallel to, Hh signals to induce Vax gene expression.

DISCUSSION

We describe the cloning and characterization of zebrafish *vax1* and *vax2*. We show that each Vax gene is required for closure of the choroid fissure and together they are synergistically required for optic nerve differentiation and to limit retinal development to the optic cup. Finally, we show that although Hh signals are perhaps the most crucial

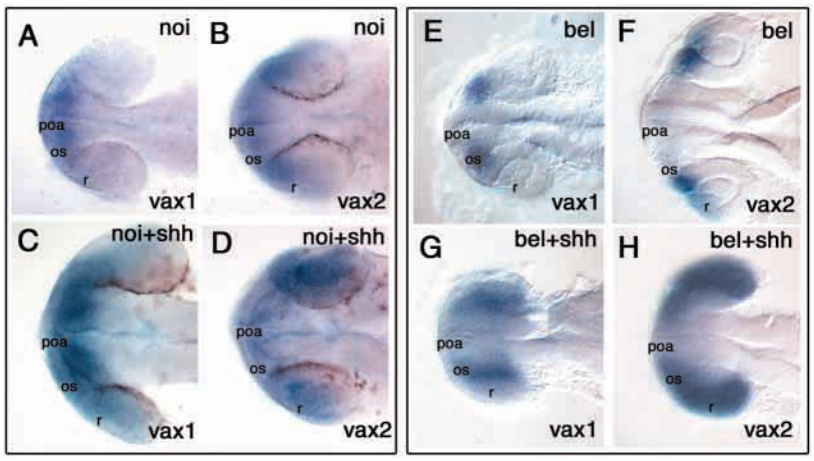


Fig. 8. Belladonna regulates Vax gene expression in the preoptic area. Anterior is to the left in the dorsal views of heads of *noi*$^{-/-}$ (*noi*) (A,B), *shh*-injected *noi*$^{-/-}$ (noi+shh) (C,D), *bel*$^{-/-}$ (bel) (E,F), and *shh*-injected *bel*$^{-/-}$ (bel+shh) (G,H) embryos. Note that the expression of Vax genes is reduced in the preoptic area but not in the optic stalk and ventral retina of *bel*$^{-/-}$ embryos (E,F, compare with Fig. 2G,J). Abbreviations: os, optic stalk; poa, preoptic area; r, retina.

regulators of Vax gene expression, other signals, including Fgfs, may also regulate Vax gene expression in a Hh-independent manner.

The expression domain of zebrafish *vax1* is nested within that of *vax2*

The presence of *vax1* and *vax2* subfamilies of genes in various vertebrates supports the idea that an ancestral Vax gene duplicated prior to the divergence of the different vertebrate classes. Despite the probable ancient nature of this duplication, *vax1* and *vax2* genes have retained conserved domains of expression between species. Thus, *vax1* genes tend to be expressed strongly in medial regions of the anterior brain, whereas *vax2* genes are expressed prominently in the ventral retina. This is presumably because of changes in the regulatory elements that control the spatial extent of expression of the genes (see Lynch and Force, 2000). The conservation of *vax1* and *vax2* expression domains between vertebrate Classes suggests that the changes in regulation of the two genes may predate the vertebrate lineage. It will therefore be of great interest to assess whether non-vertebrate chordates possess both Vax genes and if so, what their patterns of expression are.

Despite the ancient timing of the duplication that has probably established Vax1 and Vax2, the two proteins remain structurally highly related, and so probably have similar functional activity. Indeed, as Vax1 and Vax2 have highly conserved homeodomains and are expressed in overlapping regions (particularly in zebrafish in which *vax1* expression is nested entirely within the *vax2* expression domain), then it is probable that these transcription factors regulate many of the same target genes. It is possible that other factors may modulate the transcriptional activity of the Vax proteins by interacting with the two conserved domains, VCDa and VCDb, which lie outside of the Vax homeodomain. As Vax2 has shorter conserved stretches within these domains than Vax1 (data not shown), it is possible that this may impart at least some differences in functional activity between Vax1 and Vax2 proteins.

Given that Vax1 and Vax2 probably regulate the same genes in the same territory, there must be evolutionary pressure to maintain the activity of the two genes in the same cells. In other cases of functionally similar transcriptional regulators, we, and others, have found that presumed sites of expression of an ancestral gene are often split between the pair of genes that result from a duplication event, thereby minimizing co-expression (e.g. Force et al., 1999; Rohr et al., 2001). In the case of the Vax genes, we speculate that the maintenance of the *vax1* expression domain within the *vax2* expression domain will contribute to a gradient in the concentration of Vax proteins from medial regions of the brain to distal regions of the optic vesicle. As we discuss below, there is now considerable evidence of graded patterning of the optic vesicle along its proximo-distal axis, and we suggest that variations in the levels of Vax proteins may contribute to this pattern.

Vax transcription factors regulate closure of the choroid fissure, limit retinal development to the optic cup and mediate commissural axon pathfinding

Loss of function studies in mouse (Barbieri et al., 2002; Bertuzzi et al., 1999; Hallonet et al., 1999; Mui et al., 2002) and now in zebrafish have revealed requirements for Vax proteins in several different aspects of eye and forebrain midline development. The most conserved phenotype following abrogation of Vax activity is a failure in fusion of the choroid fissure. This phenotype is found in both mouse and fish lacking, or with reduced, Vax1 or Vax2 activity (with genetic background-dependent penetrance in mouse *Vax2* mutants). The severity of this coloboma phenotype is increased in fish embryos compromised in both Vax1 and Vax2 activity, strongly suggesting that both Vax1 and Vax2 co-operate to regulate fusion of choroid fissure. The proteins that mediate fusion of the retina at the choroid fissure are unknown, but possible candidates include Eph receptors and their Ephrin ligands. Members of this family of signalling proteins have been implicated in fusion events in other epithelia (Holmberg and Frisen, 2002), and several family members are expressed in the ventral retina (Holash and Pasquale, 1995; Connor et al., 1998; Eph Nomenclature Committee, 1997). Indeed, in mouse, changes in ephrinB1, ephrinB2 and EphB2 expression occur in the ventral retina of *vax2* mutants, and although these changes have primarily been considered in terms of retinal axon pathfinding (Barbieri et al., 2002; Mui et al., 2002), they could potentially contribute to the choroid fissure defects.

A second conserved phenotype in Vax mutants is disruption to commissural axon guidance and targeting of retinal axons. In *Vax1* mouse mutants, there is a severe disruption to midline development (Bertuzzi et al., 1999; Hallonet et al., 1999) and consequently, severe disruption in the pathfinding of axons as they approach the midline. In *vax2* mutants there are also variable defects in the development of ipsilateral and contralateral retinal projections (Mui et al., 2002; Barbieri et al., 2002). These may arise from incorrect assignment of identity to retinal neurons in the *Vax2* mutants (Mui et al., 2002; Barbieri et al., 2002), but the possibility that midline tissue is also disrupted has not been excluded. Indeed, in fish, commissural axon pathfinding defects are much more obvious in Vax1/Vax2 double morphants than in either single morphant. This suggests, that at least in fish, Vax2 does co-operate with Vax1 to pattern midline tissue.

The third conserved phenotype in animals compromised in Vax protein activity is a failure to limit retinal development to the optic cup. The initial indications of this phenotype came from analysis of mouse *Vax1* mutants in which expression of genes normally restricted to retinal tissue (*rx* and *pax6*) was observed to encroach into the optic nerve (Bertuzzi et al., 1999; Hallonet et al., 1999). Our study provides dramatic confirmation of the requirement for Vax protein activity to limit retinal development. In Vax1/Vax2 double morphants, there is a progressive expansion of retinal tissue along the optic nerve until by four days, neural and pigmented layers of the retina are in direct continuity with diencephalic cells of the optic chiasm. Early morphogenesis of the optic stalk and optic cup occurs relatively normally and indeed, retinal axons navigate out of the eye and along the stalk or nerve to the midline in the Vax1/Vax2 morphants. It is relatively late in development that differentiating retinal tissue expands to the midline. Our favoured hypothesis (given the spread of retina-specific gene expression into the nerve, and fate tracing experiments) is that this phenotype reflects a change in the fate of optic stalk or nerve cells to retinal tissue. We have not however, ruled out the possibility that overproliferation and evagination of retinal

cells from the back of the eye may also contribute to the phenotype.

Although there are clear similarities in the phenotypes following abrogation of Vax function in mice and in fish, there are also differences. For instance, midline defects are more severe in *vax1* mutant mice than in *vax1/vax2* morphant fish. This of course could reflect true differences in Vax protein function but there are other possibilities. Perhaps the simplest would be that the *vax1* MO does not remove all Vax1 function in fish. Although we cannot discount this possibility, the severity of the retinal expansion phenotype in the Vax1/Vax2 morphants (which is much more severe than either *vax1* or *vax2* mutant mice) and the penetrance of the coloboma phenotype suggest that the MOs probably severely abrogate Vax function. A further possibility is the presence of other Vax genes in the fish genome. Indeed, it appears that expression of the known Vax genes is initiated a little later in fish than in other vertebrates, raising the question of whether another Vax gene, perhaps with early expression and a stronger role in midline development, might exist. The ongoing sequencing of the fish genome should soon allow us to resolve this issue.

Midline signals induce Vax genes in the preoptic area, optic stalk and ventral retina

The Hh pathway is a key regulator of Vax gene expression in the preoptic area, optic stalk and ventral retina. In *smu*$^{-/-}$ embryos, Vax gene expression is lost in all these sites and so Hh signals emanating from midline tissues are required to induce Vax genes. Overexpression of Hh signals can expand Vax expression in wild-type embryos and restore Vax expression in Nodal pathway mutants, indicating that Hh signals are both necessary and sufficient to induce Vax expression within the rostral forebrain and eyes. Within the optic vesicles, Hh signalling may act in a graded way with the highest levels of activity promoting the most medial fates (preoptic area and optic stalks), with lower levels promoting choroid fissure and ventral retina. Although definitive proof of such a model is still lacking, gain of function experiments consistently show graded effects of Hh activity in proximo-distal patterning of the optic vesicles (e.g. Macdonald et al., 1995; Ekker et al., 1995; Ungar et al., 1996; this study; Hallonet et al., 1999).

The loss of Vax gene expression in Nodal pathway mutants is probably because of loss of *hh* expression in anterior regions of the mutant embryos. Indeed, activation of the Nodal pathway can lead to direct induction of Hh gene transcription and can consequently expand optic stalk *pax2.1* expression (Muller et al., 2000). Furthermore, restoration of Hh signals in Nodal mutants leads to induction of Vax genes, implying that tissue capable of Vax expression is present in the Nodal mutants but does not receive the signals necessary for induction. The loss of Hh activity in Nodal mutants may also underlie defects in patterning of ventral telencephalon (Rohr et al., 2001) and in aspects of hypothalamic development (Mathieu et al., 2002). However, Hh signals are not sufficient to induce the hypothalamus in the absence of Nodal activity (Rohr et al., 2001), and there is an absolute requirement for direct reception of Nodal signals by cells in this part of the brain (Mathieu et al., 2002). Indeed, given that the severity of optic vesicle cyclopia is greater in Nodal pathway mutants than in zygotic *smu*$^{-/-}$ mutants, we suggest that it is unlikely that the regulation of Hh gene transcription is the only route by which Nodal signals pattern the optic vesicles.

Vax genes can be induced in the absence of Hh and Nodal signals

Although Vax expression is absent in *smu*$^{-/-}$, *cyc*$^{-/-}$ and embryos that lack zygotic activity of Oep, it is present in embryos that lack both maternal and zygotic Oep activity, and in both wild-type or *smu*$^{-/-}$ embryos that overexpress the Nodal antagonist Lefty1/Atv. As both Hh and Nodal signals are severely depleted in anterior regions of these embryos, then we can conclude that under certain circumstances, Vax gene induction (and *pax2.1*) (Masai et al., 2000) can occur in the absence of both Nodal and Hh signals. Our interpretation of this result is that removal of all Nodal signals increases the level of activity of other inducers of Vax gene activity, decreases the levels of inhibitors of Vax gene expression, or may do both.

Despite the absence of ventral forebrain derivatives such as the hypothalamus (Rohr et al., 2001), the forebrain is enlarged in MZ*oep* embryos (Gritsman et al., 2000). This may be because of reduced activity of Wnt proteins emanating from the margin that posteriorize the developing neural tissue (Erter et al., 2001). One might therefore speculate that Wnt signals emanating from the marginal zone of the embryo normally inhibit Vax gene expression and the failure to induce such signals in MZ*oep* embryos relieves this inhibition. However, we argue elsewhere (Houart et al., 2002) that it is unlikely that marginally derived Wnt signals directly influence pattern within the forebrain, and instead do so by regulating the expression of other genes that act more locally within the neural plate.

The Vax-expressing cells in MZ*oep*$^{-/-}$ embryos and the cells competent to express Vax genes in Z*oep*$^{-/-}$ are located at the interface between the eye and the telencephalon. Indeed, within the neural plate, optic stalks and probably preoptic tissues, derive from cells at the interface between axial midline tissues and the anterior border of the neural plate (Varga et al., 1999). As the anterior neural plate border is a known source of signalling proteins (Houart et al., 1998; Shimamura and Rubenstein, 1997; Shanmugalingam et al., 2000; Houart et al., 2002), then such signals are very good candidates for being inducers of Vax gene expression. Furthermore, the activity of signals emanating from the anterior border of the neural plate is probably enhanced in MZ*oep* embryos (Houart et al., 2002), providing a possible explanation for the restoration of Vax gene expression in these embryos.

Our data suggests that Fgfs are good candidates for being inducers of Vax gene expression in the absence of Nodal activity. *fgf8* is expressed in the anterior border of the neural plate adjacent to the eye field, and the midline patterning defects and loss of medial *pax2.1* expression in *ace*/*fgf8* mutants suggest that Fgf8 has an important role in the development of the medial optic stalk (Shanmugalingam et al., 2000). In this study, we show that Fgf receptor activity is necessary for expression of Vax genes in the preoptic area and for the restoration of Vax expression in Nodal-depleted embryos. We therefore suggest that medial cells in the anterior neural plate express Vax genes in response to Hh signals arising from axial tissues and Fgf signals arising from the anterior margin of the neural plate (and perhaps elsewhere).

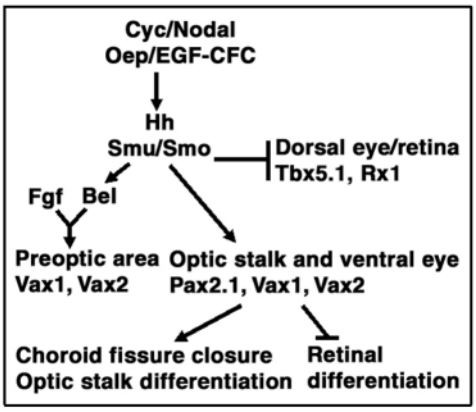


Fig. 9. Schematic indicating genetic pathways involved in the regulation of Vax genes and *pax2.1* expression and patterning of the early eye primordium. Cyc/Nodal signals from axial tissues promote medial fates in the anterior neural plate (including optic stalks and preoptic area), in part through the regulation of Hh expression. Hh proteins promote both *pax2.1* and Vax gene expression in parallel. Within the forebrain, Bel and Fgf activity is required for Hh to promote Vax expression. We currently do not know the epistatic relationship between Bel and Fgf signals. It is also probable that Fgf signals act more widely to pattern optic stalks as well as the preoptic area. Both *pax2.1* and Vax genes are required for closure of the choroid fissure, whereas Vax genes have a more prominent role than *pax2.1* in limiting retinal differentiation to the eye cup. See text for further details.

One further issue to consider is that in other regions of the CNS, Shh contributes to the establishment of pattern by relieving Gli3-mediated inhibition of Hh target genes. Thus removal of Gli3 function restores ventral spinal cord and telencephalic fates that are normally absent in the absence of Shh activity (Litingtung and Chiang, 2000; Rallu et al., 2002). It is not known if Gli3 functions within Vax-expressing territories, but this is an issue that deserves further investigation.

Belladonna and Fgf signalling regulate Vax gene expression in the preoptic area

There are striking similarities between the expression, upstream regulation and function of Vax genes and Pax2 genes within the forming optic vesicles and midline tissues of the brain (Fig. 9). For instance, the *noi*/*pax2.1* gene is expressed in the optic stalks prior to the expression of Vax genes (Macdonald et al., 1997; Pfeffer et al., 1998; this study), is regulated by Nodal and Hedgehog activity (Ekker et al., 1995; Macdonald et al., 1995; Masai et al., 2000; Varga et al., 2001), regulates choroid fissure closure and guidance of axons at the midline (Macdonald et al., 1997; Torres et al., 1996) and suppresses *pax6* expression in the optic stalk (Schwartz et al., 2000). Given these similarities, we wondered whether there might be regulatory interactions between the Pax2 and Vax genes. However as yet, no such interactions have been clearly demonstrated in either fish or mice in which Pax2 or Vax gene function is disrupted. Thus *pax2* is still expressed in embryos with reduced Vax activity (this study, Bertuzzi et al., 1999; Hallonet et al., 1999; Mui et al., 2002, Barbieri et al., 2002), whereas Vax gene expression is retained in *noi* fish mutants that lack activity of Pax2.1. These observations suggest that although *pax2* and Vax genes may be regulated in a similar way and function in the same patterning events, they may do so in parallel pathways. One caveat is that *pax2* and Vax gene expression has not been examined in embryos in which it is certain that all Pax2 activity or all Vax activity is completely absent (for instance, in *noi* mutants, a second *pax2*-related gene may still have activity in the optic stalks) (Pfeffer et al., 1998).

Bel is required to specify Vax gene expression in the preoptic tissue epistatic to Hh signals. It is probable that the activity of Bel in mediating Hh activity is very localized within the CNS as $bel^{-/-}$ embryos do not exhibit the U-shaped somites characteristic of many other Hh pathway mutants (van Eeden et al., 1996; Karlstrom et al., 1999; Schauerte et al., 1998). Conversely, not all mutants affecting Hh-dependent patterning exhibit alterations in Vax gene expression. For instance, $ubo^{-/-}$ embryos show the U-shaped somite phenotype characteristic of defects in Hh signalling (van Eeden et al., 1996), but have no defects in retinal axon pathfinding (Karlstrom et al., 1996) and no alterations to Vax gene expression (data not shown). As Vax morphants and $bel^{-/-}$ embryos share some phenotypes, this raises the possibility that the *bel* locus may encode one or other of the Vax genes. However, given the importance of Fgf activity for induction of Vax expression in the preoptic area, it is perhaps more probable that Bel functions in the Fgf signalling pathway.

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