

Reverse genetic analysis of neurogenesis in the zebrafish retina

Zac Pujic^a, Yoshihiro Omori^a, Motokazu Tsujikawa^a, Bernard Thisse^b,
Christine Thisse^b, Jarema Malicki^{a,*}

^a Department of Ophthalmology, Harvard Medical School, MEEI, r513, 243 Charles Street, Boston, MA 02114, USA

^b Institut de Génétique et de Biologie Moléculaire et Cellulaire, UMR 7104, CNRS/INSERM/ULP, 1 rue Laurent Fries, BP10142, CU de Strasbourg, 67404 Illkirch Cedex, France

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Abstract

To gain an understanding of molecular events that underlie pattern formation in the retina, we evaluated the expression profiles of over 8000 transcripts randomly selected from an embryonic zebrafish library. Detailed analysis of cDNAs that display restricted expression patterns revealed factors that are specifically expressed in single cell classes and are potential regulators of neurogenesis. These cDNAs belong to numerous molecular categories and include cell surface receptors, cytoplasmic enzymes, and transcription factors. To test whether expression patterns that we have uncovered using this approach are indicative of function in neurogenesis, we used morpholino-mediated knockdown approach. The knockdown of *soxp*, a transcript expressed in the vicinity of the inner plexiform layer, revealed its role in cell type composition of amacrine and ganglion cell layers. Blocking the function of *cxcr4b*, a chemokine receptor specifically expressed in ganglion cells, suggests a role in ganglion cell survival. These experiments demonstrate that in situ hybridization-based reverse genetic screens can be applied to isolate genetic regulators of neurogenesis. This approach very well complements forward genetic mutagenesis studies previously used to study retinal neurogenesis in zebrafish.

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Introduction

The perikarya of vertebrate retinal neurons are organized into three major layers containing photoreceptors, interneurons, and ganglion cells, respectively. These main laminae are separated by two plexiform layers, which mostly contain neuronal processes and synaptic connections. The relatively simple structure and the accessibility of the retina has made it a model of choice for the study of mechanisms involved in neuronal specification and patterning in the central nervous system (CNS). Several classes of factors, including secreted polypeptides and their receptors, transcriptional regulators, cell cycle regulators, and adhesion molecules have been shown to control retinal cell proliferation, specification, migration, and differentiation (for recent reviews, see Hatakeyama and Kageyama, 2004; Livesey and Cepko,

2001; Malicki, 2004; Ohnuma and Harris, 2003). Although a number of major regulators of retinal development are already known, it is clear that large gaps need to be filled before the genetic circuitry that orchestrates this process becomes fully understood.

The zebrafish is an attractive organism to study retinal neurogenesis (Malicki, 2000; Avanesov and Malicki, 2004). Most of genetic studies performed on the zebrafish retina so far have relied on forward genetic approaches. Nearly 200 mutants of retinal development have been isolated in three large-scale and several smaller mutagenesis experiments (Baier et al., 1996; Fadool et al., 1997; Golling et al., 2002; Malicki et al., 1996; Neuhauss et al., 1999). Following mutagenesis screens, candidate and positional cloning strategies have been successfully applied to characterize numerous regulators of eye development on the molecular level. These include, for example, the genes *one eyed pinhead*, *lakritz*, and *nagie oko*, involved in the establishment of the eye primordia, the specification of retinal ganglion cells, and the polarity of retinal

* Corresponding author. Fax: +1 617 573 4290.

E-mail address: jarema_malicki@meei.harvard.edu (J. Malicki).

neuroepithelium, respectively (Kay et al., 2001; Wei and Malicki, 2002; Zhang et al., 1998).

Although powerful, forward genetic approaches suffer from several shortcomings. First, they require laborious mutagenesis screens and positional cloning experiments. Second, late-onset phenotypes are frequently difficult to detect in forward genetic screens due to the fact that pleiotropic function of many genes results in early embryonic lethality. Finally, the same set of search criteria are usually applied throughout the entire screening experiment, making it difficult to detect a broad range of subtle abnormalities, such as defects in multiple cell classes of the retina. Some of these difficulties can be circumvented using reverse genetic approaches. The starting point of a reverse genetic experiment is some form of access to information about gene structure. Prior to functional analysis, genes are selected based on the presence of structural motifs in their sequence, or based on their expression patterns, or on both criteria combined. This initial analysis allows one to formulate hypotheses about the function of specific genes and to tailor subsequent genetic tests and phenotype detection criteria accordingly. Reverse genetic experiments in zebrafish can be performed in a high-throughput fashion and are an obvious extension of genome sequencing efforts.

Here we describe a large-scale reverse genetic screen that focuses on retinal neurogenesis in the zebrafish embryo. To isolate the regulators of neuronal development in the retina, we have taken advantage of the high fecundity of zebrafish as well as the transparency and accessibility of its embryo. ca. 8000 zebrafish embryonic cDNAs were evaluated using whole-mount *in situ* hybridization at multiple stages of development. Transcripts expressed in the developing eye in a cell-class-restricted manner were studied further in detail. In many cases, their expression correlates with the onset of neurogenesis and appears confined to a single cell class. These transcripts are excellent markers of cell identity and are good candidates for regulators of neuronal pattern formation during embryogenesis.

Materials and methods

Fish maintenance

Zebrafish (*Danio rerio*) were kept on a 14-h light/dark cycle according to standard procedures (Westerfield, 2000). Embryos were collected from pair wise matings of WIK, AB or TU strain animals and raised at 28.5°C.

cDNA library screening

To identify a broad range of transcripts during zebrafish embryogenesis, the following cDNA libraries were used: lambda ZAP oligo-dT primed cDNA library, constructed by B. Riggleman from segmentation stage embryos; lambda ZAP random primed library, prepared by C. Fromental and J.M. Garnier from a pool of 18 to 48 hpf embryos; lambda ZAP oligo-dT primed cDNA library, constructed by T. Lepage from gastrula-stage embryos; oligo-dT primed cDNA library, prepared in the pSPORT1 vector by M. Clark from 26 somite-stage embryos; oligo-dT primed cDNA library prepared in pSPORT1 vector by M. Clark from shield stage embryos; oligo-dT cDNA library constructed by L. Zon from adult kidney in the pBK-CMV vector. cDNAs have been randomly selected and used as templates to prepare RNA probes.

Sequencing and sequence analysis

5' and 3' sequence tags of each cDNA have been obtained and compared by BLAST analysis to SWISSPROT and translated EMBL databases. Other database searches were performed using the NCBI website. Once expression patterns have been determined, the entire sequence of selected cDNA clones has been obtained.

In situ hybridization

Embryos were reared in embryo medium supplemented with 0.003% 1-phenyl-2-thiourea to inhibit the development of pigmentation (Westerfield, 2000). Whole-mount *in situ* hybridization was performed as described previously, using digoxigenin-labeled antisense RNA probes and anti-digoxigenin alkaline phosphatase-conjugated antibodies as described previously (Oxtoby and Jowett, 1993; Thisse et al., 2004). To increase the throughput of the hybridization protocol, most steps of the protocol were performed in multiwell baskets (Thisse et al., 2004). Following hybridization, embryos were dehydrated, embedded in JB-4 resin (Polysciences Inc.) and sectioned. Sections were photographed using transmitted light microscopy. Detailed observations were conducted using a Zeiss Axioscope microscope, and images were recorded using a Zeiss AxioCam digital camera (Carl Zeiss Inc.) and Photoshop software (Adobe Inc.).

Morpholino microinjection

Glass micropipettes were pulled on a microelectrode puller (Narishige Co. Ltd.) and their tips were broken to 10 µm. 25-mer antisense morpholino oligonucleotides (morpholino, MO) directed against the 5' untranslated regions were obtained from GeneTools, OR: TAAGTGTGCT CAAAAGGCG CAATA (*xcr4* MO1); AGCCAACCTA CATCGTAAAA TTCCA (*xcr4* MO2); GCTCGGTTTC CATCATGTTA TACA (*soxp*, MO1); AACCGATTTT CTCGAAAGTC TACCC (*soxp*, MO2). Oligos were dissolved in 120 mM KCl, 0.04% phenol red, and ca. 3 nL of a 3 mg/mL solution (9 ng total) was injected into the yolk of 1–16 cell stage embryos. Following injections, embryos were maintained in egg water at 28.5°C with penicillin/streptomycin (Gibco Inc.) and processed for immunohistochemistry as described below. To account for nonspecific effects, in each experiment a separate group of embryos received an equivalent quantity of a standard control MO (5'-CCTCTTACCT CAGTTA-CAATTTATA-3').

Mosaic analysis

Genetically mosaic animals were generated as described previously (Avanesov and Malicki, 2004). Donor embryos were labeled with a mixture of Texas Red and biotin-conjugated dextrans. Morpholino oligonucleotides, mRNA, or both were injected together with dextran tracer. In one experiment, FITC-conjugated morpholinos were used to analyze the *xcr4* function. Wild-type embryos were used as hosts. The tracer was detected by staining of whole embryos as described (Avanesov and Malicki, 2004).

Overexpression

Full-length *soxp* and *xcr4b* cDNAs were cloned into the pXT7-myc and pXT7 expression vectors, respectively (Tang et al., 1995) and used to generate mRNA using the mMessage mMachine T7 kit (Ambion Inc.). 50–400 µg of mRNA was injected as above. For *soxp*, expression efficiency was evaluated using whole-mount immunostaining with anti-myc-tag antibodies at appropriate stages.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in PBS (pH 7.2) for 4 h at room temperature, washed in PBS, infiltrated in 30% (w/v) sucrose in PBS overnight and embedded in TBS tissue freezing medium (Polysciences Inc.). 14 µm sections were thaw-mounted on Superfrost Plus slides (Fisher Scientific Inc.) and stored at room temperature for 1 h. Sections were rehydrated in PBS

(5 min.) and blocked for 1 h in 10% (v/v) normal goat serum, 0.5% Triton X-100 (v/v) in PBS. The primary antibody was diluted in blocking solution and applied for 2 h at room temperature or at 4°C overnight. Sections were washed (3 times, 5 min. each) in PBST, stained with the secondary antibody diluted 1:500 in blocking solution for 2 h at room temperature, washed as previously, and mounted in 50% glycerol, 2% n-propyl gallate, 200 mM Tris–HCl (pH 8.0), and viewed using a Leica SP2 confocal microscope.

Immunohistochemistry was performed using the following antibodies and dilutions: Zpr-1 (formerly Fret 43, Larison and Bremiller, 1990) visualize red-green double cone photoreceptor cells (1:200, available from Oregon Monoclonal Bank); anti-carbonic anhydrase for Müller glia (1:100, Peterson et al., 2001); anti-tyrosine hydroxylase for dopaminergic interplexiform cells (1:100, Chemicon Inc.); anti-HuC/HuD for amacrine and ganglion cells (1:25, Molecular Probes Inc.); anti-neuroilin for ganglion cells and their axons (1:25, Zn8, available from Oregon Monoclonal Antibody Bank); and anti-myc to visualize myc-tagged polypeptides (1:100, Cymbus Biotechnology, Inc.). To visualize amacrine cell subpopulations, we used the following antibodies and dilutions: anti-neuropeptide Y (1:100, Chemicon Inc.); anti-parvalbumin (1:500, Chemicon Inc.); anti-GABA (1:500, Sigma Inc.); anti-somatostatin (1:25, ImmunoStar Inc.); and anti-serotonin (1:250, Sigma Inc.). Anti-mouse or anti-rabbit secondary monoclonal antibodies conjugated to Cy3 or Cy5 were obtained from Jackson ImmunoResearch Inc. Staining for γ -tubulin required the treatment of embryos for 5 min. in acetone at –20°C prior to blocking. For F-actin staining, Alexa 488-conjugated phalloidin (1:40, Molecular Probes Inc.) was included in the secondary antibody step.

Cell counts

Cell counts were performed on electronic images of transverse sections through the retina. To assure consistency, only sections that contained lens were included. 5 or more animals were used in each experiment in both experimental and control groups. As *ody* homozygous mutant zebrafish do not display obvious morphological defects, their identity was determined by the sequencing of the *cxcr4* gene. To determine the number of ganglion cells, the retinae of morphant or mutant animals were cryosectioned and stained with the Zn8 antibody (see above). To facilitate photoreceptor counts, sections were counterstained with propidium iodide.

Results

Large-scale *in situ* hybridization screen

Randomly selected cDNA clones were screened for tissue or cell-class specific expression by *in situ* hybridization on embryos aged from 12 somites to 5 dpf. Screening of ca. 8000 clones revealed 52 that are expressed in subpopulations of retinal cells. Retinal neurogenesis in zebrafish involves a gradual exit of neuronal progenitors from the cell cycle (reviewed in Easter and Malicki, 2002). Ganglion cell precursors are the first to become postmitotic between 27 and 28 hpf (Hu and Easter, 1999; Nawrocki, 1985). Following that, precursor cells of inner nuclear layer neurons initiate their cell cycle exit between 37 and 38 hpf. The last to become postmitotic are photoreceptor cell progenitors, which start to exit the cell cycle at ca. 43 hpf. To study the involvement of cell-class restricted transcripts in the specification and differentiation of retinal cell classes, we chose to evaluate their expression patterns at 36, 48, 60 and 72 hpf. The 36 hpf time point was chosen to include the early differentiation of ganglion cells (Hu and Easter, 1999). The 48 hpf time point allowed us to analyze the early differentiation of inner nuclear layer cells. This time point also made it possible to evaluate

factors involved in the earliest phases of photoreceptor development. The 60 and 72 hpf time points were used to determine the expression of transcripts in fully differentiated neurons (Easter and Nicola, 1996). When necessary, additional stages of development were examined. Numerous transcripts were found to be either entirely confined to or very much enriched in a single cell class or in a subset of cells within a single cell classes. Out of the 52 clones examined closely, the expression of 10 were found enriched in the ganglion cell layer (GCL), 9 are predominantly expressed in the inner nuclear layer (INL), 3 are mainly expressed in the photoreceptor cell layer (PRCL), 7 at the marginal zone (MZ), and 1 in the retinal pigmented epithelium (RPE). 22 clones were found to be expressed in more than one cell class. Transcription factors, transmembrane receptors, cytosolic enzymes, and cytoskeletal components are represented among protein products of these transcripts. Their expression patterns as well as their molecular nature suggest that they may play vital roles in the regulation of neurogenesis.

To determine the relatedness of the identified transcripts to known DNA sequences, we performed BLAST searches of major nucleotide and protein sequence databases using the NCBI website. This also allowed us to identify conserved structural domains and motifs. For the 52 cDNAs analyzed in detail, we determined the percentage homology to the closest DNA match (if found), and the percentage of identity and similarity to the closest protein match (if found). Each cDNA has been assigned a Genbank accession number. This analysis is summarized in Table 1.

Genes expressed in the ganglion cell layer

Ganglion cells are the output neurons of the retina. Factors that regulate the specification of these cells, their migration to form the ganglion cell layer (GCL), as well as the elaboration of axonal projections remain only partially identified. Our screen uncovered GCL-enriched cDNAs that display several types of expression. Some genes are expressed in a small subset of cells while others display expression in the majority if not all GCL neurons. Furthermore, expression occurs transiently during development, or alternatively remains high throughout the entire period that we studied. These distinct expression profiles provide clues to the potential function of these genes.

One of the most interesting patterns is displayed by the *cxcr4b* gene. This chemokine receptor is present at a high level in newly formed ganglion cells. In the eye, transcripts are detectable at ca. 30 but not at 24 hpf, correlating with the first appearance of postmitotic ganglion cells (Figs. 1A–D, and data not shown). *cxcr4b* expression is first detectable in migrating ganglion cells (Fig. 1B, inset). By 48 hpf, its expression is entirely extinguished in differentiated ganglion cells of the central retina. As the marginal zone of the zebrafish retina continues to generate cells, a small subpopulation of ganglion neurons continues to express *cxcr4b* in this region (Fig. 1D). These observations suggest that *cxcr4b* activity correlates with a brief phase of ganglion cell differentiation.

Another gene with a similar expression pattern is *gacekM* (ganglion cell expressed) (not shown). It bears homology to a human BTB/POZ domain protein 3 (Bric-a-brac, Tramtrack, Broad-complex/poxvirus and zinc finger), member of a transcription factor family implicated in neuronal development (Mitchellmore et al., 2002; Wen et al., 2000). While expressed at a high level at the retinal margin, this transcript is also absent in the central retina. Calmodulin and Neuroilin are two other transcripts characterized by an enrichment in the ganglion cell layer. Calmodulin is detectable throughout the GCL in 36 hpf retinae (Figs. 1K–M). At 60 and 72 hpf, its transcription is somewhat downregulated in the central retina but remains high in peripheral regions. The expression of neuroilin has been previously described (Laessing and Stuermer, 1996). An early and transient appearance of transcripts during GCL development is particularly interesting as it suggests a developmental role.

A very different expression pattern is exemplified by the H15 T-box-related transcription factor, *hrT* (Figs. 1H–J). At 36 hpf, its presence is detectable in a small ventral cluster of cells in the GCL. By 60 hpf, this gene is expressed by a small population of cells scattered throughout the entire GCL. *hrT*-positive cells become less frequent in the central retina by 72 hpf. As the ganglion cell class is known to contain many distinct cell types (Rockhill et al., 2002), the selective expression of *hrT* in a small subpopulation of ganglion neurons makes it a candidate for a regulator of cell type identity in this cell layer.

In contrast to previous genes, *gc12*, *gc34*, and *gc56* appear uniformly expressed throughout the ganglion cell layer (*gc34* is shown in Figs. 1E–G). All three are detected in the retina by 48 hpf and are enriched in the majority of, if not all, ganglion cells. Curiously, starting at 72 hpf, the expression of *gc12* and *gc34* genes is also present in a small subset of inner nuclear layer (INL) cells (not shown). These may represent ganglion neurons that are displaced into the INL and have been described in other vertebrate species (Linden, 1987). No sequence homology to known genes has been found for these cDNAs.

Genes expressed in the inner nuclear layer

The INL is the most heterogeneous layer of the neural retina with respect to cellular composition, containing three classes of neurons and the perikarya of Müller glia. The most numerous cell classes of the INL, amacrine and bipolar interneurons, are composed of many subpopulations. In one recent study, 13 morphological variants of bipolar cells were identified in the zebrafish retina (Connaughton and Nelson, 2000). Similarly, studies in other species reveal that amacrine cells are very diverse (MacNeil and Masland, 1998). Our in situ hybridization screen uncovered several factors that are either specifically expressed or strongly enriched in the INL. Their expression patterns suggest a role in cell fate decisions or cell differentiation. Again in this case, the expression patterns vary. Some transcripts appear expressed in entire cell classes, whereas others are confined to relatively small subpopulations, which may represent specific neuronal types.

Two clones are expressed in highly restricted cell populations in the INL. The expression of the *pou23* gene is present in a small

population of cells in the inner portion of the INL, presumably a subpopulation of amacrine cells. The expression is present by 48 hpf and is maintained until at least 72 hpf. This gene is also expressed at high levels in the CNS between 36 and 72 hpf (Figs. 2A–C) and displays high sequence similarity to the mouse *pou3f1* transcription factor, implicated in the regulation of cell differentiation (Andersen et al., 1997; Bermingham et al., 1996). Another gene characterized by a similar pattern of expression is *inlf12* (inner nuclear layer factor 12), a homolog of a TNF-induced protein. Its expression appears in the inner portion of the INL between 48 and 60 hpf and is maintained until at least 72 hpf (Fig. 2L). The localizations of *pou23* and *inlf12* are less diffuse than that of carbonic anhydrase, a marker of Müller glia, and appear confined to a smaller population of cells. Thus these two genes are likely to mark subpopulations of amacrine cells.

An interesting expression pattern is exemplified by *soxp*. This factor too is expressed in a small subpopulation of cells localized in the immediate vicinity of the inner plexiform layer (IPL). At 36 hpf, *soxp* is initially expressed in cells of the MZ, suggesting a role in cell proliferation (Fig. 2D). By 40 hpf, however, its expression appears in the region of the forming INL (Fig. 2E). This shift in its expression pattern correlates with the appearance of the first postmitotic neurons in the INL. By 48 hpf, *soxp* is confined to cells surrounding the inner plexiform layer (IPL) and its expression persists in this area until at least 72 hpf (Figs. 2F, G and data not shown). The shift in *soxp* expression domain from the zone of cell proliferation (MZ) to the region of INL formation suggests that this factor may play a role in cell-fate determination of the INL neurons. This hypothesis finds support in functional studies that are described below.

Two factors are strongly expressed in a broad band of cells at the inner margin of the INL—an area predominantly occupied by amacrine cells. They are homologous to well-characterized bHLH transcription factor genes, *ap2 α* and *ap2 β* . The onset of their expression occurs between 36 and 48 hpf and coincides with the appearance of the first postmitotic amacrine cells. A uniform level of expression is maintained by *ap2 α* throughout this layer until at least 72 hpf (Fig. 2M and data not shown). By contrast, the *ap2 β* form is downregulated in the central retina by this time (Figs. 2H–K). Both forms are present in the brain at all time points examined.

Two genes show enriched expression in the central region of the INL. This area is mostly occupied by bipolar neurons and the somata of Müller glia. *bipolin* bears homology to the family of cation-chloride cotransporters. At 60 hpf, its expression is confined to the INL cells, most likely bipolar neurons (not shown). Similar to *bipolin*, carbonic anhydrase is also present in the middle region of the INL (not shown). Its expression domain is somewhat more diffuse and most likely marks the somata of Müller glia (Peterson et al., 1997).

One factor has been found highly enriched in a narrow domain at the outer perimeter of the inner nuclear layer. This transcript, named *horizin*, appears to mark horizontal cells. At 36 hpf, its expression is fairly ubiquitous, but by 60 hpf *horizin* becomes mostly restricted to a narrow layer of the INL cells localized next to the outer plexiform layer (Fig. 2N). Its is also very weakly expressed in the inner retina at this stage. By 72

Table 1
Clones and their relatedness to known genes

Name and protein homology	DNA homology	GenBank accession	Onset (hpf)	Expression pattern
Genes predominantly expressed in the ganglion cell layer				
<i>cxcr4b</i> , G protein-coupled receptor z100/100/353 NP_571909.1	z100%/1062; NM_131834	NM_131834	<36	G
<i>gacek</i> , BTB/POZ domain protein h75/81/148 Q9Y2F9	m83%/211; BC031195.1	DW248773	<48	G
<i>neuroilin</i> (3 clones) adhesion molecule z99/100/103 NP_571075.1	z95/464; NM_131000.1	NM_131000	<36	G
<i>calmodulin</i> , cytoplasmic calcium sensor h99/100/144 P02593	k86%/408; D10363.1	DW248772	<36	G
<i>hrT</i> , T-box transcription factor z93/93/446 AAF42957	z99%/1341; AF239664.1	AF239664.1	<36	Subset of G
<i>gc12</i> -	–	DW248780	<36	G and subset of A
<i>gc34</i> -	–	DW248781	<36	G and subset of A
<i>gc56</i> -	–	DW248782	<60	G
Genes predominantly expressed in the inner nuclear layer				
<i>pou23</i> , POU transcription factor z71/71/190 P79745	z98%/951; Y07907.1	DW248798	36–48	Subset of A
<i>inlf12</i> m59/74/199 XP_146997	m84%/86; XM_146997.2	DW248791	36–48	Subset of A
<i>soxp</i> , HMG box factor c69/72/296 P48430	c85%/740; U12532.1	AB242329.1	<36	A, subset of G?
<i>ap2α</i> , bHLH factor z89/89/431 NP_789829.1	z99%/1297; AF457191.1	AF457191.1	36–48	All A
<i>ap2β</i> , bHLH factor h70/75/137 Q92481	m84%/176; NM_009334.1	DW248771	<36	All A
<i>bipolin</i> , cation-chloride cotransporter h50/60/104 Q9Y666	s87%/54; AF028807	DW248801		INL cells, probably B.
<i>carbonic anhydrase</i> z100/100/127 Q92051	z98%/624; NM_131110	NM_131110		M
<i>horizin</i> (2 clones) -	–	DW248789	<60	H, v. weak staining in A
Genes predominantly expressed in the photoreceptor cell layer				
<i>ndrg1</i> h64/78/395 Q92597	h81%/329; D87953	DW248797	36–48	PR
<i>prcb4.1</i> (2 clones), FERM-domain cytoskeletal factor h77/92/66 Q9Y2J2 h45/60/142 Q9H4G0	h83%/134; NM_012156.1	DW248799	<48	PR, single cells in INL
Genes expressed in multiple cell layers				
<i>gc rptp</i> , protein tyrosine phosphatase m56/75/161 P80560	z100%/344; XM_693534.1	XM_693534.1	<48	G and A
<i>gc src</i> , Src tyrosine kinase m51/62/264 AAH39953	v82%/342; X84074	DW248776	<72	G and A
<i>gc I4-3-3</i> , phosphoprotein regulator 50/56/199 P35214	m84%/682; BC008129	DW248774	48–60	G and A
<i>gc pak</i> , protein kinase 71/71/127	h82%/464; U24152.1	DW248775	<36	G and A
<i>gcnb</i> ion pump 70/83/37 P50851	h82%/115; NM_015678	DW248784	<36	G and A
<i>gctb1</i> , G protein, β subunit 93/100/49 P79959	x85%/195; X86969	DW248785	<36	G and A
<i>Na/K ATPase β3b</i> ion pump 94/94/203 NP_571745	z98%/690; NM_131670	NM_131670	<60	G and A
<i>gc3</i> -	–	DW248777	<36	G and A
<i>gc4</i> (2 clones) -	–	DW248778	<60	G and A
<i>gc5</i> -	–	DW248779	<60	G and A, dorsally stronger
<i>gc67</i> -	–	DW248783	36–60	G and A
<i>six3</i> , homeobox transcription factor z76/79/159 NP_571438	z98%/438; NM_131363	NM_131363	<48	G and INL
<i>ngn1</i> , transcription factor z100/100/208 NP_571116.1	z99%/627; AF017301	AF017301	<60	INL, all cell classes
<i>nr13</i> z100/100/156 AAL32471	z98/557; AF441285	AF441285	<36	INL, some in G

Table 1 (continued)

Name and protein homology	DNA homology	GenBank accession	Onset (hpf)	Expression pattern
Genes expressed in multiple cell layers				
<i>gin27</i> -	–	DW248788	<36	INL, some in G
<i>inlB7</i> -	–	DW248790	<60	INL, all cell classes
<i>inlM</i> , multiple LIM-domain factor h76/84/52 O43900	m82%/184; XM_144905	DW248792	<36	INL, all cell classes
<i>gin17</i> -	–	DW248787	<60	G and INL
<i>prag17</i> -	–	DW248802	<72	G, AC, PR(?)
<i>apoE</i> z100/100/281 NP_571173	z100%/1345; NM_131098	NM_131098	48–60	G and INL, single cells
<i>gen1</i> , RNA binding protein h66/70/208 BAA92120	r79/173; NM_053416.1	DW248786	36–48	Ubiquitous, G enriched
Genes expressed in the proliferating cell zone				
<i>her4</i> , transcription factor z75/77/149 NM_131090	z95%/487; NM_131090	NM_131090		MZ
<i>pax6b</i> , transcription factor	z100%/78; CA588064	CA588064	<36	MZ, subpopulation
<i>mz56</i> -	–	DW248796	<36	MZ
<i>mz12</i> -	–	DW248794	<60	MZ
<i>mz98</i> -	–	DW248793	<36	MZ
<i>cyclin D1</i> , cell cycle regulator z92/92/201 Q90459	z97%/700; NM_131025	NM_131025	<36	MZ
<i>mz237</i> -	–	DW248795	<36	MZ
Genes expressed in the retinal pigmented epithelium				
<i>rpe12</i> , tetraspanin?	–	DW248800	<48	RPE

Name and protein homology column provides gene name. For clones characterized by a high homology to genes previously described in zebrafish or in other species, the previously published name is provided. When known, a general description of molecular nature is provided in italics. The second line of each entry provides protein homology estimate that consists of three numbers, separated by slashes. The first number indicates amino acid identities (exact residue matches), the second number indicates similarities, and the third provides the length of the sequence used for comparison. In general, amino acid sequence homologies of greater than 40% over a stretch of at least 100 residues are provided. A few exceptions to this rule were made when high homology was detected on the nucleotide level. The accession number of the sequence showing the highest homology is also provided. DNA homology column provides homology to the closest DNA match. The first number indicates percentage homology following a BLAST alignment, the second indicates the length of the sequence used in this analysis. The accession number of the sequence showing the highest homology is provided. In both homology estimates, the species origin of the closest homology is indicated by a single letter code: h, human; c, chicken; e, *C. elegans*; x, *Xenopus*; z, zebrafish; k, killifish; r, rat; m, mouse; s, pig; v, virus. GenBank accession column provides the zebrafish sequence accession number. Onset column provides an estimate of the time when transcripts are first detectable (in hpf). Expression pattern column provides a brief summary of expression patterns. The following abbreviations are used for individual cell classes: G, ganglion cells; A, amacrine cells; B, bipolar cells; H, horizontal cells; M, Muller glia; PR, photoreceptor cells; MZ, ciliary marginal zone; RPE, retinal pigmented epithelium. Due to the high-throughput nature of cDNA analysis, sequences provided to GenBank may contain minor errors.

hpf, only HC cells near the MZ show detectable levels of *horizin* (not shown). No homology is obvious between *horizin* and any known genes.

Genes expressed in the photoreceptor cell layer

Photoreceptor cells exhibit remarkable structural features that make them conspicuously distinct from other neurons of the retina. The photoreceptor outer segments and synaptic termini display highly complex structure and function. Although many photoreceptor-specific genes have been characterized, these mostly have to do with their function as light detectors and relatively few loci are known to play a role in the specification or differentiation of this important cell class.

Two cDNAs display enriched expression in the photoreceptor cell layer of the retina. The expression of *ndrg1* is particularly interesting as very few genes are known to display such an early and specific expression pattern in photoreceptor cells. *ndrg1* is a member of the NDRG (*N*-myc downregulated gene) family of α/β -fold hydrolases implicated in cell growth and differentiation (Kalaydjieva et al., 2000; Zhou et al., 2001). *ndrg1* transcripts

are first detectable between 40 and 44 hpf in the PRCL (Figs. 3A–C). At 48 hpf, they are present throughout the photoreceptor cell layer but their expression is later downregulated in the center of the retina (Fig. 3D). Such an early expression in the photoreceptor cell layer suggests a general developmental role. Consistent with this idea, *ndrg1* is also expressed in the pineal gland (not shown). The second transcript in this group is expressed in a somewhat less specific manner. The expression of the *prcb4.1* gene, characterized by a homology to the human erythrocyte membrane band 4.1-like protein (Kim et al., 1998), is also already present at early stages of photoreceptor differentiation by 48 hpf (Fig. 3E). Transcripts are initially present throughout the PRCL and become restricted to marginal regions by 60 hpf (Fig. 3F). A small number of cells outside the photoreceptor cell layer also express high level of *prcb4.1* at 48 and 60 hpf (Figs. 3E, F).

Genes expressed in multiple cell layers

Many transcripts show expression in more than one retinal lamina or cell class (Fig. 4). A disproportionate number of

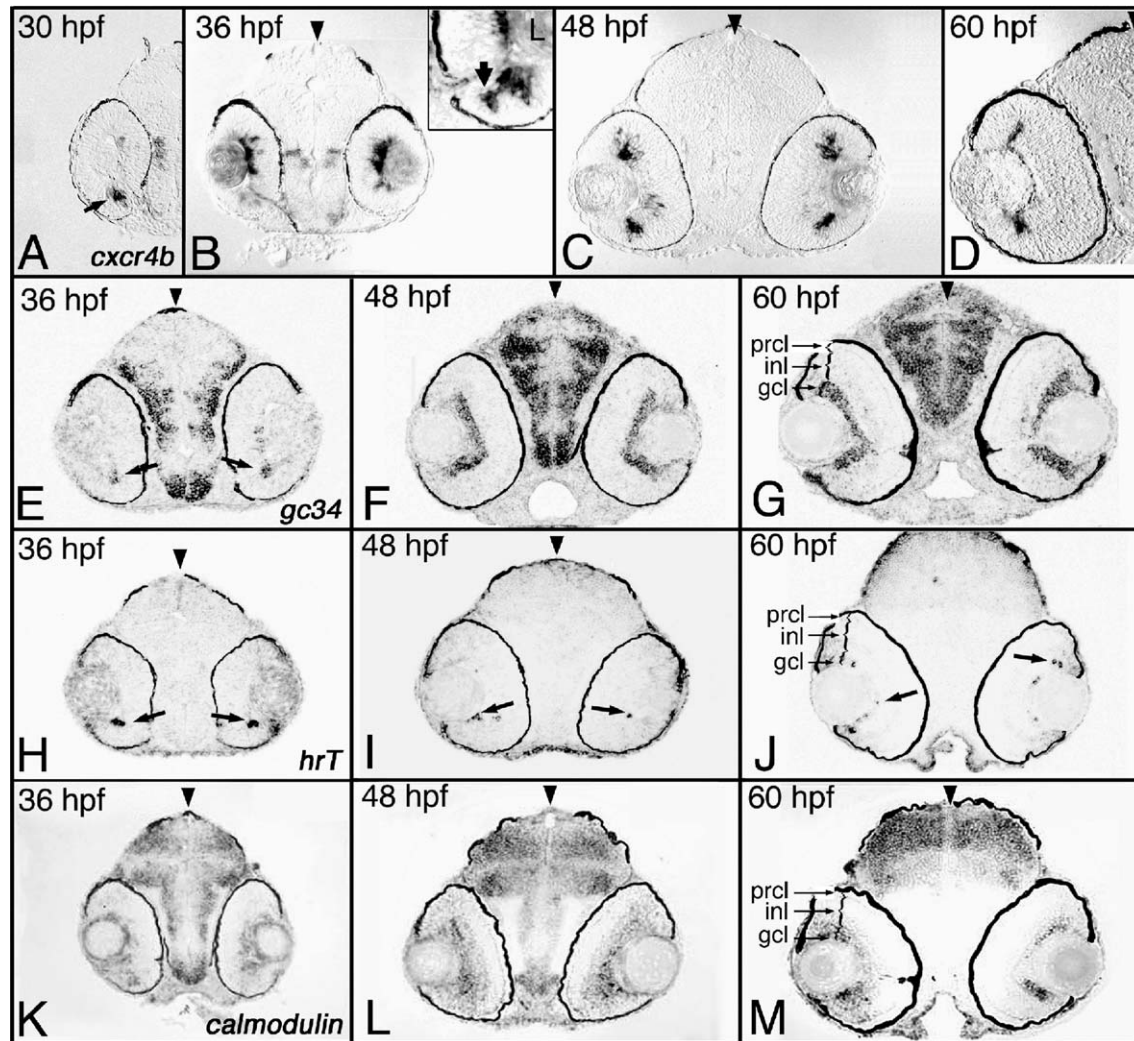


Fig. 1. Transcripts predominantly expressed in the GCL. Shown are examples of cDNAs expressed in the ganglion cell layer. (A–D) Expression of *cxcr4b* between 30 and 60 hpf. (E–G) Expression of the *gc34* gene between 36 and 60 hpf. (H–J) Expression of *hrT* between 36 and 60 hpf. (K–M) Expression of the *calmodulin* gene between 36 and 60 hpf. Expression was studied on plastic sections following whole-embryo in situ hybridization. Arrowheads mark the midline. Arrows indicate individual cells or small groups of cells that feature expression of particular transcripts. hpf, hours postfertilization; prcl, photoreceptor cell layer; inl, inner nuclear layer; gcl, ganglion cell layer.

cDNAs are expressed both in the ganglion cell layer and in the inner portion of the inner nuclear layer but not in other cell classes. These expression characteristics suggest, unexpectedly, that these two cell classes share molecular regulatory mechanisms with each other to a larger extent than with other retinal cells. Other transcripts are expressed in multiple cell classes within the INL. Expression is frequently higher in one lamina compared to others, and transcript levels frequently change in the course of development.

Among transcripts that are largely expressed in ganglion and amacrine cell layers but not in other cell classes, several clones display homology to components of signaling pathways. Four of these are implicated in kinase signaling. Synexpression of functionally related factors suggests that they may be involved in the same molecular mechanism. This may be the case, for example, for *gc src* and *gc rtp* (Fig. 4E), genes that display homology to src-like protein tyrosine kinases and receptor protein tyrosine phosphatases (RPTPs), respectively. As RPTPs

are known to regulate protein tyrosine kinases (Streuli, 1996), these two genes may play a role in the same pathway. *gc 14-3-3* (Fig. 4D), and *gc pak* are two other potential regulators of protein phosphorylation. Their possible functional relatedness to each other or to other polypeptides expressed in ganglion and amacrine cells is less clear.

Two other factors that are expressed both in amacrine and in ganglion cell layers may play a role in signal transduction cascades. *gcnb* is a plausible homologue of neurobeachin, a protein with a Beige/BEACH domain. At 36 hpf, it is expressed throughout the neuroepithelium with some enrichment at the MZ. By 60 hpf, its expression is restricted to the differentiating GCL and, by 60 hpf, it is found both in the GCL and INL. Another potential component of a signal transduction cascade, *gctb1*, displays homology to transducin β -1, a G protein subunit that functions in signal transduction in rod photoreceptors (Peng et al., 1992). *gctb1* is not enriched in the outer retina and therefore is unlikely to function in the photoreceptor

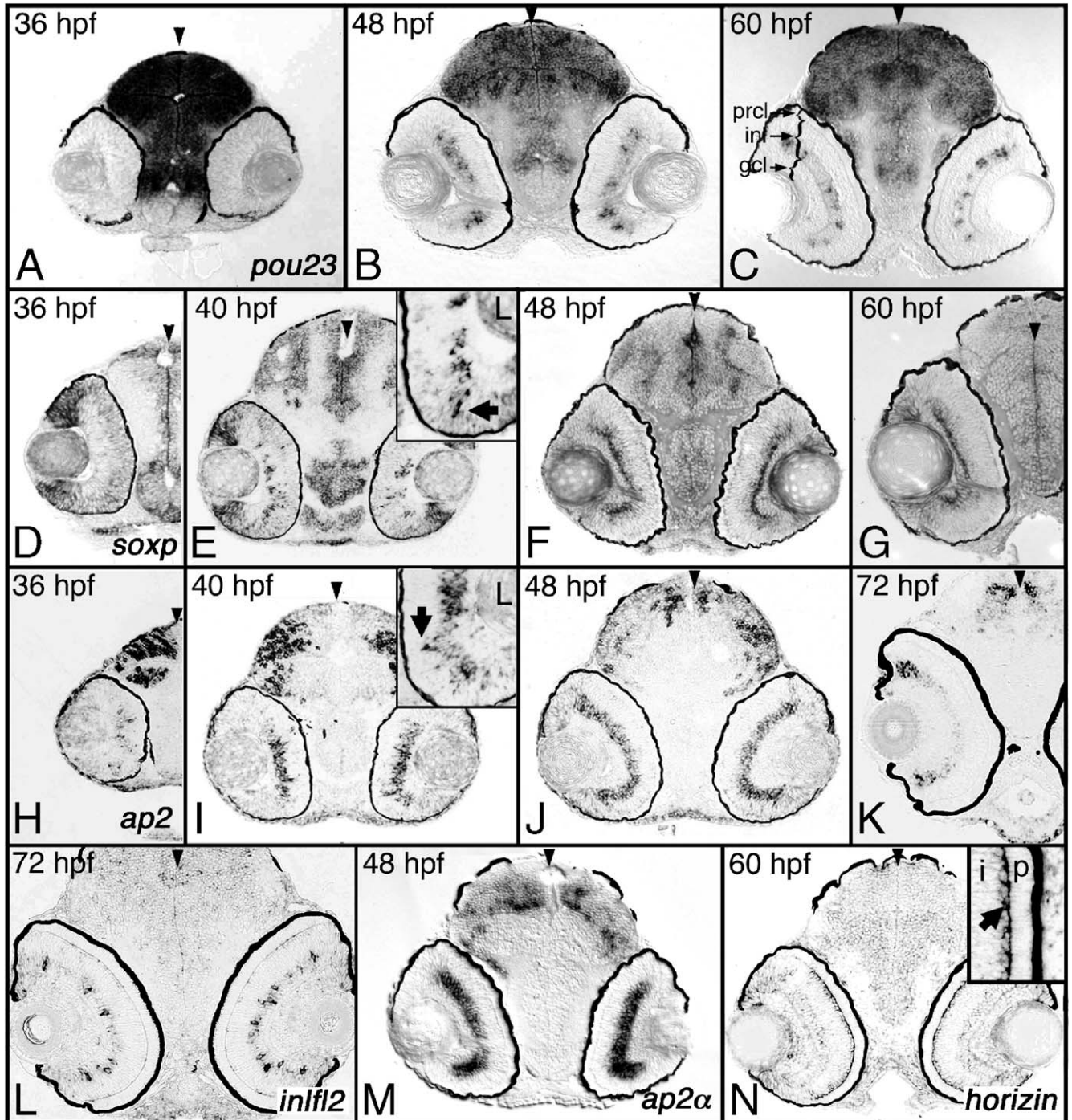


Fig. 2. Transcripts predominantly expressed in the INL. Shown are examples of cDNAs expressed in the inner nuclear layer. (A–C) Expression of *pou23* between 36 and 60 hpf. (D–G) Expression of *soxp* between 36 and 60 hpf. (H–K) Expression of *ap2β* between 36 and 72 hpf. (L) Expression of *infl2* at 72 hpf. (M) Expression of *ap2α* at 48 hpf. (N) Expression of *horizin* at 60 hpf. Expression was studied on plastic sections following whole-embryo in situ hybridization. Arrowheads indicate the midline. hpf, hs postfertilization; prcl, photoreceptor cell layer; inl, inner nuclear layer; gcl, ganglion cell layer.

phototransduction cascade. It may be, however, involved in the signal transduction of recently discovered ganglion cell-expressed opsins (He et al., 2003). Finally, in addition to the above transcripts, expression confined to amacrine and ganglion cell layers has been observed for a Na/K ATPase gene, and four transcripts with no obvious homologies, *gc3*, *gc4*, *gc5*, and *gc67*.

Among clones expressed in multiple cell layers but not specifically confined to amacrine and ganglion cells only, two

genes, *neurogenin1* and *six3*, are well-studied transcription factors. The role of *six3* in the early development of the eye primordium has been characterized extensively (Carl et al., 2002; Loosli et al., 1999). During neurogenesis, *six3* displays a complex expression pattern that suggests a developmental role (not shown). At 48 hpf, *six3* is expressed in ganglion cells and in a subset of amacrine cells. By 60 hpf, it is also expressed in other neurons of the inner nuclear layer but its expression remains the

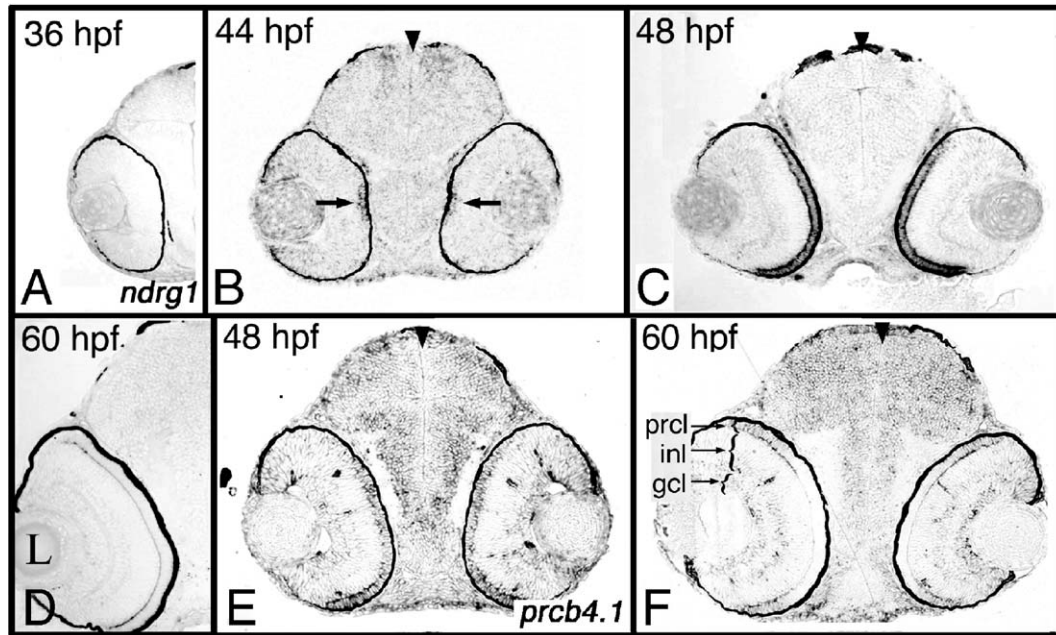


Fig. 3. Transcripts predominantly expressed in the photoreceptor cell layer. (A–D) Expression of *ndrg1* between 36 and 60 hpf. (E–F) Expression of *prcb4.1* at 48 and 60 hpf. Expression was studied on plastic sections following whole-embryo in situ hybridization. Arrowheads mark the midline. hpf, hours postfertilization; prcl, photoreceptor cell layer; inl, inner nuclear layer; gcl, ganglion cell layer.

strongest in ganglion cells. This expression is consistent with its proposed role in amacrine cell development (Inoue et al., 2002). Although neurogenin 1 (*ngn1*) plays a prominent role in neurogenesis (Korzh and Strahle, 2002), its expression in differentiating retina has not been reported to our knowledge (Korzh et al., 1998). During retinal neurogenesis, *ngn1* is expressed in INL cells, and at 60 hpf its expression is enriched in the dorsal retina (not shown).

Another potential developmental regulator is the *nr13* gene, which is known to function as an inhibitor of apoptosis in chicken development (Lee et al., 1999). It is expressed in the INL mostly, although a weaker expression in the GCL is also detectable (Fig. 4A). As cell death plays an important role in the regulation of retinal cell numbers (Bahr, 2000), *nr13* may adjust the amount of cell death required for normal development. The *gin27* transcript shows a very similar expression to *nr13*. Based on its partial sequence, homology to *nr13* has not been detected. Finally, *inlM*, a transcript predicted to encode a multiple LIM domain polypeptide, is expressed in most of INL cells. Its expression is downregulated in the center of the retina by 60 hpf (not shown). Several other transcripts display expression patterns that are unrelated to each other or to these of other genes expressed in multiple cell layers. Their distribution and molecular features are summarized in Table 1.

Genes expressed in the proliferative marginal zone

In the zebrafish eye, a population of pluripotent cells continues to divide in the peripheral region of the retina, known also as the ciliary marginal zone (MZ). Cells of the marginal zone retain many characteristics of the embryonic neuroepithelium and continue to contribute to all retinal layers (Wetts et al.,

1989). We have uncovered 7 transcripts restricted to this region. Not surprisingly, many of these are also expressed at a high level in the embryonic retinal neuroepithelium. These genes encode factors that appear to be involved in cell-cycle regulation, transcriptional regulation, and cytoskeletal structure.

Two transcription factors, *pax6b*, and *her4* display this pattern of expression. At 36 hpf, *pax6b* is strongly expressed in the neuroepithelial cells of the retina with the exception of the peripheral-most regions. By 48 hpf, its expression becomes confined to the marginal zone and the ventricular zones of the brain, and is nearly undetectable by 72 hpf (Figs. 5A–C). These findings suggest that the *pax6a* gene contributes expression that was previously reported in ganglion and amacrine cells based on antibody staining (Macdonald and Wilson, 1997). *her4* expression is similar to *pax6b*. It is initially present in the neuroepithelium and subsequently confined to the marginal zone and the ventricular regions of the brain (data not shown). These transcription factors may regulate the transition from undifferentiated pluripotent state of the neuroepithelial cells to neuronal differentiation.

Several other transcripts are present at the marginal zone. The *mz98* transcript, which displays a weak collagen homology, is expressed in a very narrow region between the MZ and the lens at all ages examined. Expression is the highest at 36 hpf and gradually declines to lower levels (Figs. 5D–F). *cyclin D1* is also expressed in the neuroepithelium. This is expected because the retinal neuroepithelium is strongly mitotically active. When cells exit the cell cycle and differentiate, its expression is downregulated (Figs. 5G–I, and not shown). Three other factors, *mz12*, *mz23*, and *mz56* do not display any obvious homologies.

It is noteworthy that marginal zone expression occurs in at least two variants. Some factors appear to be expressed in all

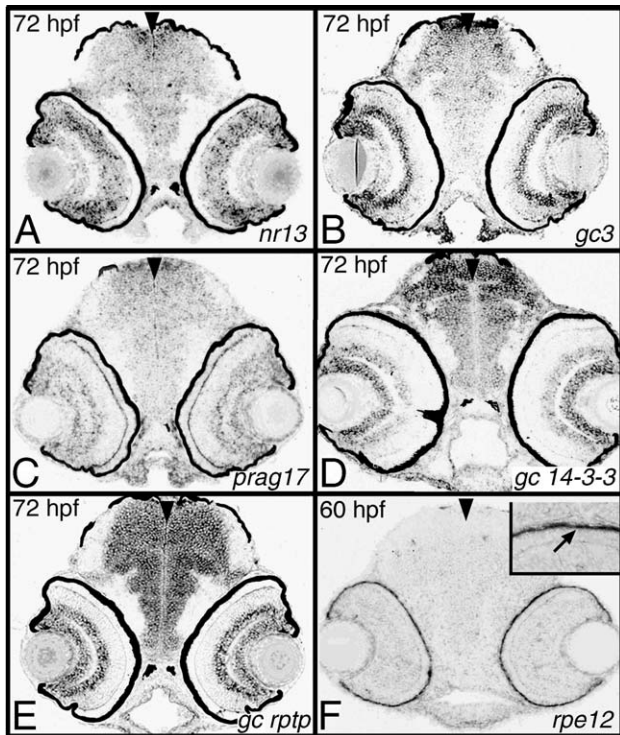


Fig. 4. Transcripts expressed in multiple cell layers or RPE. (A) *nr13* is expressed in all layers with exception of photoreceptor cells. (B) *gc3* expression is mostly detectable in the inner nuclear layer and in ganglion cells. (C) *prag17* transcript is enriched in the vicinity of the outer plexiform layer and in the inner retina. (D) *gc 14-3-3* is expressed in the ganglion cell layer and in amacrine cells at a somewhat lower level. (E) *gc rptp* is expressed at approximately the same level in both GCL and the inner portion of INL, most likely in amacrine cells. (F) *rpe12* is expressed in the retinal pigmented epithelium. The expression pattern of each clone is shown at one time point, indicated in the upper left corner of each panel. Transcripts were analyzed on plastic sections following whole-embryo in situ hybridization. To block RPE pigmentation, embryo in panel F was treated with PTU. Arrowheads mark the midline. hpf, hours postfertilization; prel, photoreceptor cell layer; inl, inner nuclear layer; gcl, ganglion cell layer.

cells in this area while others are absent at the very extremes of the marginal zone. The first type of expression is represented by the *cyclin D* and *collagen* genes, while *pax6b* and *her4* display the second mode.

Genes expressed in the retinal pigmented epithelium

The retinal pigmented epithelium (RPE) is a layer of cells which appose photoreceptor outer segments and play several important roles in photoreceptor differentiation and function. Its defects lead to photoreceptor degeneration (Bok, 1993; LaVail and Mullen, 1976; Redmond et al., 1998). We have identified a transcript that is specifically expressed in the RPE (Fig. 4F) and displays a weak homology to tetraspanins, a protein family involved in cell proliferation, adhesion, motility and differentiation (Maecker et al., 1997).

Functional analysis using morpholino-mediated knockdown

A characterization of gene expression, including its onset, duration, and tissue distribution is frequently a source of useful

insights into gene function. Many expression patterns uncovered by the in situ hybridization screen suggest a function in cell fate decisions or in the differentiation of individual cell classes. To examine whether the expression patterns of these genes are indicative of a role in retinal development, we decided to apply morpholino (MO) antisense oligonucleotide-mediated knockdown, a rapid and relatively inexpensive method (Nasevicius and Ekker, 2000). Our initial attempts to study seven genes expressed in three major layers of the retina led to the identification of function in retinal development for two: *soxp* and *cxcr4b*.

Analysis of *soxp* phenotype

The expression pattern of *soxp* suggests a role in amacrine cell development (Figs. 2D–G). To investigate *soxp* function, we treated wild-type embryos with anti-*soxp* morpholinos and evaluated cell frequency in several amacrine cell subpopulations. *soxp* knockdown results in a sharp decrease of parvalbumin-positive cells (hereafter, PV-positive) that localize to the ganglion cell layer. PV-positive processes of these cells extend into the inner plexiform layer but not into the ganglion cell layer, suggesting that these neurons represent displaced amacrine cells. A mix of two anti-*soxp* morpholinos injected into wild-type WIK strain embryos results in over 70% reduction in the number of PV-positive cells in the ganglion cell layer at 4 dpf, compared to embryos injected with a control morpholino (Fig. 6A, and image in J compared to I). The injections of single morpholinos result in ca. 50–60% reduction of PV-positive cell numbers (Table 2). Cell loss is specific to PV-positive cells, as we do not observe reduced numbers of carbonic anhydrase-positive Muller glia (Figs. 6C, J), tyrosine hydroxylase-positive interplexiform cells (Fig. 6F) or Zpr-1-positive red-green double cones (Fig. 6D). The overall number of amacrine cells as visualized with anti-Hu antibodies also remains unchanged (Fig. 6B, and image in L compared to K). Staining with phalloidin does not reveal any obvious defects in the synaptic layers of the retina (Figs. 6L, K).

A reduced number of PV-positive cells could be due to a specific delay in amacrine cell differentiation. If this was true, the number of parvalbumin-positive cells would recover to normal levels at later stages of development. To investigate this possibility, we analyzed *soxp* knockdown phenotype at 5 and 7 dpf. At both stages, the number of PV-positive cells remains well below the wild-type level. Ganglion cell layer PV-positive cells are decreased by 83% and 88% at 5 and 7 dpf, respectively (Table 2). The numbers of photoreceptor cells are not affected at either stage (Table 2). We conclude that the loss of PV-positive cells is unlikely to result from a developmental delay. A decrease in the numbers of PV-positive cells may be a consequence of a cell fate switch that favors the specification of other amacrine cell subpopulations. To determine whether this is the case, we evaluated the numbers of neuropeptide Y-, somatostatin-, GABA-, and serotonin-positive amacrine cells in knockdown animals. At 4 dpf, we have not observed statistically significant changes in the population of the NPY-positive neurons. At 5 and 7 dpf, however, the quantity of NPY-positive cells was

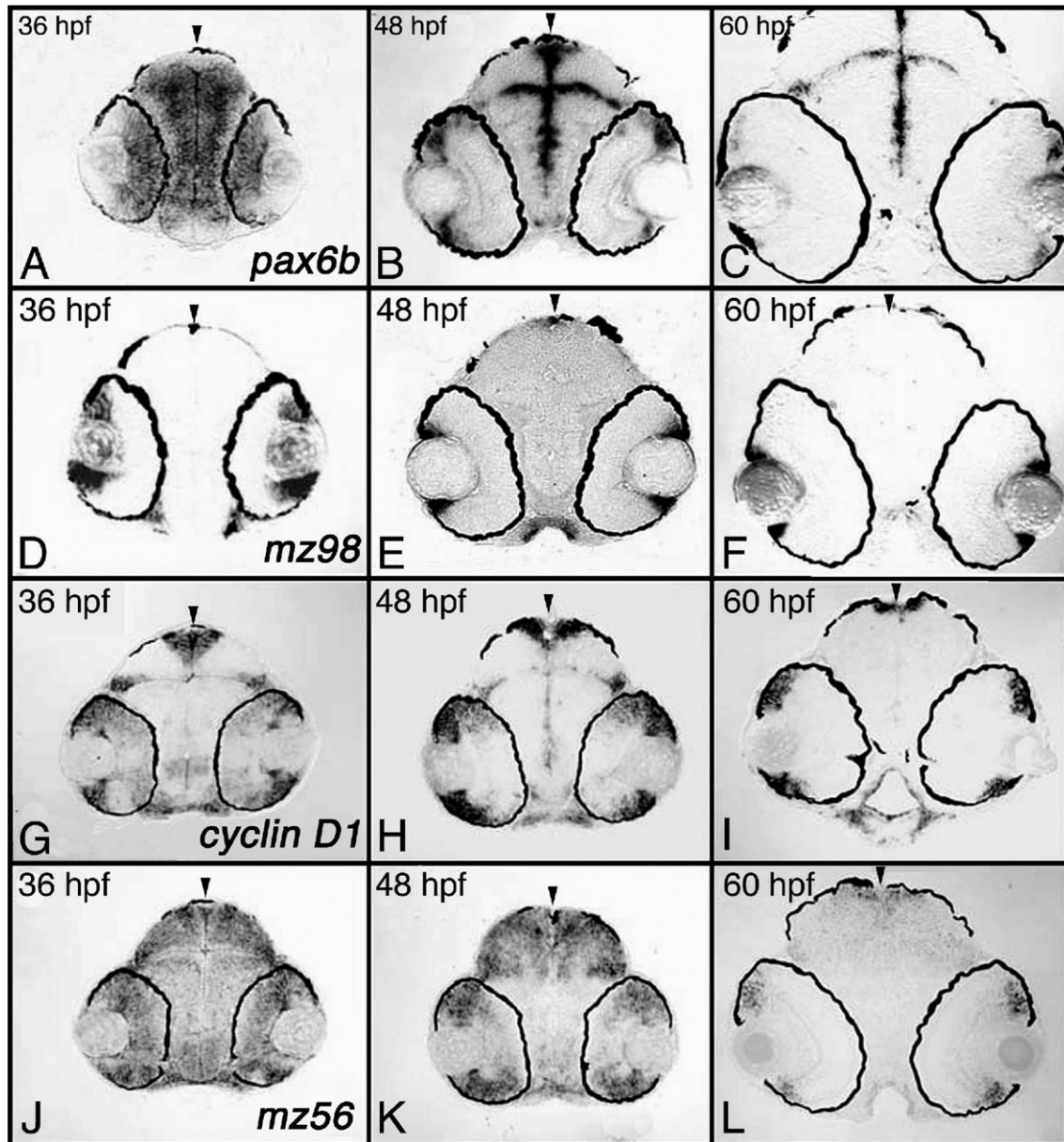


Fig. 5. Marginal zone expression. Shown are examples of transcripts expressed at the marginal zone. (A–C) *pax6b*, (D–F) *mz98*, (G–I) *cyclin D1*, (J–L) *mz56*. Transcripts were analyzed on plastic sections following whole-embryo in situ hybridization. Arrowheads mark the midline. hpf, hours postfertilization.

significantly increased (Table 2) and in some cases we observed ectopic NPY-positive cells in the ganglion cell layer. Changes in other amacrine cell subpopulations do not appear statistically significant. These experiments suggest that *soxp* plays a role in the specification of amacrine cell subpopulations. To test whether the overexpression of *soxp* affects amacrine cell development, we injected mRNA encoding a myc-tagged version of *soxp* into 1–4 cell-stage zebrafish embryos. This treatment produces variable results (3 independent experiments). We have frequently observed early embryonic lethality, malformations of eyes and the brain, and reduced numbers of multiple cell populations in the retina, including PV-positive cells and photoreceptors (data not shown). Our attempts to rescue the *soxp* knockdown phenotype also produced variable results (4 independent experiments), including early lethality

and inconsistent changes in the number of PV-positive cells. The variability of overexpression phenotypes and the lack of consistent rescue can be explained by the results of previous studies, which suggest a role for *soxp* at multiple stages of development, including early embryogenesis, optic lobe specification, and the maintenance of progenitor identity in the CNS (Avilion et al., 2003; Fantes et al., 2003; Graham et al., 2003). *soxp* overexpression is thus likely to result in a range of early phenotypes which obscure its role during neurogenesis.

Analysis of *cxc4b* phenotype

The expression pattern of *cxc4b* suggests a role in early ganglion cell development, possibly the migration of these cells to the vitreal part of the retinal neuroepithelium. To investigate

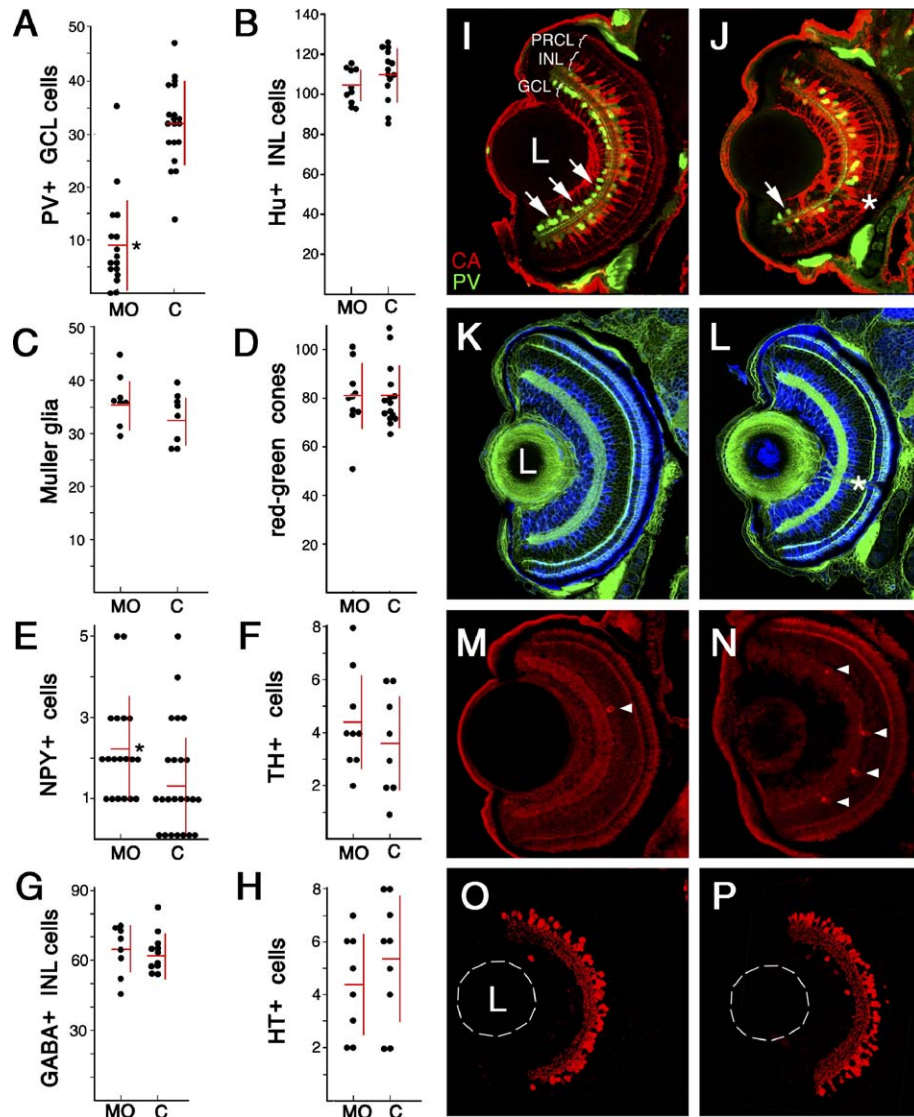


Fig. 6. Cell fate analysis in the retinae of *soxp* knockdown animals. In panels A through H, counts were performed on frozen sections following the injection of an anti-*soxp* morpholino or a control morpholino. (A) Parvalbumin-positive GCL cells in the *soxp* knockdown and control retinae. Following morpholino treatment, the numbers of parvalbumin-positive GCL cells are reduced from 32 ± 8 cells per section to 9 ± 9 cells (mean \pm SD; $P < 0.001$ *t* test). (B) HuC/HuD-positive amacrine cells. (C) Müller glia visualized with anti-carbonic anhydrase antibody. (D) Zpr1-positive red-green double cones. (E) NPY-positive amacrine cells. Following anti-*soxp* morpholino treatment, the numbers of NPY-positive amacrine cells are increased from 1.3 ± 1.1 to 2.2 ± 1.2 per retinal section (mean \pm SD; $P < 0.05$ Whitney–Mann test). (F) Tyrosine hydroxylase-positive interplexiform cells. (G) GABA-positive amacrine cells. (H) Serotonin-positive amacrine cells. (I, J) Antibody staining of parvalbumin-positive cells (green) and carbonic anhydrase-positive Müller glia (red) in *soxp* knockdown (J) and control knockdown retinae (I). (K, L) Staining of ganglion and amacrine cells with an anti-Hu antibody and of red-green cones with the Zpr-1 antibody (both blue) in control knockdown (K) and *soxp* knockdown (L) retinae. In both panels, sections are counterstained with phalloidin to visualize plexiform layers (green). (M, N) Staining of NPY-positive cells in *soxp* knockdown (N) and control knockdown retinae (M). Photoreceptor cell layer staining with the zpr-1 antibody is provided as a control (also in red). (O, P) Staining with anti-GABA antibodies in *soxp* knockdown (P) and control knockdown (O) retinae. Arrows in panels I and J indicate parvalbumin-positive cells in the ganglion cell layer. Arrowheads in panels M and N indicate NPY-positive cells in the INL. Asterisks indicate the optic nerve. C, control knockdown; L, lens; MO, *soxp* knockdown.

cxc4b function in the retina, we treated wild-type embryos with anti-*cxc4b* morpholinos and assessed the degree of ganglion cell layer development on cryosections by staining with Zn8, an antibody that recognizes neuroilin-positive ganglion cells and ganglion cell axons (Trevarrow et al., 1990). The injection of anti-*cxc4b* morpholinos into wild-type embryos results in a developmental delay. In some cases, this phenotype is accompanied by a mislocalization of ganglion neurons to ectopic locations (not shown). The ganglion cell phenotype is, however, inconsistent and its interpretation is complicated by

the overall developmental delay of the retina. To eliminate these difficulties, we decided to analyze *cxc4b* knockdown phenotype in mosaic retinae. To accomplish that, embryos were injected with *cxc4b* morpholino oligos and used as donors in standard blastomere transplantation experiments, designed to produce mosaic animals containing small *cxc4b* knockdown clones in the context of wild-type retina. If *cxc4b* is involved in ganglion cell migration, we hypothesized that its knockdown will result in ectopically localized cells. Analysis of over 40 *cxc4b* knockdown cell clones allowed us to inspect more than

Table 2
Cell population sizes following the administration of anti-*sox2* morpholinos

Cell class	4 dpf		5 dpf		7 dpf	
	Con	MO1, 2, 1 + 2	Con	MO1 + 2	Con	MO1 + 2
PV+	32 ± 8 (19)	MO1: 17 ± 4 (5) 53%	38 ± 3.9 (7)	6.6 ± 2.1 (14) 17%	40 ± 6 (7)	5 ± 2 (9) 12%
GCL		MO2: 12 ± 6 (9) 37%				
Cells		MO12: 9 ± 9 (16) 28%				
Zpr-1+	81 ± 12 (15)	81 ± 12 (15)	89 ± 8.9 (7)	93 ± 9.8 (10)	77 ± 10 (6)	69 ± 12 (8)
CA+	33 ± 4.8 (8)	36 ± 4.6 (8)				
NPY+	2.1 ± 1.2 (19)	2.4 ± 1.3 (8)	1.3 ± 1.1 (24)	2.2 ± 1.2 (19) 169%	1.1 ± 0.8 (8)	1.3 ± 1.1 (9)
	0.8 ± 1.0 (10)	0.8 ± 1.0 (9)	0.6 ± 0.5 (7)	1.6 ± 0.8 (7) 266%	0.76 ± 0.8 (17)	1.4 ± 1.1 (17) 184%
HuC/HuD+	110 ± 13 (14)	105 ± 8.7 (10)				
TH+			3.6 ± 1.9 (8)	4.4 ± 1.9 (9)		
ST+			2.4 ± 1 (8)	1.6 ± 1 (7)		
GABA+			64 ± 9 (11)	65 ± 11 (8)		
Ser+			5 ± 2 (9)	4 ± 2 (8)		

The quantitation of cell population sizes at 4, 5, and 7 dpf, following injection with control morpholino (Con), anti-*sox2* morpholino 1 (MO1), anti-*sox2* morpholino 2 (MO2), or both. Counts were performed on transverse retinal sections from multiple animals ($n \geq 5$). Values represent mean ± SD followed by the number of sections examined (in parentheses). Percentages indicate the number of cells relative to controls and are included only for statistically significant differences ($P < 0.05$). Statistical significance was evaluated using Chi-Square or Whitney–Mann tests for NPY-positive cells. The *t* test was used for other cell populations.

200 ganglion neurons. None of these were positioned in ectopic locations (Fig. 7B). As the ganglion cell layer consists of 2 to 3 tiers of cells, we hypothesized that the *cxcr4b* knockdown may slow down cell migration and bias the distribution of *cxcr4b* knockdown ganglion neurons to the more superficial tiers, compared to control experiments. This possibility is not supported by our data: both *cxcr4b* knockdown and control knockdown cells display the same distribution in the ganglion cell layer. The *cxcr4b* knockdown does, however, affect the frequency of ganglion cells in donor-derived cell clones. Approximately 30% of cells become ganglion neurons in control morpholino-injected clones. In contrast to that, *cxcr4b* knockdown clones include only 15–20% of ganglion cells (Fig. 7D). The coinjection of *cxcr4b* mRNA together with anti-*cxcr4b* morpholino restores the wild-type frequency of ganglion cells, indicating that the loss of ganglion cells in morphant animals is not due to non-specific causes (Fig. 7D).

The zebrafish *odysseus* (*ody*) locus has been reported to encode the Cxcr4b polypeptide (Knaute et al., 2003). To investigate whether zebrafish *cxcr4b* mutations produce a loss of ganglion neurons, we evaluated cell numbers in the retinae of the *ody*^{l26035} mutant animals. The *ody* mutation introduces a premature stop codon in the third intracellular loop of *cxcr4*, presumably resulting in a complete loss of function (Knaute et al., 2003). In contrast to the morpholino-induced phenotype, however, at 36, 48, and 72 hpf, the numbers of ganglion cells in *ody* mutant animals are the same as in their wild-type siblings (Table 3). As the loss of ganglion cells in the *cxcr4b* knockdown zebrafish was observed in mosaic retinae, we also performed mosaic analysis of *ody* phenotype. In contrast to the result of knockdown experiments, clones of *ody* mutant cells in wild-type retina contain the same percentage of ganglion cells as control wild-type clones in wild-type environment (Table 3). One possible explanation for the discrepancy between the morpholino-induced phenotype and the phenotype of the chemically-induced defect is that the zebrafish *ody* mutant allele retains some of the wild-type function. As another test of

cxcr4b function during retinal neurogenesis, we generated mosaic animals containing clones of *cxcr4b* mRNA over-expressing cells in otherwise wild-type retinae. In these clones, approximately 24% of cells (202 out of 822 in 17 clones)

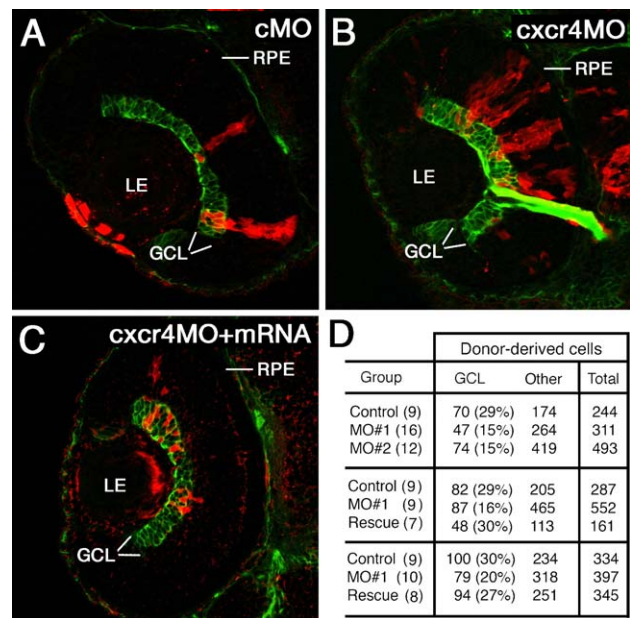


Fig. 7. Mosaic analysis of *cxcr4b* knockdown phenotype. Blastomeres were transplanted into untreated host embryos from sibling donor embryos that were injected with a control MO (A) an anti-*cxcr4b* MO (B) or a mixture of anti-*cxcr4b* MO and *cxcr4b* mRNA. The resulting mosaic retinae contain a mix of morpholino-treated donor-derived cells (red) and untreated host cells. The ganglion cell layer (GCL, green) is visualized with the Zn8 antibody. (D) The frequency of GCL cells in clones derived from donor animals treated with a control MO, anti-*cxcr4b* translation blocking MO (MO#1), anti-*cxcr4b* splice-site blocking MO (MO#2), or anti MO#1 and *cxcr4b* mRNA (Rescue). Each row of panel D table shows a separate experiment. In all experiments, morpholino injection resulted in a statistically significant loss of ganglion cells. mRNA treatment restores the wild-type frequency of ganglion cells. The numbers of retinae examined are provided in brackets. GCL, ganglion cell layer; LE, lens; RPE, retinal pigmented epithelium.

Table 3

To evaluate the survival of ganglion cells, wild-type (wt) and mutant (ody) embryonic retinæ were sectioned and stained with Zn8, an antibody that visualizes ganglion cells

	36 hpf	48 hpf		72 hpf	
	GC	GC	GC/PRC	GC	GC/PRC
<i>Ganglion cell survival in ody mutant embryos</i>					
wt	3.7 ± 1.4 (11)	108.3 ± 19 (7)	1.1	106.3 ± 6.7 (7)	1.2
ody	4.4 ± 1.6 (8)	90.7 ± 10 (11)	1.1	119.4 ± 9.7 (7)	1.0
	36 hpf	48 hpf		72 hpf	
	GC	GC	GC/Total	GC	GC/Total
<i>Ganglion cell survival in clones of cells in mosaic retinæ</i>					
wt → wt	nm	8 ± 8 (11)	0.2	nm	nm
wt → ody	nm	8.7 ± 6 (6)	0.2	nm	nm
ody → wt	nm	11.2 ± 7 (11)	0.2	nm	nm

To investigate whether changes in the ganglion cell population size are specific or reflect an overall decrease in the size of the retina, we counted the number of photoreceptor cells on the same sections and compared it to ganglion cell counts (GC/PRCL column). Ganglion cell numbers were also evaluated in clones of cells in mosaic retinæ. The number of ganglion neurons in donor-derived clones was compared to the total number of cells in these clones (GC/Total column). GC, ganglion cells; PRC, photoreceptor cells; nm, not measured.

contribute to the ganglion cell layer. This is not statistically different from GFP-overexpressing control clones, which contribute ca. 22% of cells to the ganglion cell layer (116 out of 530 in 15 clones). Cell distribution within the ganglion cell layer displays no obvious differences between *cxcr4b* and control clones. If *cxcr4b* signaling indeed plays a role in ganglion cell development, this result suggests that *cxcr4b* expression is not a limiting factor in its signaling cascade.

Discussion

Reverse genetic approach in the zebrafish embryo

As genome projects continue to generate massive amounts of sequence information, it is necessary to evaluate the function of many newly identified genes. One approach that rapidly provides useful clues to the potential activities of novel genes during embryogenesis is the evaluation of expression patterns via whole-mount in situ hybridization. In zebrafish, this can be accomplished on a large scale, owing to the high fecundity of this organism as well as the transparency and external development of its embryo. We have used this strategy to study transcripts involved in retinal neurogenesis. Following the initial evaluation of over 8000 cDNAs by whole-mount in situ hybridization, the expression of 47 transcripts (represented by 52 cDNA clones) was thoroughly analyzed at several stages of development. Approximately 40% (18/47) of them display expression predominantly confined to a single cell class and their initial appearance correlates with cell cycle exit (Table 1). Based on their expression patterns, many of these transcripts are good candidates for the regulators of cell fate decisions or the determinants of early differentiation. Even in the absence of obvious functions, many of these transcripts will provide useful markers of cell identity.

The reverse genetic approach that we present in this work very well complements forward genetic mutagenesis screens, which have been the driving force of developmental genetic

studies in zebrafish so far. One important advantage of this approach is that the familiarity with expression patterns allows one to formulate detailed hypotheses regarding the function of specific genetic loci. Consequently, functional analysis can be tailored to search for mutant phenotypes that match specific expression patterns. The phenotype of each gene can be analyzed using different criteria. This contrasts with forward genetic mutagenesis approaches, which due to labor involved are usually limited to a small set of phenotypic tests. In a reverse genetic screen, an early and specific expression in a subset of amacrine cells suggests a role in cell fate determination within this cell class. This idea can be tested using a collection of antibodies which stain subpopulations of this particular cell class. Our in situ hybridization screen has identified a number of transcripts that display restricted expression patterns in the retina. Functional tests of two transcripts suggest that they play a role in the development of individual cell classes.

Clones that display restricted expression patterns in the retina hold promise to reveal answers to questions regarding retinal development. How is the cell cycle exit of neuroepithelial cells regulated? What mechanisms are instrumental in cell-fate specification? How do retinal cell classes ultimately acquire their unique morphological features? Experiments presented here have identified factors that are likely to participate in all of these processes. At least 10 transcripts (ca. 20%) display homology to transcriptional regulators. Interestingly, many of them are confined to specific cell populations. 5 transcripts (ca. 10%) encode transmembrane polypeptides, and may function in cell–cell signaling events, ion transport, or constitute structural components of the cell membrane. Another group of 5 transcripts display homology to cytoplasmic factors involved in signal transduction cascades. In addition to the above categories, putative cytoplasmic enzymes, cytoskeletal proteins, and a cell cycle regulator have also been identified.

The wide variety of expression patterns that we identified suggests a diverse assortment of developmental roles. 7 transcripts are expressed in the retinal neuroepithelium and

later at the marginal zone. Analysis of these genes could provide insight into the mechanisms that regulate cell-cycle exit of neuroepithelial cells, a step that may constitute an essential component of cell-fate decisions (reviewed in Malicki, 2004; Ohnuma and Harris, 2003). Transcripts encoded by 8 genes that are predominantly expressed in ganglion cells (GC) may help to reveal how GC are specified, how they migrate to their appropriate locations, and how they elaborate a constellation of morphological features. Similarly, genes expressed in specific cell classes of the inner nuclear layer (8), in photoreceptor cells (2), and in the retinal pigmented epithelium (1) will answer related questions for other cell populations of the retina. Transcripts expressed in more than one cell layer may reveal processes that are shared by cells of different classes. Our analysis revealed, for example, that amacrine and ganglion cells share a disproportionately high number of transcripts, suggesting that they are more closely related to each other than to other cell populations of the retina.

Expression in the marginal zone

The marginal zone continues to generate neurons in the mature retina of teleost fish. This region is a site of dynamic changes as it contains both mitotically active self-renewing progenitor cells and differentiating neurons (Ohnuma et al., 2002). As the cells of the marginal zone are presumed to recapitulate cell fate decisions of neurogenesis, the expression patterns in this area are expected to reflect a complex array of developmental events. It is therefore not surprising that both cell cycle regulators, such as *cyclins*, as well as key determinants of developmental cell fate decisions are expressed in this region. The expression of *pax6b* in the retinal marginal zone may reflect its crucial role in retinal cell fate determination that has been demonstrated in the mouse (Marquardt et al., 2001). *pax6b* is not expressed in the most extreme areas of the retinal margin, suggesting that it is not active in maintaining the proliferative state of retinal progenitors. Instead, consistent with its role in the mouse, it is expressed at some distance from the most marginal areas, where it is likely to be involved in the regulation of cell-fate decisions that lead to differentiation of retinal cell classes (Marquardt et al., 2001). The expression of other transcriptional regulators in the marginal zone, *her4* for example, may also reflect their involvement in cell fate decisions.

Expression in specific cell classes

Ganglion cells are the first neurons to form during retinal neurogenesis. Our screen has uncovered several genes expressed predominantly in the GCL that may function during different stages of ganglion cell development. The high expression of *cxcr4b* and *gacek* in young ganglion cells suggest developmental roles. *gacek* contains the BTB/POZ domain, a protein–protein interaction motif found in factors involved in chromatin remodeling and transcriptional regulation (reviewed in Collins et al., 2001). The transient expression of this gene in young ganglion cells and its downregulation in older neurons

suggests an early developmental function. Consistent with this idea are observations that BTB/POX transcription factors are expressed in immature neurons at the time of their migration and early differentiation (Mitchellmore et al., 2002; Shim et al., 2001). Similarly, the high expression of *cxcr4b* in migrating ganglion cells and its sharp downregulation suggests a role for this chemokine receptor in early stages of ganglion cell differentiation. Another ganglion cell layer-specific expression pattern that deserves attention is represented by the *hrT* factor. Although one recent study has identified 11 types of retinal ganglion cells (Rockhill et al., 2002), the genetic basis of their specification and differentiation is unknown. Transcription factors, such as *hrT*, that are expressed in small well-defined cell subpopulations, may offer important entry points into the genetic analysis of this process.

Similar to ganglion neurons, the amacrine cell class consists of many cell types characterized by morphologically distinct dendritic trees and diverse functions (MacNeil and Masland, 1998; MacNeil et al., 1999). Also in this case, one would like to understand mechanisms that specify the common features of the entire cell class as well as factors that generate individual subpopulations. The early pan-amacrine expression patterns of *ap2 α* and *ap2 β* suggest a role in early development of the entire cell class and contrast with the restricted presence of *pou23* and *inlf12*. *pou23* is related to three *Pou4* factors that are expressed in subsets of ganglion cells. At least one of them, *Pou4f2*, is necessary for ganglion cell differentiation and survival (Gan et al., 1999). Although *Pou4* factors are not necessary for ganglion cell specification, their overexpression results in the expansion of ganglion cell numbers relative to other cell classes suggesting a role in the determination of ganglion cell identity (Liu et al., 2000). The expression of *pou23*, presumably in a subset of amacrine cells, suggests that POU-domain transcription factors may play a similar role in INL neurons.

Two transcripts that we isolated are predominantly expressed in photoreceptor cells. Importantly, both appear upregulated prior to opsin expression at ca. 50 hpf (Raymond et al., 1995). Few genes are known to display this characteristic, which hints at a role in early differentiation events. Although *ndrg1* is widely expressed in human tissues, its exact function remains obscure. It is a member of the α/β hydrolase fold superfamily without a hydrolytic catalytic site (Shaw et al., 2002). In human epithelial cells, NDRG1 immunoreactivity is found in adherens junctions (Lachat et al., 2002). NDRG1 function in zebrafish developing photoreceptors may involve the assembly cell junctions in the outer limiting membrane.

The second photoreceptor-enriched transcript, *prcb4.1*, is represented by two independent clones with a homology to the FERM domain of the protein band 4.1 superfamily (Chishti et al., 1998). Members of this family are known to connect transmembrane polypeptides to the actin cytoskeleton (reviewed in Louvet-Vallee, 2000). The FERM domain of the band 4.1 protein, for example, associates with the transmembrane protein glycophorin in erythrocytes (Anderson and Lovrien, 1984). In photoreceptor cells, a band 4.1 protein has been detected in cone myoids and an apically expressed cell polarity determinant, *crumbs*, features a band 4.1 protein binding site on its

cytoplasmic tail (Izaddoost et al., 2002; Pellikka et al., 2002; Spencer et al., 1991). The *prclb4l* polypeptide may thus play a role in the formation of complex membrane domains that subdivide the photoreceptor cell surface.

The functions of soxp and cxcr4b in neurogenesis

To test developmental roles of transcripts uncovered in the course of in situ hybridization screen, we performed antisense knockdowns of selected loci. The *soxp* gene appears to function in amacrine cell specification, while the *cxcr4b* locus may play a role in differentiating ganglion neurons. Amacrine cell development is subject to a combinatorial regulation by a group of bHLH and homeobox transcription factors that includes Six3, Math3, NeuroD, and Pax-6 (Inoue et al., 2002; Le et al., 2002; Marquardt et al., 2001; Moore et al., 2002). Genetic manipulations of these loci appear to impact the entire amacrine cell class. In contrast to most other transcription factors described so far, *soxp* appears to function in the specification of cell type diversity within the amacrine cell class, leaving the overall numbers of amacrine cells largely unaffected. We have documented changes in two amacrine cell subpopulations: a decrease in the number of PV-positive cells and an increase in the size of the NPY-positive population. As the population of NPY-positive cells is small, it is unlikely that its increase fully compensates for the loss of PV-immunoreactive cells. Changes in other neuronal population are thus very likely but so far escaped our attempts to detect them, possibly due to the lack of appropriate markers. Thus far, few genes have been shown to play a role in cell fate decisions within the amacrine cell class. One other example is the cell cycle regulator, *p57^{kip2}*, known to cause an overproduction of cabindin-positive amacrine neurons without affecting the overall number of amacrine cells (Dyer and Cepko, 2000). As amacrine cell class is very diverse, it is reasonable to expect that *soxp* is only one of many transcription factors that remain to be identified before the complex process of amacrine cell type specification is fully understood.

It has been recently shown in several model systems that *cxcr4* plays a role in the migration of several cell populations, including germ cells, and the lateral line primordium (David et al., 2002; Doitsidou et al., 2002; Knaut et al., 2003). In the context of the nervous system, *cxcr4* has been studied in the cerebellum and the hippocampus (Ma et al., 1998; Zou et al., 1998). Mouse homozygotes null for the *cxcr4* function display patterning defects in some regions of the central nervous system. While most of the cerebral cortex, midbrain, and the spinal cord are unaffected, the cerebellum of mutant animals is disorganized (Ma et al., 1998; Zou et al., 1998). The external granule cell layer (EGL) and the Purkinje cell layer are discontinuous, and ectopic cell clusters that presumably consist of granule cell progenitors are observed. These patterning defects have been hypothesized to result from premature granule cell migration from the EGL (Klein et al., 2001). *cxcr4* knockout mice also display hippocampal defects. Here, the absence of *cxcr4* function appears to lead to a premature differentiation of dentate gyrus progenitor cell (Lu et al.,

2002). In the retina, the role of *cxcr4* has not been studied extensively. One study has reported a reduced in the ganglion cell number in *cxcr4* knockout mice (Chalasanani et al., 2003). This finding is consistent with the results of mosaic analysis performed in zebrafish, which also reveals ganglion cell loss. Surprisingly, it contrasts with the analysis of retinae in the zebrafish *ody^{t26035}* mutant. The *ody^{t26035}* mutant allele contains a premature stop codon at the position 239, truncating the last two transmembrane domains (Knaut et al., 2003). One possible explanation for the discrepancy between the mouse knock-out phenotype and defects seen in the chemically-induced zebrafish mutant is that the fish mutant allele retains some of the wild-type function.

Functional tests of the remaining genes uncovered in the course of our screen are likely to reveal additional determinants of retinal differentiation. These studies may be performed using the loss-of-function knockdown approach or via overexpression analysis. Even in the absence of obvious function, many of the transcripts presented in this manuscript serve provide useful markers of cell identity.

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