The homeobox gene *Xbh1* cooperates with proneural genes to specify ganglion cell fate within the *Xenopus* neural retina

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Summary

Recent studies on vertebrate eye development have focused on the molecular mechanisms of specification of different retinal cell types during development. Only a limited number of genes involved in this process has been identified. In *Drosophila*, BarH genes are necessary for the correct specification of R1/R6 eye photoreceptors. Vertebrate Bar homologues have been identified and are expressed in vertebrate retinal ganglion cells during differentiation; however, their retinal function has not yet been addressed. In this study, we report on the role of the *Xenopus* Bar homologue *Xbh1* in retinal ganglion cell development and its interaction with the proneural genes *Xath5* and *Xath3*, whose ability to promote ganglion cell fate has been demonstrated. We show that *XHB1* plays a crucial role in retinal cell determination, acting as a switch towards ganglion cell fate. Detailed expression analysis, animal cap assays and in vivo lipofection assays, indicate that *Xbh1* acts as a late transcriptional repressor downstream of the atonal genes *Xath3* and *Xath5*. However, the action of *Xbh1* on ganglion cell development is different and more specific than that of the Xath genes, and accounts for only a part of their activities during retinogenesis.

Key words: Retinal differentiation, Ganglion cell, Photoreceptor, Retinal cell fate, Bar, *Xenopus laevis*, Homeobox

Introduction

Retinal ganglion cells (RGCs) derive from a pool of virtually identical multipotent retinal progenitor cells (RPCs) (Holt et al., 1988; Turner et al., 1990; Wetts, 1988). RPCs differentiate according to an ordered spatiotemporal pattern, largely conserved among vertebrates: ganglion cells are born first, followed by cone photoreceptors, horizontal and amacrine cells, whereas rod photoreceptors, bipolar and Müller cells are born last (Cepko et al., 1996; Stiemke and Hollyfield, 1995). Recent evidence supports the hypothesis that the commitment towards specific cell types occurs very early in RPCs (Lillien, 1998; Marquardt and Gruss, 2002; Marquardt et al., 2001). At any given time of retinal development, each RPC expresses different repertoires of transcription factors (Perron et al., 1998), which are thought to provide the cell with the intrinsic competence to respond appropriately to external stimuli and to differentiate toward particular cell fates (reviewed by Livesey and Cepko, 2001). Several transcription factors that are able to bias RPCs towards specific cell fates have been isolated (Livesey and Cepko, 2001; Marquardt et al., 2001; Vetter and Brown, 2001). Increasing evidence for similarities in retinal development between vertebrates and *Drosophila* has been of crucial importance for starting to unravel the genetic pathways regulating ganglion cell fate specification in vertebrates (Kumar, 2001; Masai et al., 2000; Neumann and Nuesslein-Volhard, 2000). Transcription factors homologous to the *Drosophila* bHLH gene *atonal*, necessary for differentiation of the first fly photoreceptor, R8, start to be expressed in RPCs just before the onset of differentiation of RGCs, the first cell type born in the vertebrate retina (Brown et al., 1998; Kanekar et al., 1997; Liu et al., 2001; Masai et al., 2000; Perron et al., 1999). In *Xenopus*, both atonal-related factors, Xath3 and Xath5, can promote ganglion cell fate when overexpressed in the retinal primordium, suggesting that they are intrinsic determinants of this cell type (Kanekar et al., 1997; Perron et al., 1999). In fact, loss-of-function analysis both in mouse and zebrafish demonstrated the requirement of a functional *ath5* (*atoh7* – Zebrafish Information Network) gene for ganglion cell fate specification (Brown et al., 1998; Kay et al., 2001; Wang et al., 2001). Despite this, *ath5* may not act as a unique intrinsic determinant of RGCs. In fact, *ath5* expression is found in a wider population of RPCs than that fated to become ganglion cells (Kanekar et al., 1997; Masai et al., 2000; Perron et al., 1999; Yang et al., 2003). Besides, forced expression of murine *ath5* (*Atoh7* – Mouse Genome Informatics) in *Xenopus* RPCs is able to promote bipolar rather than ganglion cell fate (Brown et al., 1998). Moreover, Ohnuma et al. (Ohnuma et al., 2002) have recently shown that when *Xenopus Xath5* is misexpressed in RPCs together with factors enhancing proliferation, it no longer promotes ganglion cell fate but favours later retinal fates. Similarly, when *Ath5* is misexpressed in late RPCs both in *Xenopus* and in chick, its ability to

promote early cell fates decreases (Matter-Sadzinski et al., 2001; Moore et al., 2002). Finally, the neurogenic gene *Notch* regulates the ability of *Xath5* to promote ganglion cell differentiation, by modulating in time and space the relative levels of Xath5 activity in the retinal precursors (Moore et al., 2002; Ohnuma et al., 2002). These data altogether strongly suggest that additional factors are required for proper specification of ganglion cell fate (reviewed by Vetter and Brown, 2001).

Potential factors acting downstream of *Ath5* to specify ganglion cell fate are the POU-domain transcription factors Brn3, expressed in postmitotic precursors and in differentiating RGCs (Hirsch and Harris, 1997; Hutcheson and Vetter, 2001; Perron et al., 1998; Xiang et al., 1995). All Brn3 factors are able to promote ganglion cell fate in the chick (Liu et al., 2000). In *Xenopus*, both *Xbrn3.0* and *Xbrn3d* are activated by *Xath5* in the whole embryo or in the animal cap assay (Hirsch and Harris, 1997; Hutcheson and Vetter, 2001; Perron et al., 1998). Similarly, forced expression of both chick and mouse *Ath5* in RPCs is able to activate *cBrn3c* expression (Liu et al., 2001), further corroborating the hypothesis that Brn3 genes act downstream of *Ath5* to bias retinoblasts toward ganglion cell fate. However, targeted disruption of the POU gene *Brn3b (Pou4f3)* in the mouse does not affect retinal specification, but rather blocks terminal differentiation of a subset of RGCs, causing them to die (Gan et al., 1996; Xiang et al., 1998). This suggests that Brn3 factors may instead play a later role in differentiation and survival of subsets of RGCs.

In the present work, we addressed the function of the *Xenopus* BarH1 homeobox gene *Xbh1* during RGC differentiation. Vertebrate Bar homeobox genes are related to *Drosophila BarH1* and *BarH2* genes. Mutations in these genes result in the suppression of the anterior part of the eye (Higashijima et al., 1992a; Kojima et al., 1991). Both genes act in a redundant way, and are necessary for the correct development of the external sensory organs and the eye (Higashijima et al., 1992b). In the *Drosophila* eye, *BarH1*/*BarH2* are necessary for the differentiation of the external photoreceptors (R1/R6) and primary pigment cells, where they are regulated by two other transcription factors: *lozenge* (*lz*), which modulates the expression of *BarH1*/*BarH2* in R1/R6 precursors; and *sparkling* (*spa*; *shaven*, *sv* – FlyBase), a homologue of mammalian *Pax2*, necessary for *BarH1*/*BarH2* expression in the cone and primary pigment cell precursors (Daga et al., 1996; Fu and Noll, 1997). In the developing notum, *BarH1*/*BarH2* genes are regulated by the secreted factors decapentaplegic and wingless, and exert their function by modulating the proneural *achaete-scute* genes (Sato et al., 1999). Mammalian homologues of BarH genes, *MBH1* and *MBH2* (*Barhl2* and *Barhl1*, respectively – Mouse Genome Informatics, Human Gene Nomenclature Database), have been isolated and show similar but not identical expression patterns in the central nervous system and retina (Bulfone et al., 2000; Saito, 2000; Saito et al., 1998). Though recent data show that *MBH1* acts in the specification of commissural neurones in the dorsal spinal cord (Saba et al., 2003), the role of Bar-related genes in vertebrate eye development has not been investigated. Similar to *MBH1*, *Xenopus Xbh1* and medaka *OlBar* are expressed in the retina in a spatiotemporal pattern that appears to follow the differentiation of RGCs (Patterson et al., 2000; Poggi et al., 2002). This finding suggests that vertebrate BH1 is involved in RGC type specification. Here, we show that *Xbh1* expression follows and overlaps the dorsoventral wave of *Xath5* expression during retinal neurogenesis. In the CMZ of the mature retina, *Xbh1* transcripts were found in the central-most part containing early postmitotic precursors that are about to undergo the differentiation process. We also provide functional data strongly supporting a role for *Xbh1* in promoting RGC fate. In addition, we find that *Xbh1* both enhances and is required for the ability of *Xath5* to bias retinal precursors toward ganglion cell fate. Our data suggest that *Xbh1* acts in a genetic pathway downstream of *Xath5* and upstream of *Xbrn3* in the regulation of RGC development.

Materials and methods

Xenopus laevis embryos

Xenopus females were pre-injected with 100 units of pregnant mare serum gonadotrophin (Folligon, Intervet) 4-11 days prior to egg collection, and with 800-1000 units of human chorionic gonadotrophin (Profase HP 2000, Serono) the night before collection. Eggs were fertilized with testis homogenates and cultured in 0.1×MMR (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, 0.1 mM EDTA). Jelly coats were removed in 3.2 mM DTT, 0.2 M Tris (pH 8.8). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) and fixed in MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 hour at room temperature (RT) or overnight at 4°C, dehydrated in ethanol and stored at –20°C for subsequent histological examination or whole-mount in situ hybridization.

In situ hybridization

Whole-mount in situ hybridization was performed as described by Harland (Harland, 1991). Standard RNA synthesis from linearized plasmids using SP6, T7 or T3 RNA polymerases were carried out incorporating a digoxigenin (DIG)- or fluorescein-substituted ribonucleotide. Alkaline phosphatase detection was performed with BM-purple. For histological examination, stained embryos were washed in 1×PBS several times, equilibrated in sucrose 30% (in 1×PBS) and cryostat sectioned at the thickness of 30 μm.

In situ hybridization on sections was performed using the same protocol but with the following modifications: rehydrated sections were fixed to slides using 100% methanol for 10 minutes, then rinsed in 1×PBS for 2 minutes and washed 3 times for 5 minutes in PBST (PBS+0.1% Triton). Sections were treated with 20 μg/ml proteinase K for 30 seconds with subsequent wash times reduced by half.

For double in situ hybridization, sections were hybridized simultaneously with both a DIG- and a fluorescein-labeled probe under standard conditions. After detection of the first probe with BM-purple, the alkaline phosphatase was inactivated in 100 mM glycine (pH 2.2) and 0.1% Tween-20, then the sections blocked in MAB [100 mM maleic acid, 150 mM NaCl (pH 7.5)] and Blocking Reagent supplemented with 20% lamb serum. Following incubation with the second antibody, the alkaline phosphatase reaction was performed with Magenta-Phos (Sigma).

Lipofections

DNA isolated by Qiagen maxi preps was diluted in nuclease-free water to a concentration of 1.5 μg/μl. These stocks were spun down for at least 10 minutes at 4°C before use. Each construct (1 μl) was mixed with 1 μl $pCS2^+$-GFP (green fluorescent protein) DNA to label transfected cells. $pCS2^+$-GFP with $pCS2^+$ vector alone was used as the control. DOTAP (9 μl; Roche) was added to 3 μg DNA and injected into the eye presumptive region of stage 17-18 or stage 25-26 embryos. At stage 42, embryos were fixed in 4% paraformaldehyde

for 1 hour at room temperature, sunk in 30% sucrose overnight at 4°C and cryostat sectioned (10 μm). Samples were rehydrated with two washes of 1×PBS for 5 minutes, mounted in FluorSave (CalBioChem) containing 2% DABCO (Sigma) and dried overnight at room temperature.

BrdU experiments

Stage 42 embryos were injected with BrdU (5-bromo-2′-deoxyuridine, Roche) in the gut, fixed 1 hour later and cryostat sectioned. In situ hybridization was performed on 10 μm sections as follows: DIG-labelled probes (2 ng/ml in hybridization buffer) (Shimamura et al., 1994) were heated to 70°C for 10 minutes and then incubated on sections at 60°C overnight. The rest of the protocol was performed as previously described (Myat et al., 1996). Following NBT/BCIP reaction, sections were stained for BrdU. To do this, sections were washed with 2 N HCl for 45 minutes then neutralized with several PBST washes. The anti-BrdU antibody (Molecular Probes) was added at 1:10 dilution and incubated at 37°C for 30 minutes. After three changes of PBST, Cy3 goat anti-mouse (Chemicon) secondary antibody was added at 1:500 dilution and incubated for 30 minutes at 37°C. The samples were washed three times with PBST and stained with 15 μg/μl Hoechst solution for 3 minutes at room temperature, to visualize nuclei. After three final washes in PBST, sections were mounted in FluorSave (CalBioChem) containing 2% DABCO.

DNA constructs

A partial open reading frame of *Xbh1* cloned in pGEM3Z vector was kindly provided by Dr P. Krieg (University of Texas), lacking the first two codons at the N-terminal domain. The two missing codons were restored by performing a RT-PCR reaction on cDNA from stage 31 *Xenopus* embryos. A forward primer (AA**GAATTC**TTGTGTCTGAACTGGA), with an additional *Eco*RI site, and a reverse primer (CGGTTCCATAGTGACTGATAT) were used to amplify a region spanning from nucleotide (nt) –24 to nt 265 of the published *Xbh1* open reading frame (ORF) (Patterson et al., 2000), containing a *Sac*I restriction site at nt 227. The resulting PCR fragment was *Eco*RI/*Sac*I digested and cloned into *pGEM3ZXbh1* linearized with *Eco*RI and *Sac*I, thereby restoring the complete ORF. *Xbh1* full-length ORF was afterwards subcloned into the *Eco*RI/*Xba*I site of pCS2^{+}.

The *Xbh1Vp16* construct was generated by PCR cloning by in frame fusion of an *Xbh1* fragment spanning the N-terminal domain, the two FIL peptides and the homeodomain (AA residues 1-282), into the *Cla*I site of pCS2/VP16 (Kessler, 1997) (primers used: forward, CC**ATCGAT**GAATTCTTGTGTCTGAACTGGA; reverse, CC**ATCGAT**ATAATTGCCGGCTTCGGCTAG).

The *Xbh1EngR* was constructed by in-frame PCR cloning (forward, AA**GAATTC**TTGTGTCTGAACTGGA; reverse, CG**GAATTC**AATAATTGCCGGCTTCGGCTAG) of the same *Xbh1* fragment (AA 1-282) into the *Eco*RI site of pCS2/EnR (Kessler, 1997).

Microinjection of in vitro transcribed mRNA

Capped synthetic mRNAs were generated by in vitro transcription of linearized plasmids using SP6 or T7 Cap Scribe kits (Roche). For animal caps experiments, 250-1000 pg of mRNA of *Xbh1*, *Xbh1EngR*, *Xbh1VP16*, *Xath3*, *Xath5* and *XneuroD* constructs were injected into the animal region of 2-cell-stage embryos, using a Drummond 'Nanoject' apparatus. Embryos were injected in 0.1×MMR and 4% Ficoll 400, and cultured overnight at 14°C in the same solution. Animal caps were then dissected at stage 9 and grown to stage 28 in 0.5×MMR before processing for RT-PCR. As controls for staging animal caps, sibling embryos were grown in 0.1×MMR.

RNA extraction and RT-PCR analysis

RNA extraction and RT-PCR were performed as described in Lupo et al. (Lupo et al., 2002). Primers used were as follows: *Xbh1*, ATGGAAGGATCCAGCTTTGGGATA (forward) and GATATGGGCGAAGATGGGGAG (reverse); *Xbrn3.0*, TTGATCTCTACCTCGGCCCAT (forward) and TGAGTCGCAGATAGACGCCAA (reverse); *Xbrn3d*, GATGACACTTTGCTTAGAGGA (forward) and GCCATGTGGTTAATGGCTGA (reverse); *Xath3*, GAGAGGTTCCGTGTCCGTAG (forward) and GCTTGTTGGCTGAGAAAGACC (reverse); and *Xath5*, ATCGTTACCTGCCCCAGACT (forward) and CTTGGCTTTTCCAGTGTTCC (reverse). *ODC* primers were from Bouwmeester et al. (Bouwmeester et al., 1996). PCR conditions were as described by Hutcheson and Vetter (Hutcheson and Vetter, 2001), except for *ODC* (Lupo et al., 2002).

Results

Xbh1 expression in the retina follows the spatiotemporal wave of *Xath5* expression

To elucidate in detail how *Xbh1* expression is related to retinal neurogenesis in time and space, we compared its expression with that of *Xath5*. *Xath5* expression starts in the retina at around stage 24, preceding the reported onset of retinal differentiation (Holt et al., 1988). Expression is initially present throughout most of the neural retina, but displays a dorsal to ventral gradient that is consistent with neurogenesis commencing slightly earlier in the dorsal retina than in the ventral retina (Fig. 1I,J, and data not shown) (Holt et al., 1988; Kanekar et al., 1997; Perron et al., 1998). When RGC, inner nuclear, and photoreceptor cell layers become distinct, *Xath5* expression is downregulated in differentiated neurones, but remains in the ciliary marginal zone (CMZ), where retinoblasts are generated throughout life (Fig. 1H,K,L) (Perron et al., 1998; Wetts et al., 1989). *Xbh1* expression in the retina is first detected in the dorsal inner optic cup around stage 26-27, shortly after the onset of *Xath5* expression (Fig. 1A,E, and data not shown), and subsequently spreads from dorsal to ventral (Fig. 1B,F) until it covers the entire retina (Fig. 1C,G), thus following the wave of retinal differentiation (Holt et al., 1988). At stage 38, when the three main retinal cell layers become distinct, *Xbh1* is detected in the ganglion cell layer, in some scattered cells in the inner part of the inner nuclear layer (INL), and also in the most central part of the CMZ (Fig. 1G). At stage 42, *Xbh1* expression is almost completely restricted to cells of the central differentiated ganglion cell layer, and to the central CMZ; a few cells in the INL also showed expression (Fig. 1H, and data not shown). At stage 42, double in situ hybridization shows expression of *Xbh1* and *Xath5* in the central most part of the CMZ (Fig. 1H,L). High magnification of these sections shows that, in spite of some superposition, *Xbh1* does not extend as far peripherally as *Xath5* (Fig. 1M-Q). *Xbh1* expression in the CMZ is also more central than that of *XNotch1*, predominantly restricted to proliferating cells (Perron et al., 1998; Ohnuma et al., 2002) (data not shown). Interestingly, a few cells of the ventral-most central retina still express both *Xath5* and *Xbh1* (Fig. 1H,L; arrows). These cells may be in a similar commitment state as those co-expressing the two genes in the CMZ, and may reflect the delay in differentiation in the ventral retina with respect to the dorsal retina (Grant and Rubin, 1980). After stage 42, when differentiation occurs almost exclusively in the CMZ, *Xbh1* is also progressively downregulated in the ganglion cell layer, but persists in the CMZ (data not shown) (Patterson et al., 2000).

Thus, both in the retina and in the CMZ, *Xbh1* expression strictly follows, in time and space, the dynamics of *Xath5* expression.

Xbh1 is expressed in postmitotic retinal precursors in the central CMZ

The expression of *Xbh1* in the retina is reminiscent of the dorsoventral pattern of neurogenesis, but starts at slightly later stages than has been reported for the onset of retinal differentiation (Holt et al., 1988; Stiemke and Hollyfield, 1995). The CMZ recapitulates in cellular and molecular terms the temporal sequence of retinal differentiation, and can be roughly subdivided into three main regions (Perron et al., 1998) (Fig. 2A): the peripheral-most region harbors the youngest proliferating retinal stem cells; the undetermined proliferating retinoblasts are located more towards the center; and postmitotic retinoblasts that are about to undergo the differentiation process are found in the central-most part of the CMZ. Consistently, genes expressed early during neurogenesis are expressed in the peripheral-most region of the CMZ, whereas later genes are expressed more centrally in the CMZ (Perron et al., 1998). We focused on *Xbh1* expression in the CMZ more in detail, and determined its temporal expression with respect to retinal neurogenesis, using *Xath5* and BrdU incorporation as molecular landmarks. Within the CMZ, *Xath5* is expressed in a region containing cells in transition between proliferating and postmitotic retinoblasts (Perron et al., 1998) (see Fig. 2A).

To determine whether cells expressing *Xbh1* in the CMZ are proliferating or postmitotic retinoblasts, we performed BrdU incorporation experiments to compare BrdU-positive proliferating cells with *Xbh1*- and *Xath5*-expressing cells. We therefore injected BrdU into the abdominal region of stage 41 embryos, and then fixed them after 1 hour to process them for BrdU immunodetection and in situ hybridization. Most of *Xbh1*-expressing cells did not overlap with the BrdU-positive ones in the CMZ, suggesting that *Xbh1* is predominantly expressed in postmitotic cells (Fig. 1P,Q). However, at the peripheral boundary of the *Xbh1*-expression domain, some cells were positive for both BrdU and *Xbh1*. We found that among BrdU-positive cells about 12%

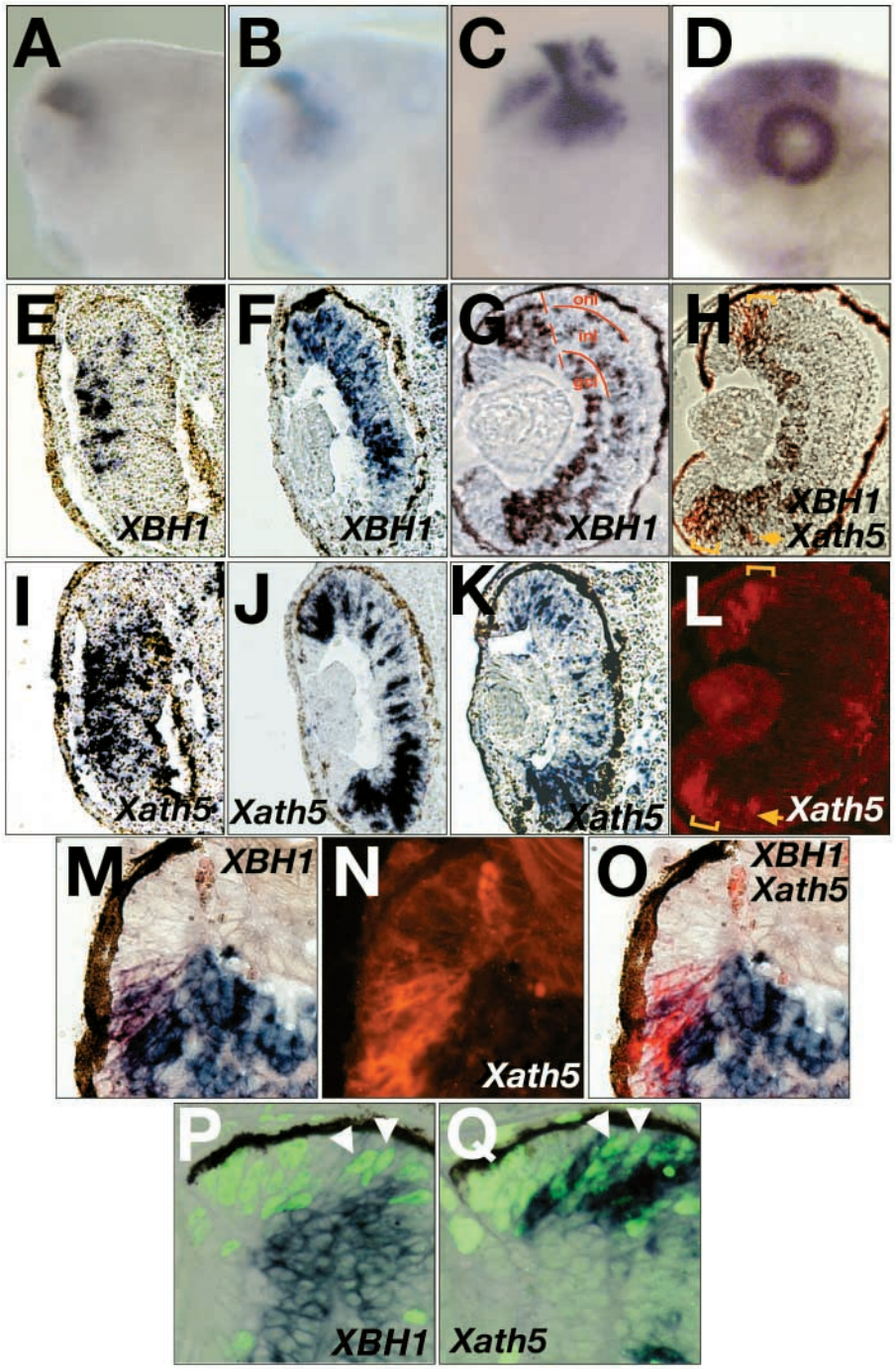

Fig. 1. (A-L) *Xbh1* expression during retinal development. In situ hybridization with either a *Xbh1* (A-H) or a *Xath5* (H-L) probe on *Xenopus* embryos at stage 28 (A,E,I), 33 (B,F,J), 38 (C,G,K) and 42 (D,H,L), respectively. (A-D) Lateral views of embryos after whole-mount in situ hybridization (anterior is on the left). (E-L) In situ hybridization on transversal retinal sections. The red hatch (G) delimitates the ciliary marginal zone (CMZ) and the three main retinal layers (ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer). (H) Cross section of a retina from a stage 42 embryo analyzed by double in situ hybridization, showing *Xbh1* (purple) and *Xath5* (red) expression. (L) The same section as in H showing *Xath5* expression alone. The yellow bracket indicates the region where *Xath5* and *Xbh1* expression overlaps in the CMZ; the yellow arrow indicates co-expression of *Xbh1* and *Xath5* in INL cells. (M-O) High magnification of CMZ following double in situ hybridization shows expression of *Xbh1* (M), *Xath5* (N), or both (O). (P,Q) Combined BrdU staining (green) and in situ hybridization (blue) performed on adjacent sections of a stage 42 retina with a *Xbh1* (P) or a *Xath5* probe (Q). Arrowheads show two identical nuclei stained on adjacent sections.

(109/881) express *Xbh1* mRNA. In adjacent sections from the same retinae, *Xath5*-expressing cells amounted to about 20% of the BrdU-positive cells (42/217), and were more peripheral than those expressing *Xbh1* (Fig. 1P,Q). In summary, *Xbh1* expression in the CMZ follows and partially overlaps that of *Xath5*, it is found in some proliferating retinoblasts, but is predominantly located in postmitotic retinoblasts.

Xath5 is able to regulate *Xbh1*, which in turn regulates *Xbrn3* genes

The fact that *Xath5* expression precedes and later partially overlaps *Xbh1* expression in the CMZ (see also Fig. 2A), suggests a possible regulatory interaction. We used the animal cap assay to investigate whether *Xbh1* can be transcriptionally regulated by *Xath5*. One-cell-stage embryos were injected into the animal pole with 1 ng of *Xath5* RNA. Animal caps were cut at blastula stage, harvested at stage 28, and processed for RT-PCR assays to detect possible activation of *Xbh1*, and of the ganglion cell markers *Xbrn3.0* and *Xbrn3d*, the earliest markers of RGCs, known as *Xath5* downstream genes (Hutcheson and Vetter, 2001; Perron et al., 1998). *Xath5* is able to activate *Xbh1*, *Xbrn3d* and *Xbrn3.0* transcription in injected animal caps, whereas none of these genes was transcribed in control caps (Fig. 2B). We also found that *Xath3* is able to activate *Xbh1*, as well as *Xbrn3.0* and *Xbrn3d* (Fig. 2B). Interestingly, injection of 500 pg of *XneuroD* mRNA, although able to trigger *Xbrn3d* in animal caps (Hutcheson and Vetter, 2001), was not able to activate *Xbh1* expression (Fig. 2B). This suggests that *Xbh1* transcription may be specifically controlled by atonal-like factors, but not by any bHLH factor.

To test whether *Xbh1* could activate *Xbrn3.0* and *Xbrn3d*, we injected 1 ng of RNA encoding *Xbh1* into 1-cell-stage embryos and assayed for the expression of *Xbrn3* genes in stage 28 animal caps. We found that *Xbh1* triggers both *Xbrn3.0* and *Xbrn3d* transcription in animal caps (Fig. 2C). We also tested whether *Xbh1* was able to activate *Xath5* and/or *Xath3* in animal caps. We found that *Xbh1* does not activate *Xath5*, but does activate *Xath3* transcription (Fig. 2C).

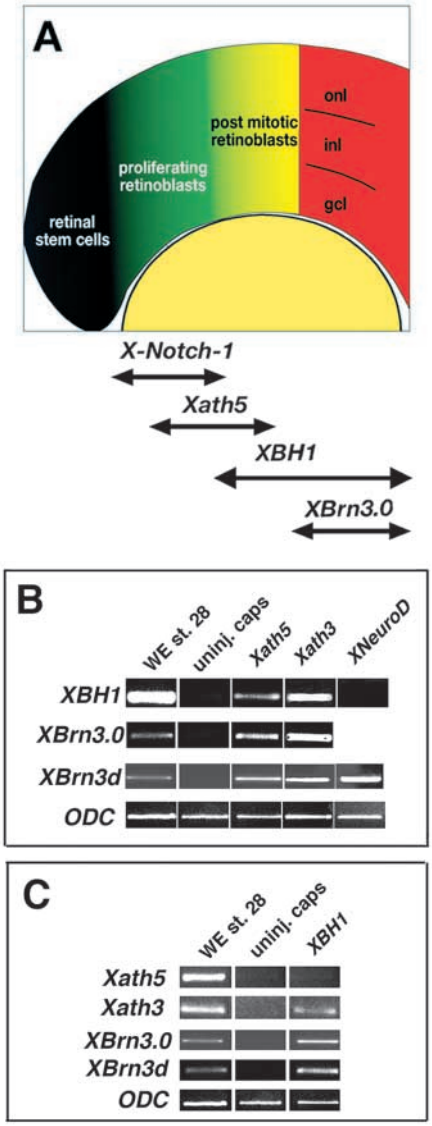

Fig. 2. (A) Representation of the domain of *Xbh1* expression in comparison with those of other genes used as morphogenetic landmarks of different regions of the CMZ and central retina. (B) *Xbh1* is positively regulated by *Xath3* and *Xath5*, but not by *XneuroD*. RT-PCR was performed on animal caps injected with 1 ng of either *Xath3* or *Xath5* mRNA, or with 500 pg of *XneuroD* mRNA. Uninjected animal caps (uninj. caps) and stage 28 whole embryos (WE st. 28) were used as negative and positive controls, respectively; *Xbrn3.0* and *Xbrn3d* were used as positive control markers. (C) *Xbh1* mRNA injection (1 ng) is able to activate Xbrn3 genes and *Xath3*, but not *Xath5*.

Xbh1 promotes ganglion cell fate and represses photoreceptor cell fate in early RPCs

The expression pattern and the results of the animal cap assay suggest that *Xbh1* may be involved in the specification of RGCs. To investigate this, we lipofected a pCS2 DNA construct encoding *Xbh1*, together with a similar construct encoding GFP, into the presumptive eye region of stage 17-18 embryos (Holt et al., 1990). We subsequently analyzed the progeny of early transfected cells in retinae of stage 42 embryos, when most cells in the central retina are postmitotic and fully differentiated (Holt et al., 1988; Stiemke and Hollyfield, 1995). Compared with controls, retinae lipofected with *Xbh1* cDNA exhibited a significant increase in the percentage of RGCs, together with a significant decrease of photoreceptor cells (Fig. 3A-C). No significant variations in the frequency of other cell types were observed instead. Significantly, *Xbh1*-lipofected cells in the RGC layer showed GFP-labeled axons clearly extending into the optic nerve, demonstrating that they are indeed ganglion cells (Fig. 3D).

Xbh1 enhances proneural function in promoting ganglion cell fate

The finding that both atonal genes can activate *Xbh1* transcription in animal cap assays, and that *Xbh1* promotes ganglion cell fate, raised the possibility that co-expression of both *Xath5* (or *Xath3*) and *Xbh1* may lead to a further increase in RGCs. Therefore, we co-lipofected stage 17-18 embryos with *Xbh1* together with either *Xath5* or *Xath3* and analyzed retinas at stage 42. When lipofected alone, *Xath5* strongly increases the proportion of RGCs while decreasing late born bipolar cells (Fig. 4C; Fig. 6A,D; and data not shown) (Kanekar et al., 1997; Moore et al., 2002; Ohnuma et al., 2002). Retinae lipofected with *Xath3* display an increase in the

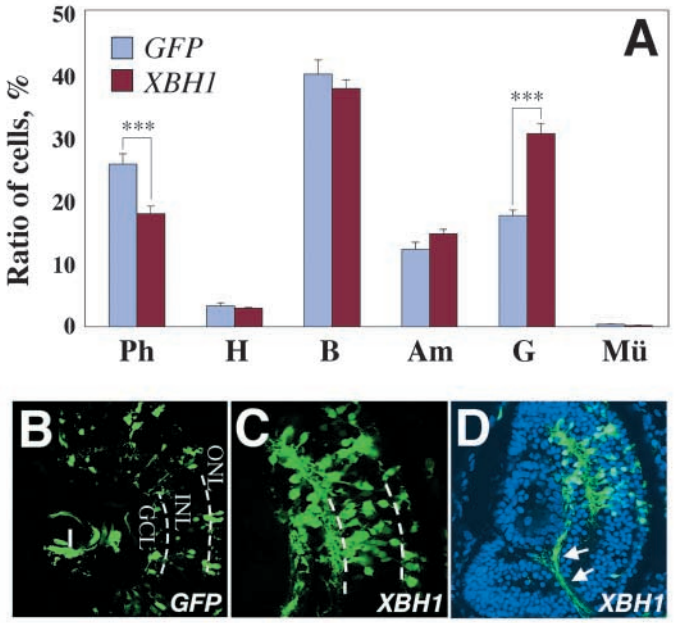

Fig. 3. (A-D) *Xbh1* promotes ganglion cell differentiation in lipofection experiments. Retinal precursors lipofected with *GFP*+vector DNA alone (B), or with *GFP+Xbh1* (C,D). Retinal layers are separated by dashed lines (B,C) and are indicated in B as follows: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; l, lens. (A) Distribution of retinal cell types in lipofected clones. Ph, photoreceptors; H, horizontal; B, bipolars; Am, amacrine; G, ganglion; Mü, Müller. The proportion of each cell type is represented as average±s.e.m. The experiment has been repeated at least three times for both *GFP* and *Xbh1*. In the experiment represented here, *n*=3907 cells from 26 retinae for *GFP*, and *n*=2799 cells from 25 retinae for *Xbh1*. Asterisks represent significant differences between *Xbh1* and *GFP*, as calculated by Student's *t*-test (**P*<0.05, ***P*<0.01, ****P*<0.001). (D) Lower magnification image of section shown in C; GFP-positive axons extend into the optic nerve (arrows).

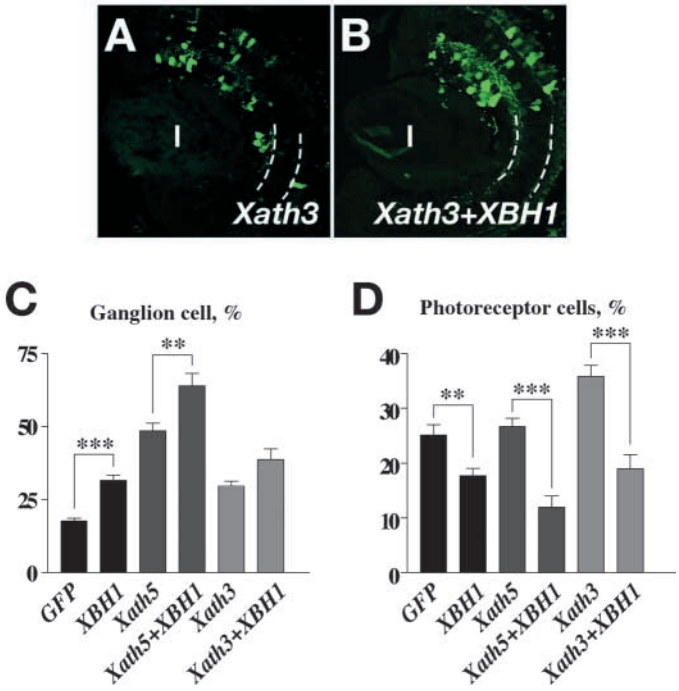

Fig. 4. *Xbh1* enhances the RGC-inducing ability of *atonal*-related genes. (A,B) Retinas lipofected with *Xath3+GFP* or *Xath3+Xbh1+GFP*. (C,D) Percentage of RGCs (C) or photoreceptors (D) in retinae lipofected with *GFP+pCS2* vector, or with *GFP* in combination with *Xbh1*, *Xath5*, *Xath5+Xbh1*, *Xath3*, or *Xath3+Xbh1*. The percent representation of each cell type was calculated as the average±s.e.m. Counted cells were: *n*=2625 cells from six retinae for *GFP*; *n*=2908 cells from six retinae for *Xbh1*; *n*=1087 cells from 10 retinae for *Xath5*; *n*=1375 cells from 11 retinae for *Xath5+Xbh1*; *n*=2018 from seven retinae for *Xath3*; and *n*=1406 from eight retinae for *Xath3+Xbh1*. Significant differences have been determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer Multiple Comparisons Test as a post-test. Black asterisks represent significant differences (***P*<0.01, ****P*<0.001).

number of RGCs, together with an increase in the number of photoreceptors, as previously described (Fig. 4A,C,D) (Perron et al., 1999). Co-lipofection of *Xbh1* with *Xath5* resulted in potentiating the effect of *Xath5* on promoting ganglion cell fate (Fig. 4C and Fig. 6A,B,D). By contrast, *Xbh1* and *Xath3* co-lipofection did not yield significantly different effects on RGCs compared with lipofection of *Xath3* alone (Fig. 4A-C). Besides, co-lipofection of either of the Xath genes with *Xbh1* significantly reduced photoreceptors compared with lipofection of either Xath gene alone (Fig. 4D and Fig. 6D). Finally, *Xbh1* did not have any effect on the *Xath3/Xath5*-induced reduction of bipolar cells (Fig. 6D), or on any other cell types (Fig. 6D, and data not shown).

The repressive *Xbh1* function is required for atonal genes to promote ganglion cell fate and activate the ganglion cell marker *Xbrn3d*

To examine the regulative activity of *Xbh1* responsible for the increase in the number of ganglion cells in lipofected retinae, activation and repressor constructs were generated by fusing the Vp16 activator or the *Drosophila* engrailed repressor (EngR) domain, respectively, downstream of the *Xbh1* homeodomain (Kessler, 1997). The resulting *Xbh1EngR* or *Xbh1Vp16* fusion constructs were injected as mRNA into animal caps, and their activities compared with wild-type *Xbh1* mRNA. Although *Xbh1* and *Xbh1EngR* mRNA were able to elicit *Xbrn3d* transcription, *Xbh1Vp16* failed to do so; rather, *Xbh1VP16* co-injection was able to suppress *Xbrn3d* activation both in *Xbh1*-injected and in *Xbh1EngR*-injected caps (Fig. 5A, and data not shown). These data suggest that *Xbh1* may promote *Xbrn3d* expression by acting as a repressor, and that *Xbh1VP16* may act as a dominant-negative construct on *Xbh1* to suppress its effect. If this were the case, we would expect *Xbh1Vp16* to decrease RGCs, whereas the opposite would be expected for *Xbh1EngR*. We therefore lipofected these DNA chimeric constructs together with *GFP* cDNA into RPCs. As expected, retinae lipofected with *Xbh1EngR* showed a significant increase in the number of RGCs, together with a significant decrease in the number of photoreceptors, similar to lipofections with wild-type *Xbh1* (Fig. 5B). By contrast, retinae lipofected with *Xbh1Vp16* showed a decrease in RGCs and an increase in photoreceptors (Fig. 5B-E; Fig. 6A,C,D). These data suggest that *Xbh1* acts as a transcriptional repressor crucially involved in the specification of ganglion cell fate.

Xbh1 is necessary for *Xath5* induction of RGCs

If the repressive activity of *Xbh1* is necessary for the ability of *Xath5* to induce RGCs, we would expect that *Xbh1Vp16* would antagonize *Xath5* induction of RGCs. To test this hypothesis, we co-lipofected retinae with *Xath5* and *Xbh1Vp16*, and analyzed them at stage 42 compared with *GFP*-lipofected control retinae and *Xath5*-lipofected retinae. In retinae co-lipofected with *Xath5* and *Xbh1Vp16*, the proportion of ganglion cells was strongly diminished compared with in *Xath5*-lipofected retinae, resulting in almost the same proportion of RGCs as in control retinae. In these co-lipofections, only an increase of photoreceptor cells was observed, as seen in single *Xbh1Vp16* lipofections (Fig. 6A,C,D). Consistently, we also observed that bipolar cells, the last-born neurone cell type, are not decreased as efficiently when *Xath5* is co-lipofected with *Xbh1Vp16* (Fig. 6D). Thus, *Xbh1Vp16* suppresses the ability of *Xath5* to promote ganglion cell fate and leads to an increase in photoreceptors. We obtained a similar result when *Xbh1Vp16* and *Xath3* were co-lipofected (data not shown). In summary, these data suggest that functional *Xbh1* is required for *Xath3* and *Xath5* to promote ganglion cell fate in vivo, but that it may inhibit the ability of these genes to promote photoreceptor fates. Furthermore, *Xbh1* may act downstream of the atonal-related bHLH factors.

To further corroborate these data, we tested whether the *Xbh1Vp16* construct was able to suppress *Xath5* induction of *Xbrn3d* in animal cap assays. We confirmed that *Xath5* is able to elicit *Xbrn3d* expression in animal caps (Hutcheson and Vetter, 2001) (Fig. 6E), and that this effect is suppressed by co-injection of *Xbh1Vp16* (Fig. 6E), suggesting that *Xath5* requires *Xbh1* function to activate *Xbrn3d*.

Xbh1 promotes ganglion cell fate in late lipofections

Timing of expression of bHLH factors is important for retinal cell fate specification. For instance, early lipofection of *Xath5* at stage 17-18 promotes ganglion cell fate in *Xenopus*, whereas lipofection at stage 26 promotes bipolar cells and photoreceptors (Kanekar et al., 1997; Moore et al., 2002). This suggests that, at least in some cases, bHLH factors may not have a strong instructive role, but rather that their in vivo action may largely depend on the temporal window of their activity (Moore et al., 2002). We therefore decided to assay whether lipofection of *Xbh1* at stage 25-26 had similar effects as early lipofections. We found that lipofection of *Xbh1* at late stage led to a significant increase in the frequency of RGCs, but had no significant effect on other cell types (Fig. 7A-C).

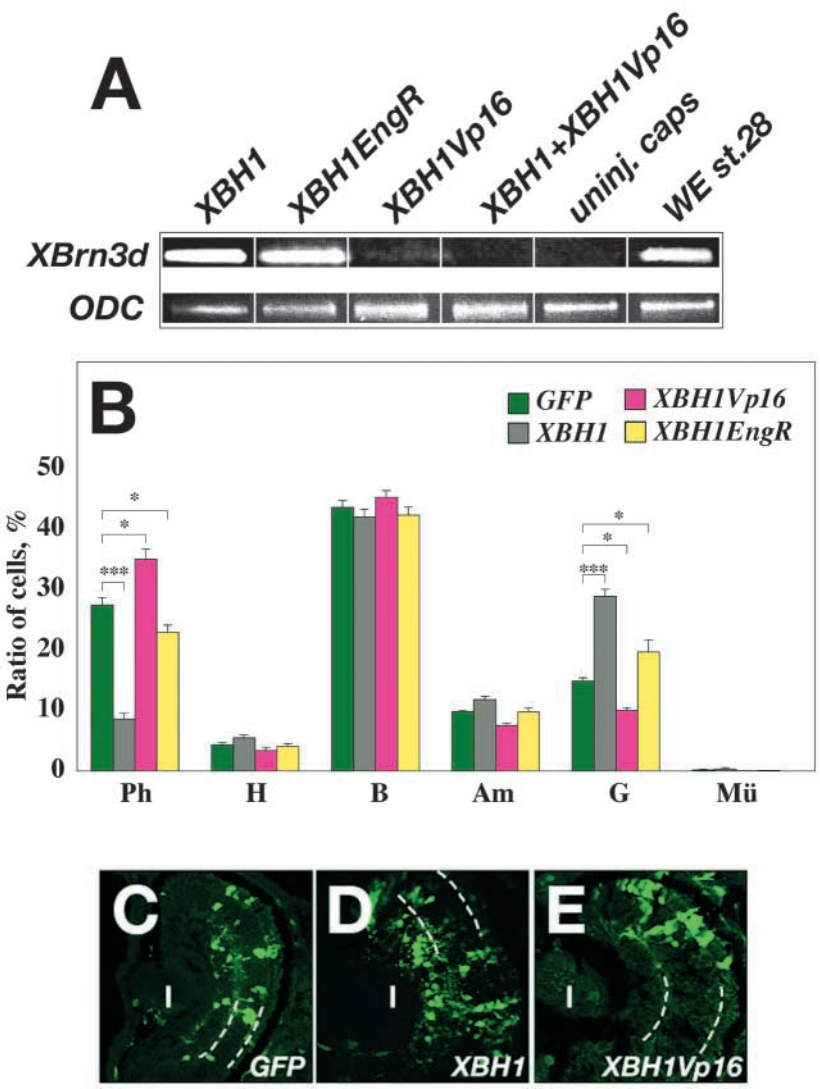

Fig. 5. (A) Comparison of the ability of *Xbh1*, *Xbh1Vp16* and *Xbh1EngR* mRNA to activate the ganglion cell marker *Xbrn3d* in animal caps. RT-PCR analysis on animal caps injected with RNA from different *Xbh1* wild-type and fusion constructs, as indicated. Injected amounts of RNA were 500 pg for each construct, either alone or in combination. (B-E) Lipofection experiments with *Xbh1*, *Xbh1EngR* and *Xbh1Vp16* constructs. Embryos were lipofected with *GFP*+pCS2 vector (C), *GFP*+*Xbh1* (D), *GFP*+*Xbh1Vp16* (E) or *GFP*+*Xbh1EngR* (not shown), and retinae were analyzed at stage 42. The percent representation of each cell type was calculated as a weighted average±s.e.m. Counted cells were: *n*=2625 cells from six retinae for *GFP*; *n*=2908 cells from six retinae for *Xbh1*; *n*=2382 from 15 retinae for *Xbh1EngR*; and *n*=3707 cells from 19 retinae for *Xbh1Vp16*. Asterisks represent significant differences as determined by one-way analysis of variance with the Tukey-Kramer Multiple Comparisons Test as a post-test (**P*<0.05, ****P*<0.001).

Discussion

Cell fate determination in the vertebrate retina results from a complex series of molecular events, whose details are just beginning to be understood. Many of these details have been inferred by studying the spatiotemporal gene-activation pattern in a specialized area of the *Xenopus* retina, the CMZ (Perron et al., 1998). Here, an important role is played by genes related to the proneural *achete-scute* and *atonal* genes of *Drosophila*

(Harris, 1997). These encode transcriptional regulators that modulate a complex genetic cascade that progressively sets up the expression of different combinations of transcription factors in retinal precursors. It is thought that these combinations are relevant to retinal cell fate decisions (Perron et al., 1998). Loss-of-function and overexpression experiments revealed the crucial importance of the atonal-related transcription factors in the determination of ganglion cell fate (Brown et al., 1998; Brown et al., 2001; Kanekar et al., 1997; Ohnuma et al., 2002; Perron et al., 1999; Wang et al., 2001). Consistent with these data, *Ath5* has been shown to regulate the expression of the Brn3 subfamily of POU genes, the earliest known markers of RGC differentiation, which are required for RGC development and survival (Gan et al., 1996; Hutcheson and Vetter, 2001; Liu et al., 2000; Liu et al., 2001; Perron et al., 1998; Xiang et al., 1995). Here, we confirm and extend these observations, showing that *Xath3* is able to upregulate Xbrn3 genes in animal caps, suggesting that both atonal homologues can positively regulate the transcription of Brn3 genes.

Although Ath5 is essential for establishing the competence of retinal precursors to acquire a RGC fate, increasing evidence suggests that other factors may be required to specify RGC fate in addition to Ath5 (Moore et al., 2002; Ohnuma et al., 2002; Yang et al., 2003). In this study, we introduce Xbh1 as a new factor potentially involved in this process. In different vertebrate species, homologues of the *Drosophila* homeobox BarH genes are expressed in ganglion cells during the retinal differentiation process (Patterson et al., 2000; Poggi et al., 2002; Saito et al., 1998), thus suggesting that they may regulate RGC formation.

We observed a tight correlation between the expression of *Xbh1* and that of the proneural gene *Xath5* during retinal neurogenesis. In the CMZ, where retinal neurogenesis is recapitulated, *Xath3* and *Xath5* mark a cell population in transition from proliferating retinoblasts to differentiating retinal neurones (Perron et al., 1998). We show that *Xbh1* expression in the CMZ is more centrally located than the expression of *Xath5* and only partially overlaps with that of *Xath5*, being restricted nearly exclusively to postmitotic cells. Because of the genetic and cellular organization of the CMZ (Perron et al., 1998), a temporal and hierarchical relationship was suggested between *Xath5*, expressed earlier and more peripherally, and *Xbh1*, expressed later and more centrally. We found that both *Xath5* and *Xath3* can indeed positively regulate *Xbh1* transcription in animal cap assays. This may not be a general effect of any bHLH, as *XneuroD* was not able to activate *Xbh1* in the same assay. Moreover, in animal caps, *Xbh1* itself is able to elicit transcription of *Xbrn3* genes.

Our functional studies demonstrate that *Xbh1* regulates RGC formation in the retina. In fact, in vivo targeted overexpression of *Xbh1* in developing RPCs strongly biases this population toward ganglion cell fate. These results are further corroborated by lipofection experiments performed with the *Xbh1Vp16* construct, which represents a dominant-negative form of *Xbh1*, and the oppositely acting *Xbh1EngR*. Early lipofections with the *Xbh1EngR* construct exhibit an increased number of ganglion cells together with a decrease in the number of photoreceptors (similar to the wild-type construct); on the contrary, retinae transfected with the *Xbh1Vp16* construct displayed an increase of photoreceptors together with a decrease of ganglion cells. Consistent with these data are the results obtained in animal caps injected with RNA from the

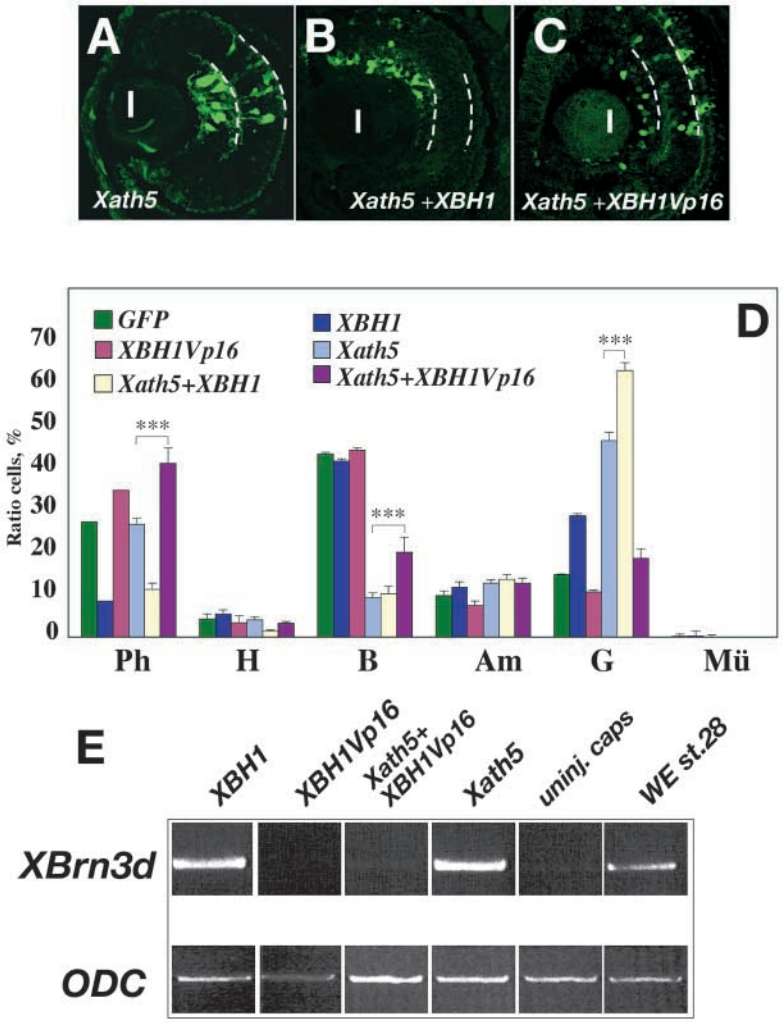

Fig. 6. (A-D) Co-lipofection of *Xath5* with *Xbh1* and *Xbh1Vp16* constructs. *Xath5* was lipofected either with *GFP+pCS2* (A), or with *GFP+Xbh1* (B) or *GFP+Xbh1Vp16* (C), and their effect analyzed on cell-type frequencies in transfected clones (D). Counted cells were: *n*=2625 cells from six retinae for *GFP*; *n*=2908 cells from six retinae for *Xbh1*; *n*=3707 cells from 19 retinae for *Xbh1Vp16*; *n*=885 from eight retina for *Xath5*; *n*=1368 cells from eight retinae for *Xath5+Xbh1*; *n*=1081 from five retinae for *Xath5+Xbh1Vp16*. Error bars represent s.e.m.; significant differences have been calculated by one-way analysis of variance with the Tukey-Kramer Multiple Comparisons Test as a post test (***P<0.001). Symbols are as in Fig. 3. (E) RT-PCR analysis of animal caps injected with different combinations of mRNA, as indicated. Caps injected with either *Xath5* (500 pg) or *Xbh1* (500 pg) show activation of *Xbrn3d*. By contrast, animal caps injected with *Xath5* (500 pg)+*Xbh1Vp16* (500 pg), or with *Xbh1Vp16* (500 pg) alone show no activation of *Xbrn3d* expression.

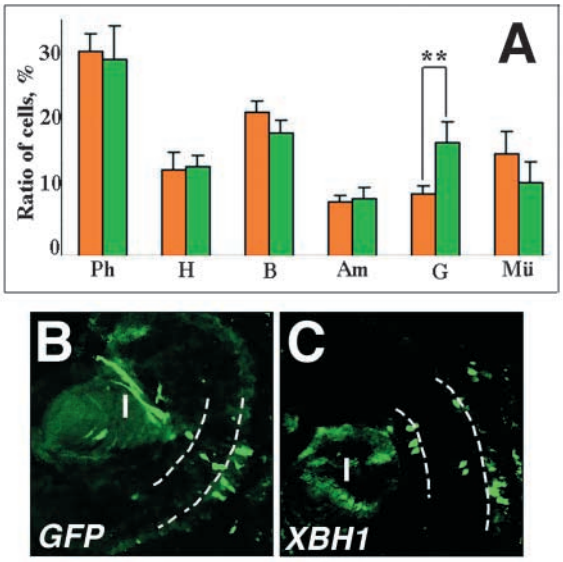

Fig. 7. (A) Lipofection of *Xbh1* at late stages promotes ganglion cell fate. Counted cells were: *n*=401 cells from 23 retinae for *GFP* (orange) (sample section shown in B); *n*=564 cells from 36 retinae for *Xbh1* (green) (C). The percent representation of each cell type was calculated as a weighted average±s.e.m. Asterisks represent significant differences between *Xbh1* and *GFP*, as calculated by Student's *t*-test (***P*<0.01).

same constructs, either singly or in combination: whereas both wild-type *Xbh1* and *Xbh1EngR* trigger *Xbrn3d* expression, a dominant-negative effect is exerted by *Xbh1VP16* on either *Xbh1* or *Xbh1EngR*. These results suggest that *Xbh1* acts as a transcriptional repressor in retinal precursors to regulate a switch towards ganglion cell fate.

Because of the particular combination of transcription factors being expressed in each region of the CMZ, as well as in each layer of the central retina, it has been proposed that retinal cell types are specified by the combinatorial action of several specific genes (Perron et al., 1998). Therefore, the competence for a particular cell fate might result from a balance between several positive and negative influences concomitantly acting at a given time on one cell. Interestingly, recent studies suggest that *Xath5* ability to promote RGC determination may be modulated by the presence of agonistic and antagonistic factors present in retinal precursors at different times of retinal neurogenesis (Moore et al., 2002; Ohnuma et al., 2002). Particularly, recent evidence shows that *Xath5* alone is not sufficient to promote RGC specification when misexpressed in later RPCs. In this respect, Xbh1 might constitute a factor cooperating with Xath5 to bias late RPCs towards a ganglion cell fate. Indeed, we show that *Xbh1* strongly enhances the RGC promoting activity of *Xath5*, but not of *Xath3*. Conversely, the *Xbh1Vp16* activator construct inhibits the ability of both *Xath5* and *Xath3* to bias RPCs towards a ganglion cell fate. Taken together, these results suggest that *Xbh1* may specifically potentiate *Xath5*, rather than *Xath3*, action to enhance ganglion cell fate, but, nonetheless, *Xbh1* also seems to be required for *Xath3* ganglion cell-promoting activity. Consistent with the lipofection results is the observation that *Xbh1Vp16* mRNA is able to block the activation of *Xbrn3d* by *Xath5* in animal caps. Altogether, these data suggest that repression of *Xbh1* target genes is required to promote RGC fate, and for the activation of *Xbrn3* genes by *Xath5*, and indicate a genetic hierarchy regulating RGCs formation, with *Xath5* being epistatic to *Xbh1*, which in turn is epistatic to *Xbrn3* factors. Finally, the observation that the effect of *Xbh1Vp16* in suppressing RGCs seems much weaker on its own than when co-lipofected with *Xath5* may suggest that some RGCs are specified in a *Xath5/Xbh1*-independent pathway; indeed, small populations of *Ath5*-independent RGCs are present in mouse and zebrafish (Brown et al., 1998; Wang et al., 2001).

Our study demonstrates important differences between *Xath5* and *Xbh1*. Whereas *Xath5* is expressed in early retinal precursors, with wider developmental potential, *Xbh1* expression is found in later precursors, whose competence is presumably more restricted (see also Harris, 1997; Livesey and Cepko, 2001). Consistently, the action of *Xbh1* is different from that of *Xath5*. In fact, *Xath5* has a broader range of effects: whereas it promotes ganglion cell fate in early RPCs, it promotes late retinal fates at later stages (Kanekar et al., 1997; Moore et al., 2002). In this sense, *Xath5* may not instruct ganglion cell fate per se, but only permit precursors to exit the cell cycle; the actual retinal cell fate would be dictated by the repertoire of other factors co-expressed in the cell (Moore et al., 2002). Consistent with this is the recent demonstration that *Math5*-expressing cells give rise to multiple retinal cell types, and not only to RGCs, in the mouse retina (Yang et al., 2003). Similar to *Xath5*, *Xbh1* promotes ganglion cell fate in early RPCs. In contrast to *Xath5*, *Xbh1* increases RGCs also at later stages, without significant variation in the other cell types. However, it may be interesting to note that if bipolar and Müller cells are ranked together, as a unique population of late born cells, their diminution becomes statistically significant (*P*=0.026), and appears to compensate for the increase in RGCs (about 9% of the total GFP-positive cells). Thus, in late lipofections, *Xbh1* favours RGCs at the expense of late retinal cell fates. If the increase of RGCs was due to a later effect on differentiation/maintenance (e.g. a selective cell death protective effect on RGCs compared with other cell types), then the observed decrease of late cell types only would be less likely. In conclusion, although we cannot exclude a later role for *Xbh1*, our lipofection data suggest that *Xbh1* regulates cell fate by restricting the state of competence in retinal precursors or by providing them with more instructive cues that commit them to a RGC fate.

Although *Xbh1* has a definite RGC-promoting activity and is regulated by *Xath5*, it may not mediate all of the abilities of *Xath5*. For example, *Xath5* has a bipolar and photoreceptor-promoting activity in later RPCs (Kanekar et al., 1997; Moore et al., 2002), which is not shared by *Xbh1*. These different activities may in part depend upon gene sets that *Xath5*, but not *Xbh1*, is able to regulate; in addition, they may in part depend on competence changes in RPCs during retinogenesis. In molecular terms, the competence could be thought of as the complement of factors that cooperate with Xath5 to refine its action. Our data suggest that Xbh1 is one such factor, acting for a more specific cell commitment to RPCs, once they have been specified to become neurones by the proneural genes. Both the results of combined *Xath5* and *Xbh1* lipofections, in which more than 60% of early RPCs are driven to a RGC fate, and of the late lipofections, are completely consistent with this.

The later activities of Xath5, and of other bHLH factors involved in cell fate specification within the retina, may instead involve other cooperative factors, such as Chx10 or Xotx2, which were shown to favour bipolar cell fate (Hatakeyama et al., 2001; Viczian et al., 2003), or Crx and Xotx5b, which are involved in photoreceptor specification and differentiation (Furukawa et al., 1997; Furukawa et al., 1999; Viczian et al., 2003).

Interestingly, a switch role played by *BarH1/BarH2* in the choice between different types of cells has been described in the external sensory organs and photoreceptors in *Drosophila*. When both Bar genes are deleted in the *Drosophila* notum, papillae are transformed into hairs, whereas the ubiquitous expression of one of them turns hairs into papillae. Analogously, overexpression of *BarH1* in the eye region transforms cones into R1/R6 photoreceptors, whereas deletion of *BarH1/BarH2* function in the eye transforms R1/R6 photoreceptors into cone cells (Higashijima et al., 1992a; Higashijima et al., 1992b; Sato et al., 1999).

In spite of some similarities in their activity as a switch for cell fate determination, the exact correspondence between the role of BarH genes in the eye of *Drosophila* and of vertebrates is not clear, as are the possible homologies of morphological components of the eye in different organisms. Recent data in *Platynereis dumerilii* have suggested that rhabdomeric photoreceptors of ancestral invertebrates may correspond to vertebrate RGCs, with which they share a common origin from *atonal*-expressing precursors and expression of orthologous *r-opsin* and *six1/2* genes (Arendt et al., 2002; Arendt and Wittbrodt, 2001). A possible interpretation of this view could be that all photoreceptors of *Drosophila*, being rhabdomeric, correspond to vertebrate ganglion cells. A different view suggests that only R8 photoreceptors of *Drosophila* are homologous to vertebrate RGCs, as only specification of R8 directly requires the *atonal* gene (Frankfort and Mardon, 2002). In this context, data on *Drosophila BarH1* may not resolve this issue, as this gene is only expressed in R1/R6, and not in other photoreceptors including the *atonal*-positive R8. However, it is possible that *Drosophila* represents a rather divergent model, and that study of *BarH1* homologues in more primitive and generalized systems will help in reconstructing the possible descendance of vertebrate ganglion cells during evolution.

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