XOtx5b and *XOtx2* regulate photoreceptor and bipolar fates in the *Xenopus* retina

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SUMMARY

Photoreceptor and bipolar cells are molecularly related cell types in the vertebrate retina. *XOtx5b* is expressed in both photoreceptors and bipolars, while a closely related member of the same family of transcription factors, *XOtx2*, is expressed in bipolar cells only. Lipofection of retinal precursors with *XOtx5b* biases them toward photoreceptor fates whereas a similar experiment with *XOtx2* promotes bipolar cell fates. Domain swap experiments show that the ability to specify different cell fates is largely contained in the divergent sequence C-terminal to the homeodomain, while the more homologous N-terminal and homeodomain regions of both genes, when fused to VP16 activators, promote only photoreceptor fates. *XOtx5b* is closely related to *Crx* and like *Crx* it drives expression from an opsin reporter in vivo. *XOtx2* suppresses this *XOtx5b*-driven reporter activity providing a possible explanation for why bipolars do not express opsin. Similarly, co-lipofection of *XOtx2* with *XOtx5b* overrides the latter's ability to promote photoreceptor fates and the combination drives bipolar fates. The results suggest that the shared and divergent parts of these homologous genes may be involved in specifying the shared and distinct characters of related cell types in the vertebrate retina.

Key words: Retinal specification, Cell fate, otd, Otx, XOtx2, XOtx5, XOtx5b, Bipolar cell, Photoreceptor, VP16, Engrailed, *Xenopus laevis*, Opsin, Lipofection

INTRODUCTION

Rod photoreceptors and bipolars are among the last cells to be born and differentiate from the late-progenitor pool (Carter-Dawson and LaVail, 1979; Chang and Harris, 1998; Holt et al., 1988; Stiemke and Hollyfield, 1995; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988; Young, 1985). As retinal development proceeds fate choices become restricted, such that late progenitors usually differentiate into rods, bipolar cells, Müller glia and certain types of amacrine cells (Belliveau and Cepko, 1999; Belliveau et al., 2000; Fields-Berry et al., 1992; Turner and Cepko, 1987; Turner et al., 1990). Retinal progenitors express many genes that affect cell fate including *Notch*, *Hes1*, *Pax6*, *Rax*, *Optx2*, *Chx10*, *p27Xic1* and *NeuroD* (reviewed by Livesey and Cepko, 2001) (Livesey and Cepko, 2001; Perron et al., 1998; Zuber et al., 1999). After differentiating, photoreceptors and bipolars both stop expressing many of these genes yet, in *Xenopus laevis*, both cell types continue to express *XRx1* and *NeuroD* (Perron et al., 1998). In mice, blue cones and bipolars both express blue opsin transgenes, indicating that these cell types share common gene regulatory mechanisms (Chen et al., 1994; Chiu and Nathans, 1994).

The link between photoreceptors and bipolars is further demonstrated by fate switching experiments. A late precursor may differentiate either as a photoreceptor or a bipolar depending on environmental factors. For example, exposing rat retinal cell cultures to the cytokine, ciliary neurotrophic factor (CNTF), causes a dramatic decrease in the number of opsin-positive cells and a compensatory increase in the number of cells expressing bipolar cell-specific markers, suggesting a change in cell fate choice from rods to bipolars (Ezzeddine et al., 1997). Similarly, when rat retinal cells are cultured at low density there is a decrease in the number of opsin-positive cells and an increase in the number of cells expressing the bipolar cell marker, 115A10 (Altshuler and Cepko, 1992). Co-culturing late with early progenitors leads to a similar shift in cell fate, which can be blocked by the CNTF antagonist, hLIF-05 (Belliveau et al., 2000). The sensitivity of late retinal precursors to extrinsic factors such as CNTF demonstrates that external cues influence the internal switches that differentially specify the fate of these two cell types.

We have found that *Xenopus* photoreceptors and bipolars also express, *XOtx5b*, which is a member of the *otd*/*Otx* family of paired-like homeodomain transcription factors (Vignali et al., 2000). *otd* (also known as *oc*; *ocelliless*) is the only *otd*/*Otx* family member identified in *Drosophila* and it contributes to head formation (Finkelstein et al., 1990). The vertebrate homologues of *otd* include *Otx1*, *Otx2*, *Otx3*, *Otx4*, *Otx5*, *Otx5b* (96% similar to Otx5) and *Crx*, which are all involved

in anterior embryo and sensory organ formation (Acampora et al., 1998a; Acampora et al., 1998b; Acampora et al., 1996; Acampora and Simeone, 1999; Andreazzoli et al., 1997; Bovolenta et al., 1997; Furukawa et al., 1997; Gammill and Sive, 1997; Kablar et al., 1996; Kuroda et al., 2000; Martinez-Morales et al., 2001; Pannese et al., 1995; Sauka-Spengler et al., 2001; Suda et al., 1999; Vignali et al., 2000). The first vertebrate homologues of *otd*, *Otx1* and *Otx2*, were characterised in mouse (Simeone et al., 1992). *Otx2*$^{-/-}$ mice lack fore- and midbrain structures, while a null mutation in *Otx1* is less severe, causing, among other defects, a reduction in brain size and lack of ciliary process next to an otherwise normal retina (Acampora et al., 1998a; Acampora et al., 1996; Acampora and Simeone, 1999; Martinez-Morales et al., 2001). Overexpression of *Xenopus Otx2* (*XOtx2*) suggests that it plays a similar role in frog (Andreazzoli et al., 1997; Blitz and Cho, 1995; Kablar et al., 1996; Pannese et al., 1995). *XOtx5b* is also expressed during early embryogenesis. Overexpression of *XOtx5b*, like *XOtx2*, produces ectopic cement gland and neural tissue as well as gross embryonic abnormalities in which posterior structures are absent or reduced and the neural tube fails to close (Kablar et al., 1996; Pannese et al., 1995; Vignali et al., 2000). Because of their roles in early embryonic development, the functions of *Xenopus XOtx5b* and *XOtx2* in retinal differentiation have not been previously determined.

Crx was isolated in a yeast one-hybrid screen using the rhodopsin promoter as bait (Chen et al., 1997; Furukawa et al., 1997). Crx can bind the rhodopsin promoter and transactivate its expression, along with a number of other photoreceptor-specific genes (Chen et al., 1997; Livesey et al., 2000). It is expressed in pinealocytes as well as rod and cone photoreceptor cells. The orthological relationships of the mammalian *Crx* genes with other gnathostome *otd*-related genes remain controversial. Phylogenetic analyses of the *otd*/*Otx* family members, however, support a relationship between the *Otx5/5b* genes characterised in amphibians or chondrichthyans, and the *Crx* genes isolated in mammals and in zebrafish (Germot et al., 2001; Sauka-Spengler et al., 2001). These results have led to the hypothesis that *Otx5/5b* and *Crx* genes might belong to a single orthology class. The identification in the zebrafish of a second, unambiguously *Otx5*-related, gene has challenged this view, suggesting that two distinct orthology classes may be present in gnathostomes (Gamse et al., 2001). However, novel phylogenetic analyses including a much wider range of amniote *Otx5* or *Crx*-related sequences as well as pufferfish sequences, indicate that the zebrafish *Otx5* and *Crx* genes have arisen through a gene duplication, which occurred in the actinopterygian lineage, and support the hypothesis of a single gnathostome *Otx5*/*Crx* class, with an acceleration of evolutionary rate of what became *Crx* early in mammalian evolution (S. Mazan, personal communication). This raises the question of whether *XOtx5b* in lower vertebrates performs the same function as *Crx* in mammals.

Crx, *Otx1*, *Otx2* and *Otx5b* are all expressed in the developing retina prior to retinal differentiation. Some *otd*/*Otx* family members are expressed in both photoreceptors and bipolar cells while others are expressed in one cell type or the other. Zebrafish *Crx* (Liu et al., 2001), mouse *Otx2* (Baas et al., 2000; Bovolenta et al., 1997) and *Xenopus Otx5b* (see below), for example, are expressed by both bipolars and photoreceptors, while mouse *Crx* is expressed only in photoreceptors cells (Chen et al., 1997; Furukawa et al., 1997) and *Xenopus Otx2* is found in bipolar cells but not photoreceptors (Kablar et al., 1996; Perron et al., 1998). We were therefore interested to know what roles *XOtx5b* and *XOtx2* play in retinal development, and specifically whether they contribute to the switch between these two cell fates. In this study, we examined the expression of *XOtx5b* and *XOtx2* during development and determined the effect of each on retinal cell differentiation. We found that both *XOtx5b* and *XOtx2* are involved in retinal cell fate specification. Next, we identified the domains of these proteins that were important in determining photoreceptor verses bipolar cell fates. Finally, we used the *Xenopus opsin* promoter to examine the relationship between *XOtx5b* and *XOtx2* in regulating photoreceptor cell specificity.

MATERIALS AND METHODS

Xenopus embryos
All embryos were the products of in vitro fertilizations of wild-type adult *Xenopus laevis* (from Nasco) maintained in the Department of Anatomy at Cambridge University.

In vivo lipofection
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 prior to use. 1 μl of each construct was mixed with 1 μl of pCS2$^+$ green fluorescent protein (GFP) DNA, to label transfected cells. GFP with pCS2$^+$ vector alone was the control. 9 μl of DoTAP (Roche) was added to 3 μg of DNA and injected into stage 17-18 embryos. At stage 41, embryos were fixed for 1 hour at room temperature, sunk in 2% sucrose overnight at 4°C and cryostat sectioned (10 μm). The samples were then rehydrated with two washes of 1× PBS for 5 minutes, mounted in FluorSave (CalBioChem) containing 2% DABCO (Sigma) and dried overnight at room temperature.

Immunocytochemistry
Cryostat sections (10 μm) were washed three times for 5 minutes in 1× PBS + 0.1% Triton X-100 (PBST), blocked 30 minutes with PBST + 5% heat-inactivated goat serum then incubated overnight at room temperature with primary antibody: XAP-1 (DSHB) at 1:1, bovine anti-rhodopsin at 1:500 (R2-12N; from Paul Hargrave) and anti-calbindin (Oncogene) 1:500. Sections were washed in PBST three times for 5 minutes then three times for 20 minutes and incubated with a 1:500 dilution of the appropriate Cy3-conjugated secondary antibody for 2 hours. The sections were washed again in PBST and mounted.

DNA construct generation
The construction of *pCS2.XOtx5b* has been described previously (Vignali et al., 2000). pCS2.XOtx2 was generated by PCR cloning of the *XOtx2* coding region into the *Eco*RI site of pCS2 with 5′XOtx2 and 3′XOtx2 (Table 1). *XOtx5bN+HD-EngR* was obtained by cloning the *Eco*RI/*Taq*I fragment of *XOtx5b* (amino acids (aa) 1-107) into the *Eco*RI site of pCS2EngN. *XOtx2N+HD-VP16* was obtained by PCR cloning the XOtx2 region spanning aa residues 1-109 into the *Cla*I site of pCS2VP16-N with 5′ClaX2 and 3′ClaX2. *XOtx5N+HD-VP16* was obtained by PCR cloning the XOtx5b region spanning aa residues 1-107 into the *Cla*I site of pCS2VP16-N using 5′Cla and 3′Cla.

Swapped domain constructs (*XOtx2/5b* and *XOtx5b/2+3′UTR*) were generated as follows. The *Bgl*II/*Spe*I fragment of T7TSXotx2 (Pannese et al., 1995) spanning the coding region plus 3′ untranslated

Table 1. Primers used to generate expression constructs

Primer name	Sequence
5′XOtx2	GG**GAATTC**GATCAAACACGAGCATGATGT
3′XOtx2	GG**GAATTC**TTCACAAAACCTGGAATTTC
5′ClaX2	GG**ATCGAT**GATCAAACACGAGCATGATGTC
3′ClaX2	GG**ATCGAT**TCTCACTTTGTTTTGGCCTCC
5′Cla	GG**ATCGAT**GATACTTGGTCACTGAAATGAT
3′Cla	CC**ATCGAT**TCGAGGCTTAGCTTGTCCAG

Bold letters indicate restriction enzyme sites used for cloning.

region (3′UTR) was cloned into the *Bam*HI/*Xba*I site of pCS2⁺. By site-directed mutagenesis, an *Asp*718 restriction site was generated into this clone and *XOtx5b* at the equivalent aa residue 61 codon, causing the CGT sequence to change to CGG. This site allowed precise swapping of the coding region C-terminal to aa 61. Because the homeodomains (HD) are identical in the region between aa 61 and the polyQ stretch at the HD end, swapping is only effective beyond aa 99 of both sequences (aa 99 to end). The 3′UTR of XOtx5b/2+3′UTR was removed by PCR cloning the coding region of XOtx5b/2+3′UTR into the *Cla*I/*Eco*RI site of pCS2⁺ using 5′Cla and 3′XOtx2, to generate *XOtx5b/2*.

In situ hybridisation

Whole-mount in situ hybridisations were performed on bleached embryos (Broadbent and Read, 1999). Dioxigenin-labelled antisense RNA probes were generated from the full-length *XOtx2* coding sequence and the 3′ untranslated region of *XOtx5b* [clone 13F8 (Gawantka et al., 1998)] as described previously (Shimamura et al., 1994). In situ hybridisation on sections was done using the same protocol 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. Sections were treated with 20 µg/ml proteinase K for 30 seconds with subsequent wash times reduced by half.

When in situ hybridisation was followed by immunohistochemistry, we performed the in situ hybridisation protocol described previously (Myat et al., 1996). After the coloration with NCT and BCIP, we washed with PBST for 5 minutes and then followed the immunohistochemistry protocol as described above.

BrdU experiments

To determine if *XOtx5b* or *XOtx2* transcripts were expressed in dividing cells, stage 41 embryos were injected with BrdU (5-bromo-2′-deoxyuridine; Labelling and Detection Kit I, Roche) in the gut, fixed 30 minutes later and cryostat sectioned. In situ hybridisation was performed on sections as follows: DIG-labelled probes (2 ng/ml in hybridisation buffer) (Shimamura et al., 1994), were heated to 70°C for 10 minutes then incubated on sections overnight at 60°C. The rest of the protocol was as previously described (Myat et al., 1996). Following NBT/BCIP staining, sections were stained for BrdU using the protocol below.

To determine if the *XOtx5b* or *XOtx2* constructs effect proliferation, lipofected embryos were injected with BrdU in the gut at stage 31. Embryos were fixed and cryostat sectioned (10 µm) at stage 41. The sections were stained with both BrdU and GFP antibodies. To do this, the cryostat sections were washed with 2 N HCl for 45 minutes then neutralised with several PBST washes. The antibodies for anti-mouse BrdU and anti-rabbit GFP (Molecular Probes, Eugene, OR) were added at dilutions of 1:10 and 1:500, respectively, and incubated at 37°C for 30 minutes. After washing with three changes of PBST, secondary antibodies [Alexa 488-goat anti-rabbit (Molecular Probes) and Cy3-goat anti-mouse (Chemicon)] were added together, both at a dilution of 1:500, and incubated for 30 minutes at 37°C. The sample were again washed three times with PBST and then stained with 15 µg/µl Hoechst solution for 3 minutes at room temperature to visualise

nuclei. After a final three washes in PBST the sections were mounted in FluorSave (Calbiochem) containing 2% DABCO (Sigma Genosys).

TUNEL staining

Embryos were lipofected with *GFP* and pCS2, *XOtx2* or *XOtx5b*-VP16, grown to stage 31, 33/34 or 37, fixed and cryostat sectioned. We used Intergen's ApopTag Kit to stain apoptotic cell nuclei and counterstained with Hoechst (15 mg/ml). Retinas with at least one lipofected, apoptotic cell were counted. Five retinas were counted for each construct at each stage. The percentage of apoptotic lipofected cells versus all lipofected cells was determined.

RESULTS

XOtx2 and *XOtx5b* expression in the developing and mature eye

XOtx5b and *XOtx2* are expressed during and after retinal differentiation (Pannese et al., 1995; Sauka-Spengler et al., 2001; Vignali et al., 2000). In situ hybridisation on whole-mount embryos is shown in Fig. 1. In stage 20 embryos, *XOtx2* expression is detected in the two eye primordia and forebrain, while *XOtx5b* expression is only observed in the epiphysis and cement gland (Fig. 1A,D). By stage 25, *XOtx2* expression remains largely unchanged, while *XOtx5b* is no longer expressed strongly in the cement gland but remains in the presumptive pineal (Fig. 1B,E). By stage 31, when cellular differentiation is underway in the eye, the expression patterns of *XOtx2* and *XOtx5b* overlap in the retina (Fig. 1C,F).

In order to compare their relative expression patterns at a more cellular level in the retina, we performed in situ hybridisation on retinal sections. At stage 25, *XOtx2* is found throughout the presumptive retinal pigment epithelium (RPE) and retina, while only a few cells in the central retina express *XOtx5b* (Fig. 2A,F). A few hours later, at stage 28, *XOtx2* expression has narrowed to the central retina and RPE, while *XOtx5b* expression has expanded (Fig. 2B,G). By stage 33, *XOtx2* and *XOtx5b* expression patterns are indistinguishable; transcripts are found throughout the developing retina except in the most peripheral regions, corresponding to the ciliary marginal zone (CMZ; Fig. 2C,H). At stage 37, *XOtx2* expression is detected in all layers of the peripheral retina, but is becoming restricted in the central retina to the outer edge of the inner nuclear layer or INL (Fig. 2D). Similarly, *XOtx5b* expression, in the central retina is narrowed to just the outer (ONL) and inner nuclear layers (Fig. 2I). In the mature, stage 41 retina, *XOtx2* is only found in the outer edge of the INL and inner edge of the CMZ (Fig. 2E). *XOtx5b* is expressed in the inner edge of the CMZ, the outer edge of the INL and the ONL (Fig. 2J).

It is difficult to identify by in situ hybridisation alone which INL retinal cell types express *XOtx2* and/or *XOtx5b*. In mouse, bipolar-specific markers helped show that *Otx2* is expressed in bipolar cells (Baas et al., 2000). Unfortunately, no bipolar cell-specific antibody is available for *Xenopus* and our attempts at staining *Xenopus* retinas with mouse protein kinase C (Greferath et al., 1990) and Chx10 (Burmeister et al., 1996) antibodies were unsuccessful. The most salient distinguishing feature of a bipolar cell, however, is its characteristic bipolar morphology with terminal arbours in the two plexiform layers. Therefore, we lipofected cDNA for GFP into *Xenopus* retinas, followed by immunohistochemistry to GFP, to reveal the
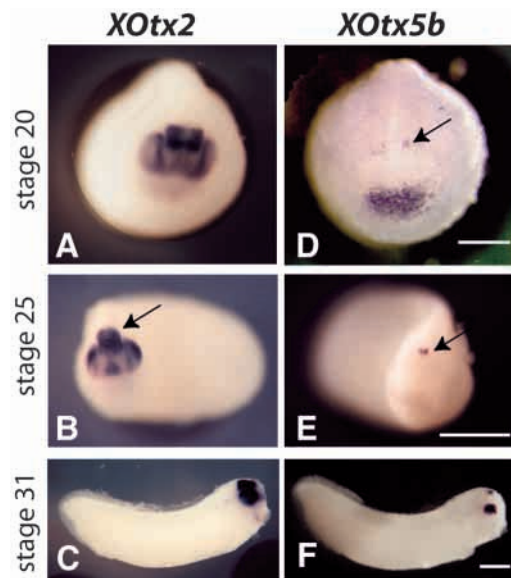
Fig. 1. Localization of *XOtx2* and *XOtx5b* in the developing *Xenopus* embryo. Whole-mount in situ hybridisation of embryos at stage 20 (A,D), stage 25 (B,E) and stage 31 (C,F) were probed with full-length cDNA of *XOtx2* (A-C) and the 3′ untranslated region of *XOtx5b* (D-F). (A) Before eye vesicle formation but after evagination, *XOtx2* is expressed in both presumptive eye and anterior neural tube. (D) At this same stage, *XOtx5b* is found in the epiphysis (arrow) and cement gland but is distinctly absent from the eye field. (B) *XOtx2* expression continues in the presumptive eye and forebrain (arrow). (E) Expression of *XOtx5b* just begins in a few cells of the eye and remains in the presumptive pineal (arrow) while cement gland expression recedes. (C) In a subsequent stage, *XOtx2* expression becomes more pronounced in both the eye and the forebrain. (F) *XOtx5b* is found throughout the eye and in the singular pineal. Bars indicate 500 μm.

co-labelled with *XOtx5b*, while 82%±5% were *XOtx2* positive (Table 2). Double in situ hybridisation with *XOtx5b* and *XOtx2* revealed that all the INL cells that express *XOtx5b* also express *XOtx2* (3G-I), indicating that 55%±8% of bipolar cells co-express *XOtx5b* and *XOtx2* (Table 2).

We also wanted to know which photoreceptors express *XOtx5b*. Using cone- or rod-specific antibodies on *XOtx5b*-stained in situ hybridisation sections, we found *XOtx5b* was expressed in all cone and rod photoreceptors (Fig. 3J-O). Thus, *XOtx5b* is preferentially expressed in photoreceptors and a subset of bipolar cells, while *XOtx2* is preferentially expressed in bipolar cells.
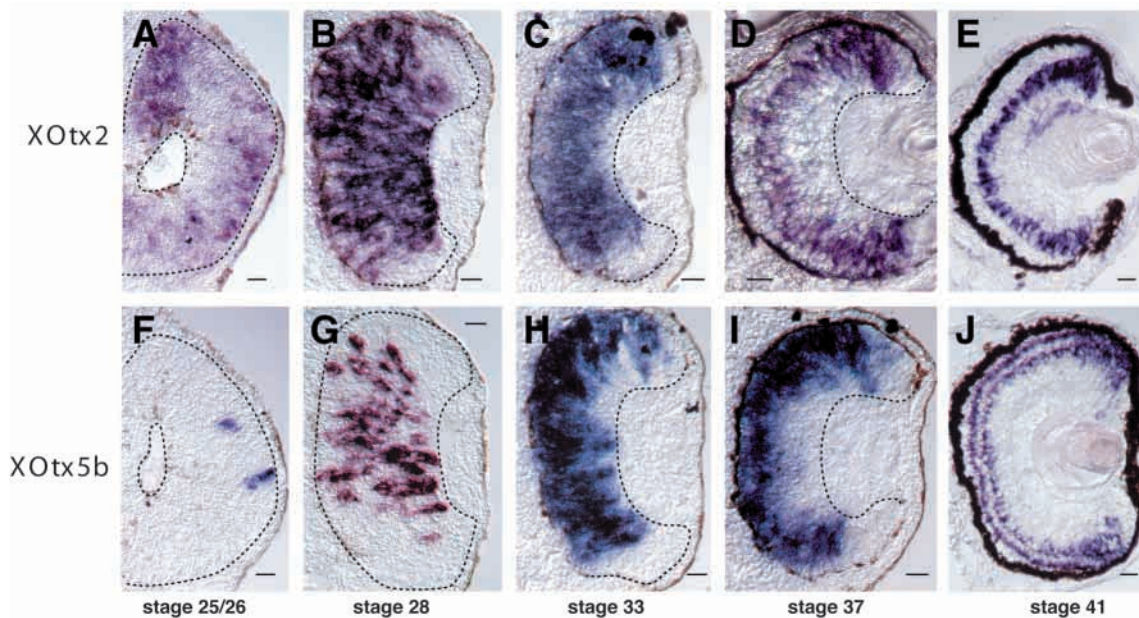
morphology of a random selection of cells. We then performed in situ hybridisations on these lipofected retinas using *XOtx2* or *XOtx5b* probes to identify the cell types expressing these *otd/Otx* family members (Fig. 3). We found that messenger RNA from both of these genes is expressed in bipolar cells (Fig. 3A-F). Of the GFP-labelled bipolar cells 55%±8% were

Fig. 2. Expression of *XOtx5b* and *XOtx2* in retinal sections. Section in situ hybridisation of *XOtx2* (A-E) and *XOtx5b* (F-J) in the *Xenopus* retina at stages 25/26 (A,F), 28 (B,G), 33 (C,H), 37 (D,I) and 41 (E,J). (A,F) *XOtx2* (A) is expressed throughout the presumptive retinal pigment epithelium and retina while *XOtx5b* (F) expression is only in the central retina. (B,G) As retinal invagination progresses, expression of both *XOtx2* (B) and *XOtx5b* (G) appear in the central retina. (C,H) Expression of *XOtx2* (C) and *XOtx5b* (H) is identical. Transcripts from either of these genes are found throughout the developing retina except in the peripheral dorsal and ventral regions. (D,I) The central retinal expression of these two transcripts is reduced to particular layers. *XOtx2* (D) is expressed in the outer edge of the inner nuclear layer (INL). *XOtx5b* (I) is also found in the outer edge of the INL and also in the outer nuclear layer (ONL). The peripheral retina still expresses these transcripts in the ganglion cell layer. (E,J) When the retina is mature, *XOtx5b* (J) is found in both the photoreceptor layer and the outer cells of the INL. Like *XOtx2* (E), it is also found in the edge of the CMZ. *XOtx2* is also expressed in cells at the outer edge of the INL. Bars: 20 μm; dashed lines indicate the extent of developing neural retina in A-D,F-I.
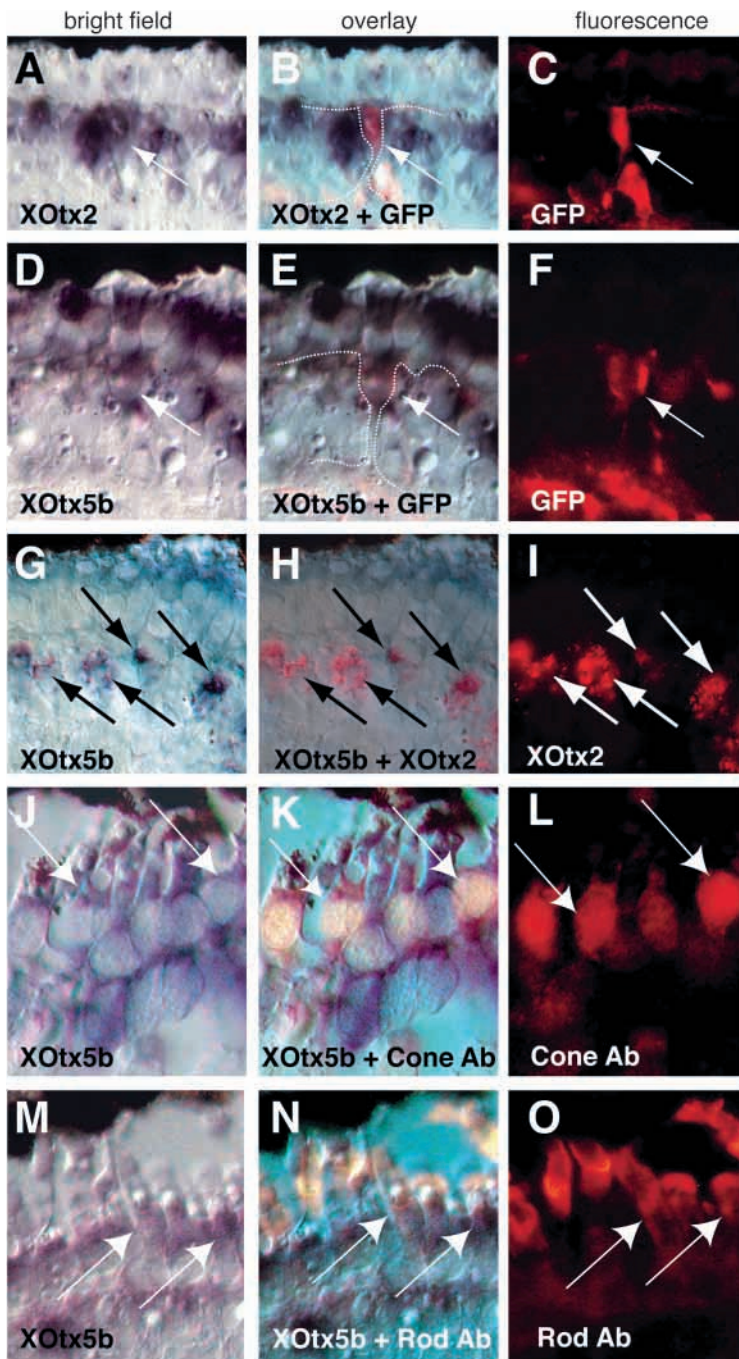
Fig. 3. Identification of cells expressing *XOtx2* and *XOtx5b*. *XOtx2* is expressed in bipolar cells. (A) Sagittal section of a stage 41 *GFP*-lipofected embryo hybridised with a DIG-labelled *XOtx2* anti-sense RNA probe. The arrow points to a cell in the INL stained with *XOtx2*. (C) Under fluorescence, the same section as in A, stained with a GFP antibody, reveals that the *XOtx2*-positive cell is a bipolar cell. (B) Superimposition of the image in A onto that in C. The arrow points to the same cell in all three panels. *XOtx5b* is also expressed in bipolar cells. (D-F) The same result is found in a retina stained for *XOtx5b*. (D) Sagittal section of a stage 41 *GFP*-lipofected embryo hybridised with a DIG-labelled *XOtx5b* anti-sense RNA probe. The arrow points to the same cell in F, which is a bipolar cell. (E) The overlay shows that the same cell is labelled for both *XOtx5b* and GFP. Cells in the INL that express *XOtx5b* also express *XOtx2*. (G-I) Stage 41 *GFP*-lipofected embryo section labelled with DIG-labelled *XOtx5b* (G: blue) and fluorescein-labelled *XOtx2* (I: red) anti-sense RNA probes. The arrows point to the same cells in all three panels, which are positive for both the *XOtx5b* and *XOtx2* probes, as shown in the overlay (H). (J-O) *XOtx5b* is also expressed by both rod and cone photoreceptors. In situ hybridisation of *XOtx5b* (blue) onto stage 41 sagittal sections co-stained for calbindin antibody (J-L) or rod opsin antibody (M-O). (J) Cells expressing *XOtx5b* also produce calbindin protein (L). The overlay (K) shows that these are the same cells, demonstrating that they are cone photoreceptor cells. (M) Similarly, *XOtx5b* is found in cells that generate rhodopsin (O). Thus, rod photoreceptors also express *XOtx5b*. (K,N) Merging panels J with L (K) and M with O (N) confirms this. Cryostat sections cut to 10 μm.

XOtx5b enhances photoreceptor cell fate while *XOtx2* enhances bipolar cell fate

The expression of *XOtx5b* in all photoreceptors and the combined expression of *XOtx5b* and *XOtx2* in a subset of bipolar cells led us to hypothesise that these genes may play a role in retinal cell fate. To test this, we lipofected stage 18 retinoblasts in vivo with either *XOtx5b* or *XOtx2* DNA expression constructs (Fig. 4). GFP DNA was co-injected with the experimental DNA in order to identify the lipofected cells. GFP DNA co-injected with vector only DNA was used as a control. *XOtx5b* significantly increased ($P<0.0001$) the proportion of photoreceptors in lipofected cells (Fig. 4A compared with Fig. 4B; quantified in Fig. 4D) when compared to the control. A slight decrease in the proportion of ganglion ($P=0.0002$), amacrine ($P=0.047$), Müller ($P=0.0016$) and horizontal ($P=0.014$) cells was also observed in the *XOtx5b* lipofected cell population, while bipolar cell numbers were unaffected. In contrast, *XOtx2* overexpression increased the proportion of bipolar cells ($P<0.0001$) at the expense of photoreceptor ($P=0.003$) and Müller cells ($P=0.0004$; Fig. 4A compared with 4C; quantified in Fig. 4E). Staining of *XOtx5b* lipofected retinal sections with the XAP-1 (anti-photoreceptor) antibody confirmed that the cells counted were indeed photoreceptors (data not shown).

XOtx5b lipofected photoreceptors are both rods and cones

Since *XOtx5b* is normally expressed in both rod and cone photoreceptors (Fig. 3J-O), we wondered if overexpression of *XOtx5b* increased the proportion of rods, cones, or both. To

Table 2. Percentage of GFP-labelled cell that also expressed *XOtx2* or *XOtx5b*

	GC	Am	BP	H	Mü	PR
XOtx2 (*n*=404)	0	0	82±5%	0	0	0
XOtx5b (*n*=381)	0	0	55±8%*	31±24%†	3±6%‡	100±0%§

n, total GFP-positive retinal cells counted. The values above represent the percentage of *XOtx2*- or *XOtx5b*-positive lipofected retinal cell types. This was done for each of the cell types listed: GC, ganglion cells; Am, amacrine cells; BP, bipolar cells; H, horizontal cells; Mü, Müller cells; PR, photoreceptor cells.

*47 /107 bipolar cells counted were positive for *XOtx5b*.
†3/12 horizontal cells counted were positive for *XOtx5b*.
‡1/15 Müller cells counted were positive for *XOtx5b*.
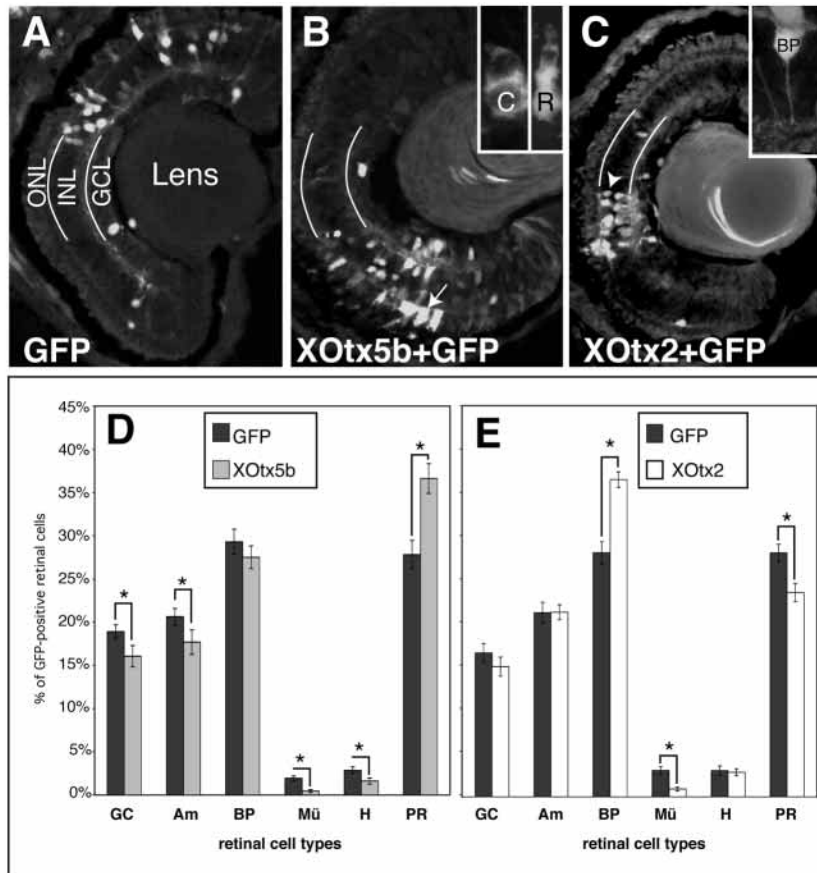§97/97 photoreceptors counted were positive for *XOtx5b*.

Fig. 4. Overexpression of *XOtx5b* or *XOtx2* in developing *Xenopus* retinoblasts causes an increase in photoreceptors or bipolars, respectively. (A) Co-lipofection of *GFP* and the pCS2$^+$ vector in the *Xenopus* retina. A diversity of retinal cell types express the fluorescent marker. White lines are drawn over the inner and outer plexiform layers to better define the outer nuclear layer (ONL), inner nuclear layer (INL) and the ganglion cell layer (GCL). (B) Retina co-lipofected with *XOtx5b* and *GFP* shows an increase in lipofected photoreceptor cells (arrow). The insets show a close-up of a cone (C) and a rod (R) photoreceptor cell. (C) Retina co-lipofected with *XOtx2* and *GFP* shows an increase in the number of lipofected bipolar cells (arrowhead). (Inset) Confocal image of a representative bipolar cell (BP). (D,E) Each of the retinal cell types (GC, ganglion cells; Am, amacrine cells; BP, bipolar cells; Mü, Müller cells; H, horizontal cells; PR, photoreceptor cells) was counted per retina (*n*) and the percentage for each was determined. The graphs show an average of the percentages obtained. (D) *GFP* + pCS2$^+$ (*n*=45) and *XOtx5b* + *GFP* (*n*=32) lipofected retinas. (E) *GFP* + pCS2$^+$ (*n*=20) and *XOtx2* + *GFP* (*n*=20) lipofected retinas. The error bars represent the s.e.m. The asterisks indicate significant difference by a Student's *t*-test between the ratios found for the control versus the experimental for that particular cell type with $P \leq 0.003$.

address this question, we used the cone photoreceptor-specific anti-calbindin antibody to identify cones in *XOtx5b* lipofected retinas. To increase the number of photoreceptors in this analysis, we used *XOtx5bN+HD-VP16* for our in vivo lipofections (see below for further details of this construct). *XOtx5bN+HD-VP16* dramatically increased the proportion of photoreceptor cells from 27% to 71% (Fig. 5C). When we stained these same sections for the cone marker, calbindin, we found approximately equal numbers of calbindin-positive and -negative photoreceptors (Fig. 5D). In control, *GFP* and vector only lipofected retinas, we also found approximately equal numbers of rods and cones (Fig. 5D), which is consistent with previous reports (Chang and Harris, 1998). Together these results demonstrate that *XOtx5bN+HD-VP16* increases the proportion of both rods and cones in equal numbers.

XOtx5b and XOtx2 N-terminal and HD fused to the engrailed repressor suggest that XOtx5b and XOtx2 have some cell fate specification activities

Transcription factors can act as activators, repressors, or both. Mouse Crx has been shown to activate *rhodopsin* but repress *NeuroD*, either directly or through a cascade of other factors (Furukawa et al., 1999; Livesey et al., 2000). We were interested to know if *XOtx5b* and *XOtx2* worked primarily as repressors or activators when modulating retinal cell fates. Therefore, we fused the powerful repressor domain of *engrailed* to the N terminus and homeodomain (HD) of *XOtx5b* (*XOtx5bN+HD-EngR)* and *XOtx2* (*XOtx2N+HD-EngR*) and

determined the effect of these fusions on cell fate (Fig. 5E-G). Lipofection of the *XOtx5bN+HD-EngR* led to fewer lipofected photoreceptor cells than controls (*P*=0.001; Fig. 5G). This suggests activation of *XOtx5b* target genes stimulates transcription necessary for photoreceptor cell specification. *XOtx2N+HD-EngR* lipofected retinas showed a decrease in the percentage of lipofected bipolar cells compared to controls (*P*=0.004; Fig. 5G), suggesting that native *XOtx2* also acts as an activator of genes necessary for bipolar cell specification. These results also suggest some control of cell fate specification by the N-terminal and HD sequences.

Engrailed fusion constructs of *XOtx5b* and *XOtx2* also gave some unexpected results. *XOtx5bN+HD-EngR* did not affect ganglion, amacrine or Müller cell populations, while the proportion of bipolar cells was slightly increased (5%, *P*<0.05). *XOtx2N+HD-EngR* increased the proportion of ganglion cells (by 4%, *P*<0.02) but did not alter the photoreceptor cell population. These results demonstrate that although full-length XOtx2 and XOtx5b may modulate photoreceptor levels, they both may act in more complex ways than as dedicated repressors or activators.

These results also suggest that the N-terminal and HD regions of these proteins are not sufficient to direct cell fate specification in the same way as the full-length proteins. This fits with a recent study that compares otd/Otx family members (Sauka-Spengler et al., 2001) and the fact that *Xenopus* XOtx5b and XOtx2 have very similar homeodomains and N-terminal regions. Indeed, *Xenopus* XOtx5b and XOtx2 homeodomains (HD) have only one

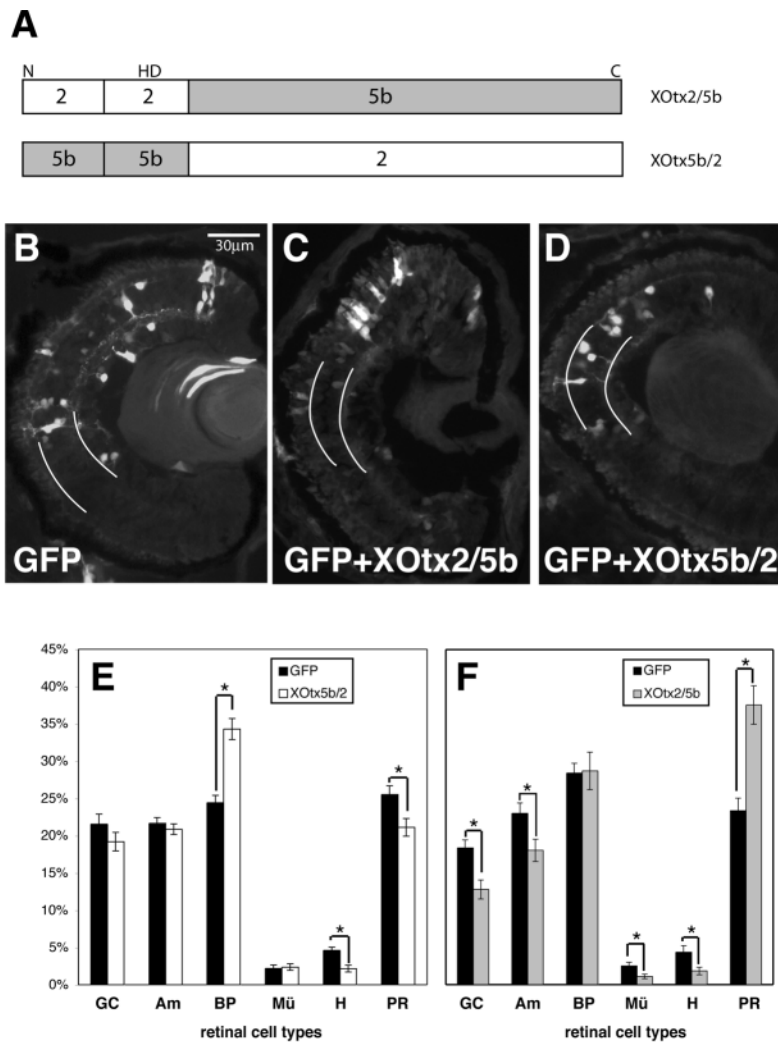**Fig. 5.** Effects of VP16 or Engrailed fusion constructs of *XOtx5b* and *XOtx2* on retinal cell fate decisions. (A,B) Cryostat sections (10 μm) of retinas lipofected with (A) *GFP* and the pCS2[+] vector or (B) *XOtx5bN+HD-VP16* and *GFP* then stained with an anti-cone photoreceptor antibody (calbindin in red). Arrowheads indicate cells that fluoresce with *GFP* alone (green) and arrows indicate cells expressing both *GFP* and calbindin (yellow). (C) Graph showing an increase in the distribution of GFP-positive photoreceptor cells in retinas co-lipofected with *XOtx5bN+HD-VP16* and *GFP* (*n*=344) when compared to *GFP* and pCS2[+] (*n*=493). Values were calculated as in Fig. 4D,E; *P*≤0.04. In C,D,G and J asterisks indicate *P* values as calculated using a Student's *t*-test; *n*=the total number of retinal cells counted; error bars, s.e.m. (D) Graph showing the types of photoreceptor cells produced in retinas co-lipofected with *GFP* and pCS2[+] (*n*=184) or *VP16-XOtx5bHD* and *GFP* (*n*=267). Cells positive for GFP (unlabelled) or GFP and calbindin (cones) were counted in one retina and the percentage of each determined. Percentages from both cones and unlabeled cells were averaged in all retinas counted. The error bar indicates s.e.m. Even though a larger number of photoreceptors were produced in *XOtx5bN+HD-VP16* lipofected retinas (C), cones and unlabeled cell numbers were similar to each other in retinas lipofected with either *XOtx5bN+HD-VP16* or the control construct. (E) Cryostat section (10 μm) of a retina co-lipofected with *GFP* and *XOtx2N+HD-EngR*, which shows a decreased number of bipolar cells. White lines (also in F-H) mark the inner and outer plexiform layers. (F) *GFP* and *XOtx5bN+HD-EngR* co-lipofected retina (10 μm cryostat section) showing a distinct decrease of fluorescently labelled photoreceptor cells. (G) The percentage of GFP-positive photoreceptor cells in the

XOtx5bN+HD-EngR+GFP (*n*=651) co-lipofected retinas is decreased while the percentage of GFP-positive bipolar cells is decreased in the *XOtx2N+HD-EngR+GFP* (*n*=474) co-lipofected retinas when compared to controls (GFP and pCS2[+], *n*=692). This is a representative experiment. Lipofection of each construct with the control was repeated in two separate experiments, resulting in identical population shifts as shown [*XOtx2N+HD-EngR+GFP* (*n*=1692); *XOtx5bN+HD-EngR+GFP* (*n*=2121)]. *P*≤0.05, (H-I) Sagittal section of stage 41 retinas that have been co-lipofected with GFP and either (H) *XOtx5bN+HD-VP16* or (I) *XOtx2N+HD-VP16*. In both, there are a large number of labelled photoreceptor cells. Scale bar: 30 μm. (J) The percentage of each retinal cell type, which is GFP-positive, in retinas lipofected with *XOtx5bN+HD-VP16* (*n*=342) or *XOtx2N+HD-VP16* (*n*=294) and *GFP*. *GFP* and the expression vector alone acted as the control (*n*=535). Nearly all of the GFP-positive cells are photoreceptors in the experimental lipofected retinas. The asterisk denotes significant differences between the control and each of the constructs lipofected (*P*≤0.02); GC, ganglion cells; Am, amacrine cells; BP, bipolar cells; Mü, Müller cells; H, horizontal cells; PR, photoreceptor cells. (K) A schematic of the constructs used in the above lipofections. HD, homeodomain. The numbers above the bars indicate the amino acid residues.
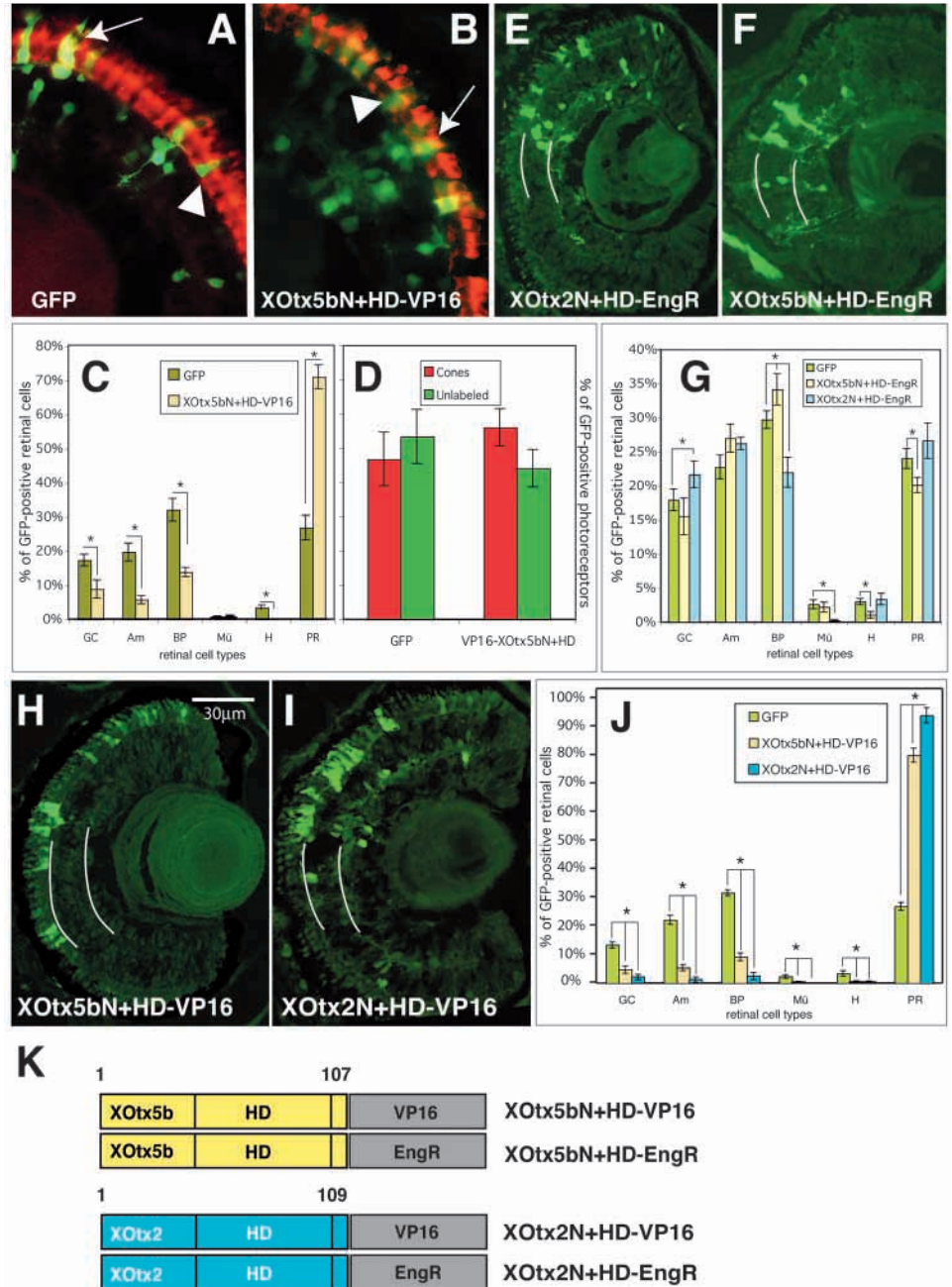
residue difference (*XOtx2*=A; *XOtx5b*=S) while the region N-terminal to the HD, which we called the N terminus, is 73% identical in these two proteins. If the N terminus and homeodomain were mostly responsible for the differences we observed in the lipofection results between these two full-length constructs, and if, as the above results suggest, that some aspects of the phenotype were due to their activity as activators rather than repressors, then a construct which fuses

A

XOtx2/5b

XOtx5b/2

GFP

GFP+XOtx2/5b

GFP+XOtx5b/2

Fig. 6. Swapped domain experiments suggest that the C terminus of XOtx2 or XOtx5b is necessary in determining bipolar or photoreceptor cell fate, respectively. (A) Schematic of constructs used in lipofection experiments. N, N terminus; HD, homeodomain; C, C terminus; 2, *XOtx2*; 5b, *XOtx5b*. (B-D) Stage 41 lipofected retinas were sagittally cryostat sectioned (10 µm) after transfection with *GFP* and pCS2+ vector, *XOtx2/5b* or *XOtx5b/2* during early stages of eye formation. Lines are drawn over inner and outer plexiform layers to define the laminated retina (see Fig. 4). (B) *GFP* lipofected retina shows all retinal cell types. (C) *GFP* and *XOtx2/5b* lipofected retinas have an increase of photoreceptor cells as do *XOtx5b* lipofected retinas. (D) GFP and *XOtx5b/2* lipofected retinas also have an increase of bipolar cells. (E-F) Quantitative analysis of retinas lipofected with *GFP* and pCS2+ compared to retinas lipofected with (E) *XOtx5b/2* and *GFP* (pCS2+, $n=24$; *XOtx5b/2*, $n=30$) or (F) *GFP* and *XOtx2/5b* (pCS2+, $n=26$; *XOtx2/5b*, $n=23$). The ratio of retinal cells per total retina was calculated for each retina and then the ratios averaged for the total retinas counted (n). The error bars, s.e.m.; $P \le 0.05$ (Student's *t*-test).

VP16 activator domain made the constructs even stronger transactivators. Hence, we lipofected retinas with just the N terminus and homeodomain of either XOtx5b or XOtx2, but in neither case was there an effect on cell fate (data not shown). These results suggest that the N-terminal and HD of both XOtx5b and XOtx2 may have a common function in retinal cell determination, and be part of the shared regulatory mechanisms of photoreceptors and bipolars (Chen et al., 1994; Chiu and Nathans, 1994). The lack cell type specificity of these domains when fused to an activator suggested that the region C-terminal to the HD may be more important in modulating cell fate.

The C-terminus of either XOtx2 or XOtx5b is involved in photoreceptor versus bipolar fate determination

C-terminal to the homeodomain, otd/Otx family member genes have several unique domains: a glutamine (Gln) rich region, a WSP domain and an OTX tail sequence (review by Morrow et al., 1998). Comparison of the entire XOtx2 and XOtx5b C termini sequence shows that they are 60.1% identical (data not shown). To test if the difference we observed in the way *XOtx2* and *XOtx5b* overexpression affects retinal cell fate maps to the C terminus, we did domain swap experiments. The N terminus and HD of *XOtx2* was fused to the C terminus of *XOtx5b* (*XOtx2/5b*) and the N-terminal and HD sequence of *XOtx5b* was fused to the C terminus of *XOtx2* (*XOtx5b/2*; Fig. 6A). Each domain swapped construct was then co-lipofected with *GFP* into developing embryos as above (Fig. 6C,D) and the ratios of each cell type calculated (Fig. 6E,F). *XOtx5b/2*, like *XOtx2* itself, increased bipolar cells ($P=0.0001$) at the expense of photoreceptor ($P=0.031$) and Müller ($P=0.0001$) cells while *XOtx2/5b*, like *XOtx5b* itself, increased photoreceptor cells ($P=0.0004$) at the expense of ganglion ($P=0.004$), amacrine ($P=0.018$), Müller ($P=0.005$) and horizontal ($P=0.021$) cells. These results clearly suggest

the N terminus and HD to the strong activator VP16 should mimic a full-length construct. To test this, we fused the N terminus and HD to the strong activator domain of VP16 for each of these genes (*XOtx2N+HD-VP16* and *XOtx5bN+HD-VP16*) and lipofected each with the tracer *GFP* into stage 18 embryos (Fig. 5H,I). In each case, the number of *GFP*-positive photoreceptor cells increased by about three times that of the control lipofected retinas (both $P<0.0001$) at the expense of all the other retinal cell types (Fig. 5J). These results suggest that the N terminus and homeodomain may be involved in regulating photoreceptor cell specification but show a striking lack of specificity between photoreceptors and bipolars. The increased percentage of photoreceptor cells in retinas lipofected with the *VP16* activator fusion constructs (*XOtx5bN+HD-VP16*=80% and GFP=27%) was even more than retinas lipofected with full-length *XOtx5b* (*XOtx5b*=37% and GFP=28%; Fig. 4D compared with Fig. 5J). We therefore had to consider the possibility that the VP16 activator alone may be responsible for photoreceptor cell fate. To test this, we injected a *VP16* activator construct fused to a nuclear localization sequence and *GFP* into developing embryos and found no effect on cell fate (data not shown). It could also be that the N terminus and homeodomain can activate photoreceptor-specific genes on their own and adding the

that the C-terminal region of *XOtx2* and *XOtx5b* is much more critical for cell fate specification affects than the N terminus and HD.

XOtx2 and *XOtx5b* transcripts are found in Brd-positive cells but do not affect retinal proliferation or apoptosis

Increases in photoreceptor or bipolar cell percentages in the population of *XOtx5b* or *XOtx2* lipofected cells, respectively, could be due cell type-specific changes in proliferation or death. Previous studies have shown that *XOtx2* is expressed in proliferating retinoblasts (Perron et al., 1998) but *XOtx5b* expression in dividing cells has not been described. To test if *XOtx5b* is normally expressed in mitotic cells, we injected embryos with BrdU and fixed them 30 minutes later. We sectioned their retinas and stained for *XOtx5b* or *XOtx2* expression by in situ hybridisation, and afterwards, for BrdU by immunostaining to look for *XOtx5b*- or *XOtx2*-positive cells that were also BrdU positive (Fig. 7A-F). We found that most of the cells that expressed *XOtx5b* or *XOtx2* were not BrdU positive. However, a few cells in the CMZ that were *XOtx5b* or *XOtx2* positive, were also labelled with BrdU. This confirmed that *XOtx2* is in some proliferating cells and indicated that *XOtx5b* can also be found in proliferating cells of the retina, as suggested by their expression during early stages of retinal development.

As these transcripts are present in BrdU-positive cells, it is possible that they cause an increase in proliferation of the respective cell types they influence. To test this, we lipofected developing retinas with GFP and each one of the following constructs in separate experiments: *XOtx5b*, *XOtx5bN+HD-VP16*, *XOtx5bN+HD-EngR*, *XOtx2*, *XOtx2N+HD-VP16* and the swapped domain constructs (*XOtx2/5b* and *XOtx5b/2*) at stage 18. At stage 31 (the beginning of photoreceptor cell differentiation), we injected the embryos with BrdU. At stage 41 (retinal maturation), the embryos were fixed and the retinas sectioned and stained for GFP and BrdU. Cells expressing just GFP or GFP and BrdU were counted. We found that none of the constructs we tested showed an increase in proliferation in any of the cell types at this stage, suggesting that these constructs do not influence proliferation (Fig. 7G,H).

To produce an increase in the percentage of lipofected bipolar cells, *XOtx2* could cause cell death in other retinal cell types, like photoreceptors or Müller cells. Similarly, *XOtx5b* lipofected retinal cells that are not photoreceptors might be more likely to undergo programmed cell death. To test if either of these constructs caused apoptosis, we lipofected developing retinas as above, fixed at stages 31, 33/34 and 37 and stained the sections for apoptotic nuclei using a TUNEL assay. The number of GFP-positive cells in each of the retinas was counted as well as those apoptotic cells that were GFP positive. In each case, the percentage of GFP-positive cells that were also apoptotic was around 2-4%. Statistical analysis using a Student's *t*-test showed no differences (data not shown). This suggests that selective cell death is also not the basis for the overexpression phenotypes. The most likely explanation for the phenotypes observed, therefore, is that these constructs cause a fate switch or conversion.

Xopsin reporter is activated by *XOtx5b* and this activation is suppressed by *XOtx2*

Crx binds to the OTX-binding consensus sequence found in the rhodopsin promoter and transactivates its expression (Chen et al., 1997; Furukawa et al., 1997). Like Crx, Otx2 also binds this sequence and has been shown to both activate and repress genes by binding to this site (Kelley et al., 2000; Morgan et al., 1999). Since *XOtx5b* has been suggested to be part of the same subfamily of otd/Otx transcription factors as Crx (Germot et al., 2001; Sauka-Spengler et al., 2001), we reasoned that *XOtx5b* might also regulate *opsin* expression. To test this hypothesis, we injected one dorsal cell of a 4-cell stage blastomere with an expression vector of GFP driven by the *Xenopus* opsin promoter [Xop-GFP (Mani et al., 2001)], capped *XOtx5b* RNA and β-gal RNA. We allowed the embryos to grow to stage 17-18, they were then fixed and the fluorescence quantified. As a negative control, Xop-GFP DNA and β-gal RNA were injected alone (Fig. 7I). All embryos that were injected with Xop-GFP DNA and *XOtx5b* RNA on the dorsal side (Fig. 7J), expressed GFP at levels higher than the negative control embryos (Fig. 7I; $P<0.0001$), suggesting that XOtx5b does activate the *opsin* promoter.

Since *XOtx5b* is expressed in a subset of bipolar cells with *XOtx2*, we were interested to know whether *XOtx2* affected the ability of *XOtx5b* to regulate opsin expression. Co-injection of equal amounts of *XOtx2* and *XOtx5b* RNA plus Xop-GFP DNA and β-gal RNA (Fig. 7K) showed that *XOtx2* buffers the effect of *XOtx5b* on Xop-GFP; i.e. levels of GFP expression are similar to those of the uninjected side (Fig. 7N). Staining for β-gal confirmed that these embryos were successfully injected with the RNA/DNA mixture. By adding double the concentration of *XOtx2* than *XOtx5b* RNA plus Xop-GFP DNA and β-gal RNA (Fig. 7L), we still observed a suppression of the *XOtx5b*-driven *opsin* activation. However, when we added the Xop-GFP DNA and β-gal and *XOtx2* RNA alone (Fig. 7M), we found that *opsin* expression was enhanced by *XOtx2*. This was not surprising since either *Crx* and *Otx2* alone activates the promoter of another photoreceptor-specific gene, interphotoreceptor retinoid binding protein (IRBP) (Bobola et al., 1999). Our results with the *Xenopus* opsin promoter suggest that the relative levels of *XOtx2* and *XOtx5b* are important in determining the activation or repression of *opsin*. It appears that *XOtx2* plays a role in suppressing the effect of *XOtx5b* on *opsin* expression when equal or double the amounts of *XOtx2* to *XOtx5b* are present in the cell.

XOtx2 suppresses the *XOtx5b* enhancement of photoreceptor cell fate

The *XOtx2* suppression of the activation of a photoreceptor-specific gene in the above assay may partly explain why bipolar cells do not normally express opsins. A similar question raised by this study is why cells that express both *XOtx5b* and *XOtx2* appear to differentiate into bipolars rather than photoreceptors. Could it be that *XOtx2* suppresses the ability of *XOtx5b* to induce photoreceptors? To test this hypothesis, we co-lipofected *XOtx2* and *XOtx5b* with GFP into the eye field of stage 18 embryos as described above. Controls were lipofected with *XOtx5b* and GFP or GFP and vector alone. *XOtx5b*-lipofected retinas had the expected increase in photoreceptor
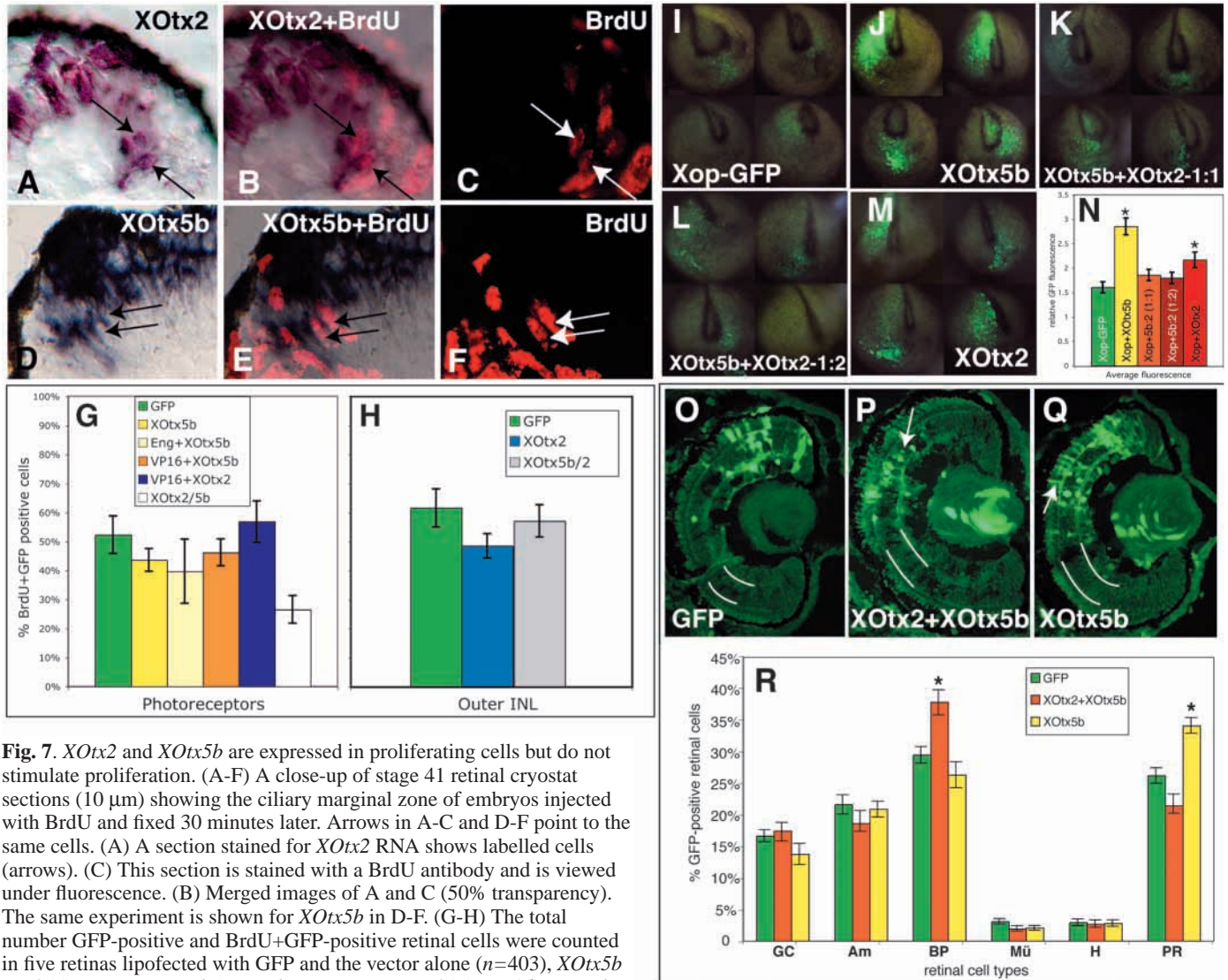
Fig. 7. *XOtx2* and *XOtx5b* are expressed in proliferating cells but do not stimulate proliferation. (A-F) A close-up of stage 41 retinal cryostat sections (10 μm) showing the ciliary marginal zone of embryos injected with BrdU and fixed 30 minutes later. Arrows in A-C and D-F point to the same cells. (A) A section stained for *XOtx2* RNA shows labelled cells (arrows). (C) This section is stained with a BrdU antibody and is viewed under fluorescence. (B) Merged images of A and C (50% transparency). The same experiment is shown for *XOtx5b* in D-F. (G-H) The total number GFP-positive and BrdU+GFP-positive retinal cells were counted in five retinas lipofected with GFP and the vector alone (*n*=403), *XOtx5b* (*n*=355), *EngR+XOtx5b* (*XOtx5bN+HD-EngR*; *n*=317), *VP16+XOtx5b* (*XOtx5b-VP16*; *n*=384), *VP16+XOtx2* (*XOtx2-VP16*; *n*=322) and XOtx2/5b (*n*=353), *XOtx2* (*n*=418), or *XOtx5b/2* (*n*=413). The percentage of BrdU-positive cells was calculated. None of the constructs tested gave an increase in proliferation of photoreceptor cells (G) or cells found in the outer edge of the inner nuclear layer (H). This analysis was done using a Student's *t*-test. *n*=the total number of cells counted. (I-R) *XOtx2* modulates *XOtx5b* activity. (I-M) Embryos at stage 18 visualised under fluorescent light that have been injected on one side with the *Xenopus* opsin promoter fused to (I) *GFP* (Xop) DNA, β-gal RNA (200 pg) and (J) 50 pg *XOtx5b* RNA (XOtx5b); (K) 25 pg *XOtx5b* and 25 pg *XOtx2* (XOtx5b+XOtx2 1:1); (L) 17 pg *XOtx5b* and 33 pg *XOtx2* (XOtx5b+XOtx2 1:2); (M) 50 pg *XOtx2* (XOtx2). (N) Embryos from each set of injections [*Xop* (*n*=35); *XOtx5b* (*n*=37); *XOtx5b+XOtx2* 1:1 (*n*=28); *XOtx5b+XOtx2* 1:2 (*n*=29); *XOtx2* (*n*=31)] were individually photographed under fluorescence and the fluorescence on both sides of the embryo was measured. The ratio of fluorescence on the injected versus uninjected side was calculated (relative GFP fluorescence) and an average for all the embryos from that set was made. Significant difference from the Xop-GFP control set using a Student's *t*-test was found for both the *XOtx5b* (*P*<0.001) and *XOtx2* set (*P*<0.001). *XOtx5b* and *XOtx2* together with *Xop* did not activate the Xop construct, suggesting that XOtx2 modulates the ability of XOtx5b to activate *opsin*. (O-Q) Sagittal section of stage 41 embryos lipofected with either GFP and pCS2[+] vector (O), *GFP*, *XOtx2* and *XOtx5b* (P), or *GFP* and XOtx5b (Q). Arrows point to an increase in bipolar cells in P and an increase in photoreceptors in Q. (R) Graph showing the percentage of GFP-positive retinal cells in retinas lipofected with *GFP* and the vector alone (*n*=1255); *GFP*, *XOtx2* and *XOtx5b* (*n*=995); and *GFP* and *XOtx5b* (*n*=1188). Asterisks: *P*<0.01 as determined by a Student's *t*-test; *n*, number of GFP-positive retinal cells counted; GC, ganglion; Am, amacrine; BP, bipolar; Mü, Müller; H, horizontal, and PR, photoreceptor cells. Error bars represent s.e.m.

cells (*P*=0.001). However, when *XOtx2* and *XOtx5b* were co-lipofected, there was no increase in photoreceptors but rather an increase in the population of lipofected bipolar cells (Fig. 7O-R; *P*=0.007). These results suggest that when *XOtx2* and *XOtx5b* are both present in progenitors, cells are more likely to take on bipolar cell fates.

DISCUSSION

XOtx5b plays a similar but not identical role to mammalian Crx

Crx, is expressed in the pineal, both rods and cones in the retina (Chen et al., 1997; Furukawa et al., 1997; Liu et al., 2001) and

has recently been localised to bipolar cells (Chen et al., 2000). It has also been demonstrated to be an activator of many photoreceptor-specific genes (Chen et al., 1997; Furukawa et al., 1997) and involved in photoreceptor cell specification (for a review, see Morrow et al., 1998). In this paper, we demonstrate in *Xenopus* that *XOtx5b*, which is highly related to mammalian *Crx* (Germot et al., 2001; Sauka-Spengler et al., 2001) has an identical expression pattern to *Crx* in later stages of embryogenesis. We confirmed its expression in the pineal (Sauka-Spengler et al., 2001; Vignali et al., 2000) and identified its expression in both rod and cone photoreceptor and bipolar cells. Misexpression of *XOtx5b* in vivo demonstrated that it is also involved in photoreceptor cell specification. Misexpression of *XOtx2* or *XOtx5b* fused to the VP16 activator in developing retinoblasts suggests that (1) *XOtx2* and *XOtx5b* target genes are involved in photoreceptor cell determination and (2) these target genes require activation in order to promote photoreceptor cell fate. Overexpression of *Crx* by retroviral infection of retinal progenitors is reported to increase the percentage of clones that contain only rods, suggesting that *Crx* influences cell fate (Furukawa et al., 1997), however *Crx* knockouts do make rods and cones (Furukawa et al., 1999) suggesting that Crx by itself is not a key determiner of photoreceptor cell fate. In the *Xenopus* retina, there are an equal number of rods and cones (Chang and Harris, 1998) unlike rodent retinas, which consist mostly of rods (Young, 1985). We have also found that the population of photoreceptors increased by overexpresssion of a *XOtx5b* activator construct (XOtx5bN+HD-VP16), is composed of both rods and cones equally. This is not surprising since it is expressed in both types of cells in *Xenopus* and zebrafish (Liu et al., 2001).

Contrary to mammalian and zebrafish *Crx* studies (Chen et al., 1997; Furukawa et al., 1997; Liu et al., 2001), *Xenopus XOtx5b* is expressed at much earlier stages of development and plays a role similar to *XOtx2* in early embryogenesis (Vignali et al., 2000). *XOtx5b* is expressed in dividing retinal cells and in all layers of the undifferentiated retina during development, which is similar to zebrafish *Crx*, but unlike mammalian *Crx,* which is expressed in photoreceptor cells just after they are born (Chen et al., 1997; Furukawa et al., 1997; Liu et al., 2001; Morrow et al., 1998). We also found that *XOtx5b-engrailed* constructs resulted in clones with a decrease in photoreceptor cells. This is also different from the *Crx-engrailed* repressor fusion construct results, which produced clones with rods lacking outer segments (Furukawa et al., 1997). The ability of *XOtx5b* to decrease the number of photoreceptors, albeit a small amount in comparison to the activation seen by the VP16 activator fusion constructs, suggests that *XOtx5b* may be playing an earlier role in photoreceptor specification than *Crx*. Phylogenetic analysis suggests that *XOtx5b* and *Crx* may be orthologous (Germot et al., 2001; Sauka-Spengler et al., 2001) suggesting that one might consider renaming *Xenopus Otx5b*, *XCrx*. Our findings that *XOtx5b* functions similarly to mammalian *Crx*, supports this idea. However, since there are clear differences between the roles played by mammalian Crx and *Xenopus XOtx5b* before eye formation, and since it is still possible that a *Xenopus* Crx gene with more sequence similarity exists as was found in teleosts, we would suggest that *Xenopus XOtx5b* is not renamed.

To date, zebrafish is the only species reported to have separate genes encoding Crx and Otx5 (Gamse et al., 2002). *Xenopus* also has two genes in the hypothesised Otx5/Crx orthology class, *XOtx5* (Kuroda et al., 2000) and *XOtx5b* (Vignali et al., 2000). Although zebrafish *Crx* and *Otx5* retinal and pineal expression are the same, their protein sequences are only 64% identical (data not shown) and they regulate pineal circadian gene expression differently (Gamse et al., 2002). This suggests that they are different genes. Although *XOtx5* retinal expression has not yet been described, the early embryonic *XOtx5* and *XOtx5b* expression patterns are virtually identical (Kuroda et al., 2000; Vignali et al., 2000) and comparison of their protein sequences show they are 96% identical (data not shown). Injections of the blastomere with either *XOtx5* or *XOtx5b* results in embryos of reduced size with posterior deficiencies and ectopic cement gland structures (Kuroda et al., 2000; Vignali et al., 2000), suggesting that the two genes function similarly in the embryo. Given the pseudotetrapoid *Xenopus laevis* genome, these results strongly suggest that *XOtx5* and *XOtx5b* arose from a gene duplication event. This is further supported by phylogenetic analysis unambiguously placing *XOtx5* and *XOtx5b* in the same orthology class (Germot et al., 2001; Sauka-Spengler et al., 2001).

XOtx2 promotes bipolar cell specification

Previously, *XOtx2* has been shown to be involved in anteriorizing the embryo and to have a role in cement gland formation (Andreazzoli et al., 1997; Blitz and Cho, 1995; Gammill and Sive, 1997; Gammill and Sive, 2001; Pannese et al., 1995). Here, we show that it is involved in specifying the bipolar cell. Recent studies on bipolar cell specification have examined several transcription factors using transgenic and mutant mice. Mice carrying a null mutation in *Chx10* produce retinas lacking differentiated bipolar cells (Burmeister et al., 1996). Similarly, *Mash1-Math3* double knockout mice retinas are also missing bipolar cells, yet neither mutation alone affects bipolar cell production (Tomita et al., 2000; Tomita et al., 1996). Viral infection experiments show that together, *Chx10*, *Mash1* and *Math3*, are all involved in bipolar cell genesis (Hatakeyama et al., 2001). However, not all cells that co-expressed *Chx10* and either *Mash1* or *Math3* become bipolars, suggesting that other factors are also necessary. Like *Chx10*, *XOtx2* is expressed throughout the undifferentiated neural retina and later is restricted to differentiated bipolar cells. Like *Chx10* and either *Mash1* or *Math3* overexpression (Hatakeyama et al., 2001), overexpression of *XOtx2* in developing retinal cells increases the number of bipolar cells in that population. Consistent with this, we found that lipofection of the N terminus and DNA binding domain of *XOtx2* fused to the *engrailed* transcription repressor decreases the percentage of lipofected bipolar cells, suggesting that *XOtx2* acts as an activator to generate an increase in lipofected bipolar cells. It will be interesting to know if these four genes together, *XOtx2*, *Chx10*, *Mash1* and *Math3,* are sufficient for directing all retinoblasts to a bipolar cell fate.

The C terminus of both XOtx2 and XOtx5b are necessary for their cell fate specific activities

Analysis of deletion constructs of *Crx* and their ability to transactivate the bovine rhodopsin promoter lead to the conclusion that regions C-terminal to the homeodomain are important for gene activation (Chau et al., 2000). Moreover,

they showed that the N terminus, homeodomain and basic region (aa 1-107) are necessary for binding the BAT-1 site on the rhodopsin promoter but are poor transactivators of rhodopsin (Chau et al., 2000). Consistent with this, we found that lipofection of this same region of *XOtx2* (aa 1-109) or *XOtx5b* (aa 1-107) does not have any effect on photoreceptor cell fate, but when fused to the *engrailed* repressor or VP16 activator, lipofection of these fusion constructs influences the cell fate of the lipofected cells. Even though this region (aa 1-107) can successfully target the rhodopsin promoter in vitro (Chau et al., 2000), it may be that removal of such a large area of the protein (aa 110-289 for XOtx2; aa 108-290 for XOtx5b) could alter the ability of the fusion constructs to correctly target DNA *cis*-acting elements in vivo. However, when we swapped the C-terminal region of these proteins onto the opposite N-terminal and homeodomain sequence, we found that the region C-terminal to the homeodomain of *XOtx5b* and *XOtx2* are capable of producing the same phenotype as *XOtx5b* and *XOtx2*, respectively, independently of which N terminus and HD they are fused to.

Clearly, the region C-terminal to the homeodomain of *XOtx5b* and *XOtx2* is not simply required for activation or repression of target genes, as overexpression of the DNA binding domain and the sequence N-terminal to the homeodomain of either *XOtx5b* (aa 1-107) or *XOtx2* (aa 1-109) fused to a VP16 activator or *engrailed* repressor produced a different labelled retinal cell distribution than overexpression with either the *XOtx2* or *XOtx5b* alone. It has been shown that XOtx2 directly activates some genes like that of the *Xenopus* transcription factor Clock (Green et al., 2001), and indirectly activates the secreted protein, XAG, while it directly represses others, like the transcription factor Brachyury and the secreted factor, Wnt-5a (Latinkic et al., 1997; Morgan et al., 1999) (reviewed by Boncinelli and Morgan, 2001).

A basic leucine zipper protein, NRL, has been shown to work synergistically with Crx to transactivate the bovine rhodopsin promoter (Chen et al., 1997). Studies done on the binding of Crx with NRL suggest that the basic domain and homeodomain of Crx are necessary for this interaction (Mitton et al., 2000). Deletion analysis on Crx shows that the region C-terminal to the homeodomain is necessary for photoreceptor-specific gene activation with or without NRL. The fact that the VP16 activator fused to either *XOtx2* or *XOtx5b* N-terminal region and homeodomain induces photoreceptor cells, suggest that these regions may be necessary for targeting genes involved in photoreceptor specification even if they are not sufficient to specify cell fate effectively. Deletion analysis also revealed that half of the transactivation function of Crx was lost upon removal of the Otx tail (Chau et al., 2000). Comparison of the Otx tail of all the Crx sequences listed to eight different species of Otx2 homologues showed an extra threonine (T) in the second repeat of the Otx tail of all the Otx2 proteins. Thus, site directed mutation analysis may clarify which residues in the C terminus of XOtx2 and XOtx5b are critical for the unique function of each of these closely related family members.

Photoreceptors versus bipolars, *Otx5b* versus *Otx2*

How are *XOtx2* and *XOtx5b* involved in retinal cell fate specification? Our results confirm other studies done on chick (Bovolenta et al., 1997) that early in retina formation, *XOtx2* transcripts are found in all layers of the neural retina. Yet, if

XOtx2 is involved in bipolar cell specification in *Xenopus*, why do not all cells become bipolar cells? Similarly, *XOtx5b* is expressed in dividing retinoblasts in all layers of the early retina during its formation but is involved in specification of photoreceptor cells. This suggests that different transcription factors may be competing or cooperating in cell fate specification in a combinatorial way. The results with the *Xenopus* rhodopsin promoter may shed light on this. Both *XOtx5b* and *XOtx2* alone can activate opsin expression but together they block activation of opsin. A similar mechanism has been shown for Barrier-to-autointegration-factor (Baf), which is expressed in bipolars with Crx in the mammalian retina. Baf interacts directly with Crx to repress Crx-mediated transactivation of a rhodopsin reporter (Wang et al., 2002). A possible mechanism for how the Otx transcription factors may interact to produce a similar result comes from recent studies of mouse Otx3, which is expressed in the developing eye, binds to the same consensus binding sequence as Crx and yet appears to act as a repressor rather than an activator (Zhang et al., 2002). This idea of cooperative specification is supported by the results of the co-lipofection experiment showing that misexpressed *XOtx5b* on its own induces photoreceptors but the combination of the *XOtx2* and *XOtx5b* overrides this activity and induces bipolar cells. We hope that these findings may stimulate insight or speculation into the developmental and evolutionary mechanisms whereby overlapping patterns of homologous genes within a tissue specify the fates of similar and possibly homologous cell types within that tissue, much as Hox genes specify segmetal identity at the tissue level.

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