

# A Role for *Xvax2* in Controlling Proliferation of *Xenopus* Ventral Eye and Brain Progenitors

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The *Vax2* homeobox gene plays a crucial role in early dorsoventral patterning of the eye. However, although *Vax2* transcripts have been detected in later differentiating eye and brain regions, its possible roles at these stages are still unclear. By immunohistochemistry and in situ hybridization, we extensively compared the expression patterns of *Xenopus Vax2* (*Xvax2*) mRNA and protein. Expression of *Xvax2* protein was found to be largely overlapping but more restricted than that of mRNA, suggesting that *Xvax2* expression may be also regulated at posttranscriptional levels. During eye and brain neurogenesis, *Xvax2* protein was detected in proliferating neural progenitors and postmitotic differentiating cells in ventral regions of both structures. Overexpression of *Xvax2* in *Xenopus* embryos by mRNA microinjection and DNA lipofection appeared to inhibit proliferation in both eye and brain cells, thus pointing to a new potential role for *Vax2* in controlling the proliferative properties of ventral eye and brain progenitors. *Developmental Dynamics* 237:3387–3393, 2008.

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## INTRODUCTION

The *Vax2* homeobox gene has been proved to play an important role in early dorsoventral patterning of the eye in different model organisms. For instance, in *Xenopus*, *Xvax2* expression is specifically restricted to the early ventral optic vesicle (Barbieri et al., 1999; Liu et al., 2001). Overexpression of *Xvax2* causes eye ventralization and expansion of the optic stalk, the ventralmost eye structure (Barbieri et al., 1999). Conversely, double knockout of both *Vax2* and the

closely related *Vax1* gene in mice causes eye dorsalization and lack of optical stalk specification (Mui et al., 2005). Single *Vax2* knockout causes a milder dorsalized phenotype with abnormal topography of axonal projections from retinal ganglion cells to the brain along the dorsoventral axis (Barbieri et al., 2002; Mui et al., 2002), indicating that *Vax2* is required for ventral eye specification, though its function is partially redundant with that of *Vax1*. Even after the completion of early eye morphogenesis and

polarity patterning events, *Vax2* continues to be expressed at high levels in restricted locations of the retina and brain, at stages when neural progenitors in these regions exit the cell cycle and differentiate (Ohsaki et al., 1999; Liu et al., 2001). During *Xenopus* metamorphosis, *Vax2* expression is maintained in the ventral aspect of the ciliary marginal zone (CMZ), a specialized region of the amphibian eye where proliferation and cell differentiation continue into adulthood (Liu et al., 2001; Casarosa et al., 2005).

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These observations suggest that, beyond early events of patterning and regionalization, *Vax2* might also be involved in controlling the proliferative properties of later neural progenitors. In this study, we have extensively compared the expression patterns of *Xvax2* mRNA and protein throughout embryonic development and in the adult eye and brain. We thought it important to compare the developmental expression of the *Xvax2* mRNA and protein in view of the recent demonstration of a posttranscriptional regulation of some homeobox transcription factors during eye development (Decembrini et al., 2006). In addition, we analyzed the effects of *Xvax2* overexpression on the proliferative properties of retina and brain precursor cells. Our results point to a possible new role for *Xvax2* in the control of cell proliferation of ventral eye and brain progenitors.

## RESULTS

### Comparison of *Xvax2* mRNA and Protein Expression During *Xenopus* Eye and Brain Development

We previously described the expression pattern of *Xvax2* mRNA during *Xenopus* embryonic development and showed that it is specifically localized to ventral forebrain and eye structures (Liu et al., 2001). To gain more insight into the possible roles of *Xvax2* and the regulation of its expression, we have extensively compared the developmental expression patterns of *Xvax2* transcripts and protein by means of a specific anti-*Xvax2* antibody (Liu et al., 2003). Consistent with the distribution of *Xvax2* transcripts revealed by in situ hybridization, *Xvax2* protein is first detectable at early neurula stage (st. 13/14) in the rostralmost neural plate (data not shown). It then appears to be located in the ventral forebrain and optic vesicle, olfactory placode and optic stalk at tail bud stages (st. 22–27; Fig. 1A,B,E,F). During early tadpole stages (st. 32–38), both *Xvax2* transcripts and protein persist in the above regions, but the expression domains of the protein appear to be more restricted than those of the transcripts; furthermore, while the signal

from the transcripts remains strong, a decrease of *Xvax2* protein expression level is observed (compare Fig. 1C,D with G,H).

At late tadpole stages (st. 42–50), when early eye morphogenesis has been completed and the four retina cell layers—retina pigmented epithelium (RPE), inner nuclear layer (INL), outer nuclear layer (ONL), ganglion cell layer (GCL)—have formed, *Xvax2* transcripts are still detectable in the ventral neural retina including the whole CMZ, but they become excluded from the ONL (Fig. 1I, and data not shown; Liu et al., 2001). At st. 42, *Xvax2* protein shows a similar distribution, except for its absence in the most peripheral CMZ (Fig. 1J). Moreover, in the later stages (st. 46, st. 50; Fig. 1K,L), expression of *Xvax2* protein becomes progressively weaker in the INL, so that by st. 50, it can be detected almost exclusively in the GCL (Fig. 1L). These observations suggest that expression of the *Xvax2* protein may be subjected to a posttranscriptional regulation during embryonic development, further restricting its localization compared with *Xvax2* mRNA.

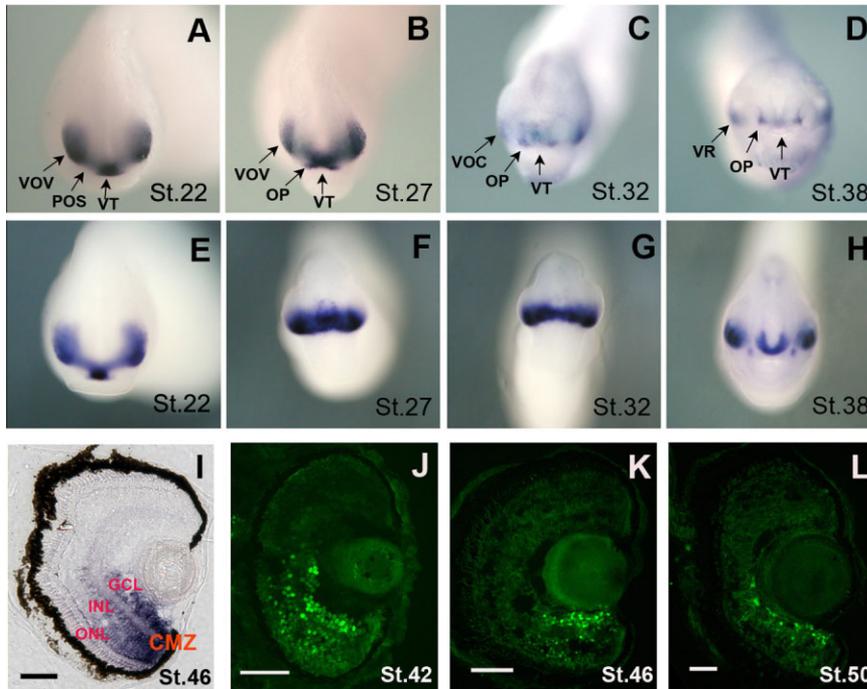
### *Xvax2* mRNA and Protein Expression in the Adult Brain and Eye

Expression of developmentally important homeobox genes in the adult central nervous system (CNS) has been little explored. Yet, the fact that localized niches of neurogenesis appear to persist even in the adult brain and eye (Lledo et al., 2006; Ohta et al., 2008) suggest that these genes may still be active in adult neural structures. Therefore, we analyzed the expression pattern of *Xvax2* transcripts and protein in the adult brain and eye of *Xenopus laevis*. In the adult brain, *Xvax2* transcripts are still clearly detectable in the derivatives of their embryonic locations in the ventral forebrain, including the olfactory bulb, ventral telencephalon, hypothalamus, hypophysis, and optic tract (Fig. 2A–E). In addition, strong hybridization signal becomes detectable in structures that do not show *Xvax2* mRNA expression at embryonic stages, such as the ventricular layer of the telencephalic pallidum, along the lateral ventricles (Fig.

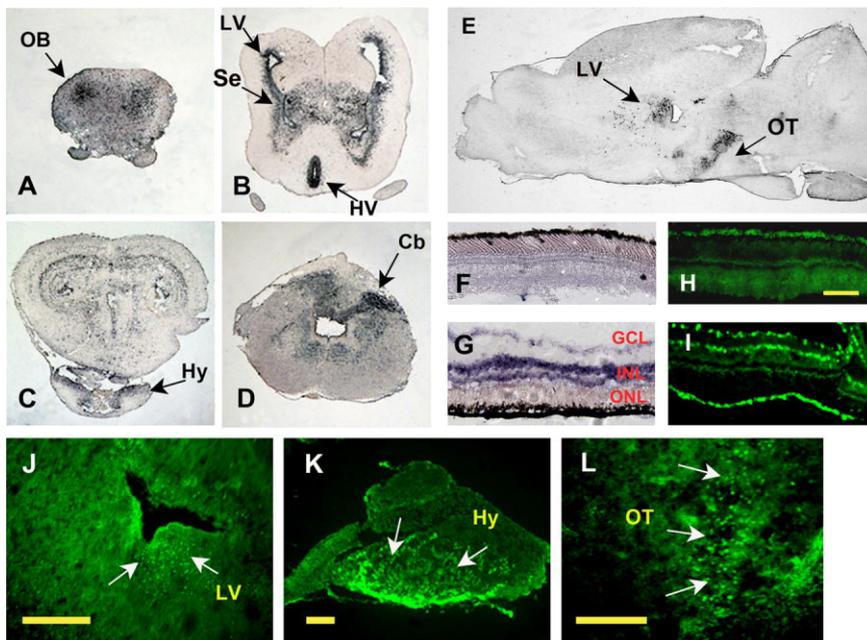
2B,E), and the cerebellum (Fig. 2D). Notably, both these structures have been associated with the presence of adult neurogenesis (Galli et al., 2002; Ahn and Joyner, 2005). *Xvax2* protein localization in the adult brain is clearly detectable in the same regions expressing *Xvax2* mRNA. (Fig. 2J–L). In the adult retina, *Xvax2* transcripts continue to be expressed in the INL and GCL of the ventral neural retina in the late tadpole stages (Fig. 2G). Expression of *Xvax2* protein is similarly detectable in the adult GCL and, differently from late embryonic stages, also in the adult INL (Fig. 2I). No expression of *Xvax2* mRNA or protein appears to be found in the adult dorsal retina (Fig. 2F,H).

### *Xvax2* Expression Colocalizes With Proliferative and Postmitotic Regions of the Late Embryonic Ventral Retina and Brain

Because *Xvax2* mRNA and protein appear to have different expression patterns in the CMZ during late tadpole stages (Fig. 1I–L), we decided to map them in more detail by comparison with the distribution of cell proliferation within the CMZ. To this aim, we co-revealed the *Xvax2* riboprobe, or the polyclonal anti-*Xvax2* antibody, with incorporated bromodeoxyuridine (BrdU) on st. 46 embryos. As previously described, the *Xenopus* CMZ can be divided into five zones: the most peripheral zone (zone I), containing retinal stem cells with low proliferation rate; the adjacent zones II–III, containing highly proliferative progenitors; the most central zones IV–V, containing postmitotic differentiating cells (Ohnuma et al., 2002). Both *Xvax2* mRNA and protein colocalize with the most central, BrdU-negative, postmitotic zone IV of the CMZ, and with the majority of the high BrdU signal region, corresponding to the highly proliferating zones II and III, (Fig. 3A,B). However, in the most peripheral zone I of the CMZ, only expression of *Xvax2* transcripts but not *Xvax2* protein is detectable. We also compared the distribution of BrdU incorporation and *Xvax2* localization in the ventral brain and found that also in this region *Xvax2* protein is ex-



**Fig. 1.** A–L: Expression of *Xvax2* as detected by immunohistochemistry (A–D, J–L) and in situ hybridization (E–I) on whole embryos (A–H) and coronal sections of eye (I–L) of *Xenopus laevis*. The developmental stages (St.) are indicated in each panel. Whole embryos are shown as anterior views, and the sections with dorsal side up. op, olfactory placode; pos, presumptive optic stalk; vov, ventral optic vesicle; voc, ventral optic cup; vt, ventral telencephalon; CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars = 100  $\mu$ m.

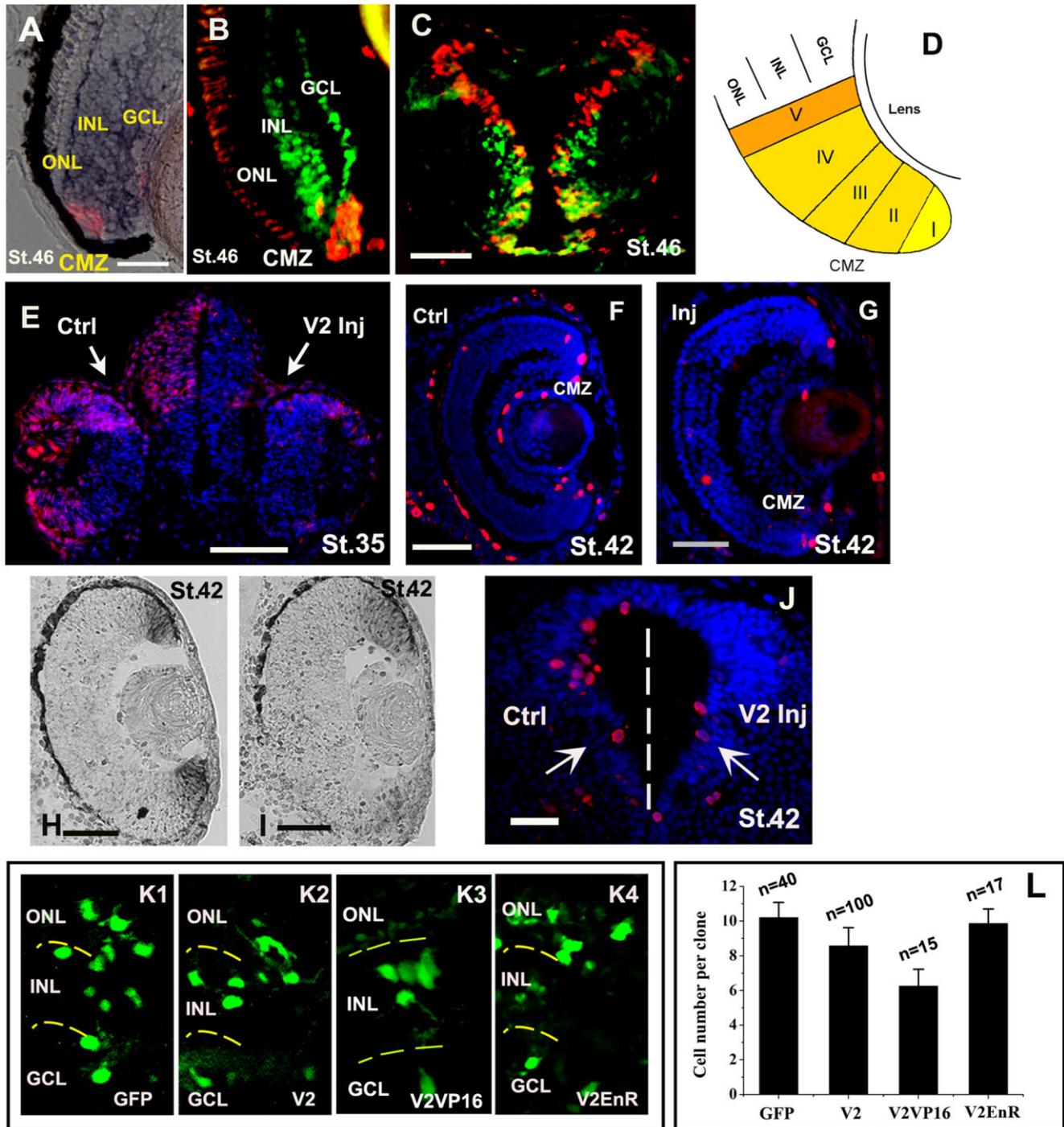


**Fig. 2.** A–L: Expression of *Xvax2* as detected by in situ hybridization (A–G) and immunohistochemistry (H–L) in the adult *Xenopus* retina (F–I) and brain (A–E, J–L). *Xvax2* mRNA localization (indicated by arrows) was detected on serial transversal sections of adult *Xenopus* brain at different anteroposterior levels (A–D) or on sagittal sections near the midline (E, J–L). F and G show the dorsal and ventral parts of coronal sections of the adult retina, respectively. H and I show sections corresponding to F and G, respectively. Cb, Cerebellum; Hy, hypophysis; HV, hypothalamic ventricle; LV, lateral ventricle; Se, Septum; OB, olfactory bulb; OT, optic tract; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars = 100  $\mu$ m.

pressed in both BrdU-positive proliferating progenitors in the ventricular zone and BrdU-negative differentiating cells outside the ventricular zone (Fig. 3C).

***Xvax2* Overexpression Inhibits Proliferation**

*Xvax2* protein expression in ventral eye and brain precursors undertaking proliferation and differentiation suggested that it might have a role in controlling the proliferative behavior of progenitor cells in these regions, a possibility that has not been explored so far. To this aim, we performed overexpression of *Xvax2* by mRNA microinjection or DNA lipofection, and analyzed its effects on cell proliferation. Injection of 100–150  $\mu$ g of *Xvax2* mRNA in embryos at early cleavage stages results in eye ventralization and coloboma (Barbieri et al., 1999; and data not shown). Therefore, to reduce these ventralization effects, we applied a lower dose of *Xvax2* mRNA (50  $\mu$ g) in one dorsal-animal blastomere of four-cell-stage embryos, leading to an almost normal eye morphogenesis on the injected side. The uninjected side served as an internal control. The effects on cell proliferation were monitored at different stages of retinogenesis by BrdU incorporation analysis or by immunohistological staining with antibody to phosphohistone-H3, a mitotic prophase marker (Fig. 3). At stage 35, both the retina and the brain on the uninjected side displayed a high level of BrdU incorporation. By contrast, a dramatic decrease in the number of BrdU-positive cells was observed in both eye and brain on the injected side (Fig. 3E, >40 sections/18 embryos), suggesting a strong repression of cell proliferation by *Xvax2* overexpression. Similar results were obtained after analyses of the effects on mitotic cells in the CMZ of retinas at st. 42. As shown in Figure 3F, at this stage, in uninjected eyes, phosphohistone-H3-positive cells are present in the CMZ and a few other retinal sites, where proliferation persists. At variance, on the injected side, almost no phosphohistone-H3 cells were observed (Fig. 3G, >40 sections/18 embryos). In agreement with the result obtained by BrdU analysis at earlier stages (Fig.



**Fig. 3.** **A–C:** Double staining for bromodeoxyuridine (BrdU) incorporation (red) and *Xvax2* expression in the ventral CMZ of *Xenopus* retina (A,B) and the ventricular zone of brain (C) at st. 46. Location of *Xvax2* mRNA (A) and protein (B,C) is shown in blue and green as detected by in situ hybridization and immunohistochemistry, respectively. **D:** A schematic model of the CMZ subdivision in *Xenopus* retina. Zone I, II–III and IV–V represent stem cells, proliferating retinoblasts, and postmitotic cells, respectively (Ohnuma S, et al., 2002, reproduced with permission of the Company of Biologists). **E–J:** Analysis of cell cycle activity in the control (Ctrl) and 50 pg *Xvax2*-injected (Inj) side of *Xenopus* embryos as detected by immunohistochemistry (E–G,J) and in situ hybridization (H,I). Cell cycle activity (red) was detected by BrdU incorporation (E), anti-phosphohistone-H3 (F–G,J) and *cyclinD1* expression (H, I) on coronal sections of eye (E–I) and forebrain (E,J) at st. 35 and st. 42. Cell nuclei are shown in blue by Hoechst staining (E–G,J). **K,L:** Gain-/loss-of-functional effects of *Xvax2* on clonal sizes of retinal cells. The fluorescent image shows lipofected cells in retinal sections at stage 42, after transfection of GFP (K1) or cotransfection of GFP with *Xvax2* (V2) construct *Xvax2* (K2), *Xvax2*-VP16 (K3), *Xvax2*-EnR (K4) at st.17/18. **L:** Quantification of retinal clonal size is shown. Error bars represent standard error of the mean (SEM). Clone counts are indicated on each column. CMZ, ciliary marginal zone; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; Scale bars = 100  $\mu$ m.

3E), a similar effect was observed at st. 42 in the anterior brain (Fig. 3J): the average phosphohistone-H3-positive cell number was 10.4 and 8.9 per section in eye and brain, respectively, on the control side, but reduced to 6.6 and 5.4 on the *Xvax2*-injected side (6 embryos, 12 sections for eye, 20 sections for brain). Furthermore, the expression of *CyclinD1*, a proliferation marker, was evidently reduced in the ventral CMZ of *Xvax2*-injected eyes (Fig. 3H,I; >40 sections/18 embryos). These data may be taken to suggest an extensive inhibition of proliferation in eye and brain cells caused by *Xvax2* overexpression. To further analyze the effects of *Xvax2* on cell proliferation during retinal development, we specifically misexpressed *Xvax2* in retinal progenitors by DNA lipofection of optic vesicles at st. 17/18. This was followed by clone size analysis at st. 42, when proliferation of neuronal precursors is almost completed in the retina, except for the CMZ (Perron et al., 1998). As shown in Figure 3K, compared with control eyes lipofected with green fluorescent protein (GFP) DNA alone, co-lipofection with *Xvax2* and GFP DNAs led to a decrease in the number of lipofected GFP-positive cells (Fig. 3K1,K2). A stronger decrease was observed after lipofection of *Xvax2-VP16* DNA, coding for a chimeric *Xvax2* protein fused to the strong transcriptional activator domain of VP16 (Stern and Herr, 1991; Fig. 3K3), while loading the VP16 construct alone has been shown to give no effect in the *Xenopus* retina (Zuber et al., 1999; Viczian et al., 2003; Wang and Harris, 2005). On the contrary, compared with controls, no obvious difference in the number of GFP-positive cells was observed after lipofection of *Xvax2-EnR*, coding for a fusion of *Xvax2* with the transcriptional repressor domain of *Drosophila* engrailed (Alexandre and Vincent, 2003; Fig. 3K4). We then quantified the clonal size of cells lipofected with different DNAs. In this analysis, a clone was defined as a cluster of transfected cells derived from an individual precursor and the clone size as the number of cells in the cluster, as previously described (Zuber et al., 1999). As shown in Figure 3L, the average clone size was 10.2 cells per clone in the control eyes lipofected with GFP only,

8.6 in *Xvax2*-lipofected eyes, 6.3 in *Xvax2-VP16*-lipofected eyes, and 9.8 in *Xvax2-EnR*-lipofected eyes, respectively. Thus, both *Xvax2* and *Xvax2-VP16* misexpression cause a decrease in clone size, with *Xvax2-VP16* producing a stronger effect than wild-type *Xvax2*. The lipofection of *Xvax2-EnR* show no obvious change in clone size compared with the GFP control. The decrease of clone size after *Xvax2* misexpression might be due to decreased proliferation of retinal progenitors, or increased cell death, or both. Therefore, we also analyzed the effects of *Xvax2* overexpression on apoptosis of retinal progenitor cells. Following *Xvax2* overexpression by 75–100 pg of mRNA injection at four-cell stage, TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling) staining of apoptotic cells and in situ hybridization for the apoptosis marker *Bcl2* were performed. Under either approach, no evident difference could be detected between the injected side and the control side within the retina (data not shown). Altogether, these results indicate that *Xvax2* overexpression can inhibit the proliferation of retina and brain progenitors and that *Xvax2* protein works as a transcriptional activator in this context.

## DISCUSSION

In this study, we show that *Xvax2* protein is expressed in restricted regions of the ventral eye and forebrain during *Xenopus* embryonic development and in the adult CNS. Within both the retina and the ventral brain, during neurogenesis, *Xvax2* protein is expressed by both proliferating progenitors and postmitotic differentiating cells. This expression pattern is largely overlapping, but more restricted than that of *Xvax2* mRNA, suggesting that *Xvax2* expression may be regulated by both transcriptional and posttranscriptional mechanisms. This appears to be particularly interesting at the level of the retinal CMZ, where the expression of *Xvax2* mRNA extends more peripherally than that of the protein (Fig. 3A,B). Within the frog CMZ, slow-dividing, self-renewing retinal stem cells are thought to be located closest to the periphery; tran-

sit-amplifying rapid proliferating retinal progenitors are found in the middle portion of the CMZ; finally, cells closer to the central edge of the CMZ have exited the cell cycle and entered the differentiation process (Perron et al., 1998). It is therefore tempting to speculate that fine spatiotemporal regulation of *Xvax2* translation in the CMZ might have a role in regulating proliferation of retinal stem/progenitor cells as they move toward the central retina during their differentiation process. Indeed, functional analysis highlighted a potential new role for *Xvax2* in controlling proliferation properties of retinal and brain progenitors. In particular, both in the retina and in the brain, *Xvax2* overexpression caused a strong decrease in cell proliferation, as shown by the much reduced numbers of BrdU and phosphohistone-H3-positive cells at the injected side of embryos injected with *Xvax2* mRNA, and the smaller size of retinal cell clones lipofected with *Xvax2* DNA (Fig. 3E–L). Previous work has shown that *Vax2* plays a crucial role in the dorsoventral patterning of the eye. In an earlier phase, *Vax2* and *Vax1* cooperate to specify the optic stalk in the ventralmost eye region (Mui et al., 2005). In a later phase, *Vax2* is involved in the establishment of the topography of retinotectal projections (Barbieri et al., 2002; Mui et al., 2002) and the arrangement of the rod photoreceptor pattern (Schulte et al., 2005), which are both asymmetric along the retinal dorsoventral axis. Although cell proliferation, cell cycle exit and differentiation are general features of neural progenitors through the CNS, they do not happen synchronously everywhere, but follow specific anteroposterior and/or dorsoventral gradients of neurogenesis and proliferation (Sur and Rubenstein, 2005; Lupo et al., 2006). In the zebrafish retina, for example, cell cycle exit and differentiation is initiated in the most ventral retina close to the optic stalk and gradually spread to more dorsal regions (Pujic and Malicki, 2004). This suggests that the same molecular mechanisms that control anteroposterior and dorsoventral CNS regionalization may also set up gradients of proliferation and differentiation along the same axis. For example, Sonic

hedgehog, a well-known ventral CNS morphogen (Lupo et al., 2006), has also been recently implicated in cell cycle regulation of neural progenitors (Cayuso et al., 2006; Locker et al., 2006). A recent study has also shown that the *Tbx2b* transcription factor is expressed with a dorsal high to ventral low gradient in the zebrafish retina (Gross and Dowling, 2005). Its inactivation impaired cell differentiation specifically in the dorsal retina, although markers of dorsal and ventral retinal compartments were not affected, thus lending further support to the hypothesis that the molecular mechanisms controlling differentiation of neural progenitors are asymmetric along the CNS dorsoventral axis. Our work provides a first indication that the homeobox gene *Xvax2*, in addition to control dorsoventral patterning of the eye, may also regulate differential proliferative properties of neural progenitors along the same axis. In future work, it will be very interesting to explore the connections between *Xvax2* activity and cell cycle regulation, and in particular whether *Xvax2* can directly regulate components of the cell cycle machinery, as suggested by the down-regulation of *CyclinD1* expression following *Xvax2* overexpression (Fig. 3H,I). In addition, because cell type determination in the retina has been shown to be closely linked to cell cycle regulation of retinal progenitors (Ohnuma et al., 2002; Casarosa et al., 2003; Cremisi et al., 2003; Cayouette et al., 2006; Decembrini et al., 2006), it will also be interesting to see whether *Xvax2* is involved in cell fate specification and differentiation of retinal progenitors, in a similar manner to the role played by *Tbx2b* in the dorsal retina (Gross and Dowling, 2005).

## EXPERIMENTAL PROCEDURES

### *Xenopus laevis* Embryos and Histology

Induction of ovulation in females, in vitro fertilization, embryo culture and staging were carried out as described (Newport and Kirschner, 1982; Nieuwkoop and Faber, 1994). For whole-mount in situ hybridization and immunohistochemistry, embryos were

fixed with MEMFA and Dent's fixative, respectively, and stored in ethanol at  $-20^{\circ}\text{C}$  before use. For cryosectioning, embryos, brains, and retinas were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), cryoprotected with 20% sucrose in PBS O/N at  $4^{\circ}\text{C}$ , embedded in OCT, then stored at  $-70^{\circ}\text{C}$  before use.

### In Situ Hybridization, Immunohistochemistry, and BrdU Incorporation

Digoxigenin (DIG) -labeled antisense RNA probes were generated from *Xvax2* (Barbieri et al., 1999) and *Cyclin D1* (Casarosa et al., 2003) plasmids. Whole-mount in situ hybridization was carried out according to Harland (1991), with modifications. In situ hybridization on cryosections was carried out as described in Kanekar et al. (1997). Whole-mount immunohistochemistry was carried out according to Lan et al. (2006), with slight modifications. A polyclonal anti-*Xvax2* antibody was generated by immunizing a rabbit with the purified bacterially overexpressed N-terminal domain of *Xvax2* (amino acids 1–104; Liu et al., 2003). Rabbit anti-phosphohistone-H3 (1:200, Upstate Biotechnology), Mouse anti-BrdU (1:200 Santa Cruz), fluorescein isothiocyanate/tetrarhodamine isothiocyanate (FITC/TRITC) -conjugated goat anti-rabbit IgG (1:200, Santa Cruz), and TRITC-conjugated goat anti-mouse IgG (1:100, Sigma) were used on cryosections. BrdU in vivo administration, labeling, and detection were carried out as described in Zuber et al. (1999). Hoechst 33258 (Sigma) was used for counterstaining of cell nuclei. Images of whole embryos were obtained with a SZX12 Olympus stereomicroscope and a C4040 digital camera, while those of sections were acquired with a IX71 Olympus inverted microscope using MicroCCD software.

### mRNA Microinjection and In Vivo DNA Lipofection

For microinjection experiments, capped mRNAs were synthesized from linearized plasmid templates using mMES-SAGE mMACHINE kits (Ambion). Embryos were injected at four-cell stage in one dorsal-animal blastomere

with 50 pg of pCS2-*Xvax2* (Barbieri et al., 1999) plus 100–500 pg of  $\beta$ -galactosidase used as a lineage tracer. pCS2-*Xvax2*-VP16 and pCS2-*Xvax2*-EnR plasmids were generated by subcloning the entire *Xvax2* open reading frame upstream of VP16 and EnR, respectively, in pCS2-VP16 and pCS2-EnR plasmids (kind gifts of Dan Kessler). In lipofection experiments, pCS2-*Xvax2*, pCS2-*Xvax2*-VP16, and pCS2-*Xvax2*-EnR DNAs were lipofected into the anterior neural region of st. 17/18 embryos as previously described (Ohnuma et al., 2002; Wang and Harris, 2005) at a final concentration of 0.25  $\mu\text{g}/\mu\text{l}$  with a 1:3 (w/w) DNA:DOTAP (Roche) ratio. pCS2-GFP reporter vector was cotransfected as a tracer. The ratio between GFP and target DNA was 2:1 (w/w). At st. 41/42, lipofected embryos were fixed, cryoprotected, and cryosectioned at 12  $\mu\text{m}$ . Sections were incubated with Hoechst 33258 for nuclear staining, washed three times for 5 min in PBS, mounted and then stored at  $4^{\circ}\text{C}$  in the dark before observation.

### Labeling for Apoptosis

Embryos were fixed with 4% paraformaldehyde in PBS, cryoprotected, and cryosectioned at 12  $\mu\text{m}$ . Apoptotic cells were detected by TUNEL using DeadEnd Colorimetric TUNEL System (Promega).

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