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SHORT COMMUNICATION

Cloning and developmental expression of the *Xenopus* homeobox gene *Xvsx1*

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Abstract In contrast to the high degree of evolutionary conservation of the *Vsx2/Chx10* gene family, vertebrate orthologues of *Vsx1* display more divergent sequences and spatio-temporal expression patterns. Here, we report the cloning and expression pattern of *Xenopus laevis Vsx1*. Differently from the mouse and zebrafish orthologues, *Xvsx1* transcription is activated at early neurula both in the evaginating eye vesicles and in the presumptive spinal cord. Compared to other retinal homeobox genes, such as *Xrx1*, *Xsix3* and *Xpax6*, *Xvsx1* is activated at a later stage; in addition, its anterior expression appears to be more specifically restricted to the retina. At tail bud stage, *Xvsx1* expression in retinal progenitors persists, and its neural tube expression, which in the spinal cord corresponds to interneurons, progressively expands anteriorly reaching the midbrain–hindbrain boundary. During retinal neurogenesis, *Xvsx1* expression is maintained in retinal progenitors and in a peripheral region of the ciliary marginal zone, while in the central retina, it becomes restricted to differentiated bipolar cells.

Keywords Vsx1 · Paired type homeobox · Retinogenesis · Ciliary marginal zone · Bipolar cells · Interneurons

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Introduction

Paired type homeobox genes, such as *Rx1*, *Pax6*, *Crx*, *Vsx1* and *Vsx2/Chx10*, play a pivotal role in eye development. In particular, in all vertebrate species analyzed, *Vsx1* and *Vsx2/Chx10* genes have been implicated in different aspects of progenitor cell proliferation and/or differentiation of retinal bipolar cells. Homologues of both *Vsx1* and *Vsx2* have been isolated in fish (zebrafish and goldfish; Passini et al. 1997), chick (*Chx10-1* and *Chx10*; Chen and Cepko 2000), mouse (*Vsx1* and *Chx10*; Liu et al. 1994; Chow et al. 2001) and humans (*Vsx1* and *Chx10*; Ferda Percin et al. 2000; Semina et al. 2000). Beside the homeodomain (HD), these proteins share another conserved domain, the CVC domain (Svendsen and McGhee 1995), located immediately carboxy-terminal to the HD. The function of the CVC domain is unknown, but apparently, all HD/CVC domain proteins share a conserved role in interneuron biology (Chow et al. 2001). The role of *Vsx* genes has been analyzed in mammals. *Vsx1* knockout in mouse displays incomplete terminal differentiation of a subtype of bipolar cells, the off-center cone bipolar cells; this phenotype is

associated with defects in retinal function, as shown by electroretinography (Chow et al. 2004; Ohtoshi et al. 2004). On the other hand, *Vsx2*/*Chx10* is responsible for the mutation *ocular retardation*, which is characterized by impaired retinal progenitor proliferation and bipolar cell differentiation (Burmeister et al. 1996). Moreover, in humans, *Vsx2*/*Chx10* mutations are associated with microphthalmia (Ferda Percin et al. 2000). According to the classification proposed by Chow et al. (2001), all HD/CVC domain proteins can be subdivided in two classes depending on their similarity to *Vsx1* or *Vsx2*. In contrast to the high level of sequence conservation observed for Vsx2 orthologues (Chow et al. 2001), comparative sequence analysis highlighted an unexpected divergence between mouse Vsx1 and human VSX1 proteins. Moreover, while *Vsx2*/*Chx10* displays a similar pattern of expression in the retina in all vertebrates analyzed, *Vsx1* expression differs among vertebrate species. In fish and mammals, no *Vsx1* expression, or only a very low level of expression, is detectable during the early phases of retinogenesis, while later in development, *Vsx1* expression appears to be restricted to bipolar cells (Passini et al. 1997; Chow et al. 2001). In contrast, in chick, *Chx10-1* (orthologue of *Vsx1*) is already expressed in early stages, in retinoblasts of the ventrotemporal region; at later stages, its expression expands to bipolar cells in the whole retina (Chen and Cepko 2000).

To extend the phylogenetic analysis of *Vsx1*, we cloned the *Xenopus laevis* orthologue of *Vsx1* and studied its developmental expression. Compared to other vertebrate *Vsx1* homologues, *Xvsx1* expression displays both similarities and distinct features.

Materials and methods

A tBlastn search identified a *Xenopus* expressed sequence tag (EST) clone (accession no. BC044049) as a cDNA highly related to *Vsx1*. Specific polymerase chain reaction (PCR) primers designed on this clone (forward 5′-CAAATACTG AGACATGACCGGGCG-3′ and reverse 5′-ACAGTGAC CACTGAACGGGGTGG-3′, 30 PCR cycles) amplified a 1,057 bp cDNA from stage-23 *Xenopus* RNA. *Xenopus* embryos were generated and staged as described (Casarosa et al. 2005). Whole-mount in situ hybridizations and in situ hybridizations on cryosections were performed as described previously (Harland 1991; Casarosa et al. 2005). Probes were generated by in vitro transcription of either an *Xvsx1* full-length plasmid or an *Nco*I deletion construct lacking the HD and most of the CVC domain. The results obtained with the two probes were indistinguishable. In vivo lipofection, immunohistochemistry [amacrine antibodies panel: anti-5-hydroxytryptamine (HT), DiaSorin, 1:1,000; anti-γ-aminobutyric acid (anti-GABA), DiaSorin, 1:1,000; anti-tyrosine hydroxylase, DiaSorin, 1:1,000], bromodeoxyuridine (BrdU) incorporation and detection were performed as described (Holt et al. 1990; Casarosa et al. 2005). Green fluorescent protein (GFP)-positive lipofected cells were detected in mature retinas (stage 42) after the procedure of mRNA detection with Fast Red (Roche) by indirect immunostaining using anti-GFP antibody (Molecular Probes, 1:2,000). For reverse transcriptase PCR (RT-PCR), total RNA was isolated from *Xenopus* embryos at different developmental stages from 4-cell (4c) stage until swimming tadpole. The corresponding cDNAs were prepared using oligo deoxythymidine primers and superscript reverse transcriptase (Gibco BRL). To amplify ornithine decarboxylase cDNA, the following primers were used: forward 5′-AATGGATTTCAGAGACCA-3′ and reverse 5′-CCAAGGCTAAAGTTGCAG-3′.

Results and discussion

Cloning and characterization of *Xvsx1*

To identify *X. laevis* homologues of *Vsx1*, we took advantage of the growing amount of information available about the *Xenopus* transcriptome. A database search initially identified a *Xenopus* EST clone highly related to *Vsx1*. This allowed us to design specific PCR primers that amplified a 1,057-bp cDNA displaying the highest sequence similarity with genes of the *Vsx1* family. The sequence analysis of the 343 amino acid predicted protein indicates the presence of a paired-type HD, a CVC domain, an RV region and an octapeptide (Fig. 1a; Chow et al. 2001). In particular, features that discriminate between Vsx1 and Vsx2, such as the presence of diagnostic amino acids within the HD and the CVC domain (Fig. 1b, arrowheads), the presence of the RV region and the absence of the OAR domain (Chow et al. 2001), allow to classify this protein as a *Xenopus* Vsx1 homologue. The Xvsx1 protein (GenBank accession no. DQ324366) shares the highest sequence identity with the deduced Vsx1 from the amphibian urodele *Cynops pyrrhogaster* (65%), followed by zebrafish (60%), chick (58%), human (52%) and mouse (49%) Vsx1.

Developmental expression of *Xvsx1*

The temporal expression pattern of *Xvsx1* was examined by semi-quantitative RT-PCR in embryos from 4c stage to tadpole (stage 45). No *Xvsx1* expression was detected in embryos younger than stage 15. This indicates that *Xvsx1* is not stored as a maternal mRNA and is not activated during the early phases of neural plate specification. *Xvsx1* expression is activated in the neurula at stage 15 and is maintained throughout the analyzed stages, reaching a peak around stage 42 (Fig. 1c).

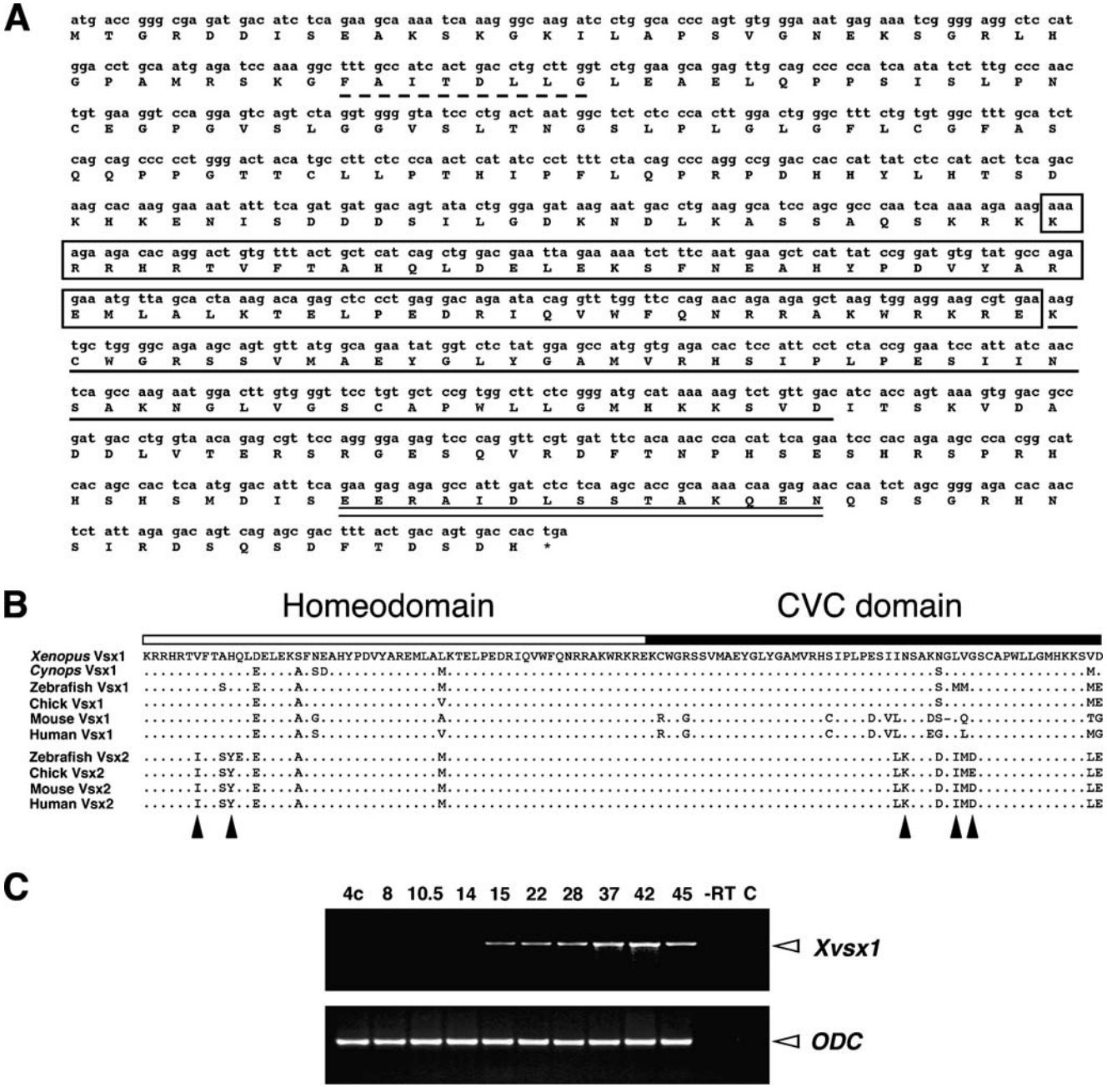

Fig. 1 **a** Nucleotide and deduced amino acid sequence of *Xvsx1* cDNA. *Dashed underline*, octapeptide; *boxed region*, homeodomain; *solid underline*, CVC domain; *double underline*, RV region. **b** Alignment of homeodomains and CVC domains from vertebrate Vsx proteins. *Arrowheads* point to diagnostic amino acids specifically found in members of the Vsx2 family. Sequences shown in this comparison are *Xenopus* Vsx1 (accession no. DQ324366), *Cynops* Vsx1 (accession no. AB119053), zebrafish Vsx1 (accession no. AAH59574), chick Vsx1 (accession no. NP_990100), mouse Vsx1 (accession no. AAL11431), human Vsx1 (accession no. Q9NZR4), zebrafish Vsx2 (accession no. NM_131462), chick Vsx2 (accession no. NM_204768), mouse Vsx2 (accession no. NM_007701) and human Vsx2 (accession no. NM_182894). **c** RT-PCR analysis to detect *Xvsx1* expression in embryos from 4c stage until stage 45 of development. *−RT* and *C* indicate reactions where reverse transcriptase or cDNA were omitted, respectively. *ODC*, ornithine decarboxylase

We analyzed the spatial localization of *Xvsx1* mRNA by in situ hybridization. In agreement with the RT-PCR results, *Xvsx1* transcripts are first detected at stage 15 in the presumptive retina territory and in two lateral stripes running along the posterior neural plate (Fig. 2a,c and d). Compared to other retinal homeobox genes, such as *Xrx1*, *Xsix3* and *Xpax6*, *Xvsx1* is activated at a later stage, thus suggesting that it may be downstream of early eye field transcription factors (Zuber et al. 2003). A comparison with *Xrx1* at stages 15 and 17 shows that the anterior *Xvsx1*

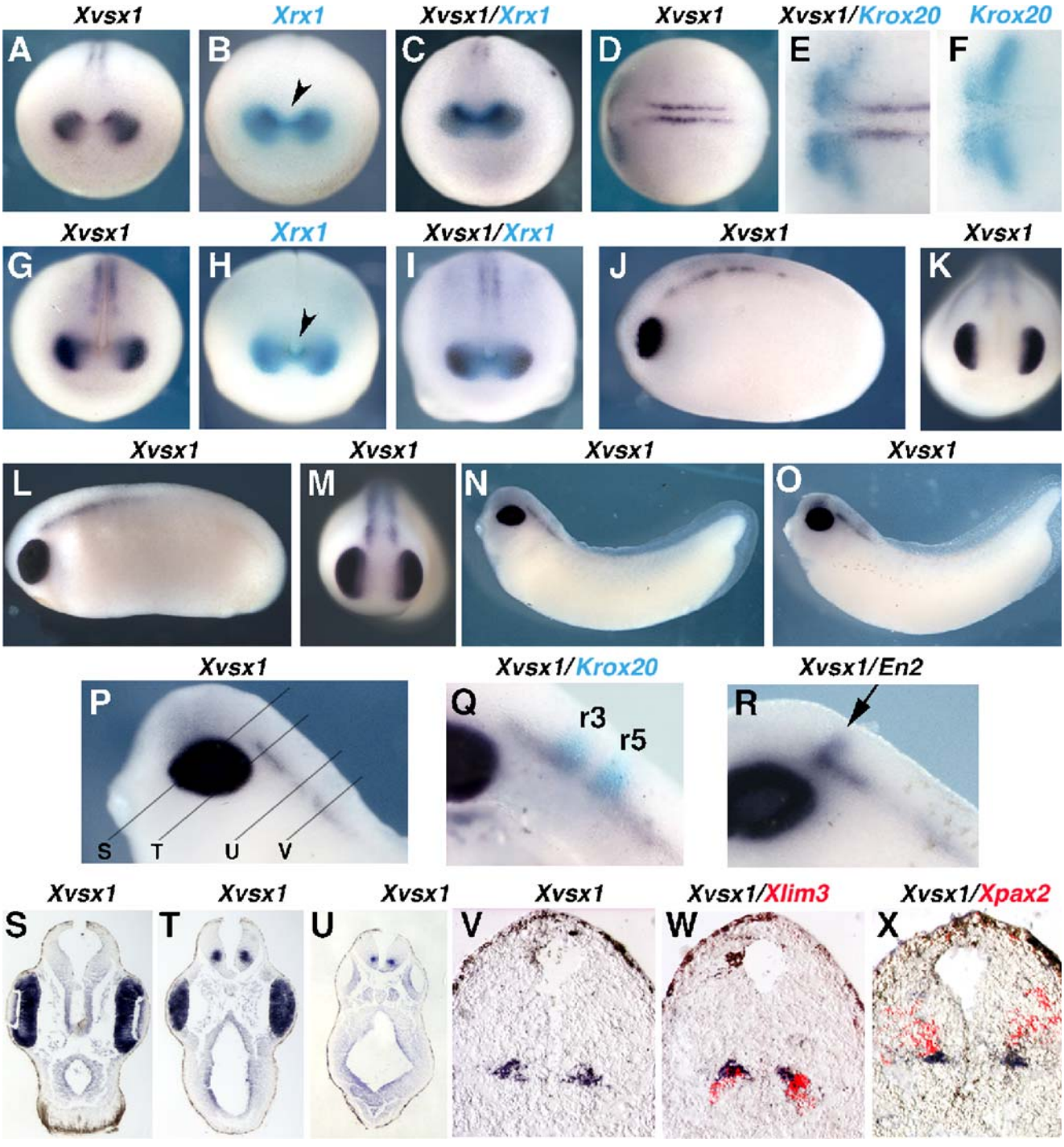

Fig. 2 Expression pattern of *Xvsx1*. The probes used are indicated and *color-coded* on the *top* of each panel. BM purple, BCIP and Fast Red (Roche) were used as alkaline phosphatase substrates. **a–r** Whole mount in situ hybridizations. **a–f** Stage 15: *frontal views*, *dorsal to the top* (**a–c**) and *dorsal views*, *anterior to the left* (**d–f**). **g–i** Stage 17: *frontal views*, *dorsal to the top*. **j**, **k** Stage 20: *lateral view*, *anterior to the left* (**j**) and *frontal view*, *dorsal to the top* (**k**). **l**, **m** Stage 25: *lateral view*, *anterior to the left* (**l**) and *frontal view*, *dorsal to the top* (**m**). **n**, **p–r** Stage 32: *lateral views*, *anterior to the left*. **o** Stage 35: *lateral view*, *anterior to the left*. **s–x** Transverse sections of stage-32 embryos. The *lines* shown in **p** indicate the approximate planes of section corresponding to diencephalon (**s**), hindbrain (**t**, **u**) and spinal cord (**v–x**). **s–u** Sections of whole-mount processed embryos. **v–x** Single (**v**) and double (**w**, **x**) in situ hybridizations performed on cryosections. The *arrowheads* in **b** and **h** point to the presumptive ventral diencephalon. r3 and r5 in **q** indicate rhombomere 3 and 5, respectively. The *arrow* in **r** indicates the *En2* staining at the midbrain–hindbrain boundary

expression is restricted to the eye vesicles (Fig. 2a–c,g–i) and does not extend to the presumptive ventral diencephalon, a medial region labelled by *Xrx1* (Fig. 2b,h, arrowheads). At stage 15, double in situ hybridizations with *Krox20*, a marker of rhombomeres 3 and 5, show that the posterior *Xvsx1* expression mainly corresponds to the presumptive spinal cord and weakly extends to the posterior hindbrain (Fig. 2e and f). During early tail bud stages (stages 20, 25, Fig. 2j–m), *Xvsx1* expression in the developing eye vesicles persists, while its posterior neural tube expression progressively expands more anteriorly. In late tail bud embryos (stage 32, 35, Fig. 2n and o), the eye expression of *Xvsx1* is clearly restricted to the neural retina (Fig. 2s). By these stages, the anterior border of *Xvsx1* expression in the posterior neural tube has reached the midbrain–hindbrain boundary, as shown by double in situ hybridizations with *engrailed-2* (*En2*), and displays a marked reduction in the posterior spinal cord (Fig. 2r). These longitudinal expression domains also show an area of reduced expression in the hindbrain at the level of rhombomere 4, which is delimited by the *Krox20* expression (Fig. 2q). To better define *Xvsx1* expression in the spinal cord, we performed double in situ hybridizations on transverse sections comparing *Xvsx1* with *Xlim3*, a marker of V2 interneurons and motoneurons, and *Xpax2*, a dorsal interneuron marker (Briscoe and Ericson 2001; Helms and Johnson 2003; Fig. 2w and x). *Xvsx1* expression is adjacent to the ventricular zone in a region located ventrally to *Xpax2* expression and that partially overlaps with the dorsal aspect of *Xlim3* expression domain. This analysis indicates that *Xvsx1* is expressed in a subpopulation of ventral interneurons probably corresponding to V2 interneurons, which have been shown to express also *Vsx2*/*Chx10* (Lee and Pfaff 2001).

In contrast to the widespread *Xvsx1* expression in the retina observed at stage 32, sections of stage 37 retinae show a restriction of *Xvsx1* expression to the outer rows of the inner nuclear layer (INL; Fig. 3a). At stage 37, *Xvsx1* is also expressed, although at lower levels, in the ciliary marginal zone (CMZ). In this area, *Xvsx1* displays a small gap in the expression, which is also maintained at stage 42 (Fig. 3b) and at metamorphic stages (Casarosa et al. 2005). The *Xvsx1* gap of expression coincides with the boundary of the highly proliferating CMZ located closer to the central retina, as shown by BrdU incorporation experiments (Fig. 3i and j). To better characterize the cells of the INL expressing *Xvsx1*, we combined in situ hybridization with immunostaining using a panel of three antibodies that, at tadpole stages, recognizes the majority of amacrine cells: anti-tyrosine hydroxylase, anti-GABA and anti-serotonin (5-HT; Huang and Moody 1998; see Materials and methods). No overlap was observed, indicating that amacrine cells do not express *Xvsx1* (Fig. 3b). Furthermore, to better define the identity of the *Xvsx1*-expressing cells, we carried out an

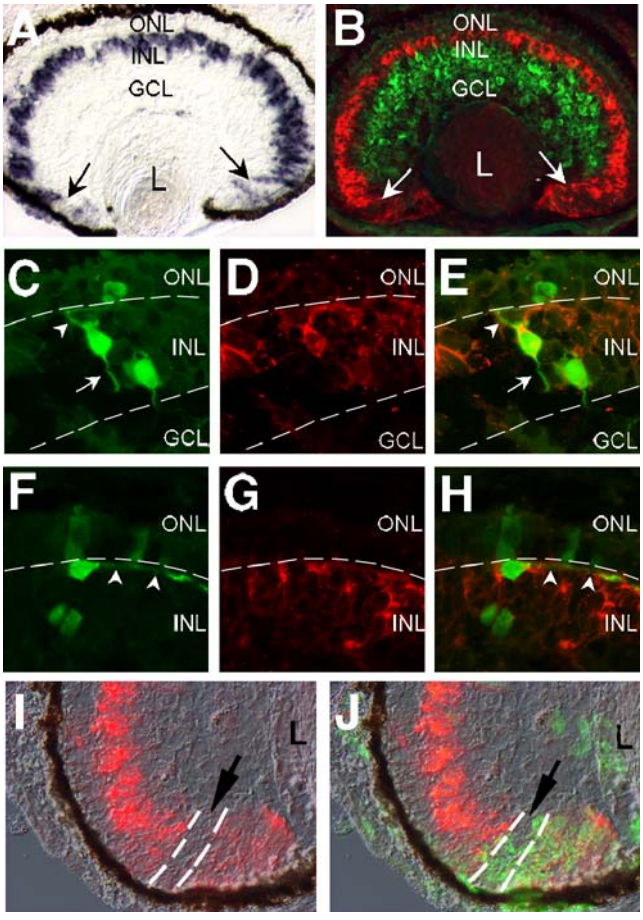

Fig. 3 *Xvsx1* mRNA expression during late retinogenesis. **a**, **b** *Xvsx1* expression on cryostat eye sections at stage 37 [**a** nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate (NBT–BCIP) detection] and stage 42 (**b** Fast Red detection), respectively. **b** The green immunofluorescence labels amacrine cells sub-types (tyrosine hydroxylase-positive, GABA-positive, 5-HT-positive). **c**–**h** Detection of lipofected GFP cDNA (**c**, **f** green immunofluorescence), *Xvsx1* probe (**d**, **g** Fast Red detection) or co-detection of both (**e**, **h**) in confocal images of stage 42 retinal cells. Images in **c**–**e** highlight a lipofected bipolar cell with apical (*arrowhead*) and basal (*arrow*) processes, which is Xvsx1-positive. **f**–**h** Xvsx1-negative horizontal cell displaying a typical process that extends along the outer plexiform layer (*arrowheads*). **i**, **j** Magnification of a stage 42 retina section at the level of the CMZ after in situ hybridization for *Xvsx1* (Fast Red detection) and BrdU immunodetection (**j** green immunofluorescence). *Arrows* in **a**, **b**, **i** and **j** indicate a gap of *Xvsx1* expression, delimited by *dashed lines* in **i** and **j** in the most central cells of the proliferating CMZ (which is labelled by BrdU incorporation in **j**). *ONL*, outer nuclear layer; *INL*, inner nuclear layer; *GCL*, ganglion cell layer. *L*, lens

analysis of GFP-lipofected retinae. GFP cell labelling by in vivo lipofection allows to distinguish among the different cell types of the *Xenopus* embryonic retina (Holt et al. 1990). In particular, among the cell types of the INL, we aimed at discriminating between bipolar and horizontal cells. Whereas horizontal cells are mostly localized in close proximity of the external border of INL and show cell bodies and processes tangentially elongated (Fig. 3f), the

body of bipolar cells usually stands in the middle of the INL and extends radially towards the outer nuclear layer (ONL) and the ganglion cell layer (GCL; Fig. 3c). *Xvsx1* hybridization signal always correlates with bipolar cells and never with horizontal cells (Fig. 3c–h, n=30 retinae, 600 lipofected cells). Altogether, these observations indicate that, as for the other described vertebrate orthologues, *Xvsx1* is expressed in bipolar cells of the INL (Passini et al. 1997; Chen and Cepko 2000; Chow et al. 2001).

In conclusion, unlike zebrafish and mouse *Vsx1* and similarly to the chick orthologue, *Xvsx1* displays an early expression in proliferating retinal progenitors until the beginning of retinogenesis when its expression becomes progressively restricted to bipolar cells and part of the CMZ. Moreover, *Xvsx1* is also expressed in the ventral hindbrain and in interneurons of the spinal cord, expression domains conserved with the zebrafish and chick orthologues.

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