

Genomes & Developmental Control

# Comparative analysis of gnathostome *Otx* gene expression patterns in the developing eye: implications for the functional evolution of the multigene family

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## Abstract

We have performed a detailed analysis of the expression pattern of the three gnathostome *Otx* classes in order to gain new insights into their functional evolution. Expression patterns were examined in the developing eye of a chondrichthyan, the dogfish, and an amniote, the chick, and compared with the capacity of paralogous proteins to induce a pigmented phenotype in cultured retina cells in cooperation with the bHLH-leucine zipper protein Mitf. This analysis indicates that each *Otx* class is characterized by highly specific and conserved expression features in the presumptive RPE, where *Otx1* and *Otx2*, but not *Otx5*, are transcribed at optic vesicle stages, in the differentiating neural retina, where *Otx2* and *Otx5* show a conserved dynamic expression pattern, and in the forming ciliary process, a major site of *Otx1* expression. Furthermore, the paralogous proteins of the dogfish and the mouse do not display any significant difference in their capacity to induce a pigmented phenotype, suggesting a functional equivalency in the specification and differentiation of the RPE. These data indicate that specific functions selectively involving each *Otx* orthology class were fixed prior to the gnathostome radiation and highlight the prominent role of regulatory changes in the functional diversification of the multigene family.

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## Introduction

Since Ohno's (1970) pioneer hypothesis, an increasing number of experimental data have convincingly shown that massive gene duplications have occurred early in the

vertebrate lineage prior to gnathostome radiation (Abi-Rached et al., 2002; Robinson-Rechavi et al., 2004). However, whether there is any relationship between the resultant increased genetic complexity and the morphological diversification of vertebrates is still controversial. Other unanswered questions concern the relative contribution of coding versus noncoding sequences in the functional evolution of vertebrate multigene families and the chronology of fixation of possible class-specific functions. These issues are more amenable to experimental testing using systematic comparisons of paralogous gene expression patterns and of the biochemical properties of paralogous proteins in a wide range of vertebrates. Knock-in experiments in mice have provided an important reference to address the equivalency of paralogous proteins in the

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context of an organism. These experiments have demonstrated that coding regions are extensively exchangeable in most genetic systems, in line with their functional redundancy or the possibilities of function shuffling often observed between paralogues (Bouchard et al., 2000; Hanks et al., 1995; Wang et al., 1996; see also below). However, there are a number of examples where class specificity for a given function is still observed (Cachaco et al., 2003; Hanks et al., 1998; Wang and Jaenisch, 1997). Comparative analyses of both expression patterns between paralogous genes and biochemical properties between paralogous proteins in a broad range of vertebrates are complementary to these approaches, since they can help to understand when specific expression territories or protein properties have been fixed.

So far, relatively few genetic systems have been compared using a detailed and systematic analysis in vertebrates. *Otx* genes provide an attractive model for such a study for several reasons. First, vertebrate *Otx* genes are derived from duplications of a single ancestral gene, after the splitting of cephalochordates, as clearly shown by comparison of their coding regions. All the vertebrate *Otx* proteins, including the highly divergent mammalian *Crx* proteins, share the repetition of a C-terminal 20–25 amino acid motif, present in a single copy in protochordates and echinoderms. This structural feature provides a strong synapomorphy of vertebrate *Otx* genes (Germot et al., 2001; Plouhinec et al., 2003; Williams and Holland, 1998). Second, the gene content of the *Otx* multigene family has been characterized in a wide range of vertebrates, including a number of osteichthyans, a group that contains all the vertebrate model organisms, a chondrichthyan, and several cyclostomes, hagfish or lampreys (Fig. 1). These studies have highlighted the presence of three orthology classes in

jawed vertebrates (*Otx1*, *Otx2*, *Otx5/Crx*). These classes do not show any clear relationships with the genes isolated in lampreys and hagfish, and therefore must have been fixed after the splitting of cyclostomes but prior to the gnathostome radiation (Germot et al., 2001; Plouhinec et al., 2003). Finally, a number of functional studies in several model organisms have provided an important reference for the physiological roles of this gene family. Thus, *Otx* genes play essential roles during gastrulation in the specification of cephalic regions, which are fulfilled by *Otx2* alone in mammals (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995). Analysis of mutant mice has shown that *Otx* genes are also involved in brain regionalization, corticogenesis, and the morphogenesis of sense organs (reviewed in Acampora et al., 2000). In particular, multiple roles of *Otx* genes have been described in the developing eye of the mouse, *Xenopus*, and zebrafish (Fig. 1). Analysis of *Otx1* and *Otx2* mutant mice and transactivation experiments in cultured cells have highlighted a cooperative role of these two paralogues in the early specification of the retinal pigmented epithelium (RPE), a role which may involve interaction with *Mitf*, a bHLH-leucine zipper transcription factor (Bovolenta et al., 1997; Martinez-Morales et al., 2001, 2003). In addition, *Otx1* and *Otx2* participate in the dorsoventral regionalization of the optic vesicle, the morphogenesis of the lens (Martinez-Morales et al., 2001), and later in the specification and differentiation of the neural retina. A tissue-specific inactivation of mouse *Otx2* in presumptive photoreceptors results in the total absence of this cell type, with a concomitant increase in number of amacrine cells (Nishida et al., 2003). In contrast, in *Crx*<sup>−/−</sup> mice, the photoreceptors are specified but their terminal differentiation fails, limiting *Crx* requirement to the final process of photoreceptor specification (Furukawa et al.,

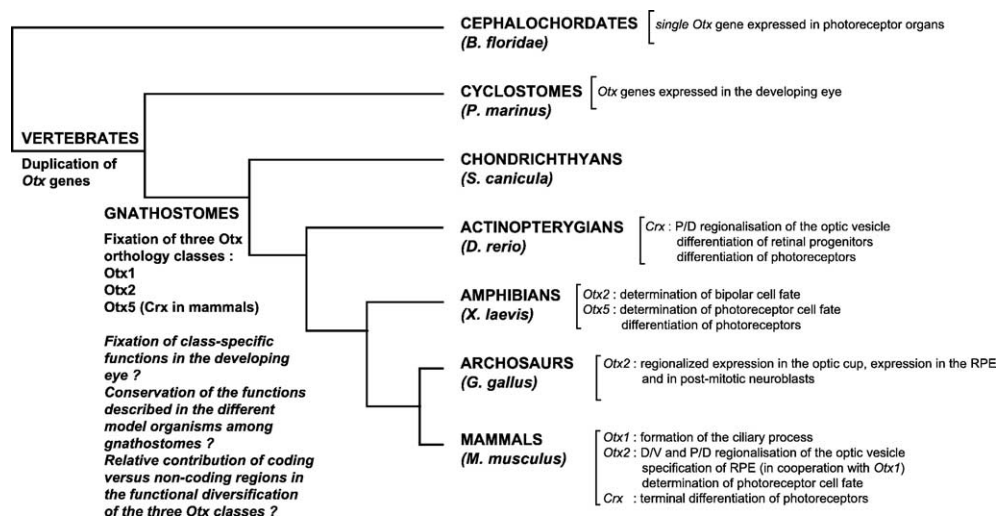


Fig. 1. Summary of the various functions fulfilled by *Otx* genes in the developing eye in vertebrates. For each phylogenetic group, the model organism considered is indicated. Functional analyses have only been performed in osteichthyans and the functions demonstrated in the different model organisms nevertheless substantially differ. The questions addressed in this study (conservation of the various functions reported in osteichthyans among gnathostomes, possible class specificities, relative involvement of coding versus noncoding regions in the functional diversification of the *Otx* multigene family) are indicated.

1999). Gain-of-function analyses in *Xenopus* have confirmed the involvement of *Otx* genes in the differentiation of the neural retina, but in a slightly different direction, since *Otx2* and *Otx5* seem to be, respectively, involved in the generation of an increased number of bipolar and photoreceptor cells, each at the expense of the other cell type (Vicizian et al., 2003). Recent knock-down experiments of one of the two representatives of the *Otx5/Crx* class in zebrafish *Crx* are consistent with its role not only in photoreceptor but also in other cell type differentiation (Shen and Raymond, 2004). Finally, *Otx1*<sup>−/−</sup> mice are devoid of ciliary process, a gnathostome characteristic, and display iris and eyelid malformations (Acampora et al., 1996).

The conserved basic structure of the eye in all vertebrate species and the multiple roles of *Otx* genes in its formation make this organ an ideal system to address the functional evolution of *Otx* genes in this taxon. To address the conservation of the multiple functions fulfilled by *Otx* genes in the developing eye and better understand the molecular mechanisms underlying the fixation of possible class-specific functions, we have performed an exhaustive expression pattern analysis of all three paralogues in this organ in an amniote, the chick, and in a shark, the dogfish *S. canicula* (Fig. 1). We have also compared the biochemical properties of the dogfish and mouse *Otx* paralogous proteins in the specification of RPE using different in vitro assays. The comparison of these data with those previously available in osteichthyans strongly suggests that the class specificity of the functions fulfilled by *Otx* genes in the developing eye was already fixed prior to gnathostome radiation.

## Materials and methods

### Whole-mount hybridizations of chick and dogfish embryos

Whole-mount hybridizations of dogfish and chick embryos were performed using digoxigenin-11 UTP-labeled antisense RNA probes according to standard protocols, as described in Sauka-Spengler et al. (2001). At stages when RPE is pigmented, an additional bleaching step was added according to the protocol reported in Broadbent and Read (1999). The chick (*Gallus gallus*, abbreviated as *Gg* hereafter) and dogfish (*Scyliorhinus canicula*, abbreviated as *Sc*) *Otx1*, *Otx2*, and *Otx5* RNA probes were transcribed from linearized pTZ19R recombinant vectors, which contain PCR-amplified fragments of the coding region. These fragments encode the C-terminal portion of *Otx* proteins, comprising helix 3 of the homeodomain and the stop codon, corresponding to the most variable part of the coding region. The use of each of these probes leads to the identification of prominent and highly specific expression territories, which remain unlabeled for the paralogous sequences, indicating the absence of cross-hybridization between them. The *Pax6*

RNA probe, which was used to hybridize chick sections was described in Turque et al., 1994. The chick *Chx10* RNA probe was synthesized starting from a pTZ18R vector containing a 687-bp fragment encoding the C-terminal part of the protein, comprising the homeodomain and the stop codon. The dogfish *Pax6* probe was described in Derobert et al. (2002).

### Histological sections following whole-mount hybridizations

Embryos were postfixed in 4% paraformaldehyde in PBS overnight at 4°C, rinsed in PBS, equilibrated in 20% sucrose overnight at 4°C and in 20% gelatin prior to inclusion. Mounting was performed after freezing in liquid nitrogen in OCT compound (Miles, Elkhart, Ind.). Cryostat sections were 10-μm thick. Photographs were taken using Nomarski optics.

### Hybridizations of cryostat sections

Hybridization of 10-μm dogfish and chick cryostat sections was performed according to Sauka-Spengler et al. (2001), using the digoxigenin-11 UTP-labeled antisense RNA probes described above.

### Transactivation and overexpression experiments in cultured neuroretina cells

The mouse *Crx*, dogfish *ScOtx1*, *ScOtx5*, mouse *NRL*, and quail *Pax6* expression vectors were constructed by inserting the corresponding full-length coding regions into the *XbaI*–*HindIII* sites of the pVNC3 vector, in phase with the C-terminal HA tag. All the clones were controlled by sequencing. The mouse *Otx2* expression vectors were described in Martinez-Morales et al. (2003). Cell cultures, transfections, and CAT assays were performed as described in Martinez-Morales et al. (2003).

### In vitro protein–protein interaction assays

The constructs used to translate *Otx* proteins are described above, and GST-, GST-Mitf, or GST-Mitf (193–413) expression vectors were reported in Planque et al. (2001). The <sup>35</sup>S-radiolabeled proteins were translated in vitro using the TNT system (Promega). The GST chimerical proteins were extracted from bacteria following the Pharmacia Biotech instructions. The labeled proteins were preincubated with empty Glutathion Sepharose beads 30 min at 4°C. The GST proteins on Glutathion Sepharose beads were preincubated with 40 μg BSA. Bead volumes were kept constant by adding empty beads. The pull down assays were performed in 25 mM HEPES pH7.5; 150 mM KCl; 12.5 mM MgCl<sub>2</sub>; 0.1% NP40; 20% glycerol. Proteins were incubated 1 h at 4°C, then the beads were washed four times in 20 mM Tris–HCl pH 8; 100 mM NaCl; 1 mM EDTA; 0.5% NP40.

## Results

### *Otx1, Otx2, and Otx5 expression in chick and dogfish embryos at optic vesicle stages*

To gain insight into the expression of *Otx* genes during early phases of eye development, we performed whole-mount hybridizations followed by cryostat sections, starting from neural plate stages (chick) or neural tube closure (dogfish) to optic cup stages. Expression patterns of the chick *GgOtx2* gene and the dogfish *ScOtx5* gene have been reported from optic evagination to optic cup stages (Bovolenta et al., 1997; Sauka-Spengler et al., 2001), and we therefore focused on *GgOtx1*, *GgOtx5*, *ScOtx1*, and *ScOtx2*.

#### *Chick*

*GgOtx1* is first transcribed at gastrula stages (not shown). At neural plate stages (HH7–HH8), its signal is prominent in both the neural plate and the surface ectoderm, with a sharp posterior border (Figs. 2A–B), but it is largely excluded from the ventral midline and neural crest cells (Figs. 2A'–B'). Upon neural tube closure, the expression of *Otx1* in the neuroectoderm is restricted to the developing forebrain and midbrain, as observed in all gnathostomes (Figs. 2C–D). The staining is observed over the whole optic evagination at stage HH10 (Fig. 2C'), but it becomes mainly restricted to the dorsal part of the optic vesicle at stage HH12 (Fig. 2D'). The adjacent surface ectoderm is also labeled. This expression pattern largely overlaps with the one reported for *GgOtx2* (Bovolenta et al., 1997 and data not shown).

#### *Dogfish*

*ScOtx1*, *ScOtx2*, and *ScOtx5* expression patterns were studied by whole-mount hybridization at stages 15, 18 (Figs. 2E–H), and 22 (Figs. 3J–L). As previously published (Sauka-Spengler et al., 2001), only faint *ScOtx5* expression, excluding the developing eye, can be detected before stage 22. At stage 15, *ScOtx1* and *ScOtx2* transcripts are detected in the anterior neuroectoderm, the surface ectoderm, and the developing gut (Figs. 2E, E', G, and G'). The expression domain of the two genes completely overlaps in the entire neuroectoderm. However, their expression only partially coincides in the primitive gut, where *ScOtx1* transcripts are restricted to dorsal territories, excluding the axial mesendoderm, while those of *ScOtx2* are equally distributed in the entire endoderm and axial mesendoderm (Figs. 2E' and G'). In the surface ectoderm and neural crest cells, *ScOtx1* and *ScOtx2* transcripts also show different distributions, with the first predominant in the dorsal and the second in the ventral portions of the ectoderm (Figs. 2E' and G'). At stage 18, *ScOtx1* signal in the optic vesicle becomes restricted to dorsolateral territories (Fig. 2F') and *ScOtx2* transcripts are mostly present in the dorsalmost part of the vesicle neuroepithelium (Fig. 2H'). At this stage, *ScOtx2* transcripts become undetectable in the surface ectoderm,

where *ScOtx1* expression persists, adjacent to the optic vesicle (Figs. 1H and 2F').

### *Otx1, Otx2, and Otx5 expression in chick and dogfish embryos at optic cup stages*

At optic cup stages, *GgOtx1* is transcribed in the surface ectoderm and, as *GgOtx2* (Bovolenta et al., 1997 and Fig. 3E), in the prospective pigmented epithelium (Fig. 3D). However, *GgOtx1* and *GgOtx2* expression territories do not overlap completely, since *GgOtx1* expression, but not that of *GgOtx2* (Figs. 3B and E; Bovolenta et al., 1997), is prominent in the distalmost optic cup and in the developing choroid fissure (Figs. 3A and D). Expression of the third paralogue *GgOtx5* is first detected at optic cup stages (stage HH15). It is restricted to the prospective pigmented epithelium, with a very similar pattern to that of *GgOtx2* but with a reproducibly lower signal intensity (Figs. 3C and F). Expression of the three paralogues is maintained in the prospective pigmented epithelium until stage 23 (Figs. 3G–I). At this stage, *GgOtx1* transcripts are also detected in the surface ectoderm and presumptive cornea. At later stages (E5 to E7), *GgOtx2* in the RPE remains prominent and a fainter *GgOtx5* expression is also detectable. In contrast, no *GgOtx1* expression can be observed outside the margin (Figs. 4A–D and data not shown). As in the chick, the presumptive RPE is a major site of *Otx* gene expression in the dogfish at optic cup stages (stages 22–24). Both *ScOtx1* and *ScOtx2* display strong signals in the entire prospective pigmented epithelium (Figs. 3J and K) while *ScOtx5* transcripts are only detected in its dorsal portion, as previously described (Sauka-Spengler et al., 2001, Fig. 3L). Expression of *ScOtx1*, but not of *ScOtx2* or *ScOtx5*, is also visible in the marginal zone of the neural retina (Figs. 3J–L). In older embryos (3.5–4.5 cm), *ScOtx5* expression becomes prominent in the RPE, while only a faint *ScOtx2* signal is observed (Figs. 5A–B and D–E). As in the chick, no *ScOtx1* expression is observed in the RPE at these stages except at marginal levels.

### *Expression of the chick Otx genes in the differentiating neural retina*

The localization of *GgOtx1* and *GgOtx5* transcripts in the developing neural retina was studied by hybridization of cryostat sections starting from E 3.5 to postnatal stages. *GgOtx2* expression has already been reported (Bovolenta et al., 1997) but was systematically included for comparisons. *Pax6* and *Chx10* were used as markers for prospective amacrine and bipolar cells, respectively. In the neural retina, *GgOtx5* transcripts were first detected at E5, while the onset of *GgOtx1* expression takes place substantially later, at E11. Between E5 and E7, *GgOtx5* and *GgOtx2* expression domains largely overlap (Figs. 4A–D). In both cases, the transcripts are restricted to the neuroblast layer and the retinal pigmented epithelium. The staining shows a marked



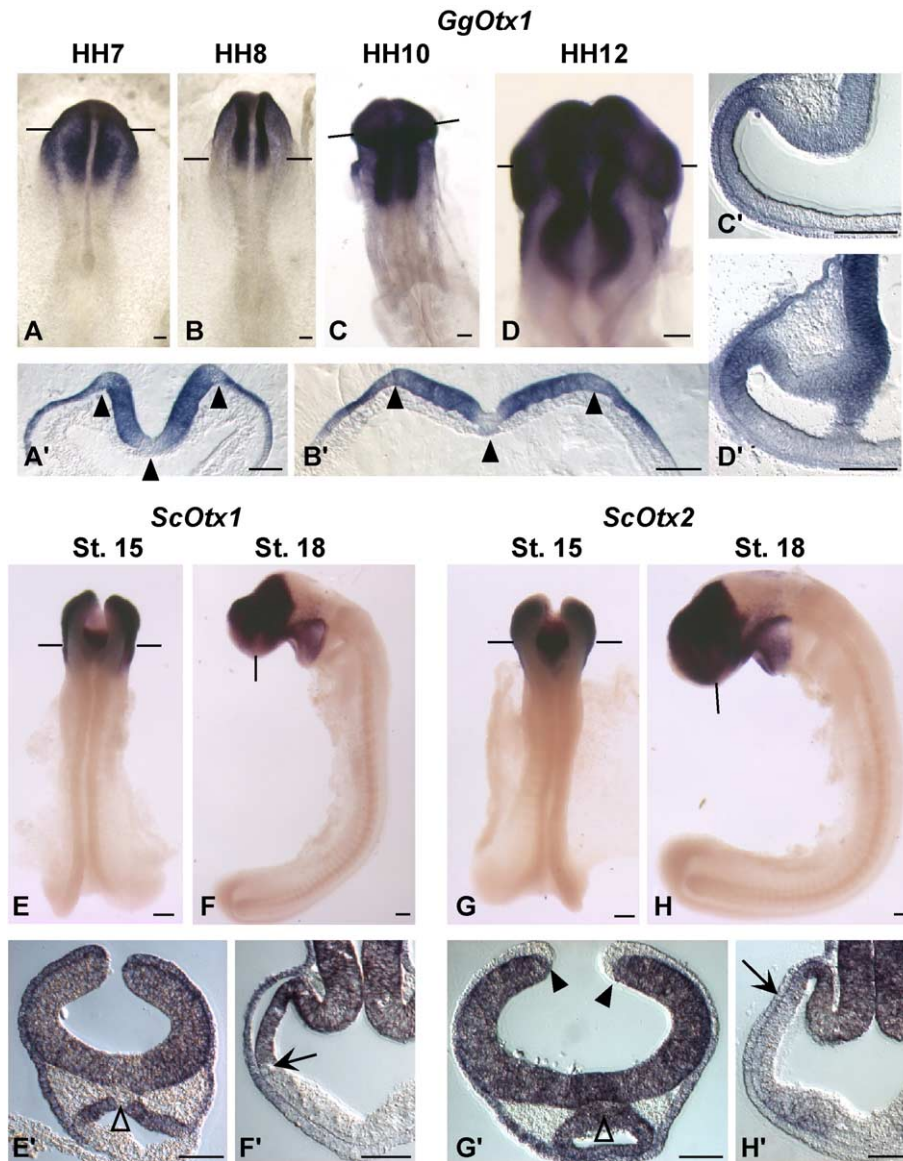


Fig. 2. Expression patterns of *GgOtx1*, *ScOtx1*, and *ScOtx2* genes during early eye development. (A, B, C, and D) Dorsal views of chick embryos following whole-mount hybridization with the *GgOtx1* probe at stages HH7, HH8, HH10, and HH12, respectively. A', B', C', and D' show transverse sections of the embryos photographed in A, B, C, and D, respectively. The planes of sections are shown by thin lines in A–D. At neural plate stages, the labeling is present in both the surface and neural ectoderm, but it excludes the midline and neural crest (black arrowheads in A' and B'). The signal becomes restricted to dorsal territories at optic vesicle stages (C' and D'). A faint labeling becomes visible at the level of the otic placodes (stars in D) at stage HH12. E and F) and G and H) dogfish embryos hybridized with *ScOtx1* and *ScOtx2* probes, respectively. E and G show dorsal views of stage 15 embryos and F and H lateral views of stage 18 embryos. E', F', G', and H' show transverse sections of the embryos photographed in E, F, G, and H, respectively. The planes of sections are indicated by thin lines in E–H. The midline mesendoderm (opened arrowheads in E' and G') is negative for the *ScOtx1* probe but positive for *ScOtx2*. A sharp boundary of *ScOtx2* expression (black arrowhead in G') is observed between the labeled neuroectoderm and the adjacent ectoderm. Expression of the paralogous genes becomes restricted to the dorsal part of the optic vesicle at stage 18, albeit with different ventral boundaries (arrows in F' and H'). Scale bar = 100  $\mu$ m.

gradient of intensity across the neural retina, reaching background levels in its innermost aspect, and overlaps with *Pax6* expression domain, which exhibits an inverse labeling gradient (Figs. 4A–E). At E11, when the inner and the outer nuclear layers (INL and ONL) segregate, *GgOtx2* expression is prominent in both the ONL and the outer half of the INL, with a sharp inner boundary (Fig. 4G). The *GgOtx5* expression domain is largely overlapping, although the signal intensity is substantially lower in the outer half of

the INL (Fig. 4H). *GgOtx1* transcripts also become detectable in the INL at this stage, in the proximity of the optic nerve within the *GgOtx2*- and *GgOtx5*-positive territory (Fig. 4F). In the INL, the expression domains of the three paralogues are included in the *Chx10*-positive territory and are largely complementary to the *Pax6*-expressing domain (Figs. 4I and J). These *Otx* expression patterns persist at E14 (Figs. 4K–M) and E16 (data not shown), with increased *GgOtx1* signal intensity in the outer

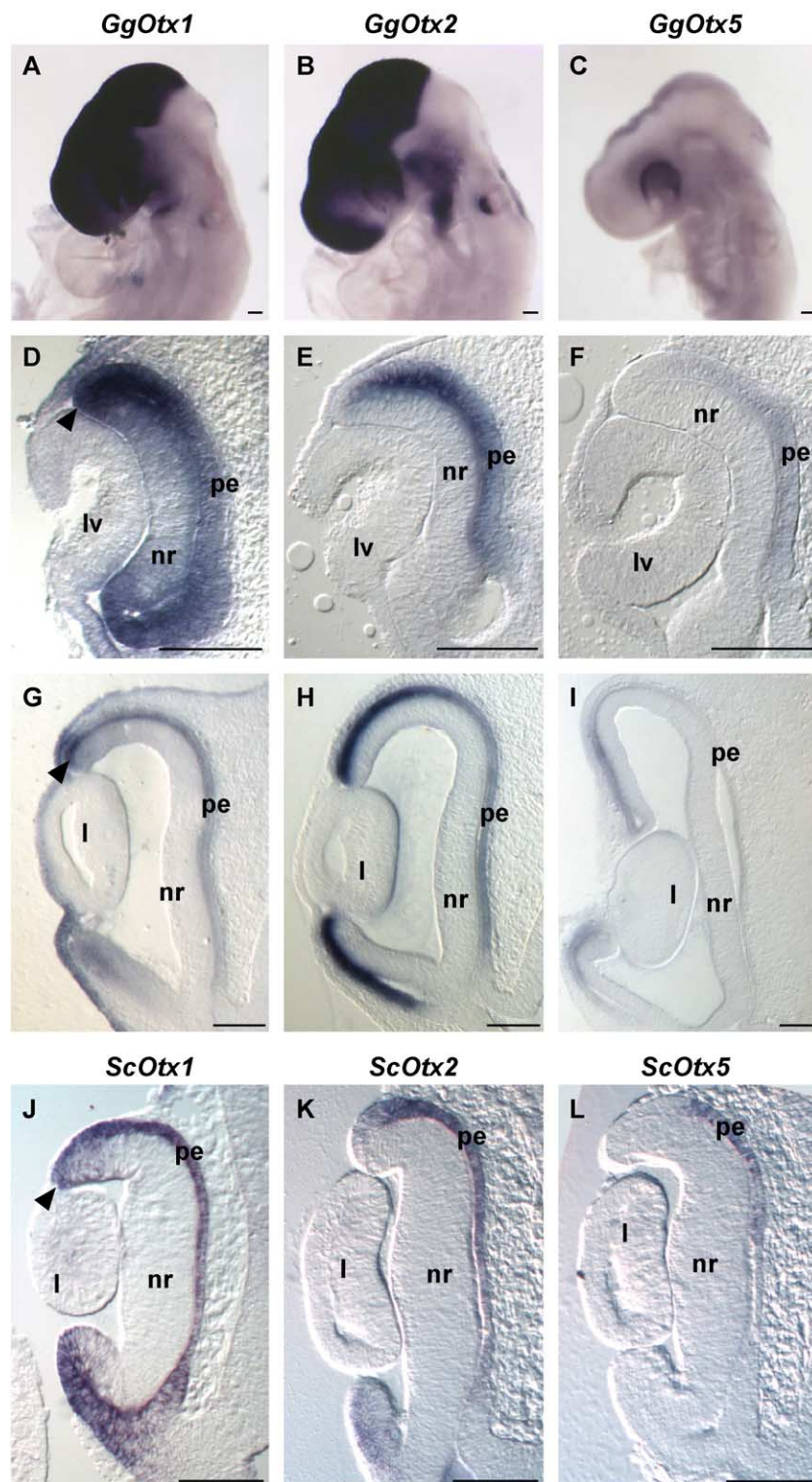


Fig. 3. Expression patterns of the three *Otx* classes in the developing optic cup of chick and dogfish embryos. (A, B, and C) Lateral views of stage 15 (A) and 16 (B and C) chick embryos after whole-mount hybridizations with *GgOtx1* (A), *GgOtx2* (B), and *GgOtx5* (C) probes. D, E, F and G, H, I are transverse sections of the optic cup after whole-mount hybridizations of stage 16 and stage 22 chick embryos, respectively, using probes for *GgOtx1* (D and G), *GgOtx2* (E and H) and *GgOtx5* (F and I). (J, K, L) Transverse sections of stage 22 (J and L) or stage 23 (K) dogfish embryos at the optic cup level after whole-mount hybridizations with *ScOtx1*, *ScOtx2*, and *ScOtx5* probes, respectively. In both species, the transcripts are mainly restricted to the prospective pigmented retina (pe) and absent from the neural retina (nr). The marginal zone of the neural retina expresses *Otx1*, but not the paralogous genes, in both species (marginal *Otx1* signal indicated by a black arrowhead in D, G, and J). A transient *ScOtx1* signal is visible in the dogfish lens (l) when it closes. l, lens; lv, lens vesicle; nr, neural retina; pe, prospective pigmented epithelium. Scale bar = 100  $\mu$ m.



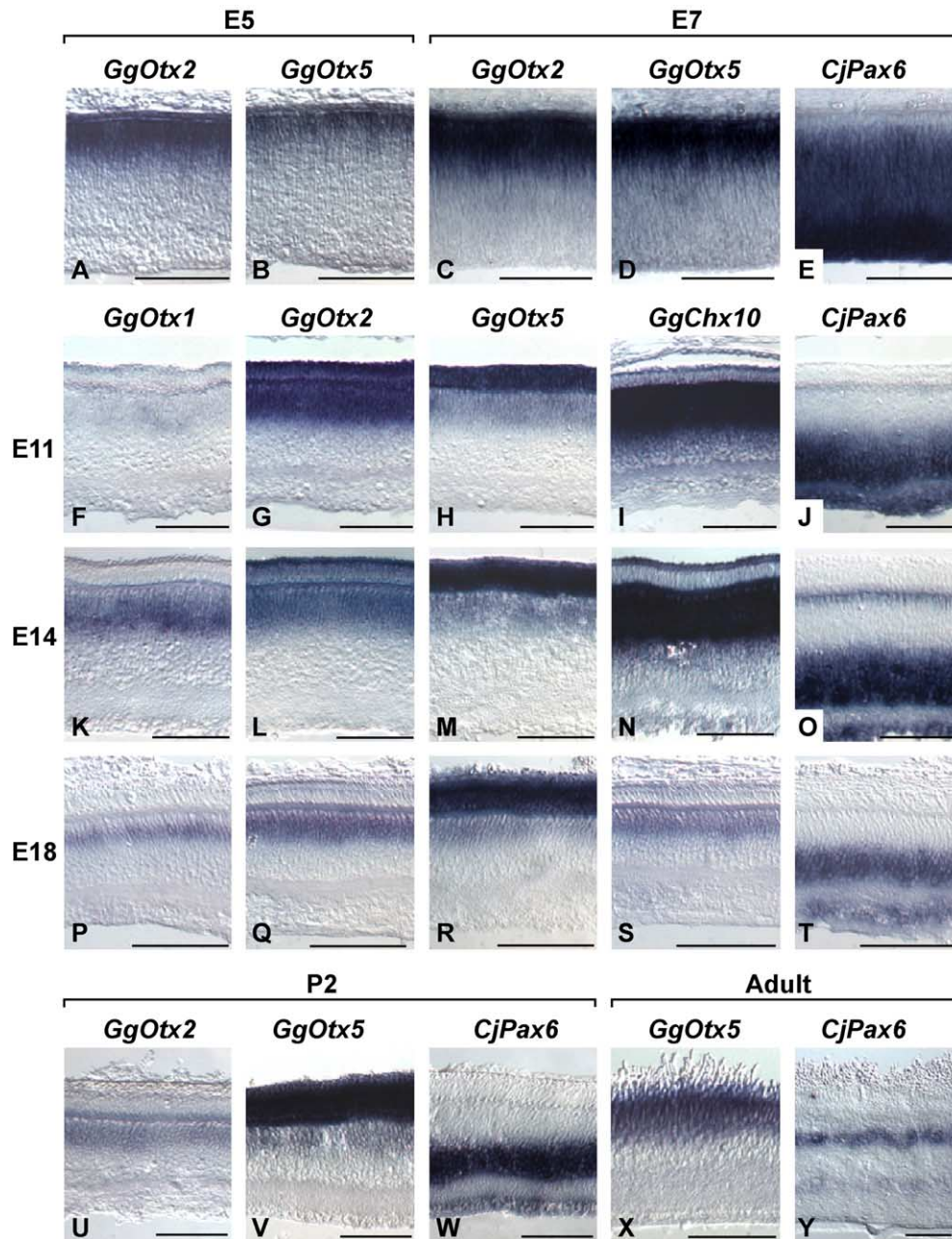


Fig. 4. Expression patterns of *GgOtx1*, *GgOtx2*, and *GgOtx5* in the differentiating neural retina of the chick, comparison with *Chx10* and *Pax6*. A, B, C–E, F–J, K–O, P–T, U–W, and X–Y show sections of chick retina at E5, E7, E11, E14, E18, P2, and P60, respectively, after hybridizations with the following probes. (F, K, and P) *GgOtx1*; (A, C, G, L, Q, and U), *GgOtx2*; (B, D, H, M, R, V, and X) *GgOtx5*; (E, J, O, T, W, and Y), *CjPax6*; (I, N, and S), *GgChx10*. *Gg*: *Gallus gallus*. *Cj*: *Coturnix japonica*. In each case, the sections shown are located in the central part of the retina. Scale bar = 100  $\mu$ m.

half of the INL, excluding the prospective horizontal cells (compare Figs. 4K and O), where *Pax6* is transiently transcribed (Fig. 4O compared with Figs. 4J and T). At E18, the outer segments of the photoreceptors are fully developed. At this stage, *GgOtx2* expression persists in the INL, but substantially declines in the ONL (Fig. 4Q), while *GgOtx1* and *GgOtx5* distribution is basically unchanged (Figs. 4P and R). At postnatal stages, the expression of *GgOtx1* becomes undetectable, while that of *GgOtx2* and *GgOtx5* persists (Figs. 4U–V). In adults, only *GgOtx5*

expression is maintained in the photoreceptor cell layer and at lower level in the inner nuclear layer, excluding the *Pax6*-positive amacrine cells (Figs. 4X and Y).

#### *ScOtx2 and ScOtx5 expression in the neural retina*

In chondrichthyans, the mature retina broadly contains the same cell types as in osteichthyans and shows the same topological organization in three nuclear layers, separated by plexiform layers. The chronology of retinal differ-

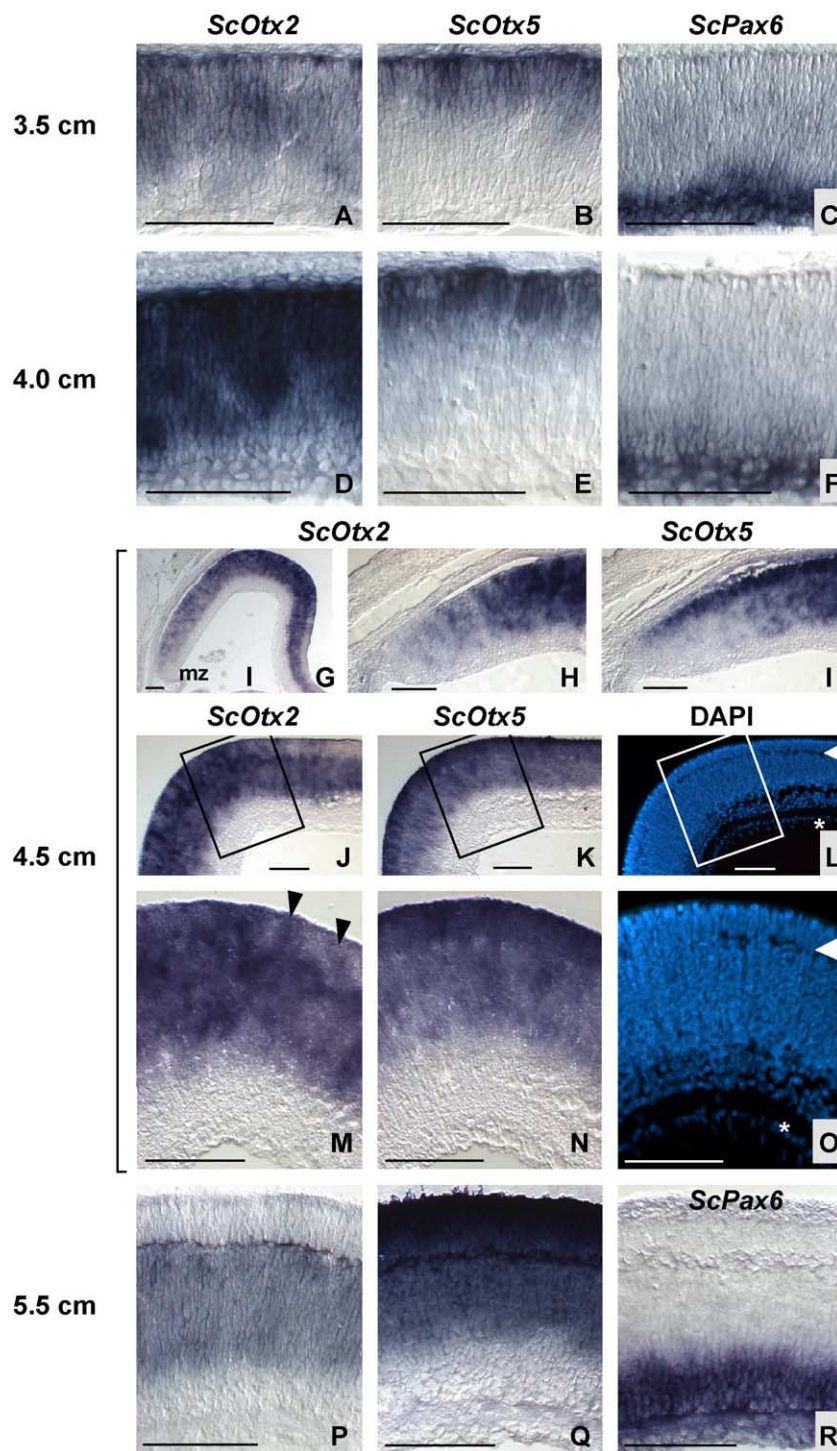


Fig. 5. Expression patterns of *ScOtx2* and *ScOtx5* in the differentiating neural retina of the dogfish, comparison with *ScPax6*. A–C, D–F, G–O, and P–R show retina sections in 3.5-, 4.0-, 4.5-, and 5.5-cm dogfish embryos, respectively, after hybridizations with the following probes: (A, D, G, H, J, M, and P) *ScOtx2*; (B, E, I, K, N, and Q) *ScOtx5*; (C, F, and R) *ScPax6* or after DAPI staining (L and O). G is a low magnification view of the dorsal part of the retina of 4.5-cm embryos, showing different distributions of *ScOtx2* transcripts in central and more peripheral parts of the retina. H and I are high magnification views of the retina margin of 4.5-cm embryos after hybridization with *ScOtx2* and *ScOtx5* probes. At this level, a recapitulation of earlier expression patterns can be observed in the dogfish as in other vertebrates. J, K, and L show retina sections at the level of the transition zone between the central retina, where the three nuclear layers have segregated, and more peripheral regions, where ONL and INL are not yet visible. M, N, and O show higher magnification views of this transition zone at the levels shown squared in J, K, and L. A white arrowhead in L and O shows the forming outer plexiform layer, between the ONL and INL, a white star shows the level of the ganglion cell layer. Black arrowheads in M point to *ScOtx2*-positive cells in the newly formed ONL. l, lens; mz, marginal zone; *Sc*: *Scyliorhinus canicula*. Scale bar = 100  $\mu$ m.



entiation remains unknown in this species, but histological analyses and Hoechst staining show that in 3.5-cm dogfish embryos, the inner ganglion cell layer is morphologically visible (Figs. 5A–C and data not shown). While no indication of segregation of the ONL and INL is visible in 4.0-cm embryos (Figs. 5D–F), these two layers become distinct in the central half of the neural retina of 4.5-cm embryos, suggesting that as in osteichthyans, differentiation starts in the central retina and progressively expands toward more peripheral levels (Figs. 5G–O). In 5.5-cm embryos, the three cell layers are established over the whole retina except at marginalmost levels (Figs. 5P–R and data not shown). In order to test the conservation of the chick *Otx* expression patterns among gnathostomes, we analyzed *ScOtx1*, *ScOtx2*, and *ScOtx5* expression by hybridization of eye cryostat sections at four stages (3.5, 4.0, 4.5, and 5.5 cm embryos; Fig. 5). No *ScOtx1* expression could be observed in the neural retina at these stages. In contrast, in 3.5- to 4.0-cm embryos, *ScOtx2* and *ScOtx5* are both transcribed in the neuroblasts located in the outer part of the neural retina, excluding the *Pax6*-positive ganglion cell layer (Figs. 5A–F). As in the chick, a decreasing gradient of intensity is observed from outer to inner parts of the labeled territory, which extends over the whole neuroblast layer for *ScOtx2* (Figs. 5A and D), while *ScOtx5* transcripts are restricted to its outer half (Figs. 5B and E). At these stages, the *ScOtx2* and *ScOtx5* expression patterns are observed over the whole circumference of the neural retina. In slightly older embryos (4.5 cm), these signals are maintained for both paralogues in the undifferentiated neuroblast layer, now restricted to the peripheral half of the retina (Figs. 5G, J, and K). *ScOtx5* expression is also very similar in the central retina where ONL and INL have segregated, with a prominent signal not only in the ONL but also in the outer part of the INL (Figs. 5K and N). In contrast, at this level, *ScOtx2* transcripts are still present in the outer part of the INL but become undetectable in the ONL (Figs. 5G and J). A careful examination of the transition zone between the central retina, where the three layers are now visible, and more peripheral levels suggests that *ScOtx2*-positive cells persist in the newly formed ONL, but only during a very transient time window (compare Figs. 5J and L; Figs. 5M and O). In 5.5-cm embryos, the signals observed for *ScOtx2* and *ScOtx5* in the central retina now extend over the whole neural retina except in the marginal region (Figs. 5P–R), whereas in *Xenopus* (Perron et al., 1998), a gradient of retinal differentiation can be observed, together with a recapitulation of the expression patterns previously described (data not shown; Figs. 5H–I in 4.5 cm embryos).

*Nonneural sites of Otx1 expression in the eye: the marginal zone and its derivatives are conserved sites of Otx1 expression in the chick and the dogfish*

Both *GgOtx1* and *ScOtx1* are transcribed in the marginal zone of the retina at optic cup stages (Figs. 3D and G;

Mazan et al., 2000). In the chick, this expression (Figs. 6A–D), but not that of *GgOtx2* and *GgOtx5* (Figs. 6E, G, and H), is maintained throughout development of the ciliary process, which derives from this territory. The transcripts are detected in both the inner and, to a lesser extent, in the outer epithelial layers lying in the continuity of the neural and pigmented retina, respectively, but are absent from the adjacent mesenchyme, also part of the ciliary body (Figs. 6B–D). At E14, a marginal unlabelled territory is clearly visible between the *GgOtx1*-positive forming ciliary process and the more central *GgOtx1*-expressing retina (Fig. 6F). *Pax6*, *GgOtx2*, and *GgOtx5* are all expressed in this *GgOtx1*-negative territory with a layered distribution that resembles that observed in the central retina (Figs. 6I, G, and H). This regionalization is maintained at E18 (Figs. 6D–E and data not shown).

Although *ScOtx1* distribution in the ciliary process of the dogfish is similar to that observed in the chick (Figs. 6J–L), faint *ScOtx2* expression was additionally transiently observed in the outer, but not in the inner layer of this structure in 3.5- to 4.0-cm embryos (Figs. 6P–R). No expression of *ScOtx5* was observed in this structure at any of the stages studied. Other prominent *ScOtx1* expression sites include the epidermis at the tip of the eyelid (Figs. 6J and M), epidermal placodes, and the adjacent epidermis observed in the proximity of the eye of 3.5-cm dogfish embryos (Fig. 6M). At slightly later stages, structures reminiscent of invaginating placodes and vesicles presumably derived from the former, which also strongly express *ScOtx1*, can be observed in the same location (Figs. 6N and O). At these stages, the signal becomes restricted to these structures, excluding the adjacent epidermis. Some of these vesicles appear connected to pores opened to the outside, suggesting that they may correspond to Lorenzini ampullae, which are sensitive electroreceptor organs uniquely present in sharks.

#### *Otx genes and pigment cell differentiation*

The mouse *Otx2* and *Otx1* (m*Otx2* and m*Otx1*) proteins synergize with the bHLH-leucine zipper transcription factor *Mitf* to activate the promoters of melanogenic genes, such as *Tyr*, *Trp1*, and *QNR-71*. Furthermore, transfection of *Otx2* induces pigment cell differentiation in cultured neural retina cells (Martinez-Morales et al., 2003). We therefore asked whether these functional properties were also shared by the paralogous mouse protein *Crx* (which is not expressed in the RPE, Nishida et al., 2003), its dogfish orthologue *ScOtx5*, and the dogfish *ScOtx1* protein that substantially differs in sequence from osteichthyan *Otx1* proteins. We focused on the promoter of *QNR71*, which encodes a transmembrane glycoprotein specifically targeted to the melanosomes (Le Borgne et al., 2001). CAT-coupled *QNR71* promoter vectors were cotransfected in neuroretina cells either alone or in combination with constructs containing the coding sequence of mouse *Otx2* and *Crx*, dogfish *ScOtx1* and *ScOtx5*, and

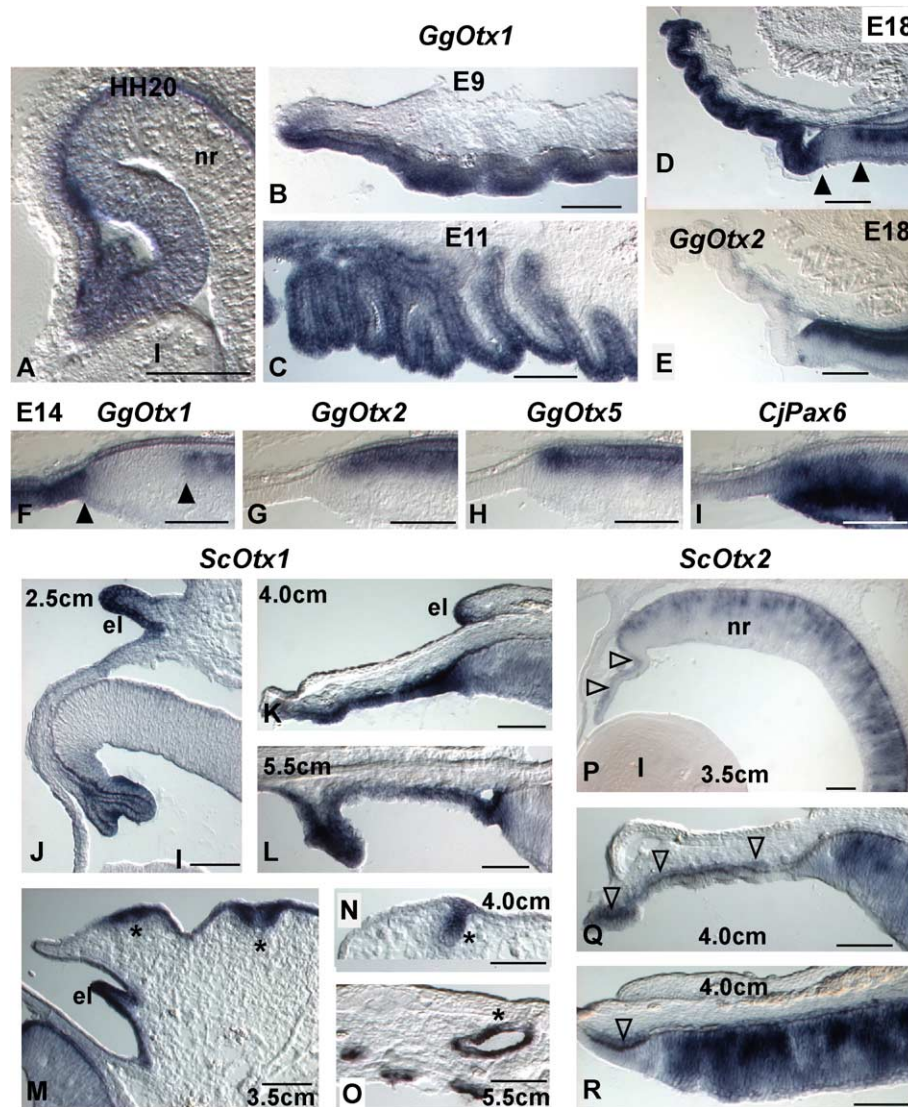


Fig. 6. Expression of *Otx1* at the retina margin in the developing ciliary process and other nonneural structures in the chick and the dogfish. Comparison with *Pax6* and paralogous genes. (A–I) Sections of chick embryos at the level of the margin of the retina after hybridizations with a *GgOtx1* probe (A–D and F), a *GgOtx2* probe (E and G), a *GgOtx5* probe (H), or a *CjPax6* probe (I). A shows a magnification of the folding, *GgOtx1*-positive, retina margin at HH20. B shows the prospective iris (unfolded) and ciliary process (folded), both of which express *GgOtx1* at E9. C shows the *GgOtx1*-positive developing ciliary process at E11. D and E show adjacent sections of the developing ciliary process and retina margin at E18. Black arrowheads delimit a *GgOtx1*-negative but *GgOtx2*-positive territory at the margin of the neural retina. F, G, H, and I show adjacent sections of the retina margin at E14. The marginal *GgOtx1*-negative territory (delimited by black arrowheads as in F) is positive for *GgOtx2* (G), *GgOtx5* (H), *CjPax6* (I), and *GgChx10* (not shown). (J, K, and L) Sections of 2.5-, 4.0-, and 5.5-cm dogfish embryos hybridized with the *ScOtx1* probe and shown at the level of the developing ciliary process. (P, Q, and R) Sections of the retina margin of dogfish embryos hybridized with the *ScOtx2* probe. Opened arrowheads point to the *ScOtx2* signal in the external epithelial layer that lies in continuity with the retina pigmented epithelium. (M, N, and O) Nonneural sites of *ScOtx1* expression in the eye region in 3.5-, 4.0-, and 5.5-cm dogfish embryos. The eyelid (el) (also see J) and the placodes around the eye (black stars in M, N, and O) show a prominent labeling. The placodes invaginate in 4.0-cm embryos and form vesicles that still express *ScOtx1* and are connected to the outside at later stages (O). I, lens. Scale bar = 100  $\mu$ m.

mouse *Mitf*, under the control of the CMV promoter. Since *Nrl*, a leucine zipper transcription factor, has been reported to act synergistically with *Crx* in the control of the rhodopsin promoter (Chau et al., 2000; Mitton et al., 2000), we also included an expression vector encoding this factor. Two days after transfection, cell lysates were collected and the levels of CAT activity determined. All *Otx* encoding vectors enhanced the basal activity of the QNR71 promoter in a dose-dependent manner (Fig. 7A and

data not shown). In addition, as previously shown (Martinez-Morales et al., 2003), mouse *Otx2* and *Otx1* showed synergistic effects when assayed with *Mitf*, supporting the idea that coordinated activity with *Mitf* may be necessary for the full activation of these target genes. The mouse *Crx* and dogfish *ScOtx1* and *ScOtx5* showed the same synergistic effects on the transactivation of QNR71 when assayed together with *Mitf* (Fig. 7A). In contrast, there was no synergism between *Nrl* and *Crx* on the activation of QNR-





Table 1

Pigment foci number	Control	mOtx2	ScOtx1	ScOtx5	mCrx	mMitf	mMitf + mOtx2	mMitf + mCrx
0		38	15	15	5	150	–	–
1		–	12	–	7	125	650	500

ScOtx5 as well as the more divergent mouse Crx proteins were also interacting with Mitf, we performed pull-down experiments. Affinity columns containing a glutathione-Sepharose matrix coupled to GST or GST-Mitf and GST-Mitf carboxyl terminus (amino acids 193–413) were used to pull down *in vitro* radiolabeled ScOtx1, ScOtx5, mOtx2, and Crx. The percentages of labeled proteins that bound the columns were determined after SDS-polyacrylamide gel electrophoresis by PhosphorImager measurement. Similar levels of labeled mOtx2 retention were observed using GST-Mitf and the GST-Mitf carboxyl terminus (Fig. 8), whereas no retention was evident using a radiolabeled GFP protein (data not shown) or when GST alone was used. Identical results were obtained using labeled ScOtx1, ScOtx5, and mouse Crx proteins, indicating a strong interaction between MITF and all Otx proteins tested (Fig. 8).

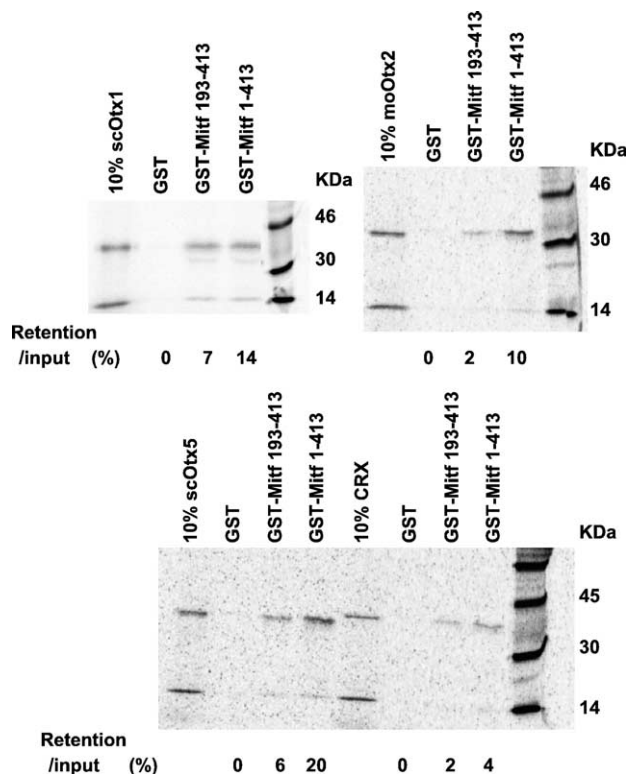


Fig. 8. Protein–protein interactions between Mitf and Otx family members: PhosphorImager analysis of the GST pull-down assay. For each assay (ScOtx1, mOtx2, ScOtx5, Crx), autoradiograms of the gels loaded with 10% of the  $^{35}\text{S}$ -labeled Otx proteins translated in reticulocyte lysates used and each of the fractions retained after incubation with GST alone, GST-Mitf (193–413), or GST-Mitf (1–413) are shown. Percent of retention (shown below the autoradiograms) was calculated from PhosphorImager quantification.

## Discussion

Exhaustive analyses of all paralogues in as broad a range of gnathostomes as possible are important to gain insight into the functional evolution of a multigene family in this taxon. The detailed comparative analysis of the three *Otx* classes in the developing eye in an osteichthyan, the chick, and a chondrichthyan, the dogfish, completes the data already available in other model organisms and provides new clues to understand how the partitioning of the various *Otx* functions has been achieved in different gnathostome taxa.

*Highly specific expression patterns were fixed for each Otx orthology class prior to gnathostome radiation*

The results of the comparison of gnathostome *Otx1*, *Otx2*, and *Otx5* expression patterns in major territories requiring an Otx activity during development are summarized in Fig. 9. The first conclusion, which emerges from this comparison, is that most of the expression sites studied involve a well-defined orthology class or combination of classes. For instance, consistent with a conserved cooperative role of both paralogues in the regionalization of the optic cup and early specification of the RPE, a dorsal restriction of *Otx1*, *Otx2*, or a combination of both is observed in all osteichthyans studied thus far, as well as in the dogfish (Fig. 9A; Bovolenta et al., 1997; Kablar et al., 1996; Li et al., 1994; Martinez-Morales et al., 2001; this study). In contrast, no *Otx5/Crx* expression has been observed at optic evagination to early optic vesicle stages in any of the species thus far studied (Furukawa et al., 1997; Sauka-Spengler et al., 2001; Vignali et al., 2000). Comparisons of *Otx* gene expression in the presumptive lens ectoderm also suggest that the early specification of the lens placode might involve *Otx1* or *Otx2*, but never their paralogue *Otx5/Crx* in all gnathostomes (Fig. 9D; Martinez-Morales et al., 2001; Walter et al., 2004; Zygar et al., 1998). Similarly, in all gnathostomes studied thus far (zebrafish, mouse, chick, and dogfish; Li et al., 1994; this study), only *Otx1* shows prominent expression in the ciliary marginal zone of the optic cup and at later stages, the marginal epithelial layers, which give rise to the mature ciliary process (Fig. 9C; Johnston et al., 1979). This again strongly suggests that the formation of this structure may selectively involve *Otx1* in all gnathostomes. Finally, the best example of this conservation may be provided by *Otx2* and *Otx5* expression patterns in the differentiating neural retina. Our results highlight the succession of at least two distinct phases of *Otx2* and *Otx5/Crx* expression in the

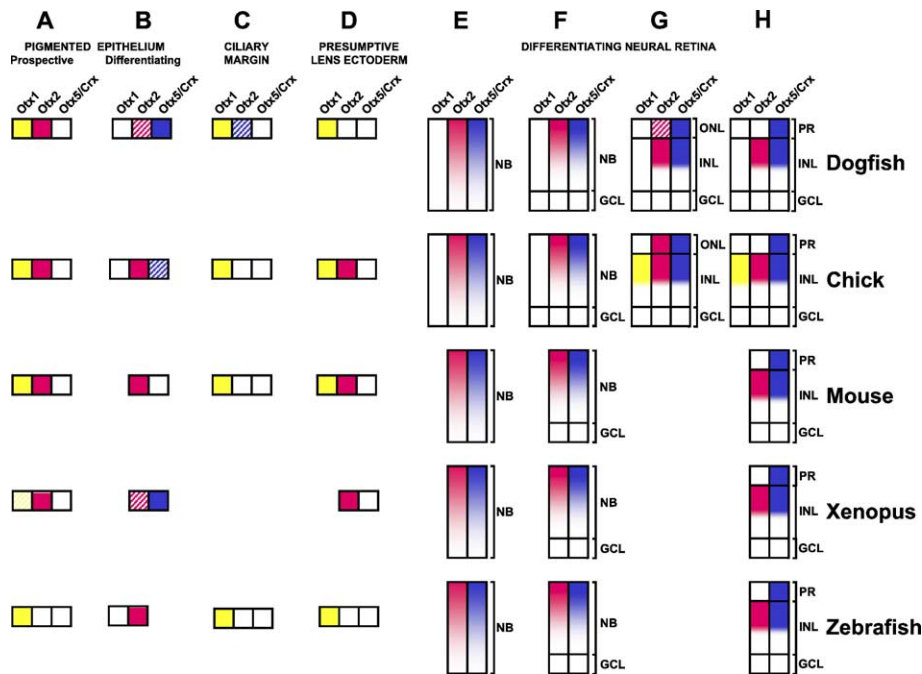


Fig. 9. Summary of the expression data available for each *Otx* class in gnathostome model organisms. The different territories considered are indicated in A (prospective RPE at optic vesicle stages), B (RPE starting from optic cup stages), C (ciliary margin at retina differentiation stages), D (presumptive lens ectoderm), E (proliferating neural retina), F (two-layered neural retina, GCL individualized), G (newly formed three-layered neural retina), and H (differentiated neural retina). The different layers of the neural retina have been shown (NB, neuroblast layer; GCL, ganglion cell layer; ONL, outer nuclear layer; INL, inner nuclear layer; PR, differentiating photoreceptor layer). For each species considered, expression of one class in a given territory is indicated by a colored box (yellow for *Otx1*, red for *Otx2*, and blue for *Otx5/Crx*; hatched boxes for transient or faint signals), an empty box corresponding to an absence of expression. Only the phases, which have been clearly observed, are included in the figure and the absence of a box therefore means an absence of data for the gene and territory considered. The references used for this comparison are cited in Discussion.

differentiating neural retina of the chick and the dogfish (Figs. 9E–H). The first phase is characterized by an extensive overlap of both expression territories initially in the outer aspect of the neuroblast layer (Figs. 9E–G). The second phase is characterized by the maintenance of both *Otx2* and *Otx5* expressions in the INL, but the disappearance of *Otx2* transcripts from the ONL, where *Otx5* remains prominent (Fig. 9H). Comparison of these data with those reported in the mouse, *Xenopus*, and zebrafish shows that all of these species share these expression phases (Nishida et al., 2003; Shen and Raymond, 2004; Viczian et al., 2003). One difference, however, may be sustained *Otx2* expression in the newly formed ONL, which we observe in the chick but which has not been reported in other osteichthyans. We nevertheless note that similar expression is observed in the dogfish, albeit during a very narrow time window, suggesting that this transient phase may be particularly short and hardly detectable in some species. Another clear difference is the INL *Otx1* expression observed in the chick, but absent in the dogfish. In this case, the most straightforward interpretation is that highly specific expression in the outer part of the INL may be ancestral to all *Otx* genes and largely lost by the *Otx1* class, except in some taxa such as archosaurs. Taken together, these data strongly suggest that highly specific features of expression were already fixed for each orthology class in the developing eye, prior to the gnathostome radiation.

#### *The three gnathostome Otx protein classes are equivalent in their ability to drive pigment cell differentiation*

In the mouse, *Otx1* and *Otx2* can largely complement each other in the developing eye of knock-in animals (Acampora et al., 1999, 2003). In contrast, the functional equivalency of *Otx1* or *Otx2* proteins with the paralogous form *Otx5/Crx* has not been addressed so far, either in knock-in mice or overexpression experiments. The induction of a pigmented phenotype in neural retina cells and the ability to transactivate pigment-specific genes in cooperation with *Mitf*, both demonstrated for mouse *Otx2*, provide experimental tests to address this question in the developing RPE. In these tests, we could not detect significant differences between ScOtx1, ScOtx5, mOtx2, and even the highly divergent mouse *Crx* proteins, which indicates that the ability to drive pigment cell differentiation is shared by all *Otx* proteins, whatever the gnathostome species and the orthology class considered. This function is not shared by all the transcription factors expressed during RPE development, as shown for *Pax6*. In addition, in contrast to *Otx* family members, *Pax6* reduced the *Mitf* pigmentation-inducing ability, further confirming the specificity of *Otx* family members, even though all paralogous proteins were able to interact with *Mitf* (this study and Planque et al., 2001). This suggests that the functional evolution of *Otx* genes in the RPE has

essentially relied on changes in regulatory cis-acting signals. In this respect, it should be noted that *Otx* functions in the RPE may not be restricted to its early specification, since *Otx* transcripts are also detected in the RPE during retina differentiation stages in the mouse, chick, *Xenopus*, and dogfish. As described above, only *Otx1* and *Otx2* are involved in the early specification of the RPE (Fig. 9A). However, possible roles of *Otx* genes in the later RPE may provide examples of function shuffling such as those reported in other genetic systems (Locascio et al., 2002; Sefton et al., 1998). For instance, prominent expression of *Otx2* was observed in the late RPE of the mouse, where *Crx* transcripts remain undetectable (Fig. 9B.; Martinez-Morales et al., 2001; Nishida et al., 2003). In contrast, *Otx5* expression prevails in the late RPE of the dogfish and *Xenopus*, and is also transiently observed in the chick (Fig. 9B; Perron et al., 2003 and personal communication).

*The dynamic Otx2 and Otx5/Crx expression patterns in the differentiating neural retina suggest conserved roles among gnathostomes*

The functional conservation of *Otx* genes in the differentiation of the neural retina remains an open question, since experiments carried out in all vertebrate model organisms each highlight substantially different functions. The high conservation of the dynamic *Otx2* and *Otx5/Crx* expression patterns, which we observe among gnathostomes, is consistent with the conservation of important *Otx* functions in the differentiating neural retina and suggests that discrepancies between available functional data may largely result from differences in the experimental approaches used. Thus, the *Otx2* and *Otx5* expression observed in all gnathostomes in the outer part of the neuroblast and outer nuclear layers (Figs. 9E–G) is consistent with a conserved *Otx* role in promoting photoreceptor and bipolar cell fates, as demonstrated in the mouse and *Xenopus* (Nishida et al., 2003; Viczian et al., 2003). In the mouse at least, the early specification of photoreceptors is uniquely fulfilled by *Otx2*, possibly due to the extensive divergence of *Crx* over the coding region. Functional redundancies with the paralogous gene *Otx5* may however occur in other species, as suggested by overexpression analysis carried out in *Xenopus* (Viczian et al., 2003). Similarly, the prominent *Otx5/Crx* expression observed in the differentiating photoreceptor layer of all studied gnathostomes suggests that the *Crx* function demonstrated in the mouse in the terminal differentiation of photoreceptors (Furukawa et al., 1999) is a conserved *Otx* role, selectively fulfilled by the *Otx5/Crx* class in all gnathostomes. In addition, in *Xenopus*, *Otx2* can suppress both the capacity of *Otx5* to induce a photoreceptor phenotype in vivo and to transactivate the rhodopsin gene, indicating that *Otx2* may negatively regulate the terminal differentiation of photoreceptors

(Viczian et al., 2003). In our study, we observed that in the chick and the dogfish as in the mouse and *Xenopus*, *Otx2* expression withdraws from the ONL at advanced stages of retina differentiation (Fig. 9H). In the chick, this decrease of *Otx2* expression in the ONL roughly correlates with the onset of rhodopsin expression (Bruhn and Cepko, 1996). These data are consistent with the hypothesis that withdrawal of *Otx2* expression from the ONL might be required for the terminal differentiation of photoreceptors in all gnathostomes.

## Conclusion

Changes at the level of regulatory cis-acting sequences are often considered as the driving force in the functional evolution of genetic systems. In the particular context of gnathostome multigene families, our results are largely consistent with this idea. However, this does not preclude a possible involvement of changes at the level of coding regions for some particular functions, such as the modulation of *Otx5* activity by *Otx2* in the differentiation of photoreceptors or the role of *Otx1* in the formation of the ciliary process (Acampora et al., 1999; Viczian et al., 2003). It will be important to test how far these conclusions apply to other genetic systems to better understand vertebrate evolution.

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