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DIFFERENTIAL AND OVERLAPPING EXPRESSION PATTERNS OF *X-dll3* AND *Pax-6* GENES SUGGEST DISTINCT ROLES IN OLFACTORY SYSTEM DEVELOPMENT OF THE AFRICAN CLAWED FROG *XENOPUS LAEVIS*

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Summary

In *Xenopus laevis*, the formation of the adult olfactory epithelium involves embryonic, larval and metamorphic phases. The olfactory epithelium in the principal cavity (PC) develops during embryogenesis from the olfactory placode and is thought to respond to water-borne odorants throughout larval life. During metamorphosis, the PC undergoes major transformations and is exposed to air-borne odorants. Also during metamorphosis, the middle cavity (MC) develops *de novo*. The olfactory epithelium in the MC has the same characteristics as that in the larval PC and is thought to respond to water-borne odorants. Using *in situ* hybridization, we analyzed the expression pattern of the homeobox genes *X-dll3* and *Pax-6* within the developing olfactory system. Early in development, *X-dll3* is expressed in both the neuronal and non-neuronal ectoderm of the sense plate and in all cell layers of the olfactory placode and larval PC. Expression becomes restricted to the neurons and basal cells of the PC by mid-metamorphosis. During metamorphosis, *X-dll3* is also expressed throughout the developing MC epithelium and becomes restricted to neurons and basal cells at metamorphic climax. This expression pattern suggests that *X-dll3* is first involved in the patterning and genesis of all cells forming the olfactory tissue and is then involved in neurogenesis or neuronal maturation in putative water- and air-sensing epithelia. In contrast, *Pax-6* expression is restricted to the olfactory placode, larval PC and metamorphic MC, suggesting that *Pax-6* is specifically involved in the formation of water-sensing epithelium. The expression patterns suggest that *X-dll3* and *Pax-6* are both involved in establishing the olfactory placode during embryonic development, but subtle differences in cellular and temporal expression patterns suggest that these genes have distinct functions.

Key words: olfaction, development, patterning, gene expression, *X-dll3*, *Pax-6*, *Xenopus laevis*, thyroid hormone, homeobox gene.

Introduction

The African clawed frog *Xenopus laevis* belongs to the Pipidae family of frogs, all of which have an aquatic lifestyle in adulthood. They rarely leave the water and have several adaptations to aquatic life including loss of the tongue (Sokol, 1969) and the presence of lateral line organs (Russel, 1976). Furthermore, in *X. laevis*, the olfactory system has adapted to accommodate this primarily aquatic lifestyle. Together with a nasal cavity that is exposed to air, the main olfactory system of the adult has an additional cavity that is exposed to water (Altner, 1962; Venus et al., 1998). The formation of this new adult nasal cavity occurs at metamorphosis concomitant with the remodeling of the larval nasal cavity (Föske, 1934; Paterson, 1939; Paterson, 1951).

The formation of the olfactory epithelium involves embryonic, larval and metamorphic phases. The olfactory epithelium develops during embryogenesis from a thickening of the anterior ectoderm that comprises neuronal and non-neuronal ectodermal cells. This thickening forms the olfactory placode that will give rise to all the cells forming the olfactory epithelium: basal cells, olfactory receptor neurons and supporting cells (Klein and Graziadei, 1983). The olfactory epithelium of the larval principal cavity (PC; medial diverticulum) is exposed to water and is thought to respond only to water-borne odorants throughout larval life (Elepfandt, 1996; Venus et al., 1998). At metamorphosis, the PC undergoes major cellular and molecular transformations (Föske, 1934; Reiss and Burd, 1997a) apparently in preparation for sensing air-borne odorants. Also at metamorphosis, the middle cavity (MC; lateral diverticulum) develops *de novo*, is exposed to water (Venus et al., 1998) and is likely to sense water-borne odorants (Elepfandt, 1996; Venus et al., 1998). The functional segregation, however, is not as well defined as

the anatomical one, as it appears that olfactory receptor neurons from the MC are able to respond to both water-borne and air-borne odorants (Iida and Kashiwayanagi, 1999).

The olfactory epithelium of the adult MC displays similar cellular and biochemical characteristics to those of the olfactory epithelium of the larval PC, which suggests a conserved functional role for these two cavities. At the cellular level, both epithelia contain ciliated and microvillar olfactory receptor neurons whose axons project to the ventral olfactory bulb (Reiss and Burd, 1997b; Hansen et al., 1998). At the biochemical level, both epithelia display identical expression patterns for the lectin soybean agglutinin (SBA) (Key and Giorgi, 1986; Hofmann and Meyer, 1991; Reiss and Burd, 1997b) and the monoclonal antibody E7 (Petti et al., 1999). *X. laevis* possess a gene repertoire encoding two classes of olfactory receptor gene; one class is related to the olfactory receptor genes of fish and is called Class I and the other class is related to the olfactory receptor genes of mammals and is called Class II (Freitag et al., 1995). Finally, at the molecular level, both epithelia express the fish-like Class I olfactory receptor genes (Freitag et al., 1995). In contrast, the olfactory epithelium of the adult PC has only ciliated olfactory receptor neurons whose axons project to the dorsal bulb (Fritz et al., 1996; Reiss and Burd, 1997b; Hansen et al., 1998), does not stain with either SBA or E7 (Key and Giorgi, 1986; Hofmann and Meyer, 1991; Petti et al., 1999) and expresses the mammalian-like Class II olfactory receptor genes (Freitag et al., 1995). The cellular, biochemical and molecular differences between olfactory epithelia that are exposed to water or to air are likely to be the result of differential expression of developmental control genes. We therefore hypothesized that differences in expression of developmental control genes exist between the formation of the larval PC and adult MC and the remodeling of the adult PC.

The *Distal-less* and *Pax-6* genes are homeobox genes whose differential expression has been shown to mediate patterning, morphogenesis and histogenesis in vertebrate development (Callaerts et al., 1997; Bendall and Abate-Shen, 2000). In vertebrates, there are several members of the *Distal-less* gene family. Unfortunately, the nomenclature for vertebrate *Distal-less* genes is confusing. *Distal-less* genes are expressed in the olfactory placodes of zebrafish, chick, mouse and *Xenopus laevis* but different numerical names have been used for homologous genes. For example, the homologous *Distal-less* genes expressed in the nasal tissues are *zdfdlx4* in zebrafish (Akimenko et al., 1994), *Dlx3* in chick (Pera and Kessel, 1999) and *Dlx5* in mouse (Simeone et al., 1994). Expression of *Dlx5* is required for the development of the olfactory epithelium, as demonstrated by the phenotypes of homozygous *Dlx5* mouse mutants lacking exons I and II (Depew et al., 1999; Acampora et al., 1999). These mice display a reduction or absence of olfactory epithelium. Since the construct used by Acampora et al. (Acampora et al., 1999) lacked the start codon, Dlx5 protein synthesis should be abolished in these mutant mice. Similarly, *Pax-6* is expressed in the olfactory placode and developing olfactory bulb of human, mouse, chick, quail and zebrafish (Ton et al., 1991; Walter and Gruss, 1991; Goulding et al., 1993; Martin et al., 1992; Puschel et al., 1992). In humans and mice, homozygotic mutants lacking the *Pax-6* gene fail to form olfactory tissues, resulting in the absence of both olfactory epithelium and olfactory bulb (Glaser et al., 1994; Grindley et al., 1995).

In *X. laevis*, both *Distal-less* and *Pax-6* are expressed in the developing olfactory system. *X-dll3*, the orthologue of the mouse *Dlx5*, and *Pax-6* are both expressed early during embryogenesis in the anterior ectoderm, tissue that gives rise to the prospective olfactory placodes (Papalopulu and Kintner, 1993; Swiergiel et al., 1994; Burd et al., 1995; Hirsch and Harris, 1996). Since *X-dll3* and *Pax-6* are in a position to specify the early olfactory placode in *X. laevis* embryos, we predicted that these two genes might also be involved in remodeling the olfactory epithelium at metamorphosis. Using a combination of *in situ* hybridization and immunocytochemical procedures, we show that *X-dll3* is expressed in all regions of olfactory epithelium throughout embryogenesis and metamorphosis, whereas *Pax-6* is expressed only in a subset of structures at specific stages in the embryonic olfactory placode, larval PC and vomeronasal organ and adult MC. This differential expression pattern suggests that *X-dll3* is likely to be involved in the patterning of olfactory epithelia exposed to both air and water, whereas *Pax-6* involvement seems to be restricted to the formation of olfactory epithelia exposed to water.

Materials and methods

Animals

Xenopus laevis (Daudin) embryos were obtained from gonadotropin-induced breedings (HCG; Sigma), and tadpoles and adult frogs were purchased from NASCO (Fort Atkinson, WI, USA). Animals were reared at 22 °C on a 12 h:12 h L:D cycle in 6 l polypropylene tanks containing *X. laevis* rearing solution (Burd, 1991). Animals were given daily feedings of either boiled nettle (tadpoles; Wunderlich-Diez Corp., Hasbrouck Heights, NJ, USA) or frog brittle (frogs; NASCO). Animals were staged according to established developmental criteria (Nieuwkoop and Faber, 1994).

Probes for in situ *hybridization*

Digoxigenin-labeled RNA probes were synthesized from linearized DNA templates using either T3 or T7 RNA polymerase. A partial-length *X-dll3* clone containing base pairs +1 to +753 was obtained from Dr Nancy Papalopulu (Papalopulu and Kintner, 1993). The *X-dll3* antisense probe was obtained by digestion with *Eco*RI and subsequent transcription with T7 RNA polymerase in the presence of digoxigenin-labeled UTP following recommended protocols (Boehringer Mannheim); the *X-dll3* sense probe was obtained as above using *Bam*HI and T3 RNA polymerase. For *Pax-6* probes, a partial-length *Pax-6* clone was isolated from a *X. laevis* cDNA library provided by Dr Richard Harland (Hemmati-Brivanlou et al., 1991). The probe used to screen the

library was a radiolabeled 1035 base pair *Eco*RI–*Nde*I restriction fragment of the zebrafish *Pax-6* cDNA clone pn108 (Puschel et al., 1992). The *Pax-6* antisense probe was obtained by digestion with *Bst*XI and subsequent transcription with T7 RNA polymerase in the presence of digoxigenin-labeled UTP following recommended protocols (Boehringer Mannheim); the *Pax-6* sense control probe was obtained as above using *Eco*RV and T3 RNA polymerase. The nucleotide identity of the probes was confirmed by sequencing the clones used to generate the probes. All probes were hydrolyzed for 10–15 min in 40 mmol l^{-1} sodium bicarbonate/60 mmol l^{-1} sodium carbonate at 60 °C.

In situ *hybridization*

Whole-mount *in situ* hybridization using digoxigenin-labeled RNA probes was performed as described by Harland (Harland, 1991). Briefly, embryos were fixed in MEMFA (0.1 mol l^{-1} MOPS, pH 7.4, 2 mmol l^{-1} EGTA, 1 mmol l^{-1} $MgSO_4$, 3.7 % formaldehyde) for 1–2 h. For storage, embryos were kept at –20 °C in 100 % methanol and were then rehydrated by washes in 75 % ethanol, 50 % ethanol and 25 % ethanol in PBS-T (0.1 mol l^{-1} phosphate-buffered saline with 0.1 % Tween 20) for immediate use. Embryos were then incubated for 10–15 min in 10 μg ml^{-1} proteinase K (Fisher), washed in 0.1 mol l^{-1} triethanolamine (TEA, pH 7–8) and incubated in 0.1 mol l^{-1} TEA, 0.25 % acetic anhydride. Embryos were then washed in PBS-T and prehybridized for at least 6 h at 60 °C in 50 % formamide, 5× SSC (1× SSC is 0.15 mol l^{-1} sodium chloride, 0.015 mol l^{-1} sodium citrate), 1 mg ml^{-1} torula RNA, 100 μg ml^{-1} heparin, 1× Denhardt's solution (0.02 % Ficoll 400, 0.02 % polyvinyl pyrrolidone, 0.02 % bovine serum albumin), 0.1 % Tween 20, 0.1 % CHAPS, 5 mmol l^{-1} EDTA. Hybridization was carried out at 60 °C overnight in prehybridization solution containing 0.5–1 μg ml^{-1} digoxigenin-labeled probes. Following hybridization, embryos were washed at 60 °C in prehybridization solution, rinsed at 60 °C in 2× SSC, treated at 37 °C in 20 μg ml^{-1} RNAse A and 10 μg ml^{-1} RNAse T1 in 2× SSC, and rinsed at 60 °C in 0.2× SSC. Embryos were then rinsed in maleic acid buffer (MAB; 100 mmol l^{-1} maleic acid, 150 mmol l^{-1} NaCl, pH 7.5), blocked in MAB containing 20 % heat-inactivated lamb serum and 2 % blocking reagent (Boehringer Mannheim) at room temperature (24 °C) for 2 h, and incubated overnight in the same solution containing a sheep alkaline-phosphatase-conjugated, anti-digoxigenin antibody (1:2000 dilution of the Fab fragment; Boehringer Mannheim).

For the colorimetric detection, embryos were rinsed in MAB and washed three times in alkaline phosphatase (AP) buffer (100 mmol l^{-1} Tris-HCl, pH 9.5, 50 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 0.1 % Tween 20 and 5 mmol l^{-1} levamisol). The colorimetric reaction was performed using 0.34 μg ml^{-1} Nitro Blue Tetrazolium (NTB; Sigma) and 0.18 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Sigma). The staining was stopped by washing the embryos several times in AP buffer. Embryos were then refixed in MEMFA and stored in methanol at –20 °C. Stained animals were photographed, and selected embryos were embedded in paraffin, sectioned at 10 μm, cleared in xylene, coverslipped and photographed with a Nikon Biophoto microscope.

In situ hybridization on tissue sections was performed as described by Strahle et al. (Strahle et al., 1994). Animals were anesthetized using 0.02 % 3-aminobenzoic acid ethyl ester (MS222, Sigma), fixed by perfusion with 4 % paraformaldehyde, 4 % (w/v) sucrose in PB buffer (0.1 mol l^{-1} phosphate buffer, pH 7.4) and stored in the fixative solution overnight at 4 °C. After a series of rinses in fixative solution minus paraformaldehyde, tissues were embedded in 4 % agarose and soaked overnight at 4 °C in PB containing 30 % (w/v) sucrose. Alternatively, tissues were frozen in Tissue-Tek (OCT compound, Miles Inc., Elkhart, IN, USA) for immediate sectioning. The tissues were sectioned at 14 μm using a cryostat (Microm, Heidelberg) and collected on either TEPSA- (Sigma) or Vectabond-coated (Vector Labs) slides. Sections were air-dried at room temperature for at least 1 h and stored at –20 °C for subsequent analysis. Sections were directly hybridized overnight at 58 °C with 0.5–1 μg ml^{-1} RNA probe in 50 % formamide, 0.3 mol l^{-1} NaCl, 10 mmol l^{-1} NaH_2PO_4, 10 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl, pH 7.5, 10 % dextran sulfate, 1 mg ml^{-1} rRNA, 1× Denhardt's solution. Slides were coverslipped and placed into a sealed box containing Whatman filter paper soaked with the hybridization solution minus the RNA probe. The box was then placed in an incubator overnight at 58 °C. The coverslips were then removed, and the sections were washed first at 65 °C in 25 % formamide, 1× SSC, 0.5× PBS and then at room temperature in PBS. Sections were then blocked in PBS-T, 0.2 % (w/v) bovine serum albumin, 2 % heat-inactivated lamb serum for 2 h at room temperature, incubated in alkaline-phosphatase-conjugated anti-digoxigenin antibody, rinsed and reacted with the colorimetric solutions as described above for the whole-mount procedure except that, for some reactions, the BM Purple AP Substrate (Boehringer Mannheim) replaced the NTB/BCIP solution. The staining was analyzed with a Nikon Biophoto microscope.

Immunocytochemistry

Localization of the Distal-less protein was carried out using indirect immunofluorescence. The cross-reacting polyclonal antibody against the Distal-less protein was generated against the butterfly *Precis coenia* and subsequently affinity-purified. This antibody was provided by Dr G. Panganiban (University of Wisconsin, Madison, WI, USA). Tissue sections were prepared as described above for *in situ* hybridization on frozen sections. Sections were treated for 30 min at room temperature in blocking solution (3 % normal goat serum, 0.4 % Triton X-100) in PB (0.1 mol l^{-1} sodium phosphate buffer, pH 7.4). Sections were then incubated for 2 h at room temperature in blocking solution containing the polyclonal antibody against Distal-less (0.45 μg ml^{-1} final concentration). After three washes in PB, the sections were incubated for 1 h at room temperature in PB containing the Cy3-conjugated goat anti-rabbit antibody (1:500

dilution; Jackson ImmunoResearch Laboratories). In all experiments, sections were counterstained for 15 min at room temperature with the nuclear stain 4′-6-diamidino-2-phenylindole (DAPI; Sigma). The slides were then coverslipped using AquaPolymount (Polysciences Inc). The fluorescence was analyzed under a Zeiss Universal epifluorescence microscope. Negative controls consisted of staining in the absence of the primary antibody. The positive control for Distal-less staining was the demonstration of staining in tissues known to contain Distal-less (e.g. larval limb tissues); the positive control for Pax-6 staining was the demonstration of staining in the olfactory bulb with our antibody and a Pax-6 antibody generated by Davis and Reed (Davis and Reed, 1996).

Processing of histological images

Animals and tissues were photographed onto transparency films. Films and images were digitized and figures were processed using Adobe Photoshop (Adobe Systems Incorporated).

Results

General information

The olfactory placodes can be fate-mapped from cells located in the anterior lateral portion of the anterior neural ridge, a strip of tissue that lies anterior to the open neural plate at stages 14–16 (Eagleson and Harris, 1989; G. D. Burd, A. Collazo and S. E. Fraser, unpublished observations) (Fig. 1A). When the neural tube closes, the anterior neural ridge folds back over the anterior neural tube; as the right and left sides of the anterior ectoderm fuse over the anterior neural tube at stage 20, the sense plate is formed (Burd, 1999) (Fig. 1A). At stage 23, the olfactory placodes form from bilateral thickenings in the sense plate (Nieuwkoop and Faber, 1994; Klein and Graziadei, 1983). The olfactory placode remains a flattened thickening of tissue until stage 31, when the nasal pit begins to form (Nieuwkoop and Faber, 1994) (Fig. 1A). At stage 37/38, the nasal pit begins to differentiate into the principal cavity (PC) and the vomeronasal organ (VNO) (Nieuwkoop and Faber, 1994; Burd, 1999). At stage 52, the onset of metamorphosis, the middle cavity (MC) forms *de novo* (Burd, 1999). After metamorphosis and in the adult, the nasal cavity contains the PC, MC and VNO (Fig. 1B).

Embryonic expression of X-dll3 *and* Pax-6

The tissue fated to give rise to olfactory placodes (Eagleson and Harris, 1989; Burd et al., 1995) expresses *X-dll3* by stage

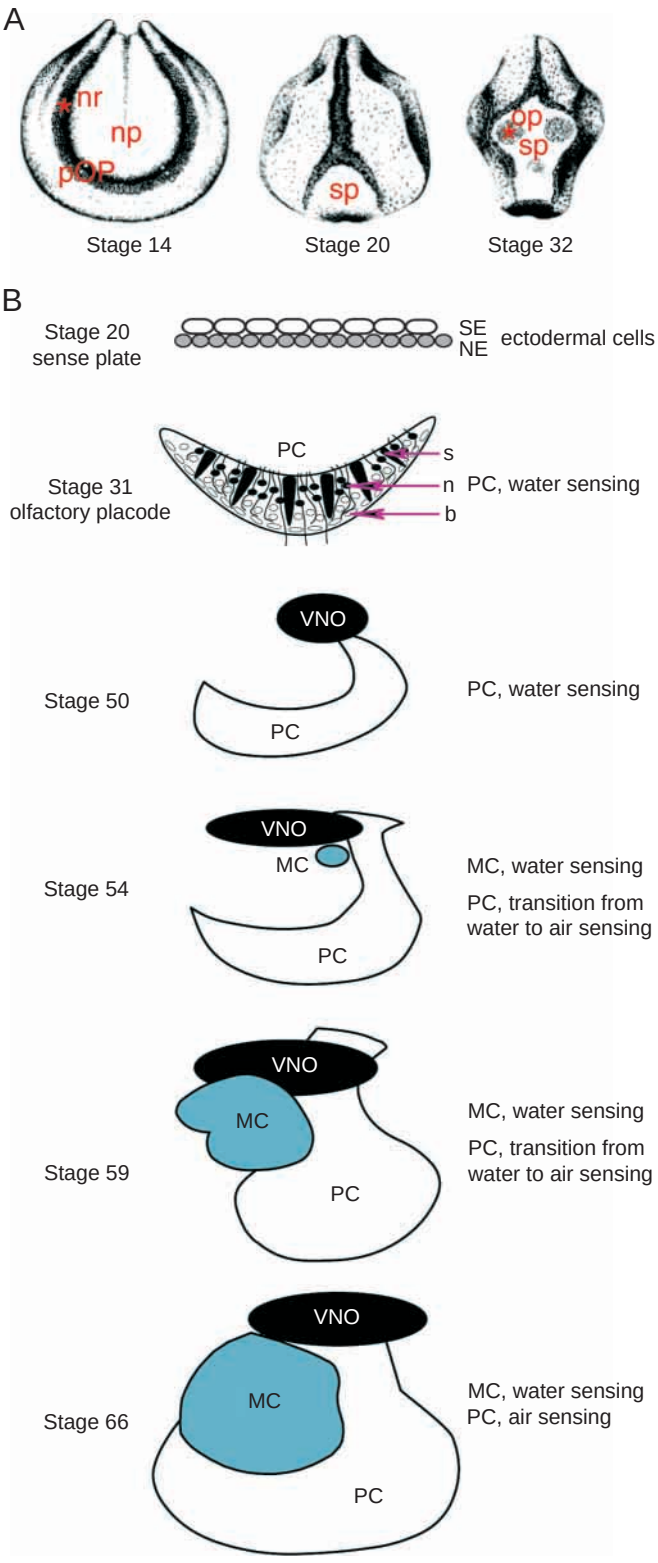

Fig. 1. Schematic diagrams of the olfactory epithelium during embryonic, larval and metamorphic development in *Xenopus laevis*. (A) Front views of whole-mount embryos at stages 14, 20 and 32. nr, neural ridge; np, neural plate; pOP, tissue fated to become placodes; op, olfactory placode; sp, sense plate. The asterisks indicate the location of nr and op. (B) Stages 20 and 31 are views from horizontal sections. Stages 50–66 are schematic diagrams of the nasal capsule viewed as whole mounts in the horizontal plane with anterior at the top. By stage 20, superficial ectodermal cells (SE) (precursors of the supporting cells) and neuronal ectodermal cells (NE) (precursors of the olfactory receptor neurons and basal cells) have formed. 'Water sensing' and 'air sensing' refer to the presumed function of the olfactory receptor neurons. MC, middle cavity; PC, principal cavity; VNO, vomeronasal organ; b, basal cells; n, olfactory receptor neurons; s, supporting cells. Diagrams are not to scale.

15, the open neural plate stage of development (Papalopulu and Kintner, 1993) (Fig. 2A). The expression pattern continues in pre-olfactory placode tissue during closure of the neural tube at stages 18–19 (Fig. 2B). *X-dll3* is widely expressed in the sense plate between stages 20 and 25 before and during the initial formation of the olfactory placodes (Fig. 2C,J), but staining is more intense in the developing olfactory placodes. Ultimately, *X-dll3* staining is lost from the sense plate by stage 35, but remains in the olfactory placodes and developing olfactory epithelium (Fig. 2D,L). In tissue sections, it is clear that *X-dll3* expression is present in both the non-neuronal and neuronal layers of the sense plate at stage 26 (Fig. 2J). In stage 35 animals, staining for *X-dll3* expression is present throughout all cell types of the olfactory placode (Fig. 2L). Cells fated to

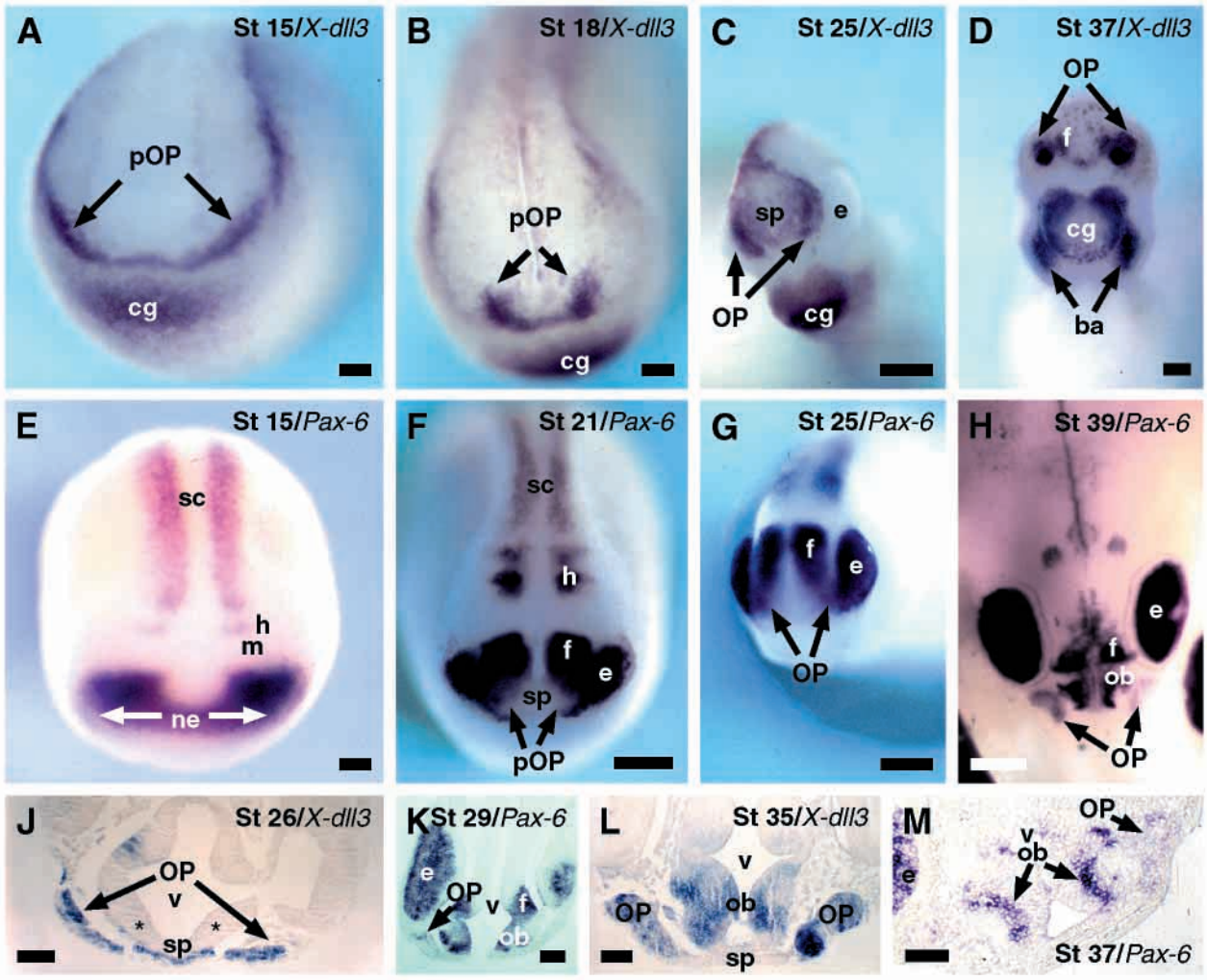

Fig. 2. Embryonic expression of *X-dll3* and *Pax-6* by whole-mount *in situ* hybridization. (A) *X-dll3* expression at stage (St) 15 showing stained anterior neural ridge and future cement gland. (B) *X-dll3* expression at stage 18 in the presumptive olfactory placodes and cement gland. (C) *X-dll3* expression at stage 25 showing stained olfactory placodes, sense plate and cement gland. (D) *X-dll3* expression at stage 37 showing stained olfactory placodes, branchial arches and forebrain. By stage 37, *X-dll3* expression begins to disappear in the cement gland. (E) *Pax-6* expression at stage 15 showing stained anterior neural ectoderm, hindbrain anlage and spinal cord. (F) *Pax-6* expression at stage 21 showing staining in an area of the presumptive olfactory placodes and darker staining in the eye, forebrain, hindbrain and spinal cord. (G) *Pax-6* expression at stage 25 showing staining in the eye, forebrain and hindbrain. Note that the olfactory placodes are only very lightly stained at this stage. (H) *Pax-6* expression at stage 39 showing stained olfactory placodes, olfactory bulb, forebrain and eyes. (J–M) Paraffin sections of whole-mount stained embryos. (J) *X-dll3* expression at stage 26 in all layers of the olfactory placodes and in the non-neuronal and neuronal ectodermal cells of the sense plate. The asterisks indicate the regions of the anterior neural tube that will give rise to the olfactory bulb. (K) *Pax-6* expression at stage 29 in the olfactory placodes and in the olfactory bulb, forebrain and eyes. (L) A stage 35 animal showing *X-dll3* expression in all layers of the olfactory placodes and olfactory bulb. At this stage, expression has disappeared from the sense plate and a darker stain is visible in the vomeronasal organ of the olfactory placode on the right side. (M) A stage 37 embryo showing *Pax-6* expression in the olfactory placodes and olfactory bulb. Staining in the olfactory placodes is light, and staining in the olfactory bulb is primarily located in differentiating mitral cells. Whole-mount photographs are dorsal/anterior views. Sections are horizontal with anterior facing down. ba, branchial arches; cg, cement gland; e, eye; f, forebrain; h, hindbrain; m, midbrain; ne, neuroectoderm; ob, olfactory bulb; OP, olfactory placode; pOP, tissue fated to become olfactory placodes; sc, spinal cord; sp, sense plate; v, ventricle. Scale bars, 150 μm.

give rise to the olfactory bulb in the anterior neural tube do not express *X-dll3* at stage 26 (Fig. 2J), but begin to express *X-dll3* by stage 27, at a time before olfactory axons contact the neural tube (Byrd and Burd, 1991). In embryos, staining for *X-dll3* occurs throughout the neural tube cells that form the olfactory bulb (Fig. 2L), but becomes restricted to a subset of bulb cells in the larval stages (see below).

Pax-6 expression is first seen at the small yolk plug stage (stage 12) as patches of stain dorsal to and flanking the receding yolk plug (data not shown). This region corresponds to the posterior sensory layer of the neuroectoderm (Nieuwkoop and Faber, 1994). At the open neural plate stage (stage 15), *Pax-6* is expressed as a broad stripe in the anterior neural ridge and anterior neural plate, tissue fated to give rise to the olfactory placodes, olfactory bulb, eyes and various forebrain tissues (Fig. 2E). Two posterior stripes of stained cells are fated to give rise to the spinal cord and hindbrain (Fig. 2E). The gap in staining in the neural tube corresponds to the presumptive midbrain (Eagleson and Harris, 1989). *Pax-6* expression is observed primarily in the most anterior sense plate cells at the neural tube stage (stage 21) (Fig. 2F), while the presumptive olfactory placodes are fated to form from all regions of the sense plate at this stage (G. D. Burd, A. Collazo and S. E. Fraser, unpublished observations). *Pax-6* expression is very light during the early stages of olfactory placode development (Fig. 2G,K). *Pax-6* expression increases in the olfactory placodes by stage 39 but remains light (Fig. 2H). Throughout the early embryonic stages, precursor cells that are fated to become olfactory bulb cells stain for *Pax-6* (Fig. 2K). However, by stage 37, *Pax-6* expression in the precursor cells around the edge of the ventricles is reduced, and most of the *Pax-6* expression in the developing olfactory bulb is in differentiating neurons (Fig. 2M).

Metamorphic expression of X-dll3 *in the olfactory epithelium and olfactory bulb*

Metamorphosis of the olfactory epithelium includes the remodeling of the larval PC and the *de novo* formation of the adult MC. The remodeling of the larval PC appears to start at stage 49 with the expression of the genes encoding the mammalian-like Class II odorant receptors (Mezler et al., 1999). The formation of the adult MC begins at stage 51 as a small bud at the anterior end of the larval PC (Föske, 1934; Petti et al., 1999).

Analysis of the spatio-temporal pattern of *X-dll3* expression during early larval development shows a similar pattern to that seen at embryonic stages 35 and 37 (Fig. 2D,L). The PC in young larvae expresses *X-dll3* throughout the cavity including the supporting, neuronal and basal cell layers (data not shown). The onset of metamorphosis (stage 51) marks the beginning of a series of changes in the pattern of expression of *X-dll3*. At stage 51, *X-dll3* expression is lost from the supporting cell layer of the PC, as shown by the absence of purple staining from the apical-most layer of the olfactory epithelium (Fig. 3B). *X-dll3* expression in the PC remains the same until premetamorphosis (stage 54) (Fig. 3E). By the onset of metamorphic climax (stage 58), *X-dll3* expression has become restricted to the deepest cell layer of the PC that contains basal cells and some olfactory receptor neurons (Petti et al., 1999) (Fig. 3H). By the end of metamorphosis (stage 64), only clusters of cells close to the basal lamina of the PC express *X-dll3* (Fig. 3K). These deep clusters are likely to be basal cells and a subpopulation of olfactory receptor neurons (Petti et al., 1999). The expression pattern of *X-dll3* within the developing MC is similar to that of the remodeling PC during metamorphosis; however, the expression is delayed temporally, as illustrated in Fig. 3D,G,J. At stage 54, *X-dll3* is expressed primarily in the deeper layers of the MC epithelium (Fig. 3D), but by stage 59 *X-dll3* expression is restricted to the neuronal and basal cell layers, as illustrated by the absence of purple staining from the supporting cell layer of the MC (Fig. 3G). By the end of metamorphosis, *X-dll3* expression becomes lighter and but remains in the deeper layer of the MC (Fig. 3J). In addition, we analyzed *X-dll3* expression within the developing olfactory bulb. *X-dll3* is expressed mainly around the lateral ventricles and in the granular cell layer of the olfactory bulb, and the pattern of expression does not change during metamorphosis (Fig. 3C,F,I,L).

Metamorphic and adult expression of the Distal-less protein in the olfactory epithelium

The potential functional role for Distal-less in the development of the olfactory epithelium, as revealed by the expression pattern of *X-dll3* mRNA, was supported by the finding that Distal-less protein has a similar expression pattern. Using immunocytochemistry, we show that Distal-less protein is expressed throughout the MC and in the neuronal and basal cell layers of the PC at stage 53 (Fig. 4A,B). At the adult stage, Distal-less is expressed primarily in the neuronal and basal cell layers of both the MC and PC, and the overall pattern of expression has moved towards the basal lamina of both cavities (Fig. 4C–F).

Metamorphic expression of Pax-6 *in the olfactory epithelium and olfactory bulb*

The pattern of expression of *Pax-6* during early larval development and metamorphosis was analyzed using *in situ* hybridization. Analysis of *Pax-6* mRNA distribution during early larval development shows that *Pax-6* expression decreases in the larval PC around stage 44 and disappears from the PC by stage 49 (Fig. 5B). Interestingly, the ability of the olfactory placode to regenerate when removed is also lost at approximately stage 44 (Stout and Graziadei, 1980; Byrd and Burd, 1993). *Pax-6* expression remains absent from the PC throughout metamorphosis, as illustrated for stages 58, 61 and 66 (Fig. 5D,F,H). In contrast, *Pax-6* is expressed in the MC at the onset of its formation (data not shown) and during metamorphic climax (Fig. 5A,E). In the MC, *Pax-6* is expressed primarily in the neuronal and basal cell layers (Fig. 5A,E), whereas in the VNO, *Pax-6* is expressed only in the supporting cell layer (Fig. 5A). In the developing forebrain, *Pax-6* is expressed in the granular cells and in some mitral cells in the olfactory bulb

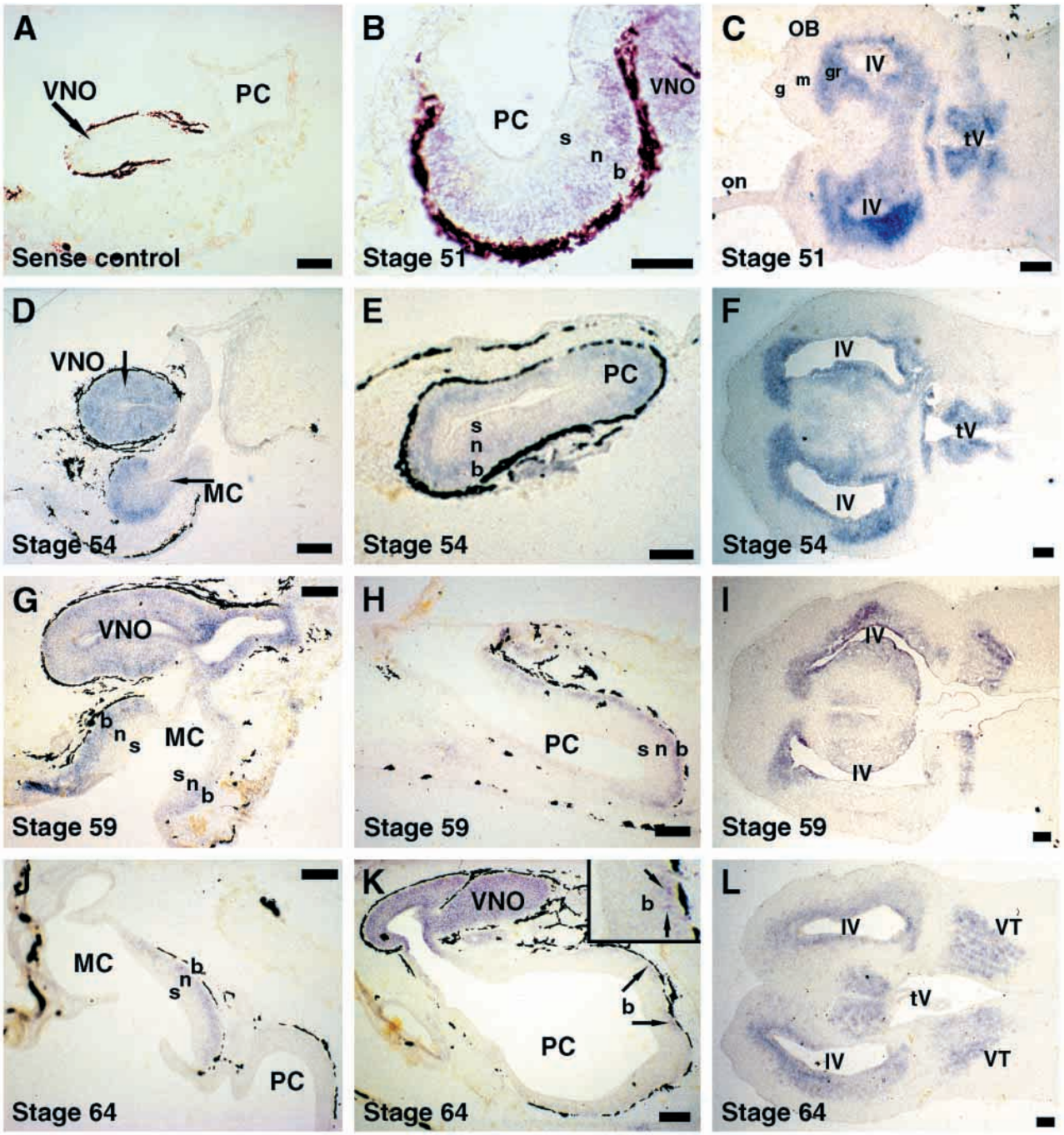

Fig. 3. Expression of *X-dll3* in the olfactory epithelium and forebrain during metamorphosis. (A) The principal cavity (PC) and vomeronasal organ (VNO) of a stage 57 tadpole hybridized with the sense *X-dll3* riboprobe as a negative control. (B–L) Sections through the nasal cavities and forebrain hybridized with the antisense *X-dll3* riboprobe. (B,E) The PC at stages 51 and 54, respectively, showing *X-dll3* expression in the neuronal and basal cell layers. (C,F,I,L) Sections through the forebrain at stages 51, 54, 59 and 64 respectively. *X-dll3* expression is seen around the lateral and tectal ventricles, in the granular cell layer and in the ventrothalamus. (D) At stage 54, *X-dll3* expression occurs throughout the VNO and middle cavity (MC). (G) At stage 59, *X-dll3* expression is present throughout the VNO and in the neuronal and basal cell layers of the MC. (H) The PC at stage 59 with *X-dll3* expression in deep neuronal and basal cell layers. (J) The MC and PC at stage 64 showing *X-dll3* expression in the basal cell/deep neuronal layer of the MC. (K) The PC and VNO at stage 64 showing *X-dll3* expression in a few basal cells or deep olfactory receptor neurons and throughout the VNO. The inset at the upper right is an enlargement of the basal cell layer showing arrows pointed to individually stained cells. Nose sections are transverse sections of the olfactory cavity with ventral facing up and lateral to the left except for B and E, in which dorsal is upwards. Dense black material delimiting regions of the olfactory epithelium in the nasal cavities is composed of pigment granules in A, B, D, E, G, H, J and K. Brain sections are horizontal, with anterior facing the left. b, basal cell layer; g, glomerular cell layer; gr, granular cell layer; lV, lateral ventricle; m, mitral cell layer; n, neuronal cell layer; OB, olfactory bulb; on, olfactory nerve; s, supporting cell layer; tV, tectal ventricle; VT, ventral thalamus. Scale bars, 200 μm.

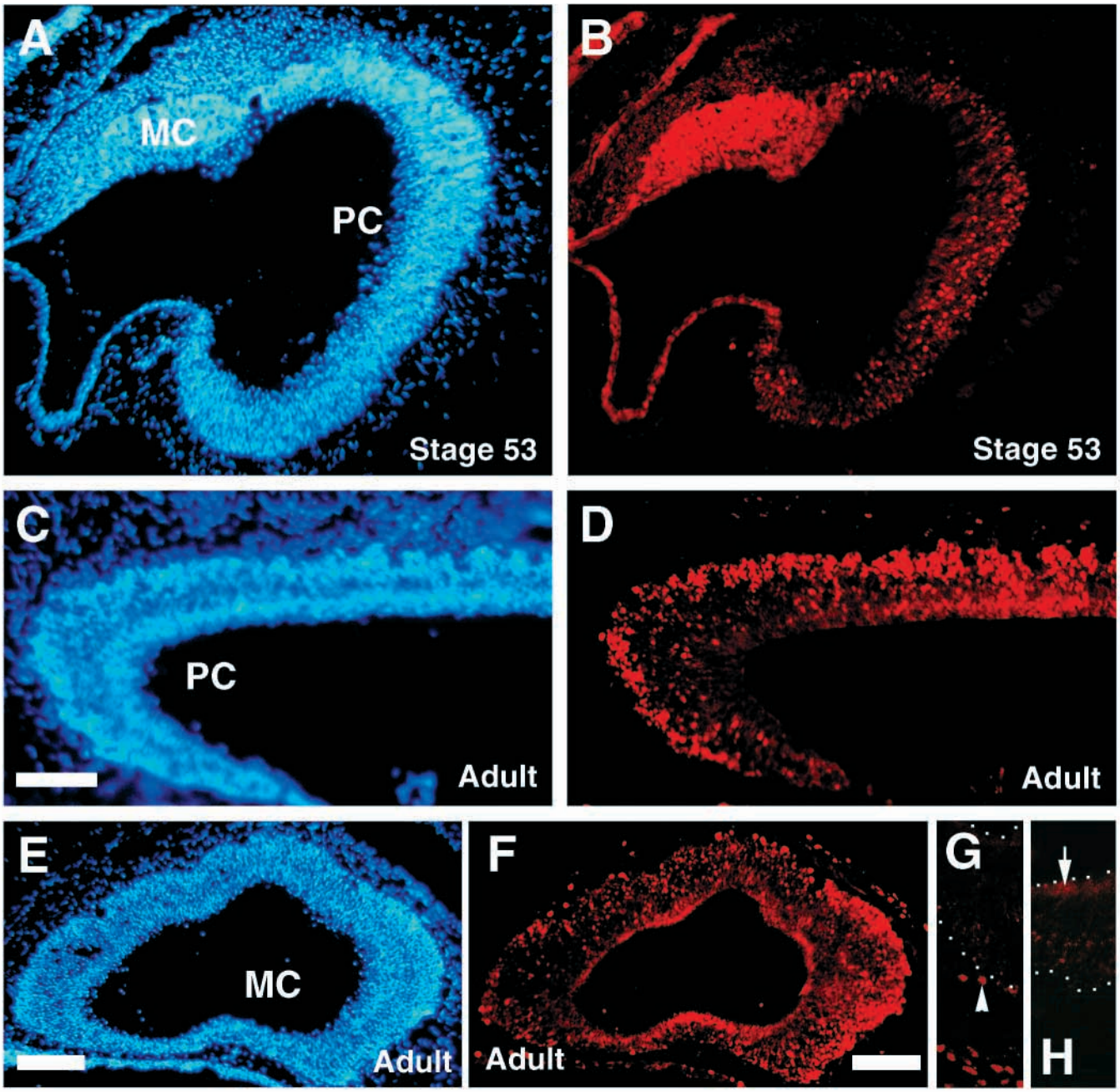

Fig. 4. Immunocytochemistry showing metamorphic and adult expression of Distal-less protein in the olfactory epithelium. Sections in A, C and E are stained with the blue nuclear stain DAPI and sections in B, D and F are stained with anti-Distal-less antibody followed by Cy3-labeled secondary antibody. (A,B) Staining in the olfactory epithelium of a stage 53 tadpole, (C,D) staining in the principal cavity (PC) of an adult, and (E,F) staining in the middle cavity (MC) of an adult. Note that the staining in the PC at stage 53 is scattered throughout the neuronal cell layer and in the basal cell layer, while in the adult PC, most of the staining is concentrated in the deeper neuronal and basal cell layers. In the MC, the staining is distributed throughout the olfactory epithelium at stage 53, but also becomes concentrated in deeper layers in the adult. (G,H) Negative control (no primary antibody) that illustrates areas of background staining at the most apical surface of the olfactory epithelium (arrow) and in the region at and below the basal lamina (arrowhead); the dots define the superficial (top) and basal (bottom) surfaces of the olfactory epithelium. Scale bars, 100 μm.

throughout metamorphosis. Ventricular cells do not express *Pax-6* during this same period (Fig. 5C,G,I). Apparently, the amount of Pax-6 protein produced in the olfactory epithelium during metamorphosis is low since we were unable to demonstrate protein expression using immunocytochemistry with a Pax-6 antibody generated against the last 18 amino acid residues at the carboxy terminus; the peptide we used was identical to that used by Davis and Reed (Davis and Reed, 1996). Both our antibody and that of Davis and Reed gave the same pattern and intensity of staining in the olfactory bulb.

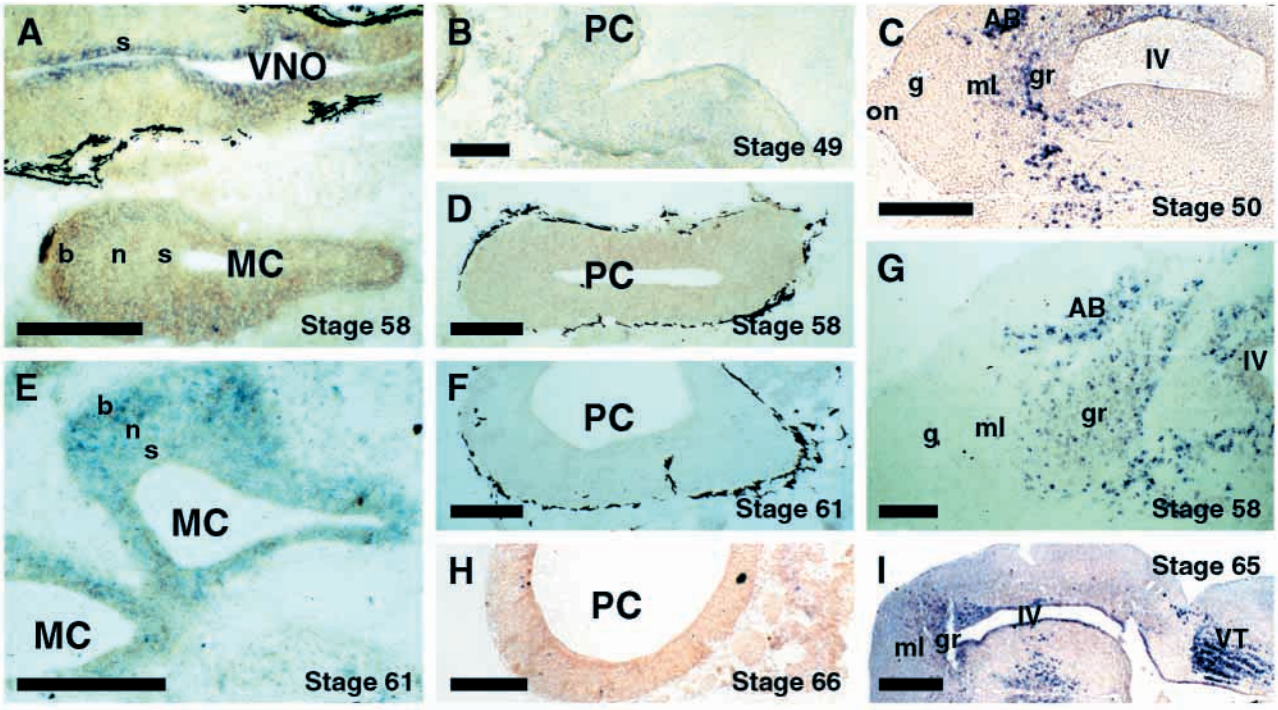

Fig. 5. Expression of *Pax-6* in the olfactory epithelium and forebrain during metamorphosis using the antisense *Pax-6* riboprobe. (A) Middle cavity (MC) and vomeronasal organ (VNO) at stage 58 showing *Pax-6* expression in the supporting cell layer of the VNO and in the neuronal and basal cell layers of the MC. (B,D,F,H) Sections through the principal cavity (PC) at stages 49, 58 61 and 66, respectively, demonstrate that *Pax-6* is not expressed in the remodeling olfactory epithelium. (C,G,I) Sections through the forebrain at stages 50, 58 and 65, respectively, showing *Pax-6* expression mainly in the granular cell layer of the olfactory bulb, in a few mitral cells and in very few cells around the ventricles. (E) The MC at stage 61 showing *Pax-6* expression in the neuronal and basal cell layers. Sections A, B, D, E, F, G are transverse sections of the olfactory cavity with ventral upwards and lateral to the left; B, dorsal upwards. Dense black material delimiting regions of the olfactory epithelium in the nasal cavities is composed of pigment granules in A, D and F. Brain sections are horizontal with anterior facing the left and lateral facing upwards. AB, accessory olfactory bulb; b, basal cell layer; g, glomerular cell layer; gr, granular cell layer; lV, lateral ventricle; ml, mitral cell layer; n, neuronal cell layer; on, olfactory nerve; s, supporting cell layer; VT, ventrothalamus. Scale bars, 150 μm.

Differential and overlapping expression patterns of X-dll3 *and* Pax-6

A direct comparison of the expression patterns of *X-dll3* and *Pax-6* during the embryonic, larval and metamorphic phases of olfactory system development is illustrated in Fig. 6. Fig. 6A shows that *X-dll3* is expressed in all cells of the olfactory placode and in all cells of the young larval PC. As the larval PC remodels, *X-dll3* expression becomes restricted to the deeper layers of the olfactory epithelium. By the end of metamorphosis, *X-dll3* is expressed only in deep neurons and basal cells. A similar spatial pattern of expression is found in the MC; *X-dll3* is expressed in all cells of the newly forming MC, and expression becomes restricted to deep neurons and basal cells at the end of metamorphosis. Fig. 6B shows that *Pax-6* is expressed in neurons and basal cells of larval PC and metamorphic MC. *Pax-6* expression then completely disappears from the larval PC as functional remodeling begins at stage 49.

Discussion

The olfactory system of the African clawed frog *Xenopus laevis* is a valuable model in which to study the pattern of expression of developmental control genes because there are two developmental stages. During early embryonic development, the body plan is established. Later, at metamorphosis, many of the same regions are remodeled and other structures are formed *de novo*. From the perspective of developmental biology, it is interesting to compare these two developmental stages to determine whether any of the molecular signals used to establish a particular tissue are reused to remodel that tissue at metamorphosis. Clearly, the major signal involved in the initiation of metamorphosis is thyroid hormone, which stimulates the adaptive body changes associated with the normal amphibian transition from aquatic to terrestrial life (White and Nicoll, 1981). Thyroid hormone exerts its effect on metamorphosis through its nuclear receptors, the thyroid hormone receptors (Evans, 1988). These receptors are thyroid-hormone-dependent transcription factors that induce metamorphosis by activating a cascade of changes in gene expression in most tissues of the larvae (Shi, 1996). While thyroid hormone is responsible for stimulating tissue changes at metamorphosis, it does not play a role in early embryonic development in frogs. Thus, we were interested in determining whether similar patterning genes might participate in the formation of specific olfactory tissues in the absence and presence of thyroid hormone.

Fig. 6. Summary of the spatial and temporal expression of *X-dll3* and *Pax-6* in the developing olfactory epithelium. The presumptive water-sensing epithelia of the larval principal cavity (PC) and the adult middle cavity (MC) are represented in blue and the presumptive air-sensing epithelium of the adult PC is represented in green. The time course is not to scale. b, basal cell layer; n, neuronal cell layer; s, supporting cell layer. Stages are according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

The molecular mechanisms involved in embryonic patterning have been extensively studied in recent years and have been shown to involve at least four types of molecule: the signaling molecules, the growth factors, the cell adhesion molecules and the transcription factors. Examples of these four types of molecule have been shown to participate in the embryonic development of the olfactory system (see Reiss and Burd, 1997a). In contrast, little is known about the molecular mechanisms involved in post-embryonic patterning. In this report, we show that genes encoding the transcription factors X-dll3 and Pax-6 are expressed during both embryonic and post-embryonic development of the olfactory system. Subtle differences in their spatio-temporal expression patterns suggest that they play distinct roles in forming the adult peripheral olfactory system.

Several studies have suggested that *X-dll3* orthologues are involved in the patterning of the olfactory system through expression in the developing olfactory placodes of *X. laevis*, mice, zebrafish and chicks (Papalopulu and Kintner, 1993; Akimenko et al., 1994; Simeone et al., 1994; Pera and Kessel, 1999). Recently, the generation of mice homozygous for a targeted deletion of the *Dlx5* gene has confirmed the role of these genes in the development of olfactory placodes (Depew et al., 1999). In the most severe cases, mice with *Dlx5* deletions have nearly complete aplasia of the nasal capsule, and in these mice the olfactory epithelium and vomeronasal organs are absent (Depew et al., 1999). During early development (E8.0–E8.5) in mice, *Dlx5* is expressed in a thin stripe of cells at the neural/non-neural boundary of the neural plate that contains olfactory and otic placodes (Depew et al., 1999). At a similar developmental stage (stage 15), this thin stripe of cells, termed the neural ridge in *X. laevis*, displays a similar pattern of expression when stained with the *X-dll3* orthologue. Our report supports the involvement of *X-dll3* in the early patterning because we show that *X-dll3* is expressed in cells fated to form the olfactory placodes as early as stage 15 (Eagleson and Harris, 1989). In addition, we show that *X-dll3* is expressed in the MC when it starts to develop at stage 51, suggesting that *X-dll3* is involved not only in embryonic patterning but also in the morphological changes that occur at metamorphosis. In both larval PC and MC, the spatio-temporal expression of *X-dll3* is similar, suggesting a common role in the formation of these two cavities. These results also suggest that *X-dll3* expression is not entirely under the control of thyroid hormone since *X-dll3* is expressed in the absence (embryogenesis) and in the presence (metamorphosis) of thyroid hormone.

In vertebrates, the *Distal-less* genes have been correlated with specific functions throughout development (for a review, see Bendall and Abate-Shen, 2000), ranging from neural induction (Papalopulu and Kintner, 1993; Yang et al., 1993; Pera et al., 1999) to cell differentiation (Porteus et al., 1994; Liu et al., 1997). The pattern of *X-dll3* expression in the peripheral olfactory system changes during development, suggesting that *X-dll3* may have multiple functions in establishing the adult olfactory system. Indeed, during embryogenesis, *X-dll3* is expressed in both supporting and neuronal cell layers, suggesting that *X-dll3* has a role in patterning the epithelium.

However, during metamorphosis, *X-dll3* expression is

restricted to the deeper cell layers, suggesting that X-dll3 is involved in neurogenesis. In addition, the intensity of the expression decreases as the PC and MC cavities form, correlating with the decrease in the number of new neurons generated at these later stages. Specifically, *X-dll3* expression in the PC becomes restricted to the olfactory receptor neurons and basal cells by mid-metamorphosis. Similarly, *X-dll3* is expressed throughout the olfactory epithelium of the developing MC, and its expression later becomes restricted to olfactory receptor neurons and basal cells at metamorphic climax. We also show that the Distal-less protein is expressed in young olfactory receptor neurons and basal cells of both adult PC and MC. Taken together, these results suggest that *X-dll3* is first involved in the patterning and genesis of all cells forming the peripheral olfactory tissue and is later involved in neuronal maturation and neuronal turnover in putative water- and air-sensing epithelia. Indeed, in most vertebrates, the development of the olfactory system requires initial patterning and continuous replacement of the olfactory neurons throughout adult life (Graziadei and Metcalf, 1971; Graziadei and Monti Graziadei, 1979).

Unlike *X-dll3*, which seems to be involved in the formation and maintenance of all nasal cavities, *Pax-6* seems to have a narrower role in the development of the peripheral olfactory system of the frog *X. laevis*. Our analysis of the expression pattern at stage 15 suggests that *Pax-6* is likely to be involved in early patterning of the olfactory system, but the level of expression during subsequent stages of early olfactory placode development is very low. In contrast, during these same stages of placode development, there is strong expression of *X-dll3*. In addition, while *X-dll3* is expressed in the sensory epithelium of the PC, MC and VNO during and following metamorphosis, suggesting a role in development and maintenance, *Pax-6* expression appears to be restricted to the formation of olfactory epithelia since it is expressed only during placodal stages in the PC, and in the developing MC. Since these two olfactory tissues are responsible for sensing odors in water, *Pax-6* appears to be involved in forming water-sensing olfactory epithelia. Alternatively, *Pax-6* could be involved in early and *de novo* non-remodeling events during the formation of the main olfactory epithelium, independent of water-sensing function.

The pattern of *Pax-6* expression in the VNO also differs from the pattern of VNO *X-dll3* expression. In addition, the patterns of expression of *X-dll3* and *Pax-6* differ in the olfactory bulb. *X-dll3* expression is restricted to bulb precursor cells and neurons in the early stages of differentiation both in embryos and through metamorphosis, while the expression pattern for *Pax-6* is primarily in differentiating and mature neurons during embryonic development, throughout metamorphosis and into adulthood. Thus, the spatial and temporal patterns of expression suggest different roles for these two transcription factors in the development of the olfactory epithelium and olfactory bulb.

Pax-6 expression in the olfactory epithelium differs between mice and *X. laevis*. In mice, Pax-6 protein is expressed only in basal and supporting cells of the main olfactory epithelium and in the proliferative zone of the VNO (Davis and Reed, 1996; Behrens et al., 2000), leading to the suggestion that Pax-6 is involved in the regulation of non-neuronal, lineage-specific genes (Davis and Reed, 1996). Our findings show that, in contrast, *Pax-6* is expressed in the supporting cells of the VNO and in basal cells and olfactory receptor neurons of the larval PC and MC, the equivalent tissues to the mammalian olfactory epithelium. Thus, *Pax-6* appears to play different roles in the developing olfactory epithelium of *X. laevis* and mice.

During the development of the olfactory system of *X. laevis*, there is an interesting spatio-temporal correlation between the patterns of expression of *Pax-6* and at least one of the fish-like Class I odorant receptor genes. The expression of fish-like odorant receptor genes begins at stage 32 in the larval PC (Mezler et al., 1999). Although diminishing by stage 50, their expression remains in the PC (Mezler et al., 1999) until the onset of the cellular remodeling of the PC at stage 57 (Reiss and Burd, 1997a; Reiss and Burd, 1997b; Hansen et al., 1998). Although some fish-like olfactory receptor genes continue to be expressed in the PC until stage 57 (Mezler et al., 1999), the neurons expressing them are probably the neurons that are fated to die to allow a complete remodeling of the PC (Hansen et al., 1998; Higgs and Burd, 2001). At stage 49, overlapping with the loss of the fish-like Class I olfactory receptor genes in the PC is the onset of expression of the mammalian-like Class II odorant receptor genes in the larval PC (Mezler et al., 1999). The absence of *Pax-6* expression during this period of remodeling in the PC suggests that *Pax-6* is not involved in establishing the air-sensing olfactory epithelium of the PC. In contrast, both *Pax-6* and the fish-like olfactory receptor genes (Freitag et al., 1995) are expressed in the MC, a cavity presumably specialized for sensing odorants in water (Venus et al., 1998). Taken together, these observations suggest that the fish-like receptor genes are co-expressed with *Pax-6* in the presumed water-sensing epithelia and that *Pax-6* expression never overlaps with expression of the mammalian-like receptor genes. This correlation in gene expression favors a role for *Pax-6* in the specific formation of presumed water-sensing epithelium.

Thyroid hormone plays a causative role in amphibian metamorphosis and has been shown to have a major influence over the remodeling of the olfactory system in the frog *X. laevis* (Burd, 1990; Burd, 1992; Petti and Burd, 1995; Burd, 1999). Thyroid hormone function is mediated through the regulation of gene expression (Shi, 1996), and many thyroid-hormone-regulated genes have been identified during hind limb transformation (Buckbinder and Brown, 1992), intestine remodeling (Shi and Brown, 1993) and tail resorption (Wang and Brown, 1991; Wang and Brown, 1993; Brown et al., 1996). Another gene, the transcription factor NF-I, is regulated by thyroid hormone (Shi and Brown, 1993) and has been reported to participate in establishing olfactory neuron phenotype in the rat (Baumeister et al., 1999; Behrens et al., 2000). These results suggest that thyroid hormone is likely to be involved in regulating the transcription factors associated with olfactory

system plasticity. Thus, it is likely that thyroid hormone is involved in regulating some of the changes in expression of *Pax-6* and *X-dll3* in the olfactory epithelium at metamorphosis. However, *Pax-6* and *X-dll3* cannot be regulated by thyroid hormone during embryogenesis since this hormone is not present until approximately stage 54, at the onset of metamorphosis (Leloup and Buscaglia, 1977).

This report provides new insight in the involvement of two transcription factors, *X-dll3* and *Pax-6*, in the patterning the adult olfactory system of the frog *X. laevis*. Our results suggest that *X-dll3* is involved in the formation and maintenance of olfactory epithelia exposed to either air or water and that *Pax-6* is specifically involved in the formation of olfactory epithelium exposed to water. That *Pax-6* expression can be correlated with the onset of expression of the fish-like Class I receptor genes in the larval PC and adult MC and that *Pax-6* expression in the MC is coincident with rising levels of thyroid hormone suggest that *Pax-6* is likely to be involved in a network of transcriptional control involving thyroid hormone at metamorphosis.

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