Development 122, 3381-3391 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2084



# *Pax2* contributes to inner ear patterning and optic nerve trajectory

**Miguel Torres[1], Emilia Gómez-Pardo and Peter Gruss***

Abteilung Molekulare Zellbiologie, Max-Planck-Institut für Biophysikalische Chemie, Göttingen 37077, Germany

[1]Present address: Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología, Universidad Autónoma, Madrid 28049, Spain
*Author for correspondence

## SUMMARY

**During gestation, the paired box-containing gene *Pax2* is expressed in the mid-hindbrain area, developing eye and inner ear. We generated *Pax2* null mutant mice, which show the requirement of *Pax2* for the establishment of axonal pathways along the optic stalks and ventral diencephalon. In mutant brains, the optic tracts remain totally ipsilateral due to agenesis of the optic chiasma. Furthermore, *Pax2* mutants show extension of the pigmented retina into the optic stalks and failure of the optic fissure to close resulting in coloboma. In the inner ear, *Pax2* mutants show agenesis of the cochlea and the spiral ganglion, i.e., the parts of the organ responsible for auditory function and in whose primordium *Pax2* is expressed. Our results identify *Pax2* as a major regulator of patterning during organogenesis of the eye and inner ear and indicate its function in morphogenetic events required for closure of the optic fissure and neural tube.**

Key words: *Pax2*, gene targeting, midbrain, hindbrain, sense organ, mouse

## INTRODUCTION

In vertebrates, the nose, the eye and the ear house the highly specialized sensory apparatuses for olfaction, vision, balance and hearing. Classical embryological studies have shown that both autonomous and inductive cues contribute to specify and regionalize the developmental fields that constitute the complex architecture of sense organs (for review see Noden and Van de Water, 1986; Saha et al., 1992). However, very little is known about the molecular nature of the processes involved. Several families of transcription factors have been found in a variety of organisms, which provide tissue- or region-specific autonomous determination during development and thus represent valid candidates for autonomous regionalizing signals. The *Pax6* gene, encoding a paired domain-containing transcription factor, is the only example of a molecule with such a role in sense organ patterning (Walther and Gruss, 1991; Hill et al., 1991; Ton et al., 1991; Quinn et al., 1996). Its function has been strikingly conserved throughout evolution, as demonstrated by the ability of the mouse *Pax6* to promote eye development in flies (Halder et al., 1995). In general, Pax genes are specifically expressed during the development of a wide range of structures and organs (Gruss and Walther, 1992). As indicated by the phenotypes resulting from specific mutations in both man and mouse, members of the Pax family have critical morphogenetic functions during the development of complex tissues and organs in vertebrates (for review see Chalepakis et al., 1992; Strachan and Read, 1994). Two of the Pax genes; *Pax2* and *Pax6*, are restrictedly expressed in developing sense organs (Nornes et al., 1990; Walther and Gruss, 1991). While *Pax2* is expressed in the developing inner ear and *Pax6* is expressed in the developing nose, both are expressed in the developing eye. During development, the embryonic primordium of the eye and optic tracts, consisting of the optic evagination and the contacting surface ectoderm, undergoes morphogenetic movements that delineate the different fields composing the eye (Saha et al., 1992). The different fields subsequently differentiate into the definitive components of the eye and optic tracts. Expression of *Pax6* in the visual system is restricted to regions fated to constitute the eye deriving from the distal optic vesicle and the ectodermal lens placode. In contrast, *Pax2* expression is initially confined to the ventral optic vesicle, without proximodistal restriction, later being confined mostly to the proximal regions destined to contribute to the optic nerve and optic chiasm. Interestingly, *Pax2* is also regionally expressed within the otic vesicle primordium confined mostly to regions of the vesicle fated to have an auditory function. Characterization of spontaneous mouse and human *Pax6* mutations has demonstrated a critical role for *Pax6* in the differentiation of structures derived from the distal optic vesicle and lens as well as nasal structures (Hill et al., 1991; Ton et al., 1991; Quinn et al., 1996). Spontaneous mutations affecting *Pax2* function have been described in both humans and mice (Krd mutants) (Keller et al., 1994; Sanyanusin et al., 1995). Heterozygosity for these two mutations produced eye and kidney defects, suggesting the relevance of *Pax2* in eye and kidney development, showing the functional conservation between species and demonstrating haplo-insufficiency for the locus. However, the study of the mutation only in the heterozygous condition in the mentioned reports, precluded the complete definition of *Pax2* functions. Mutation of *Pax2* by homologous recombination (Torres et al., 1995) has allowed its detailed analysis in the complete loss-of-function condition, showing the important role of this gene during urogenital development, and repro-

ducing Krd and human *Pax2* haplo-insufficient phenotype for kidney development.

We present here for the first time the analysis of the phenotype of *Pax2* null mutants in the central nervous system (CNS) and sense organ development. Our results show that *Pax2* plays important roles in morphogenesis and regional specification of the eye, in which the heterozygous phenotype partially reproduces the human syndrome. In addition, we uncover novel functions for *Pax2* in the development of optic tracts, auditory regions of the inner ear and closure of the neural tube at the midbrain level.

## MATERIALS AND METHODS

### Mice

A previously described *Pax2* null mutation obtained by homologous recombination was used for this analysis (Torres et al., 1995).

Embryo dissections: fetus were collected in PBS and yolk sacs or tails were removed for DNA analysis. Genotyping of embryos and adults was performed by Southern blot as previously described (Torres et al., 1995).

### Histology

E12.5 embryos were dissected out in PBS and embryos were fixed in 4% paraformaldehyde (PFA) and, after dehydration, embedded in paraffin and sectioned at 8 to 10 μm. Sections were stained with Haematoxylin and Eosin. E17.5 embryos were treated similarly except they were fixed in Bouin's fixative and stained by Mallory's tetrachromic procedure.

### Antibody staining

E13.5 embryos were fixed overnight in PFA, immersed in sucrose 30% for 24 hours, embedded in Tissue tek and cryostat sectioned at 10 μm. Primary antibodies: anti-Pax2 (Dressler and Douglas, 1992), anti-Neurofilament 160 (Sigma) and anti-E-Cadherin (Sigma). Secondary antibodies: Cy3-conjugated (Jackson ImmunoResearch Laboratories) and peroxidase-conjugated (Vectastain) antibodies.

### Whole-mount in situ hybridizations

Embryos were processed as described (Wilkinson, 1992) using probes previously described for *Wnt-1* (Wilkinson et al., 1987), *Pax5* (Asano and Gruss, 1992), *En-1* (Davis and Joyner, 1988), *Pax2* (Dressler et al., 1990), *Shh* (Echelard et al., 1993). After in situ hybridization embryos were dissected and photographed as whole mounts.

## RESULTS

### *Pax2* is required for the closure of the cephalic neural tube

*Pax2* is dynamically expressed during neurulation in the prospective brain neuroepithelium. Initially *Pax2* is expressed at E7.5 (embryonic day 7.5) in a broad region encompassing most of the prospective forebrain down to the prospective anterior hindbrain (Püschel et al., 1992; Rotwich and McMahon, 1994). As neural tube closes, the expression rapidly narrows in the anteroposterior (A-P) direction and, by E9.5, it is reduced to a thin stripe at the mid-hindbrain boundary. *Pax2* homozygous mutant embryos show exencephaly, resulting from the failure of the neural folds to close at the midbrain region (Fig. 1). At E9.0, when the neural tube has just closed in control embryos, the normal disposition of early A-P brain

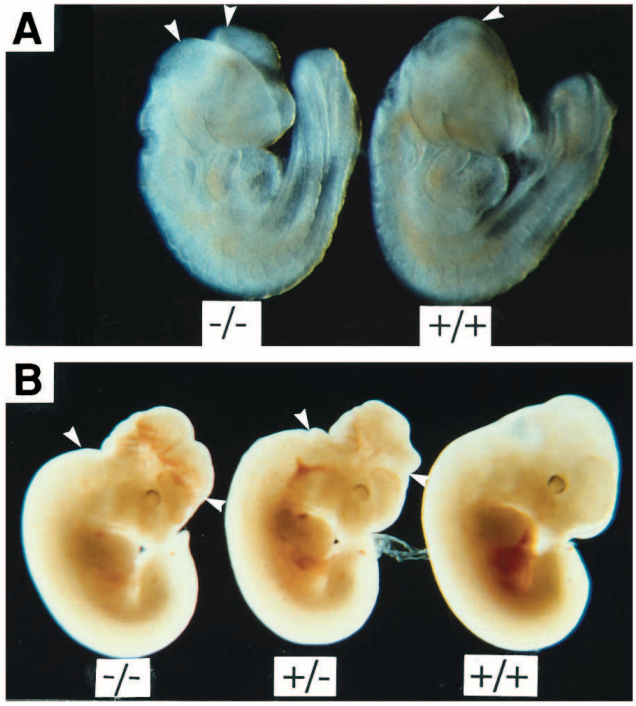


**Fig. 1.** *Pax2* mutants fail to close the neural tube around the midbrain region. At around 9.0 days of development in the mouse, the neurulation process finishes in the head region by the complete closure of the neural folds into a tube. (A) Control (right) and mutant (left) E9.0 embryos. The arrow in the control embryo shows the approximate point in the midbrain where the last portion of the tube is closed at the head region. In the mutant embryo, the head folds (arrowheads in the mutant embryo) do not come together and therefore fusion does not take place. As a result of the failure of the neural tube to close, the embryos develop exencephaly at later stages (B) from the anterior hindbrain region to the posterior forebrain region. (B) Exencephalic homozygous and heterozygous mutant and control embryos at E11.5.

subdivisions are found in mutant embryos, but the neural folds are not fused (Fig. 1). At later stages, the non-fused neural folds proliferate abnormally and overgrow outwards. The exencephaly is also found in heterozygous embryos, albeit with a low penetrance and in a background-dependent manner. Among the defective embryos, the degree of exencephaly is not dependent on the genotype, appearing as an all-or-nothing phenomenon. The incidence of exencephaly in heterozygous embryos is 11 out of 59 cases analyzed in a 129sv × NMRI mixed background, 1 out of 29 in a 129sv × C57/B6 mixed background, 2 out of 11 in a 129sv × C3H mixed background and none out of 14 in a 129sv inbred background. The extension of the exencephaly shows a low degree of variation among individuals that does not correlate with the genotype. The severity of the phenotype, when present, is similar between homozygous and heterozygous animals.

*Wnt-1*, a member of the wingless family of signalling molecules, *Pax5*, a member of the paired box family closely related to *Pax2*, and the homeobox genes *En-1* and *En-2* are expressed around the mid-hindbrain boundary area (Wilkinson et al., 1987; Davis et al., 1988; Asano et al., 1992). Loss-of-function mutations in these genes result in deletions affecting different portions of the meten-mesencephalic areas

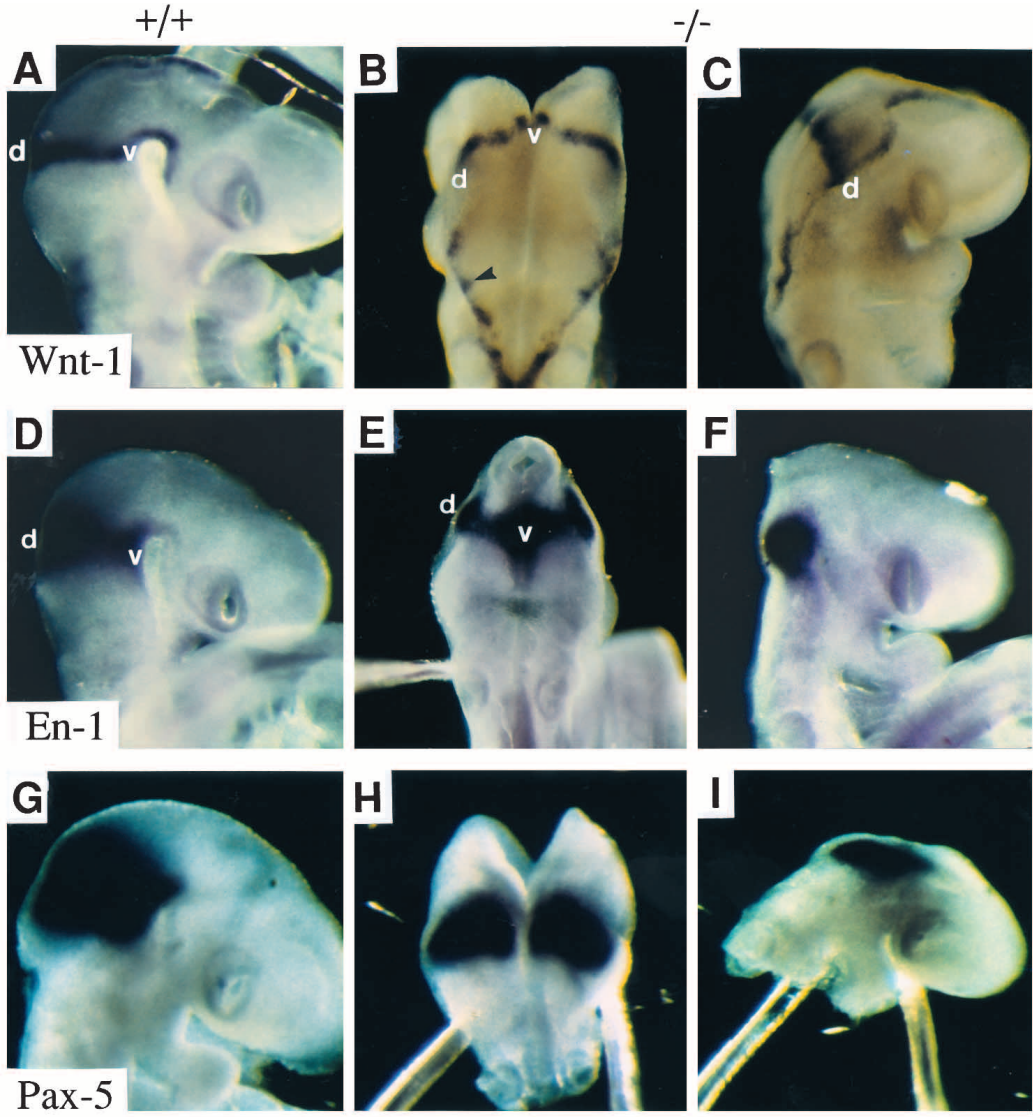


**Fig. 2.** *Pax2* mutation does not alter early regional gene expression in the brain. Plates show the expression by whole-mount in situ hybridization of different regulatory genes expressed at the mid-hindbrain region of wild-type and *Pax2* mutants at E9.5. (A,D,G) The heads of control embryos hybridized with probes for, respectively, *Wnt-1*, *En-1* and *Pax5*. (B,E,H) Lateral views and (C,F,I) dorsal views of homozygous mutant embryos hybridized with *Wnt-1*, *En-1* and *Pax5* probes, respectively. d, dorsal; v, ventral

(McMahon and Bradley; 1990, Joyner et al., 1991; Urbanek et al., 1994; Wurst et al., 1994). *Pax2* is expressed in the same region and its expression precedes the onset of the above mentioned genes. We have analysed the expression of *Wnt-1*, *En-1* and *Pax5* in mutant embryos in order to establish whether *Pax2* activates the expression of these genes in this region. Despite the abnormal opening of the neural tube in mutant animals, anteroposterior and dorsoventral patterns of expression of *Wnt-1*, *Pax5* and *En-1* appear normal at E9.5 (Fig. 2). Examination of the expression of these markers at later stages is not accessible due to the gross alteration of the brain morphology in midbrain and anterior hindbrain.

### *Pax2* is required for eye morphogenesis

Eye formation in vertebrates is mediated by multiple early inductive events. After these initial inductive events, eye formation involves two distinct processes: firstly, morphogenetic processes, through which the basic elements of the eye are produced and shaped, and, secondly, differentiation processes, through which cells allocated to the different regions during the first phase, differentiate into the appropriate fate according to their position. One of the main events during the morphogenetic phase is the invagination of the ventral portion of the optic vesicle. At the distal-most part of the stalk, the invaginated tissue encircles the ectoderm-derived forming lens. The result of this process is the formation of the optic globe with its three main components, the outer pigmented retina, the neural retina and the lens. Ventrally, the eye remains transiently open at the optic fissure through which vascularization occurs. The ventral invagination and the presence of the optic fissure also extends into the optic stalk region. Closure of the optic fissure marks the end of the morphogenetic phase. Early in morphogenesis, *Pax2* is expressed in the ventral half of the optic vesicle (Dressler et al., 1991). The expression is very dynamic, being repressed in the prospective neural retina shortly after its invagination but persisting during the whole morphogenetic phase at the lips of the optic fissure (Fig. 3E) and extending ventrally into the optic stalk up to the brain. We have examined the evolution of the morphogenetic phase of the eye in the mutants. Formation of the optic stalk, invagination of the prospective neural retina and lens segregation occurs normally. However, homozygous

*Pax2* mutants fail to close the optic fissure, showing complete bilateral coloboma at birth (Fig. 3A-D). During normal eye development, the optic fissure closes by contact-dependent dissolution of the basal lamina at the contacting neuroepithelial lips. We have followed the basal lamina in mutant embryos by the presence of laminin at the lips of the optic fissure (Fig. 3F,G). We found that, despite the apparent contact between the lips, the basal lamina persists precluding the closure of the optic fissure. Despite the open optic fissure, both retinal layers appear normal at birth with regard to cell type composition (data not shown). Therefore the requirement for *Pax2* in the optic cup appears to be restricted to morphogenetic events occurring in the most ventral cells around the optic fissure, where its expression is persistent throughout all the morphogenetic period.

### *Pax2* sets the limit between the pigmented retina and the optic stalk epithelium

Although *Pax2* expression is initially restricted to the ventral half of the optic evagination, after the optic cup is formed it extends dorsally in the optic stalk. As a result, it labels the prospective optic nerve, stopping sharply at the prospective pigmented retina-optic nerve boundary (Fig. 4A-D). After the morphogenetic phase, the prospective outer retinal layer is continuous with the optic stalk epithelium, nevertheless they both give rise to very different cell types during the differentiation phase. While the prospective outer retinal cells differentiate into pigmented cells, the optic stalk cells proliferate and differentiate into glial cells that populate the optic nerve. Pigmentation of the outer retina cells progresses in a distal-to-proximal direction and stops at the *Pax2*-expressing cells in the optic stalk. The limit is sharply defined such that no cell expressing *Pax2* shows pigmentation (Fig. 4B,D). In agreement with this expression pattern, we have observed defects in the differentiation of stalk cells. In *Pax2* mutant animals, the pigmentation does not stop at the eye-stalk limit but rather extends abnormally into the stalk region. Thus in the mutants, cells located in the stalk region differentiate into cells that can not be morphologically distinguished from pigmented retinal cells (Fig. 4E-H). The extension of the pigmentation into the optic stalk affects, depending on the genetic background, from 50% to practically all of the stalk extension up to the diencephalon.

Optic nerve genesis starts when the axons coming from the retina grow into the stalk region on their way to the base of the diencephalon. Growing axons interact tightly with stalk cells, which in turn, differentiate into glial cells whose processes hold the axon bundles and provide the outer cell layer of the nerve (Horsburgh and Sefton, 1986). Optic nerve glial cells originate from the in situ proliferation and differentiation of optic stalk cells, except for the oligodendrocytes, which migrate postnatally into the optic nerve from diencephalic regions. At the time of the analysis, the only glial cells expected to be in the optic nerve are those derived from the optic stalk. In the mutant, axons migrate as expected through the optic disc, however, we do not detect any cell body in the optic nerve between the axons (Fig. 4F,H). The mutant optic nerve consists of a single bundle of axons and has a reduced diameter, probably because of the absence of cell bodies between the axons, but a lower number of axons is also possible. Therefore, all glial cells derived from the optic stalk are lost in the mutant optic nerves; the outer sheath of glial cells is replaced by pigmented cells and the stalk-derived inner glia is missing.

### Optic fibers project exclusively to the ipsilateral side in *Pax2* mutants

We have examined the trajectory of the optic fibers in *Pax2*

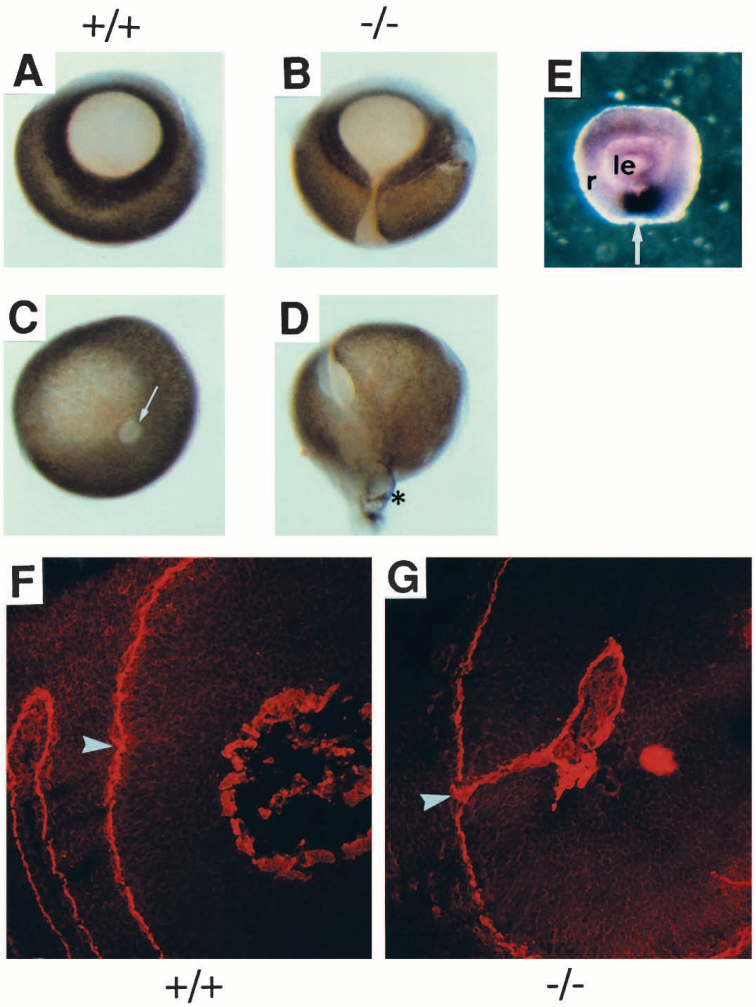


**Fig. 3.** *Pax2* is required for the closure of the optic fissure. A to D show whole-mount eyes dissected from E17 fetus. A and B show front views of, respectively, wild-type and mutant embryonic eyes and C and D show back views of the same eyes. Note the open optic fissure in the mutant and the absence of a defined boundary (arrow in C and asterisk in D) limiting the pigmented retina at the back of the eye. E shows a front view of a whole-mount dissected eye hybridized with a *Pax2* probe at E11.5, when the closure of the optic fissure starts. Note that *Pax2* transcripts at this stage are restricted to the converging lips (arrow) of the retina on both sides of the optic fissure. F shows detection of laminin by immunofluorescence to identify basal lamina in sagittal sections of a wild-type E13 embryo. Note how basal lamina has dissolved in the contact regions of the converging lips of the prospective retina at the former optic fissure (arrowhead) so that it is now continuous around the lens. At a similar stage, the mutant eye shows persistence of the basal lamina as judged by the presence of laminin (shown in G), even though the converging lips seem to contact each other. r, retina; le, lens.

mutants as they travel towards the midline to insert into the brain. Normally, leading migrating optic fibers reach the midline at the optic recess region at the base of the diencephalon (Horsburgh and Sefton, 1986; Marcus and Mason, 1995). At this point, axons make a 'decision'; some fibers will cross the midline and constitute the contralateral projection and some will turn and form the ipsilateral projection; altogether they constitute the optic chiasm (Godement et al., 1990). In the mouse, roughly 95% of the fibers project contralaterally in normal animals. In contrast, *Pax2* mutants have no optic chiasma and all axons project ipsilaterally without reaching the midline (Fig. 5). The optic fibers appear to turn without reaching the region of the optic chiasm, such that the insertion of the nerve occurs more laterally than in the wild type. We have also performed retrograde labelling experiments at E16.5, to explore the possibility of the presence of a minority of axons projecting contralaterally, but were unable to identify any (data not shown). The observed phenotype is not likely to result from defects in the choice between the ipsilateral or the contralateral side that takes place at the midline, since axons never reach the optic chiasma region. Rather, it appears to result from the abolishment of the ventral pathway to the midline so that the fibers never have the chance to choose sides and all project ipsilaterally.

### Defects in the ventral diencephalon may abolish the pathway of the axons towards the midline

Cellular cues in the optic stalks and at the ventral midline of the diencephalon have been implicated in the guidance of growing retinal axons towards the optic recess area to form the optic chiasma (Marcus and Mason, 1995). We have analyzed genes expressed at the ventral midline of the diencephalon in

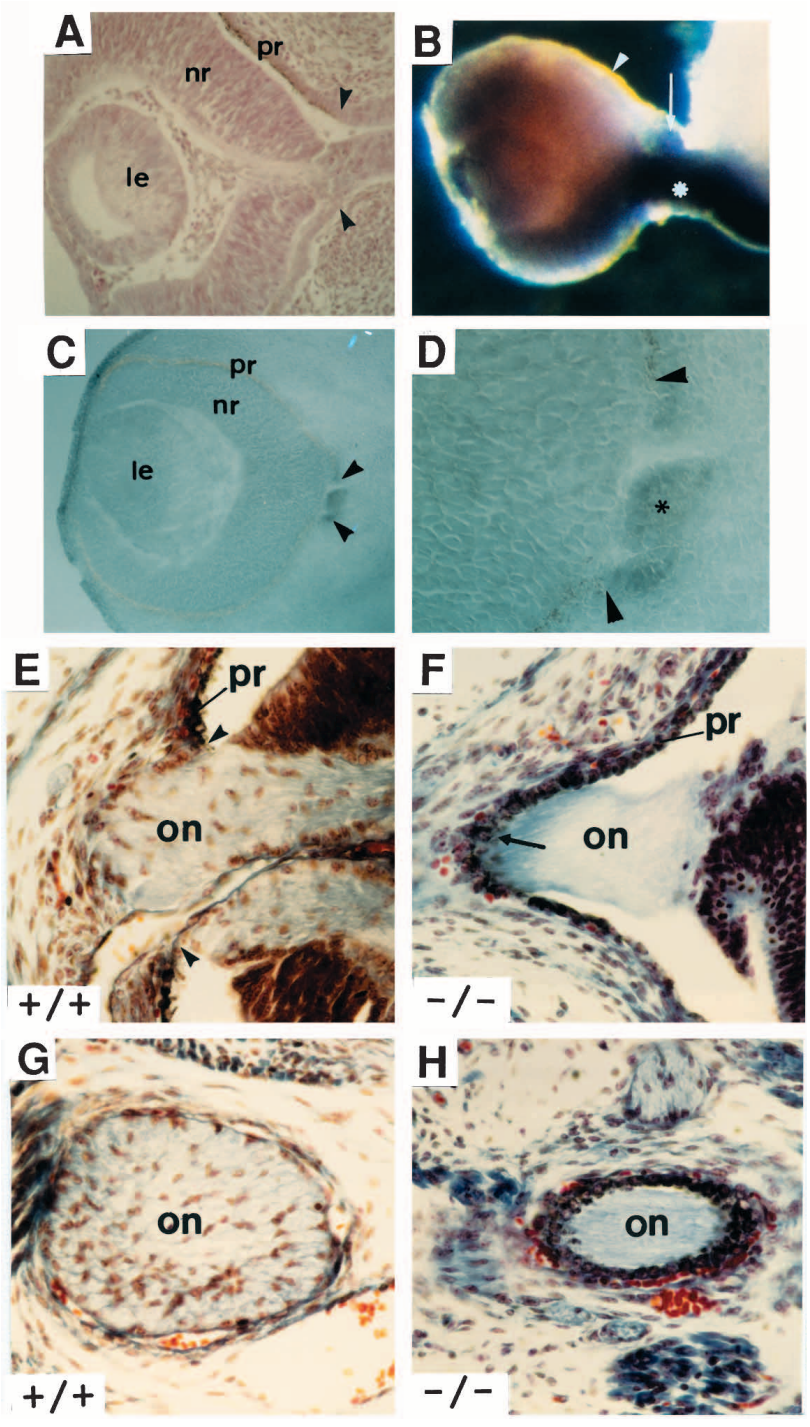


**Fig. 4.** The absence of *Pax2* results in distalization of the optic stalk. After the morphogenetic events that establish the basic fields of the eye, the outer eye layer is continuous with the optic stalk epithelium. However, distal regions of this layer differentiate into pigmented retina while proximal regions support axonal growth and differentiate into glial cells of the optic nerve. The limit between the cells destined to either fate lies between the optic cup and the optic stalk. At E12.5 pigmentation of the dorsal retinal cells has progressed until this limit (see A). (B) The distribution of *Pax2* messenger by whole-mount in situ hybridization in dissected eyes together with the optic stalks and the diencephalon. Note how the ventral expression of *Pax2* at the optic fissure (asterisk), extends from the distal regions to the basis of the diencephalon. Only in the optic stalk region, contiguous to the pigmented epithelium of the optic cup (arrowhead), the expression extends to dorsal regions of the stalk (arrow), in coincidence with the observed distribution of the protein. (C) Peroxidase immunodetection of Pax2 protein in those optic stalk cells contiguous to the pigmented retinal cells (arrowheads). (D) A magnification of B, note the presence of Pax2 protein in the invaginated tissue of ventral origin (asterisk) and in the outer epithelial layers continuous with the pigmented epithelium. Note how pigmentation avoids Pax2-expressing cells (arrowheads). (E) A histological section at E17 at the insertion of the optic nerve into the eye cup in a control embryo. The pigmented retina shows a clear limit with respect to the optic nerve (arrowheads). Cells of the former optic stalk have proliferated and differentiated into Glial cells of the optic nerve. A similar section of the mutant eye (F) shows that the pigmented cell phenotype has extended into the optic stalk region and thus surrounds the optic nerve. (G,H) Sections of, respectively, control and mutant optic nerves in their way to the diencephalon. Note the abnormal coating of the mutant optic nerve by pigmented cells as well as the absence of glial cells in F and H. le, lens; nr, neural retina; pr, pigmented retina; on, optic nerve.

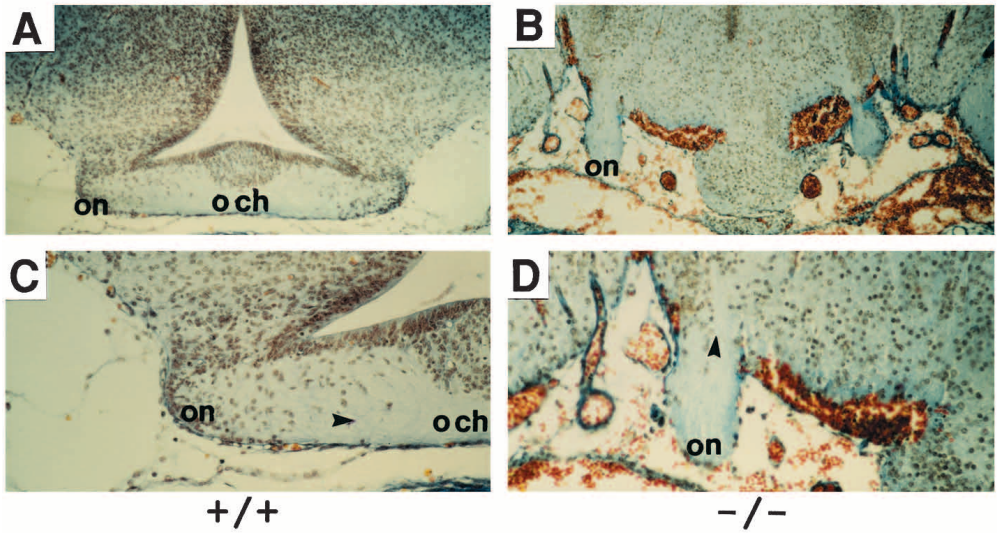


**Fig. 5.** *Pax2* mutants optic tracts lack contralateral projections. (A,B) Histological frontal sections of, respectively, control and mutant embryos at the optic chiasm level. (C,D) Respective magnifications of A and B. Note the ipsilateral insertion (arrowheads) of the mutant optic nerve, associated with abnormal morphology of the basis of the diencephalon, where no optic chiasm is appreciated. on, optic nerve; och, optic chiasm.

order to determine the possible defects in this area. Hedgehog proteins are a family of signalling molecules with patterning activities in a wide range of organisms (Fietz et al., 1994). Some of the members of this family are expressed in the ventral midline of the vertebrate neural tube. We have analyzed the expression of one of the members of the family, *Sonic-hedgehog (Shh)* (Echelard et al., 1993), which is expressed in the ventral forebrain area and compared it to *Pax2* expression. At E9.5, *Shh* is expressed in the ventral forebrain from the ventral hypothalamus continuously up to the preoptic area. *Pax2* is expressed in the optic stalks, stopping sharply at the limit with the diencephalon, thus avoiding the hedgehog-expressing area (Fig. 6C). At this early stage, we did not find any difference in the expression pattern of *Shh* between mutant and control embryos (Fig. 6A,B). At E11.5, just before the first axons start to migrate out from the retina towards the optic recess, the expression patterns of both *Shh* and *Pax2* change drastically. *Shh* expression has split in two, leaving a gap devoid of expression, whereas *Pax2* expression has extended medially into the gap left by *Shh*, so that the expression pattern is now continuous ventrally, yet still not overlapping the *Shh* expression domain (Fig. 6D,F). This gap is the only interruption of the *Shh* expression domain along the A-P axis and corresponds to the optic recess, the area through which the first retinal axons cross the midline. Strikingly, in *Pax2* mutants, the gap, which is normally devoid of *Shh* expression, is lost, so that the *Shh*-expressing regions at the preoptic area and the ventral hypothalamus join together and there is no longer an interruption in *Shh* expression along the A-P axis (Fig. 6E,F). Furthermore, some examples of ectopic *Shh*-expressing cells in the ventroproximal region of the optic stalks can be identified (Fig. 6I). This phenomenon occurs in the absence of any obvious A-P extension of the normal *Shh* expression domains, either in the preoptic area or in the ventral hypothalamic area, suggesting that both domains come together as a consequence of the loss or displacement of the *Shh* non-expressing cells from the optic recess area.

### *Pax2* is essential for differentiation of auditory regions of the inner ear

*Pax2* begins to be expressed very early during inner ear development. From the onset of its expression, it is restricted to the ventral half of the developing vesicle from which the cochlear and saccular regions derive (Nornes et al., 1990; Torres and Gruss, unpublished data). Invagination of the otic vesicle progresses normally in the mutants and neuroblastic precursors of the vestibulo-acoustic neurons segregate normally from the vesicle (not shown). However, as shaping of the vesicle proceeds to give rise to the different elements of the ear, the vestibular regions of the ear differentiate properly with only minor shape differences compared to controls, but the cochlear duct fails to extend from the ventral portion of the vesicle, thus remaining unshaped (Fig. 7G,H). As cell differentiation progresses, sensory cells of the vestibular portion of the inner ear differentiate normally and establish their innervation with the vestibular ganglion (Fig. 7A,B). In contrast, the cochlear region remains as a ball-shaped simple epithelium, where none of the organ of Corti cell types can be recognized (Fig. 7C,D). In addition, the cochlear ganglion is absent and therefore no innervation of the cochlear region occurs (Fig. 7E,F). The defects correlate with homozygosity for the *Pax2* mutation and not with the exencephaly, since heterozygous exencephalic embryos do not show obvious alterations of inner ear development.

## DISCUSSION

We have analyzed the phenotype of a complete loss-of-function mutation for the paired box-containing gene *Pax2* in the central nervous system and sense organs. The defects observed in mutant mice severely affect the regionalization and morphogenesis of the eye, inner ear and midbrain. *Pax2* is required within these organs for at least two apparently unrelated functions: morphogenetic functions, such as the closure of the neural tube and the optic fissure, and regional specification, such as the determination of fates along the proximodistal axis of the optic evagination or the development of auditory regions of the inner ear. Outside the nervous system, *Pax2* function is essential for the differentiation of all intermediate mesoderm-derived components of the urogenital system (Torres et al., 1995). Multiplicity of functions is not

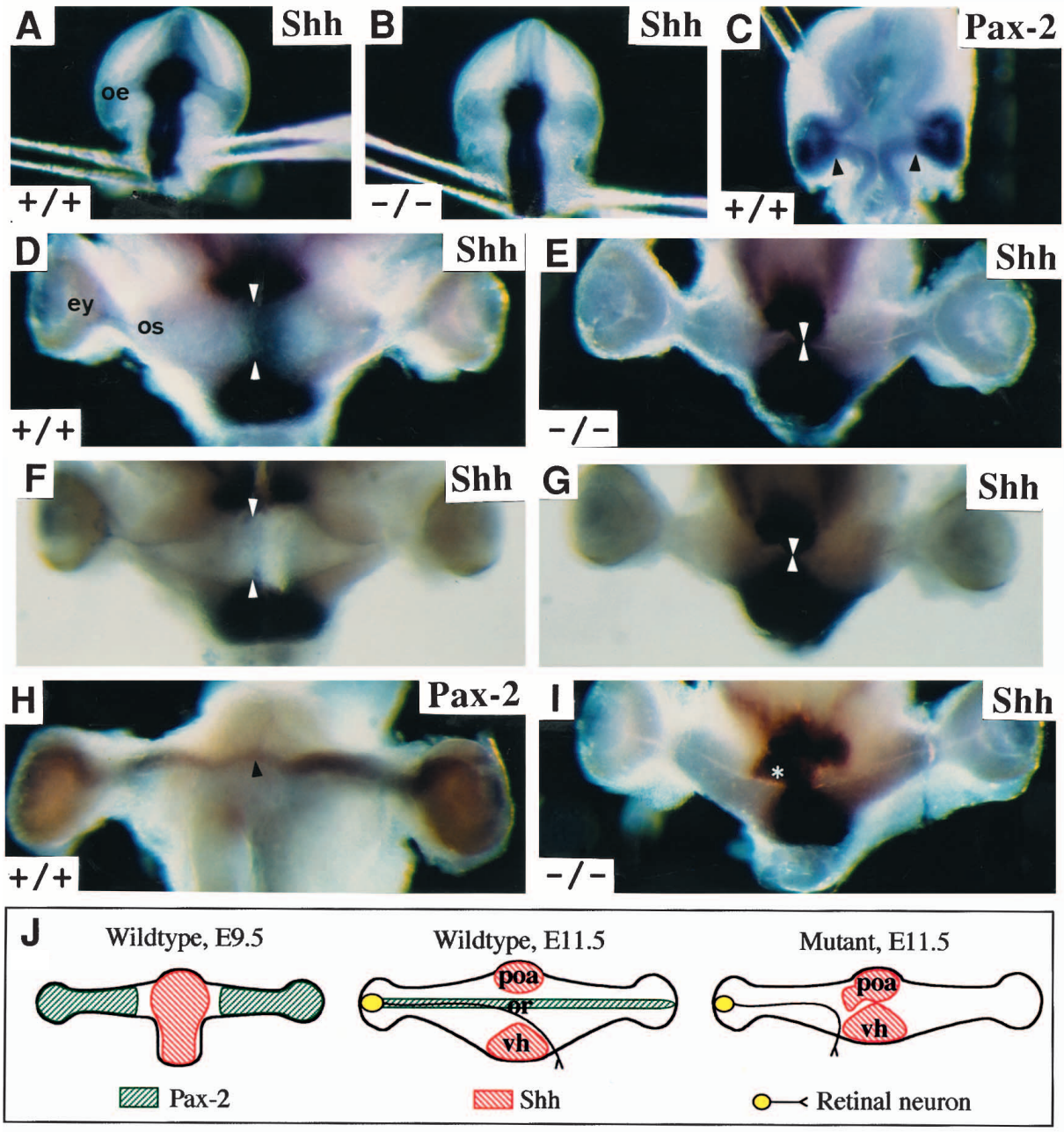


**Fig. 6.** *Pax2* mutants show alterations in the distribution of *Shh-expressing* cells at the basis of the diencephalon. (A-C) A ventral view of the forebrain region dissected out of E9.5 embryos after whole-mount in situ hybridization. (A,B) Control and mutant E9.5 embryos hybridized with a Sonic hedgehog probe. (C) A similar embryonic stage hybridized with a *Pax2* probe, arrowheads point to the limit of *Pax2* expression at the insertion of the optic stalk in the basis of the diencephalon. (D-I) Ventral views of the diencephalon, together with the optic stalks and developing eyes, dissected out of E11.5 embryos after whole-mount in situ hybridization. (D,F) A control specimen hybridized with *Shh* and photographed under dark-field and bright-field illumination, respectively. (E,G) Dark- and bright-field photographs of a mutant specimen hybridized to *Shh*. (I) A different mutant specimen hybridized with *Shh* under dark-field illumination. (H) A control embryo hybridized with a *Pax2* probe. Arrowheads in D to G point to the limits of the presence of *Shh*-expressing cells in the preoptic area (upper arrowhead) and basis of the hypothalamus (lower arrowhead). Asterisk in I shows the presence of *Shh*-expressing cells in the optic stalk. Arrow in H points to the *Pax2*-expressing cells across the midline. (J) Scheme summarizing the observations. oe, optic evagination; ey, eye; os, optic stalk; poa, preoptic area; or, optic recess; vh, ventral hypothalamus.

infrequent among transcription factors with roles in embryonic development and suggests that many of them may act as multipotent switches such that a single molecule is able to instruct cells into different pathways depending on the cell history and position in the embryo. *Pax* genes represent a particularly good example of this multiplicity since many of its members are involved in multiple apparently unrelated developmental functions (see Chalepakis et al., 1992 and Strachan and Read 1994 for review). *Pax6* has been shown to be essential for eye and nose development (Hill et al., 1991; Ton et al., 1991). *Pax3*

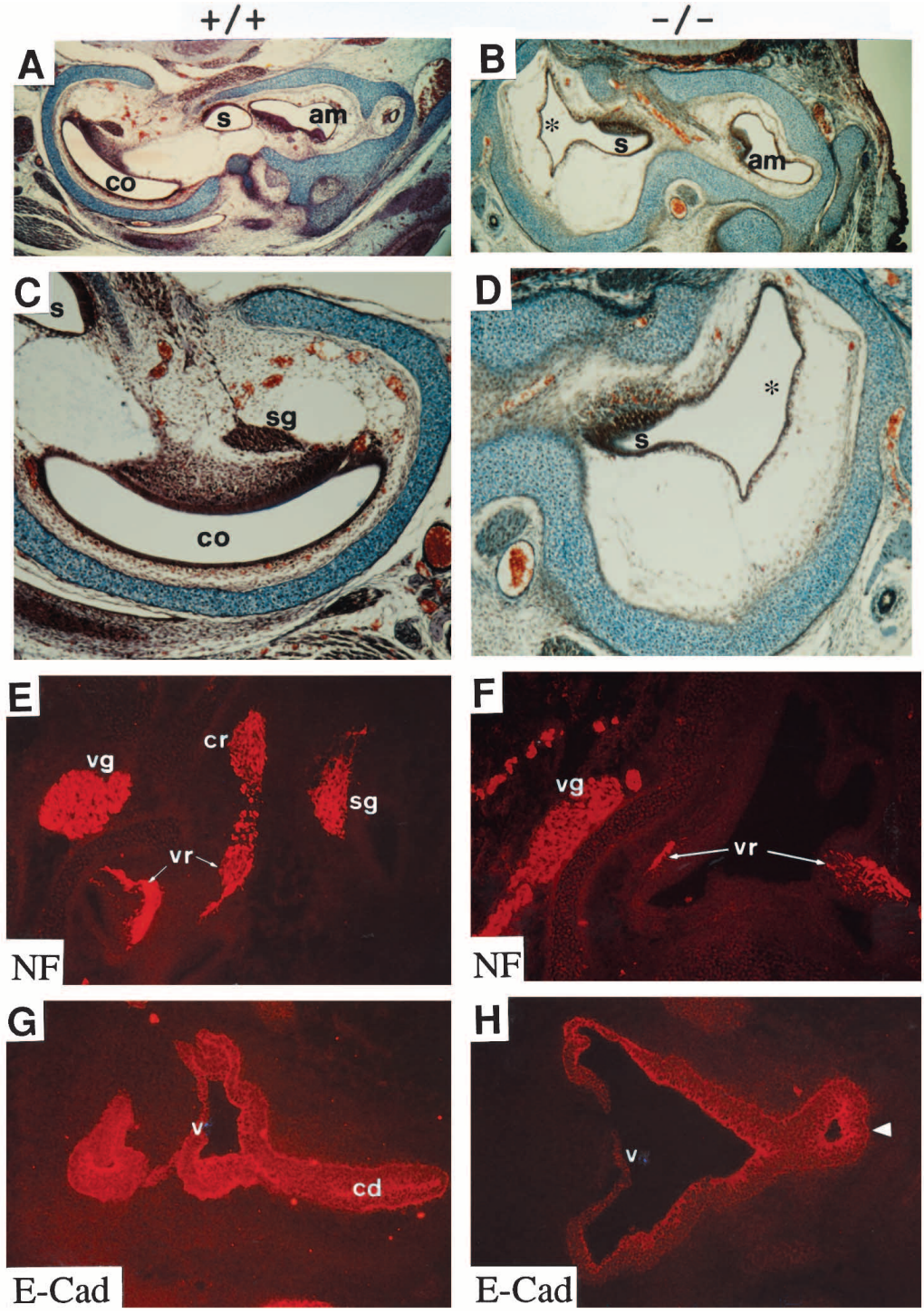


**Fig. 7.** *Pax2* is required for the differentiation of cochlear regions of the inner ear.
(A-D) Histological frontal sections of the inner ear of E17 fetus: (A), control embryo; (B) mutant. (C,D) Cochlear regions of, respectively, control and mutant embryos at higher magnification. Note that, while vestibular and saccular portions of the inner ear develop rather normally, the cochlea is completely agenic (asterisk in D) and the spiral ganglion is absent in the mutant. (E,F) Immunofluorescent detection of Neurofilament in cryostat frontal sections of the inner ear of E13.5 embryos: (E) control; (F) mutant. Note the absence of cochlear root of the vestibulo-acoustic nerve and spiral ganglion in the mutant. (G,H) Similar sections with immunofluorescent detection of E-Cadherin which stains all the inner ear epithelium: (G) control; (H) mutant. Note the absence of elongation of the cochlear duct in the mutant. co, cochlea; s, saccule; am, ampula of the anterior semicircular canal; sg, spiral ganglion; vg, vestibular ganglion; vr, vestibular root of the vestibulo-acoustic nerve; cr, cochlear root of the vestibulo-acoustic nerve; v, vestibular portion of the inner ear; cd, cochlear duct.

is required for neural crest and premyoblast migration (Epstein et al., 1991; Bober et al., 1994). *Pax5* is required for midbrain patterning and B cell development (Urbanek et al., 1994) and *Pax1* is required for paraxial mesoderm (Balling et al., 1988) and thymic development (Wallin et al., 1996). Phenotypic analysis of *Pax2* mutations show that this gene also performs multiple functions in the development of the CNS, sense organs and the urogenital system and, in addition, that *Pax2* locus is haplo-insufficient (Keller et al., 1994; Sanyanusin et al., 1995; Torres et al., 1995 and this report). Haplo-insufficiency is a characteristic shared by other three members of the family, suggesting that proper dosage of Pax proteins is essential to provide wild-type function.

Comparison between the three *Pax2* mutant models is hampered by the different nature of the mutations, the constrain to the heterozygous condition in two of the models and the different level of the analysis. In the three cases, the neural phenotype found in the heterozygous condition is extremely variable and background-dependent. The main defect observed in the human mutant condition is the optic

nerve coloboma, a defect present in some $Pax2^{+/-}$ mice (data not shown) but not found in the Krd mouse. Both the Krd mice and the human mutants show abnormal electroretinograms indicating retinal defects, which appear to be more extensive in the mouse, and related to hypocellularity in the nuclear layers of the retina (Keller et al., 1994; Sanyanusin et al., 1995). In addition, we have found background-dependent exencephaly in $Pax2^{+/-}$ mice, a defect not found in either the Krd mice or in humans. A possible explanation for these differences is that, besides *Pax2*, other genes deleted in the *Krd* mutation are contributing to its mutant phenotype. In addition, species-specific characteristics may explain the differences observed between mice and humans. Finally, it is likely that the contribution of genetic modifiers, differently represented in mouse lines, enhance different aspects of *Pax2* function. This aspect is clearly illustrated by the different phenotypes found in the urogenital and nervous systems for *Krd* and *Pax2*-targeted mice under different backgrounds (Keller et al., 1994; Torres et al., 1995).

### The role of *Pax2* in morphogenesis of the inner ear

Our analysis uncovers new functions related to the regional specification within the eye and the inner ear. Inner ear development is extremely complex as it depends on both morphogenetic events and a variety of cell differentiation programs. Added to this complexity, very little is known about the early patterning molecules involved. *Pax2* mutation completely blocks the differentiation of the auditory portion of the inner ear, where under normal circumstances, it is expressed from the otic placode stage onwards. Several homeobox-containing genes expressed in the vestibular portion of the inner ear have been described (Ekker et al., 1992; Deitcher et al., 1994; Rinkwitz-Brandt et al., 1995); however, *Pax2* is the only transcription factor expressed in the entire auditory part of the ear. Interestingly, a vestibular-specific gene, *Nkx-5.1* is expressed very early in the otic placode in a complementary manner to *Pax2* (Rinkwitz-Brandt et al., 1995). In vitro culture of explanted portions of the early otic vesicle, and assesment of their developmental potential, has suggested the early segregation of the domains and developmental programs for vestibular and auditory regions of the inner ear (Streeter, 1914; Fell, 1928; Li et al., 1978). Even though *Pax2* is expressed in some vestibular regions, like the sacculus, we have not observed defects in the differentiation of saccular macula and its innervation; therefore, the role of *Pax2* appears restricted to cochlear regions and it is likely that other transcription factors are specifically involved in the development of vestibular regions. The strong agenic phenotype of the *Pax2* mutation and its early expression in the auditory primordium suggests a high position for this gene in the hierarchy of genes controlling the developmental program of this region. In addition, our results strongly suggest that the hearing defects observed in the human syndrome (Sanyanusin et al., 1995) are due to *Pax2* malfunction.

### *Pax2* has multiple roles in the development of the eye and optic nerve

As in the inner ear, *Pax2* is expressed in a restricted manner beginning very early in the development of the eye primordium. Its expression is initially confined to the ventral half of the optic evagination, a region that does not correlate with any of the classical developmental compartments of the eye. The expression is very dynamic: in the optic cup, it is rapidly restricted to the cells arround the optic fissure as it forms. The phenotype observed suggests that the only role of *Pax2* in the optic cup is related to the closure of the optic fissure and the ventral restriction of its early expression only indicates the future position of the fissure. Besides this role in the closure of the optic fissure, the functions of *Pax2* in eye development appear mainly related to the development of the optic stalk into the optic nerve. After the formation of the optic cup, *Pax2* is expressed in the entire optic stalk with a sharply defined limit at the pigmented retina. The mutant phenotype shows that *Pax2* defines the optic stalk domain, restricting the pigmented retinal cell type to the optic cup. This may result from a transformation in the fate of cells located in the optic stalk region. Alternatively, it may be due to overproliferation of the retinal compartment, with subsequent invasion of the optic stalk region. In either case, *Pax2* is required in stalk cells for them to originate glial cells, either by determining the precursors or by building up the region from which those precursors derive.

Most interestingly, our results show a role for *Pax2* in the establishment of axonal pathways along the optic stalks and ventral diencephalon. The expression of *Pax2* in the optic fissure extends well into the optic stalk where it strongly labels the invaginated ventral cells. These cells play an important role in the formation of the optic nerve since the first ingrowing axons from the retina interact tightly with their membranes on their way to the optic chiasm (Horsburgh and Sefton, 1986). Retinal neurons transplanted to different regions of the head project their axons directly to the tectum without crossing the optic chiasma, unless the transplantation is made close to the optic stalk, in which case some are incorporated into the optic stalk and cross the midline at the optic chiasma. Therefore, cellular cues rather than long-range attractant substances appear to guide the retinal axons to the optic chiasma (Harris, 1989). *Pax2* expression correlates well with the distribution of stalk cells involved in the interaction with the first retinal axons. Moreover, its expression extends across the ventral midline at the optic recess area just before the axonal growth into the midline. In *Pax2* mutants, optic tracts do not grow towards the midline but grow directly towards the midbrain. A possible explanation for this phenotype is the extension of pigmented cells into the optic stalk. Pigmented cells are repulsive for retinal axons (Silver and Sapiro, 1981) and, therefore, it is not surprising that axons grow in the mutant as a single bundle without interaction with the surrounding pigmented cells. The situation in *Pax2* mutant mice would therefore be similar to retinal transplantation experiments, where axons lack the proper cellular environment and choose the shortest way to the tectum. This explanation would not imply any direct function for *Pax2* in the establishment of the optic pathway, instead the abnormal trajectory would be a consequence of the extension of the pigmented cells into the stalk region. However, the pigmentation of optic stalk cells is only partial in some genetic backgrounds, but still the projection of the optic fibers is completely abnormal. In addition, *Pax2* expression appears specifically in those optic stalk cells that interact tightly with pioneering axons and extends into the optic recess region just before axons enter it. Besides, pigmentation of the optic stalks never extends into the optic recess, but still there is a deletion or displacement of tissue at

the optic recess region that brings together the preoptic area with the ventral hypothalamic region. Therefore, the results do not support a major involvement of the abnormal pigmentation in the aberrant projection of the nerves. The agenesis of the optic chiasma in *Pax2* mutants is most likely due to the early deletion of the ventral region of the optic stalks and diencephalon, illustrated by the collapse of the *Shh*-negative area at the optic recess region.

We summarize our conclusions in a model for the role of *Pax2* in the development of the optic chiasma represented in Fig. 6J. According to this model, *Pax2*-expressing cells represent a favourable environment to sustain axonal growth. Before axons reach the optic recess area, a *Pax2*-expressing domain has to build up across the midline in order to allow axons to reach the chiasma region. Based on the dynamics of *Pax2* and *Shh* expression and on the mutant phenotype, we propose that the generation of the chiasma domain occurs by intercalation of *Pax2*-expressing cells from the optic stalks across the midline resulting in a separation of the *Shh* expression domain. In the absence of *Pax2*, cells from the stalk are not be able to intercalate due to either migration or proliferation defects. Besides creating a favourable environment for axonal growth, the establishment of a bridge of *Pax2*-expressing cells across the midline may build up a region devoid of signals inhibitory for retinal axon growth. In fact, negative signals have been shown to play a critical role in determining the pathway of the growing fibers into the chiasma region. The cell adhesion molecule CD44 is expressed at E12 in the ventral diencephalon just posterior to the optic recess region in an inverted V shape, strikingly coincident with the expression domain of *Shh* (Sretavan et al., 1994; Marcus and Mason, 1995). CD44 represents a repulsive signal for retinal axons that grow along the edges of the CD44-positive region thus forming the typical X-shaped optic chiasma. The deletion of the optic recess area in the mutants places the *Shh*-expressing cells, and presumably those expressing CD44, at the region where axons should cross. Considering the repulsion that CD44 exerts on retinal axons, the presence of CD44-positive cells in that region would be sufficient to explain the mutant phenotype; however, with *Shh* being a signalling molecule, we can not exclude that it may also represent a negative signal for retinal axon growth.

Ectopic expression of *Shh* in zebrafish has suggested a model for long-range patterning of the optic vesicle (Ekker et al., 1995; Macdonald et al., 1995). In this model, *Shh* would promote fates proximal to the midline by activating *Pax2*. The situation appears to be more complicated than suggested in this model since it does not account for the observation that, at least in mice, *Pax2* expression extends all along the proximodistal axis. In addition, we show here that the mRNA expression domains of *Pax2* and *Shh* do not overlap at any time of development suggesting that *Shh* expression may result in switching off *Pax2*.

### The role of *Pax2* in brain development

The boundary between hindbrain and midbrain is a major landmark within the developing neural tube. It is characterized by both morphological traits and the remarkable segmental expression of several regulatory molecules in the area like *Wnt-1*, *En-1*, *En-2* and *Pax5* (Wilkinson et al., 1987; Davis et al., 1988; Asano et al., 1992). Targeted mutations for these genes produced deletions of different portions of tissue around the meten-mesencephalic area, demonstrating their role in the regional specification of this area of the brain (McMahon and Bradley, 1990; Joyner et al., 1991; Urbanek et al., 1994; Wurst et al., 1994). The early expression of *Pax2* in the mid-hindbrain region led to the proposal that it may act on the top of the hierarchy of genes involved in the regional specification of the area (Rotwich and McMahon, 1995). Our results challenge this view, since the *Pax2* mutation does not produce any alteration in the early expression pattern of regulatory genes expressed in the area. Besides, the *Pax2* mutant phenotype results in the opening of the neural tube, showing that the main function of *Pax2* in the brain is related to the morphogenetic movements of the neural plate that allow its closure. In fact, the expression of *Pax2* is extraordinarily dynamic in the area and disappears as the neural tube closes. Tissue recombination experiments have shown that the onset of the expression of *En* genes is dependent on the interaction with the underlying mesendoderm (Ang and Rossant, 1993). Similarly, it may be possible that the initial activation of other genes expressed in this area is achieved independently by extrinsic factors, and subsequent regulatory cross-talk between them, refines and stabilizes the expression patterns. Our results do not exclude a possible role for *Pax2* in the maintenance of gene expression at this boundary. The identification of such a role would only be possible in the analysis of a later stage, not accessible in *Pax2* mutants due to the exencephaly.

We are grateful to S. Mashur, R. Scholz and S. Geisendorf for invaluable technical assistance and R. Altschäffel for excellent photographic work. We thank E. Stuart and P. Bovolenta for critical reading of the manuscript and A. Stoykova for stimulating discussions. M. T. received support from EMBO and European Community Human Genome Analysis program.

## REFERENCES

**Ang, S. L. and Rossant, J.** (1993). Anterior mesendoderm induces mouse Engrailed genes in explant cultures. *Development* **118**, 139-149.

**Asano, M and Gruss, P.** (1992) *Pax5* is expressed at the midbrain-hindbrain boundary during mouse development. *Mech. Dev.* **39**, 29-39.

**Balling, R., Deutsch, U. and Gruss, P.** (1988). *undulated*, a mutation affecting the development of the mouse skeleton has a point mutation in the paired box of *Pax1*. *Cell* **55**, 531-535.

**Bober, E., Franz, T., Arnold, H. H., Gruss, P. and Tremblay, P.** (1994). *Pax3* is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* **120**, 603-612.

**Chalepakis, G., Tremblay, P., and Gruss, P.** (1992). Pax genes, mutants and molecular function. *J. Cell Sci.* **Supplement 16**, 61-67.

**Davis, C. A. and Joyner, A. L.** 1988. Expression patterns of the homeobox-containing genes *En-1* and *En-2* and the proto-oncogene *int-1* diverge during mouse development. *Genes Dev.* **2**, 287-298.

**Deitcher, D. L., Fekete, D. M. and Cepko, C. L.** (1994). Asymmetric expression of a novel homeobox gene in vertebrate sensory organs. *J. Neurosci.* **14**, 486-498.

**Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P.** (1990). *Pax2*, anew paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

**Dressler, G. R. and Douglas, E. C.** (1992). *Pax2* is a DNA-binding protein expressed in embryonic kidney and Wilms tumor. *Proc. Nat. Acad. Sci. USA.* **89**, 1179-1183.

**Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., McMahon, A. P.** (1993). *Sonic hedgehog*, a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. *Cell* **75,** 1417-30.

**Ekker, M., Akimenko, M. A., Bremiller, R. and Westerfield, M.** (1992). Regional expression of three homeobox transcripts in the inner ear of zebrafish embryos. *Neuron* **9**, 27-35.

**Ekker, S. C., Ungar, A. R., Greenstein, P., Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. B.** (1995). Patterning activities of the vertebrate *hedgehog* proteins in the developing eye and brain. *Current Biology* **5**, 944-955.

**Epstein, D. J., Vekemans, M. and Gros P.** (1991). *Splotch ($Sp^{2h}$)*, a mutation affecting the development of the mouse neural tube, shows a deletion within the paired homeodomain of *Pax3*. *Cell* **67**, 767-774.

**Fell, H. B.** (1928). The development in vitro of the isolated otocyst of the embryonic fowl. *Arch. Expt. Zellforsch.* **7**, 69-81.

**Fietz, M. J., Concordet, J. P., Barbosa, R., Johnson, R., Krauss, S., McMahon, A. P., Tabin, C. and Ingham, P. W.** (1994). The *hedgehog* gene family in Drosophila and vertebrate development. *Development* **1994 Supplement**, 43-51.

**Godement, P., Salaün, J. and Mason, C. A.** (1990). Retinal axon pathfinding in the optic chiasm: divergence of crossed and uncrossed fibers. *Neuron* **5**, 173-186.

**Gruss, P. and Walther, C.** (1992). Pax in development. *Cell* **69**, 719-722.

**Hadler, G., Callaerts, P. and Gehring, W. J.** (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. *Science* **267**, 1788-1792 (1995)

**Harris, W. A.** (1989). Local positional cues in the neuroepithelium guide retinal axons in embryonic Xenopus brain. *Nature* **339**, 218-221.

**Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and Van Heyningen, V.** (1991). Mouse *Small eye* results from mutations in a paired-like homeobox-containing gene. *Nature* **354**, 522-525.

**Horsburgh, G. M. and Sefton, A. J.** (1986). The early development of the optic nerve of the rat. *J. Comp. Neurol.* **243**, 547-560.

**Joyner, A. L., Herrup, K., Auerbach, A., Davis, C. A. and Rossant, J.** (1991). Subtle Cerebellar Phenotype in mice homozygous for a targeted deletion of the *En-2* Homeobox. *Science* **251**, 1239-1243.

**Keller, S. A., Jones, J. M., Boyle, A. Barrow, L. L., Killen, P. D., Green, D. G., Kapousta, N. V., Hitchcock, P. F., Swank, R. T. and Meisler, M. H.** (1994). Kidney and retinal defects (Krd), a transgene-induced mutation with a deletion of mouse chromosome 19 that includes the *Pax2* locus. *Genomics* **23**, 309-320.

**Li, C. W., Van de Water, T. R. and Ruben, R. J.** (1978). The fate mapping of the eleventh and twelfth day mouse otocyst: an in vitro study of the sites of origin of the embryonic inner ear sensory structures. *J. Morph.* **157**, 249-268.

**Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W.** (1995). Midline signalling is required for *Pax* gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

**Marcus, R. C. and Mason, C. A.** (1995). The first retinal axon growth in the mouse optic chiasm: Axonal Patterning and the cellular environment. *J. Neurosci.* **15**, 6389-6402.

**McMahon, A. P. and Bradley, A.** (1990). The *Wnt-1* (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

**Noden, D. M. and Van de Water, T. R.** (1986). The developing ear: Tissue origins and interactions. In *The Biology of Change in Otolaryngology* (ed. R. W. Ruben et al.) pp 15-46. Amsterdam: Elsevier Science Publishers.

**Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U. and Gruss, P.** (1990). Spatially and temporally restricted expression of *Pax2* during murine neurogenesis. *Development* **109**, 797-809.

**Püschel, A. W., Westerfield, M. and Dressler, G. R.** (1992). Comparative analysis of *Pax2* protein distributions during neurulation in mice and Zebrafish. *Mech. Dev.* **38**, 197-208.

**Quinn, J. C., West, J. D. and Hill, R. E.** (1996). Multiple functions for *Pax6* in mouse eye and nasal development. *Genes Dev.* **10**, 435-446.

**Rinkwitz-Brandt, S., Justus, M., Oldenettel, I., Arnold, H. H. and Bober, E.** (1995). Distinct temporal expression of mouse *Nkx-5. 1* and *Nkx-5. 2* homeobox genes during brain and ear development. *Mech. Dev.* **52**, 371-381

**Rotwich, D. H. and McMahon, A. P.** (1995). *Pax2* expression in the murine neural plate precedes and encompasses the expression domains of *Wnt-1* and *En-1*. *Mech. Dev.* **52**, 3-8.

**Saha, M. S., Servetnick, M. and Grainger, R. M.** (1992). Vertebrate eye development. *Curr. Opin. Genet. Dev.* **2**, 582-588.

**Sanyanusin, P., Schimenti, L. A., McNoe, L. A., Ward, T. A., Pierpont, M. E. M., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R.** (1995). Mutation of the *Pax2* gene in a family with optic nerve colobomas, renal anomalies and vesiculoureteral reflux. *Nature Genet.* **9**, 358-363.

**Silver, J., and Sapiro, J.** (1981). Axonal guidance during development of the optic nerve: the role of pigmented epithelia and other extrinsic factors. *J. Comp. Neurol.* **202**, 521-538.

**Strachan, T. and Read A. P.** (1994). Pax genes. *Curr. Op. Dev. Biol.* **4**, 427-438.

**Streeter, G. L.** (1914). Experimental evidence concerning the determination of posture of the membranous labyrinth in amphibian embryos. *J. Exp. Zool.* **16**, 149-176.

**Sretavan, D. W., Feng, L., Puré, E. and Reichardt, L. F.** (1994). Embryonic neurons of the developing optic chiasm express L1 and CD44, cell surface molecules with opposing effects on retinal axon growth. *Neuron* **12**, 957-975.

**Ton, C. C. T., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., Jordan, T., Van Heyningen, V., Hastie, N. D., Meijers-HeijBoer, H., Dreschler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C. and Saunders, G. F.** (1991). Positional cloning and characterization of a paired box- and homeobox-containing gene from the Aniridia region. *Cell* **67**, 1059-1074.

**Torres, M., Gómez-Pardo, E., Dressler, G. R. and Gruss, P.** (1995). *Pax2* controls multiple steps of urogenital development. *Development* **121**, 4057-4065.

**Urbanek, P., Wang, Z., Fetka, I., Wagner, E. F. and Busslinger, M.** (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking *Pax5*/BSAP. *Cell* **79**, 901-912.

**Wallin, J., Eibel, H., Neubuser, A., Wilting, J., Koseki, H. and Balling, R.** (1996). *Pax1* is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development* **122**, 23-30

**Walther, C. and Gruss, P.** (1991). *Pax6*, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

**Wilkinson, D. G., Bailes, J. A. and McMahon, A. P.** (1987). Expression of the proto-oncogene *int-1* is restricted to specific neural cells in the developing mouse embryo. *Cell* **50**, 79-88.

**Wilkinson, D. G.** (1992). Whole mount in situ hybridization of vertebrate embryos. In *In situ hybridization, A Practical Approach.* (Ed. D. Wilkinson) pp 75-84. Oxford: IRL Press.

**Wurst, W., Auerbach, A. B. and Joyner, A. L.** (1994). Multiple developmental defects in *Engrailed-1* mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120**, 2065-2075.

(Accepted 14 August 1996)