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PhD thesis

Eye evolution and development: an insight from jellyfish and mouse

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I thereby declare that this thesis has been composed by Jana Růžičková, the undersigned, for the degree of Ph.D. at the Charles University of Prague. This work has not been presented in any previous application for a degree. All verbatim extracts have been clearly distinguished by quotation marks and tha sources of all information have been specifically acknowledged in the text.

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This work would never be done without kind help of several people:

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CONTENTS

ABBREVIATIONS

APC	adenomatous polyposis coli
bHLH	basic helix-loop-helix
CKI	casein kinase I
c-opsin	ciliary opsin
Dkks	Dikkopf proteins
E10.0	embryonic day ten
EE	ectoderm enhancer
EST	expressed sequence tag
HD	homeodomain
GCL	ganglion cell layer
GSK3β	glycogen synthase
INL	inner nuclear layer
NR	neuroretina
OCA2	oculocutaneous albinism type 2
OM	ocular mesenchyme
ONL	outer nuclear layer
OV	optic vesicle
PD	paired domain
PDE	phosphodiesterase
PRCs	photoreceptors
RGC	retinal ganglion cell
r-opsin	rhabdomeric opsin
RPC	retinal progenitor cell
RPE	retinal pigment epithelium
RGCs	retinal ganglion cells
SFRPs	secreted frizzled-related proteins
TCFs	TCF/LEF1 family

I. AIMS OF THE STUDY

Vision is one of the most crucial senses in higher vertebrates and perhaps the most important sense for humans. The eye morphogenesis has been studied for a long time and especially in the last two decades to address the function of individual genes duing eye development became one of the most interesting topic.

Although most of our knowledge about the genetic program underlying eye formation comes from vertebrates, valuable new information about eye evolution has recently been obtained by studies of cnidaria. Therefore elucidation of the biological role and function of eye components of jellyfish *Tripedalia cystophora* will enhance our knowledge of specific aspects of both eye evolution and development.

Specific aims of my PhD thesis were as follows:

1. to describe a new crystallin gene in jellyfish *Tripedalia cystophora* with respect to the evolution of its regulatory sequences;
2. to characterize genes required for the assembly of camera-type eyes in jellyfish *Tripedalia cystophora*;
3. to study the role of Wnt/β-catenin during lens induction and formation by employing a new mouse tissue-specific Cre-line;
4. to investigate the functional properties of jellyfish *PaxB* by expressing it in the developing lens and retina of transgenic mice.

II. INTRODUCTION

II.1. Overview of vertebrate eye development

Vertebrate eye is a complex neurosensory organ. During the embryonic development individual tissues interact in order to create functional visual organ. Abnormal lens and retinal development can cause several eye defects (e.i cataracts, aphakia, retinitis pigmentosa etc.). Murine eye development begins at the embryonic day 8 (E8) with the separation of the eye field and evagination of the ventral forebrain into bilaterally symmetrical optic vesicles (OV) (Chow and Lang, 2001). The region of head surface ectoderm immediately adjacent to the optic vesicle becomes thickened and thereafter is called lens placode (Fig.1). Formation of a morphologically apparent lens placode coincides with the expression of specialized tissue-preferred proteins, the so called crystallins (Wistow and Piatigorsky, 1988).

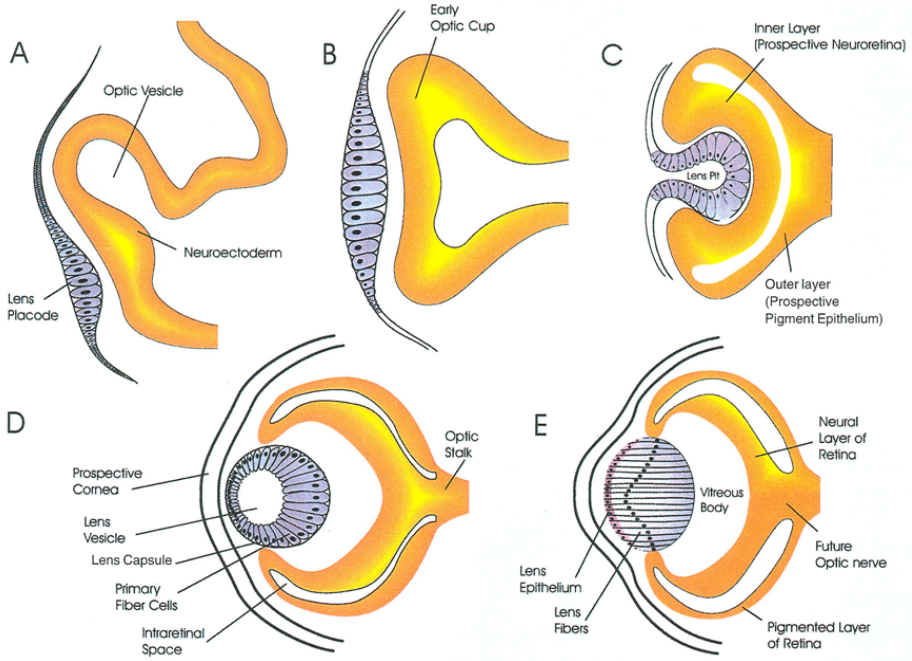

Fig.1. ***Highly schematic diagram of the early events in mouse eye development.***

(A) *At embryonic day 9 to 9.5 (E9.0-9.5), the optic vesicle is attached to the ventral wall of the prosencephalon via the optic stalk. The lens placode (prospective lens) becomes apparent as a thickened area of the surface ectoderm.* ***(B)*** *At E9.5-10.0, the area of the lens placode has enlarged.* ***(C)*** *At E10.5, the central part of the lens placode invaginates to form the lens pit and concurrently the optic vesicle invaginates to form the optic cup.* ***(D)*** *At about E11.5, the lens pit is converted into the lens vesicle, which is surrounded by a capsule.* ***(E)*** *At E13.5, the lens comprises the anterior cuboidal epithelial cells and the posterior elongationg fiber cells. The neural retina layer behind the lens begins to differentiate and the primitive cornea develops in front of the lens (Adopted from Cvekl and Piatigorsky 2006).*

The inductive interactions between head surface ectoderm and the optic vesicle are necessary for the lens placode formation and subsequent lens development. While the lens placode internalizes to form the lens vesicle, the distal part of the OV invaginates to form the optic cup with the inner layer developing into the neuroretina (NR) and the outer layer forming the retinal pigment epithelium (RPE) (Fig.1) (Lang, 2004). Thus, the pigmented RPE cells now lie in the inverted position (in terms of the path of incident light). The optic cup is incomplete inferiorly at the so-called embryonic (choroidal) fissure, which is used by the hyaloid artery to pass into the optic cup. This artery supplies nutrients to the inner layer of the cup and the lens vesicle during ocular development.

The transition part between the NR and the RPE is called the ciliary margin and gives rise to the iris, the ciliary body and the ocular drainage structures.

II.1.1. Retinal pigment epithelium (RPE)

Retinal pigment epithelium (RPE) is the pigmented monolayer of cells intervening between the retina and the choroidal circulation that serves multiple functions, e.g. synthetizes the shielding pigment melanin which than absorbs the excess of light that passes through the retina and thus prevents the degradation of the visual image; recycles the chromophore retinal to its 11-cis form; protects against free radicals; phagocytoses the apical tips of the outer segments of photoreceptors and thus ensures their renewal (Bok, 1993, Boulton and Dayhaw-Barker, 2001, Chow and Lang, 2001, Martinez-Morales *et al.*, 2004, Bharti *et al.*, 2006).

The importance of RPE and melanin is well documented. The reduction or absence of melanin pigment causes oculocutaneous albinism, a heterogeneous group of autosomal recessive disorders (Carden *et al.*, 1998). Besides the hypopigmentation in skin and hair colour it exhibits ocular phenotypic characteristics which are rather constant and include reduced visual acuity, nystagmus, high risk of strabismus, pale irides that transilluminate, hypopigmented fundi, hypoplastic foveae, and lack of stereopsis (Oetting *et al.*, 1996, Carden *et al.*, 1998).

II.1.1.1. Melanin synthesis

The production of melanin is tightly controlled and compartmentalized. Melanin biosynthesis occurs in melanosomes; endosome-derived organelles that share some features with lysosomes (Oliver *et al.*, 1995, Dell'Angelica *et al.*, 2000). The melanogenic complex

of the melanocyte consists of tyrosinase (OCA1, oculocutaneous albinism type 1), tyrosinase related protein 1 (TRP-1), dopachrome tautomerase (TRP-2), OCA2 (oculocutaneous albinism type 2, *P* protein, *pink eye dilution* protein), lysosome associated membrane protein 1, and melanocyte stimulating hormone receptors (Orlow *et al.*, 1994, Carden *et al.*, 1998). Tyrosinase, the product of the mouse *Tyr* gene (Kwon *et al.*, 1987), is the rate-limiting enzyme in the biosynthesis of melanin from tyrosine (Orlow, 1995). It catalyses three reactions in the melanin pathway (Zhao and Boissy, 1994).

Oca2 is an essential gene for melanin biosynthesis which has been suggested to be a tyrosine transporter (Rinchik *et al.*, 1993, Gahl *et al.*, 1995), nevertheless its exact function is not clear and it may be important for melanosomal pH (Brilliant, 2001). It is the most commonly mutated gene in cases of human albinism (Rinchik *et al.*, 1993, Oetting *et al.*, 1996). In addition, mutations in *Oca2* are responsible for pigmentation defects in mouse (Rinchik *et al.*, 1993), medaka (Fukamachi *et al.*, 2004), and independently arisen populations of the cave fish, *Astyanax* (Protas *et al.*, 2006).

II.1.1.2. Transcriptional regulation of RPE development

In vertebrates, the development of melanin-producing cells and specification of retinal pigment cells require several transcription factors; *Pax6, Mitf, Otx2*. Among those *Mitf* (microophthalmia-associated transcription factor) plays an essential role (Hodgkinson *et al.*, 1993, Opdecamp *et al.*, 1997, Nguyen and Arnheiter, 2000). Mitf regulates expression of *tyrosinase, TRP1* and *TRP2* (Hemesath *et al.*, 1994, Yasumoto *et al.*, 1994, Yasumoto *et al.*, 1997, Aksan and Goding, 1998). In mouse *Mitf* null mutants the RPE transdifferentiates into the NR (Nguyen and Arnheiter, 2000).

II.1.2. Differentiation of murine lens

The lens placode invaginates and resulting lens pit subsequently forms the lens vesicle later on. As the lens placode detaches from the surface ectoderm to form the lens vesicle, the surface ectoderm above the lens vesicle forms the future corneal epithelium, conjunctiva, limbus and eyelid epidermis. The lens vesicle is almost spherical and has a large central cavity which becomes filled by the primary lens fibre cells elongating from the posterior part of the lens vesicle. The cells located in the most anterior part of the lens vesicle remain as epithelial cells. Mitotically active cells in the central region of the lens epithelium move into the equatorial region, where they elongate and differentiate into

secondary lens fibres. They form concentric layers around the primary fibers of the lens nucleus. The lens continues to develop throughout life, albeit at a slower rate, with new fibers successively added and retained for the entire life of the organism. Finally, the terminally differentiated lens fibre cells lose their mitochondria and cell nuclei to allow lens transparency (Graw, 2003). For the primary fibers this process takes place in mice at E17/18 and is finalized two weeks after birth, when the mice open their eyelids (Vrensen *et al.*, 1991). The secondary fiber cells, which encircle the primary fiber cells, lose their organelles after moving from the outer to the inner cortex (Kuwabara and Imaizumi, 1974).

II.1.2.1. Crystallins

Up to 90% of the soluble protein in the postmitotic lens cells consists of proteins, which are referred to as α-, β-, and γ-crystallins. Crystallins are abundant, water-soluble proteins responsible for the optical properties of the transparent lens both in vertebrates and invertebrates. In contrast to the conservation of visual pigments (opsins) in the retina, the lens crystallins are diverse, multifunctional and taxon-specific, i.e. different proteins function as crystallins in different species (Wistow and Piatigorsky, 1988, de Jong *et al.*, 1989, Piatigorsky and Wistow, 1989). Many of the crystallins are identical or related to common, ubiquitously expressed metabolic enzymes or physiological stress proteins. For instance, vertebrate α-crystallins are small heat shock proteins (Ingolia and Craig, 1982, de Jong *et al.*, 1993) and the β/γ-crystallins are related to microbial stress proteins (Wistow, 1990, D'Alessio, 2002). The α-crystallins are effective chaperones that protect partially denatured proteins from agregating in the lens (Horwitz, 1992) and hence the transparency of the lens is maintained.

Although many crystallins are related to stress proteins or metabolic enzymes, many different proteins could potentially function as crystallins when expressed at high levels in the lens. This possibility is supported by the diversity of crystallins throughout the animals (Wistow and Piatigorsky, 1988, Tomarev and Piatigorsky, 1996).

This hallmark of being recruited from diverse multifunctional proteins led to the idea that crystallins are unified more by their high, lens-preferred expression than by their structure *per se* (Piatigorsky and Wistow, 1991, Piatigorsky *et al.*, 1993, Carosa *et al.*, 2002) and they play more or less just the refractive role in the lens. In addition to their role as crystallins, most if not all crystallins also have non-lens functions. The dual use of a single protein encoded in one gene is called, „gene sharing“ (Piatigorsky *et al.*, 1988, Piatigorsky and Wistow, 1989, Piatigorsky, 2007). An important implication of gene sharing illustrated

by the lens crystallins is that a protein can evolve a new role, without losing its original function, by a change in gene expression in the presence or absence of gene duplication (Piatigorsky and Wistow, 1991, Piatigorsky, 2003, Piatigorsky, 2007).

II.1.2.2. Other structural lens proteins

In the vertebrate lens, each cell is coupled to its neighbors via gap junctions, resulting in a network of cell-cell contacts important for the maintenance of ion flux and for metabolic cooperation between the peripheral lens cells and the interior fiber cells (Goodenough, 1992). The cell-to-cell communication performed by gap junctions is crucial for the development, differentiation and growth of the lens (Jiang *et al.*, 1994, Yu *et al.*, 2005). Three connexin genes are expressed in the vertebrate lens; epithelial cells express α1 (Cx43) connexin; fiber cells express α3 (Cx46) and α8 (Cx50) connexin (Paul *et al.*, 1991, Church *et al.*, 1995). Expression patterns of different cadherins indicate that this class of adhesion molecule plays an important role in the lens development (Xu *et al.*, 2002).

The major intrinsic protein (MIP) belongs to the aquaporin family of water channels (Reizer *et al.*, 1993). Mip forms specialized junctions between the fiber cells and can be first detected in the primary fiber cells of the early lens vesicle. Mip expression is highest in the elongating fiber cells in the bow region of the lens; Mip antiserum specifically decorates fiber cell membranes, highlighting their regular anterior to posterior organization (Shiels and Griffin, 1993, Zhou *et al.*, 2002).

In addition other noncrystallin genes expressed in lens during development have been suggested to contribute to the formation of lens cytoskeletal structure. CP49 (phakinin) and CP95/115 (filensin) are lens-specific beaded filaments that form a meshwork underneath the plasma membrane of the lens fiber cells (Sandilands *et al.*, 1995, Georgatos *et al.*, 1997).

All above mentioned structural lens proteins if mutated cause the cataracts, ei. lens opacities. Nevertheless the congenital eye diseases (including cataracts) can be caused by impaired function of transcription factors which acts upstream of these structural lens proteins.

II.1.2.3. Transcriptional regulation of lens development

Growth factors are known to influence cell behaviour and cell fates during development. Some of the major growth factor families, including FGFs (Faber *et al.*, 2001, Govindarajan and Overbeek, 2001), TGFb/BMPs (Furuta and Hogan, 1998, de Iongh *et al.*, 2001a, de Iongh *et al.*, 2001b, Belecky-Adams *et al.*, 2002, Faber *et al.*, 2002), and Wnt

signalling (Stump *et al.*, 2003, Ang *et al.*, 2004) are involved in regulating lens developmental processes.

Several genes play important roles in the development of the lens. A key component of the lens forming cascade is the homeodomain-containing transcription factor *Pax6* (Williams *et al.*, 1998, Kammandel *et al.*, 1999, Dimanlig *et al.*, 2001). Several Pax6 downstream targets have been identified. Mutations of these genes frequently lead to abnormal development of the anterior eye segment (Chow and Lang, 2001, Cvekl and Tamm, 2004). In mouse, the downstream targets of *Pax6* are the genes *Foxe3* (Blixt *et al.*, 2000, Brownell *et al.*, 2000, Medina-Martinez *et al.*, 2005) and *Mab21l1* (Yamada *et al.*, 2003). Animals with mutated *FoxE3* or *Mab21l1* possess rudimentary lenses with persistent connection between lens and cornea (Blixt *et al.*, 2000, Yamada *et al.*, 2003, Medina-Martinez *et al.*, 2005).

Prox1, a vertebrate homologue of *Drosophila prospero,* (Doe *et al.*, 1991, Vaessin *et al.*, 1991, Oliver *et al.*, 1993), another transcription factors important for lens development, is one of the earliest markers expressed in developing lens placode as early as E9.0-E9.5 (Duncan *et al.*, 2002). Homozygous *Prox1* knockout mice have defects in the elongation of lens fiber cells and cell cycle exit (Wigle *et al.*, 1999).

In addition, three known homeodomain transcription factors direct lens development, Six3, Meis1 and Meis2 (Oliver *et al.*, 1995, Oliver *et al.*, 1996, Purcell *et al.*, 2005, Liu *et al.*, 2006). Conditional knockout of *Six3* in the presumptive lens ectoderm disrupts lens formation and is accompanied by downregulation of *Pax6* (Liu et al., 2006). Meis1 and Meis2, the TALE (three amino acid loop extension) homeoproteins, are direct regulators of *Pax6* expression in prospective lens ectoderm (Zhang et al., 2002). *Meis1* mutant animals revealed eye defects (Hisa *et al.*, 2004).

It has been also demonstrated that transcription factors Sox1, 2, 3 and Maf, play important role in lens formation (Kamachi *et al.*, 1998, Ogino and Yasuda, 1998, Reza *et al.*, 2002). Three large Maf family proteins, L-Maf, c-Maf, and MafB, have been found to take part in the lens developmental program (Reza and Yasuda, 2004). Maf-recognition elements (MAREs) within the promoter/enhancer region of the *crystallin* gene family have been identified. *In vitro* as well as *in vivo* studies have shown that crystallins are regulated by Maf through these regulatory sites (Ogino and Yasuda, 1998, Muta *et al.*, 2002, Yoshida and Yasuda, 2002). Studies on knockout mice for *c-maf* have revealed a hollow lens with reduced crystallin expression (Kawauchi *et al.*, 1999, Kim *et al.*, 1999, Ring *et al.*, 2000). It has recently been demonstrated that *Maf* mutation results in cataract, microcornea, iris

coloboma, and anterior segment dysgenesis in human (Jamieson *et al.*, 2002) and in cataract in mouse (Lyon *et al.*, 2003).

The Sox-family of transcription factors is characterized by HMG domain (high mobility group). The genes *Sox1*, *Sox2* and *Sox3* (Kamachi *et al.*, 2000) show expression in mouse central nervous system and in the sensory placodes. In particular, *Sox2* is expressed during early eye development in the lens placode and in the presumptive lens vesicle. Lens placode invagination coincides with the onset of *Sox1* expression in the mouse. The 'turning on' of the transcriptional regulators Sox2/3 in the *Pax6*-expressing ectoderm is an essential molecular event in lens induction (Kamachi *et al.*, 1998).

At later stages, Sox2 is downregulated while Sox1 is upregulated and its continued activity is essential for further development of the lens (Kamachi *et al.*, 1998). A targeted deletion of Sox1 in mice caused microphthalmia and cataract. Mutant lens fiber cells fail to elongate, probably as a result of an almost complete absence of Cryg (γ-crystallin) transcripts (Nishiguchi *et al.*, 1998). The phenotype of the homozygous *Sox1* deletion mutant is very similar to the most severe *Cryg* mutation. In contrast to Sox1, mutations in the human *Sox2* gene cause anophthalmia (Fantes *et al.*, 2003). The heterozygous knockout mice of *Sox2* appeared normal, while the homozygous mutation is lethal (Avilion *et al.*, 2003).

II.1.3. Anterior eye segment

Normal functioning of the eye is dependent on a variety of highly specialized structures in the anterior segment of the eye. These include the cornea and lens, which provide both transparency and light refraction; the iris, which protects the retina from excess of light; and the ciliary body as well as ocular drainage structures, which secrete the aqueous humour required for cornea and lens nutrition and for the regulation of intraocular pressure. The intraocular pressure is needed to stabilize the shape of the eye and to keep constant distances between the retina and refractive surfaces of the lens and cornea. Development of these tissues involves coordinated interactions between surface and neural ectoderm and periocular mesenchyme that is derived from the neural crest. Failure of these interactions results in multiple developmental eye disorders, such as Axenfeld-Rieger's anomaly, which consists of small eyes (microphthalmia), hypoplastic irises, polycoria (iris tears), and abnormal patterning of the chamber angle between the cornea and the iris. Axenfeld-Rieger's anomaly is also associated with a high prevalence of glaucoma (Alward, 2000).

Shortly after the lens vesicle has become detached from the surface ectoderm, mesenchymal cells start to migrate into the space between the anterior epithelium of the lens vesicle and the surface ectoderm. With increasing number of cells between the lens and surface ectoderm, the cells condense to form several layers of flat mesenchymal cells that are separated from each other by a loose fibrillar extracellular matrix. Later in the mouse development (E14.5–E15.5), the posterior mesenchyme cells closest to the lens flatten and extend to form apicolateral contacts with adjacent cells (Pei and Rhodin, 1970, Kidson *et al.*, 1999, Reneker *et al.*, 2000, Flugel-Koch *et al.*, 2002). Finally, the cells become connected to each other through continuous bands of junctional complexes forming an endothelial monolayer. At the end of this process, all layers of the future cornea have been defined. The endothelial monolayer that has been formed from posterior mesenchyme cells will become the corneal endothelium, the surface ectoderm that covers the anterior side of the mesenchyme will become the corneal epithelium. Mesenchyme cells between the corneal epithelium and endothelium start to differentiate into stromal fibroblasts or keratocytes, which are responsible for the synthesis of the highly specialized extracellular matrix resulting in corneal transparency. Finally the cornea consists of three disctinct layers: an external stratified non-keratinizing epithelial cell layer, a neural crest-derived collagenous stroma and a single cell layer endothelium.

It should be stressed out that the mature anterior segment develops from four embryonic lineages: neural ectoderm (components of the ciliary body and iris), ocular surface ectoderm (lens, epithelial components of the cornea, limbus, conjunctiva, and the harderian and lacrimal glands, and eyelid epidermis), neural crest (corneal stroma and endothelium, conjunctival and eyelid mesenchyme), and mesoderm (schlemm's canal and blood vessel endothelium).

II.1.3.1. Transcriptional regulation of anterior eye segment during development

Development of the anterior eye segment depends on the proper function of two transcription factors in the periocular mesenchyme, the forkhead/winged-helix factor Foxc1 and the paired-like homeodomain factor Pitx2. In humans, hypomorphic and overactivating mutations in either gene leads to Axenfeld-Rieger's anomaly (Alward, 2000). In mice mutation of either *Foxc1* or *Pitx2* results in defects in the anterior eye segment, similar to that seen in human Axenfeld-Rieger's anomaly (Kume *et al.*, 1998, Lu *et al.*, 1999, Holmberg *et al.*, 2004). Whereas downstream targets of FOXC1 expressed in the human eye are supposedly involved in modulating intraocular eye pressure and ocular development

(Tamimi *et al.*, 2004), PITX2 target genes are associated with extracellular matrix synthesis and stability (Hjalt *et al.*, 2001).

It was also shown that defective TGFβ signalling interferes with neural crest cell differentiation and survival of anterior eye structures (Ittner *et al.*, 2005).

II.1.4. Differentiation of murine neuroretina

The mouse mature neural retina contains six types of neurons and one type of glial cells forming three cellular layers: the rod and cone photoroceptors in the outer nuclear layer (ONL); the inner nuclear layer (INL), containing bipolar, horizontal, amacrine interneurons and Müller glia; and finally ganglion as well as displaced amacrine cells in the ganglion cell layer (GCL). All these cell types are produced from common progenitors residing in the inner layer of the optic cup in an general order conserved among species, with ganglion cells being born first and rods and bipolar cells being born late (Young, 1985).

Retinal progenitor cells (RPCs) undergo a series of changes in competence to give rise to the various retinal cell types (Harris, 1997, Cepko, 1999). Recent studies have demonstrated that basic helix-loop-helix (bHLH) and homeobox transcription factors contribute to the intrinsic properties of retinal precursors (Kageyama and Nakanishi, 1997, Cepko, 1999, Livesey and Cepko, 2001). For the differentiation of each retinal cell type the specific transcription factors have to be co-expressed in the RPCs. As retinal neurons differentiate, they exit the cell cycle and migrate towards the inner (vitreal) side of the optic cup. For example, retinal ganglion cells‘ (RGCs) specification requires *Math5* and *Pax6* expression (Brown *et al.*, 2001, Marquardt *et al.*, 2001, Wang *et al.*, 2001). RGCs are formed at late E12.5/ early E13.0 stage and differentiated RGCs are clearly visible within a central domain of retina, marked by strong expression of Pax6 protein. The early progenitor cells that coexpress *Math3* and *NeuroD* (Inoue et al., 2002) together with *Pax6/Six3/Prox1* (Dyer et al., 2003) or *Pax6/Six3/Lim1* (Liu et al., 2000) adopt the amacrine or horizontal cell fate.

Adjacent to the pigmented epithelium, the light-sensitive rod and cone photoreceptor cells are aligned in the outer nuclear layer. Photoreceptor cells connect through interneurons (bipolar, horizontal, and amacrine cells) of the inner nuclear layer to the ganglion cells that project their axons along the optic nerve to the brain.

Amacrine (AM) cells are interneurons in the vertebrate neural retina. Typical AM cell somata are located in the inner nuclear layer (INL) and displaced AM cell somata are

located in the ganglion cell layer (GCL), adjacent to the inner plexiform layer (IPL). AM cells form synapses with bipolar cell terminals and the dendrites of retinal ganglion cells, thereby modulating the synaptic transmission between these two cell classes (Wassle, 2004).

II.1.4.1. Transcriptional regulation of retinal development

The patterning of the forebrain and eye development is at the molecular level characterized by activation of several homeobox-containing genes including *Otx2*, *Pax6*, *Six3*, *Six6* and *Rx*. One of the earliest genes expressed in the anterior neural region is the homeobox gene *Otx2*. Otx2 is required for the formation of the anterior neural region, as mice lacking functional Otx2 form neither forebrain nor midbrain (Acampora *et al.*, 1995). The Otx2 activity is suppressed in the center of the presumptive eye field, however, its expression remains in the periphery. This differential inactivation of Otx2 is of functional significance, as the center of the eye field develops into the neuroretina, while the periphery of the eye field develops into the retinal pigment epithelium (Martinez-Morales *et al.*, 2003). In the absence of *Otx2* function, the retinal pigment epithelium differentiates into the neuroretina (Martinez-Morales *et al.*, 2004). In the postnatal and adult eye, *Otx2* has been shown to be expressed not only in RPE cells but also in a restricted subset of retinal neurons including ganglion cells and bipolar cells. Moreover it is present in the cytoplasm of rod photoreceptors (Baas *et al.*, 2000).

Six3, a homologue of the *Drosophila sine oculis* (*so*) gene, is also expressed in the anterior neural plate (Oliver *et al.*, 1995) and has a critical role in the formation of the forebrain as mutations in the human Six3 cause holoprosencephaly (Wallis *et al.*, 1999, Pasquier *et al.*, 2000). Furthermore, mouse embryos with non-functional Six3 lack most of the head structures anterior to the midbrain (Lagutin *et al.*, 2003). Six3 is also involved in the proximo-distal patterning of the optic vesicle (Carl *et al.*, 2002).

Another homeobox-containing gene, *Six6 (Optx2),* is expressed in the precursors of the retina (Jean *et al.*, 1999, Toy and Sundin, 1999) and seems to regulate the proliferation of retinal cells (Zuber *et al.*, 1999). The targeted elimination of this gene in mice confirmed that Six6 has a role in the proliferation of retinal progenitor cells (Li *et al.*, 2002).

Recently a small family of paired-like homeobox genes that is critical for eye formation, the Rx/Rax (for Retinal homeobox) family, has been identified (Casarosa *et al.*, 1997, Furukawa *et al.*, 1997, Mathers *et al.*, 1997, Eggert *et al.*, 1998, Ohuchi *et al.*, 1999, Loosli *et al.*, 2001). *Rx* is one of the first retinal patterning genes to be expressed during development (Mathers *et al.*, 1997). *Rx*-deficient mouse embryos lack eye „anlagen“ and do

not express *Pax6* in the eye field indicating that *Pax6* upregulation in these tissues is dependent on functional Rx (Mathers *et al.*, 1997). However, *Rx* expression in ocular tissues is relatively independent of *Pax6* (Zhang et al., 2000).

The homeobox transcription factor *Chx10* is expressed in proliferating retinal progenitor cells throughout retinal development (Liu *et al.*, 1994, Burmeister *et al.*, 1996, Ferda Percin *et al.*, 2000). Its expression is first detected in the presumptive neural retina of the invaginating optic vesicle. As progenitor cells exit the cell cycle and differentiate, *Chx10* expression is maintained only in the mitotic layer of the retina. In the postmitotic retina *Chx10* expression persists only within bipolar cells of the inner nuclear layer. In the absence of *Chx10* eye development is severely impaired (Burmeister *et al.*, 1996, Ferda Percin *et al.*, 2000).

II.2. The role of *Pax* genes during eye development

All the above mentioned developmental steps are directed by several evolutionary conserved transcription factors (Pax6, Six3, Meis1, Meis2, Sox1-3, Rx etc.). Among those the most outstanding roles play the homeobox-containing genes *Pax*.

Pax genes encode transcription factors critical for metazoan development. Members of the Pax protein family can be classified based on the presence and characterictic features of their three evolutionarily conserved domains: a paired domain, a paired-type homeodomain and an octapeptide. The paired domain and the paired-type homeodomain encode two independent DNA-binding domains, while the octapeptide plays an important role in protein-protein interactions. Pax proteins interact with similar DNA sequences although differences in specificity exist. In particular, three amino acids (at positions 42, 44, and 47) within the N-terminal half of paired domain are responsible for the difference in the DNA-binding specificities between Pax6 and Pax2/5/8 subfamilies (Czerny and Busslinger, 1995). The amino acids IQN at these positions specify Pax6, whereas amino acids QRH determine Pax2/5/8 specificity (Czerny and Busslinger, 1995). Pax2 of the Pax2/5/*8* subfamily has a DNA binding paired domain and an octapeptide, but only a truncated homeodomain. It has been implicated in eye development both in mice and *Drosophila.*

The *Drosophila Pax2* orthologue has a key role in the development of ommatidial cone and pigment cells (Fu and Noll, 1997). In mice, *Pax2* deficiency results in eye, kidney and inner ear defects (Favor *et al.*, 1996, Torres *et al.*, 1996, Gehring and Ikeo, 1999).

Pax6 is an evolutionarily highly conserved transcription factor that has been considered as a key regulator of the eye development among metazoans (Favor *et al.*, 1996, Torres *et al.*, 1996, Gehring and Ikeo, 1999, Gehring, 2002, Gehring, 2005, Kozmik, 2005). *Pax6* mutations are associated with aniridia in humans and the Small eye (Sey) phenotype in mice (Hanson and van Heyningen, 1995). *Drosophila* possess two *Pax6* homologs, *eyeless* (*ey*) and *twin of eyeless* (*toy*), both of which are able to induce ectopic eyes (Quiring *et al.*, 1994, Halder *et al.*, 1995, Czerny *et al.*, 1999). Pax6 directly activates rhodopsin genes in *Drosophila* (Sheng *et al.*, 1997, Papatsenko *et al.*, 2001) and lens crystallins of vertebrates (Cvekl and Piatigorsky, 1996, Duncan *et al.*, 2004). In addition to the paired domain, Pax6 contains a second DNA-binding domain, the homeodomain (HD) but it lacks the octapeptide, which is responsible for repression abilities of Pax2/5/8 proteins mediated through interaction with Groucho co-repressors (Eberhard *et al.*, 2000, Kozmik *et al.*, 2003).

The *Pax6* gene encodes three isoforms of Pax6 protein, and these may play different roles in eye development. The so-called canonical Pax6 protein contains the paired domain (PD) and a homeodomain (HD). Alternative RNA splicing of the Pax6 primary transcript in mice and humans leads to a second Pax6 isoform, Pax6(5a), where a peptide encoded in an additional exon is inserted within the paired domain (Epstein et al., 1994). Both isoforms are encoded by transcripts that initiate from P_1- and P_0- *Pax6* promoters (Kammandel *et al.*, 1999, Xu *et al.*, 1999).

Finally, third isoform of Pax6 has been recently described (Kammandel *et al.*, 1999, Kleinjan *et al.*, 2004, Kim and Lauderdale, 2006). It lacks the paired domain and is encoded by the transcript starting from the P_{alpha} promoter.

Paired-less Pax6 is likely involved in the cell fate decision leading to the generation of amacrine cells since its immunoreactivity can be detected in the subset of GABAergic amacrine cells (Lakowski et al., 2007). Overexpression of Pax6ΔPD in the distal retina results in a microphthalmic phenotype (Kim and Lauderdale, 2006). In general, *Pax6* function depends on a proper expression level; any change in the dosage of Pax6 protein disrupts eye development (Schedl *et al.*, 1996, Collinson *et al.*, 2000, van Raamsdonk and Tilghman, 2000, Davis-Silberman *et al.*, 2005, Kim and Lauderdale, 2006). The expression of *Pax6* in lens is driven by an ectoderm enhancer (EE) (Williams *et al.*, 1998, Kammandel *et al.*, 1999). Targeted deletion of EE is accompanied by distinctive defects at every stage of lens development (Dimanlig et al., 2001). In addition the exact dosage of Pax6 protein is required for lens placode formation (Collinson *et al.*, 2000, van Raamsdonk and Tilghman, 2000, Davis-Silberman *et al.*, 2005). Moreover *Pax6* activity in the lens primordium is

necessary for correct placement of the retina in the eye (Ashery-Padan et al., 2000). *Pax6* expression is maintained throughout retina development, from early optic vesicle formation to specification of neuroretina and differentiation and timing of distinct retinal cell types. Pax6 protein is abundant in proliferative zones of the neuroretina and subsequently in three types of retinal neurons: retinal ganglion cells (RGC), amacrine and horizontal cells. Null mutations in *Pax6* arrest optic vesicle formation early in eye development (Hogan *et al.*, 1986, Grindley *et al.*, 1995). Nevertheless retinal progenitor cells (RPCs) differentiate in the optic vesicle of *Pax6* mutant mice, earlier than in wild-type, although exhibiting reduced proliferation (Philips et al., 2005). *Pax6* is known to control the proliferation rate of neuroepithelial progenitors (Duparc et al., 2007) and retinal stem cells in the mouse optic vesicle (Xu et al., 2007). Conditional inactivation of *Pax6* in RPCs of the distal retina prior to initiating retinogenesis resulted in reduced RPCs proliferation and exclusive differentiation of amacrine neurons (Marquardt et al., 2001). By contrast, differentiating neurons were identified in the absence of amacrine cells in *Pax6* mutant optic vesicles of Sey^{1Neu} mice (Philips et al., 2005).

It is apparent that in mice, *Pax6* and *Pax2* play distinct as well as redundant roles during the eye development. *Pax6* is expressed in the developing mouse eye in the prospective lens, retina and pigmented epithelium and *Pax6* mutations cause several eye defects. *Pax2* is initially co-expressed with *Pax6* within the optic vesicle, but soon after invagination of the optic cup *Pax2* expression becomes restricted to the optic stalk (Nornes *et al.*, 1990, Torres *et al.*, 1996). In contrast to Pax6, the Pax2 protein can not be found in lens at any time during eye development. However, redundant activities of Pax2 and Pax6 specify the optic cup/optic stalk boundary (Schwarz et al., 2000) and determinate the retinal pigmented epithelium (RPE) in the mouse eye (Baumer et al., 2003). There are Pax2- and Pax6- binding sites on the retina enhancer of the *Pax6* gene and on the *Pax2* upstream control region (Schwarz et al., 2000). The Pax2 protein cooperates with Vax transcription factors to repress *Pax6* expression in the ventral region of the optic vesicle and the optic stalk (Mui et al., 2005).

II.3. Wnt proteins

Several signaling molecules, in particular the members of the FGF and BMP families of growth factors, have been shown to be involved in lens induction and differentiation (Lovicu and McAvoy, 2005). More recent studies implicated Wnt signaling in the lens epithelium (Stump *et al.*, 2003) and lens fiber cell differentiation (Lyu and Joo, 2004). A number of Wnt signaling molecules, their Frizzled (Fz) receptors, as well as Wnt antagonists such as secreted frizzled-related proteins (Sfrps) and Dickkopfs (Dkks), were shown to be expressed during mammalian lens development (Liu *et al.*, 2003, Stump *et al.*, 2003, Ang *et al.*, 2004, Chen *et al.*, 2004).

Wnt proteins belong to the family of secreted signaling molecules which play crucial role during development, regeneration and disease. Wnts acts in a paracrine fashion by activating diverse signaling cascades inside the target cells. Somatic mutations in the Wnt pathway are associated with different tumors, mainly of gastrointestinal origin (Nusse, 2005). The intracellular mediator of Wnt signaling is β-catenin, a molecule with a dual function. In so-called non-canonical Wnt pathway β-catenin is a key component of cadherin-mediated cell adhesion complex, where it links cadherins to α-catenins, which in turn anchors the adhesion complex to actin cytoskeleton (Aberle *et al.*, 1996). In canonical Wnt signaling (Fig.2) β-catenin serves as an intracellular mediator of Wnt pathway, controling the transcriptional properties of DNA-binding proteins of the TCF/LEF1 family (TCFs) (Huelsken and Behrens, 2002).

In the absence of Wnts, the level of β-catenin is kept low through degradation of cytoplasmic β-catenin in proteasomes. β-catenin is targeted for ubiquitination by paired phosphorylation through serine/threonine kinases: glycogen synthase 3β (GSK3β) and casein kinase I (CKI) (Polakis, 2002). Phosphorylation of β-catenin occurs in a multiprotein complex containing the scafold protein axin, tumor supressor adenomatous polyposis coli (APC), GSK3β, and diversin (Cadigan and Nusse, 1997a, Cadigan and Nusse, 1997b, Polakis, 2000). Canonical Wnt signaling pathway is initiated when Wnt molecule binds to seven transmembrane receptor Frizzled and its co-receptor LRP (low density lipoprotein receptor-related protein). Receptor activation leads to inhibition of GSK3β activity, resulting in stabilization and accumulation of cytoplasmic β-catenin and subsequent translocation into the nucleus and activation of TCF/LEF1 transcription factors followed by induction of target genes expression (Fig.2) (Cadigan and Nusse, 1997a, Cadigan and Nusse, 1997b, van de

Wetering *et al.*, 1997). A variety of Wnt/β-catenin target genes have been identified. These include regulators of cell proliferation, development and genes implicated in tumor progression. Mouse embryos lacking β-catenin exhibit early gastrulation patterning defects (Haegel *et al.*, 1995, Huelsken *et al.*, 2000).

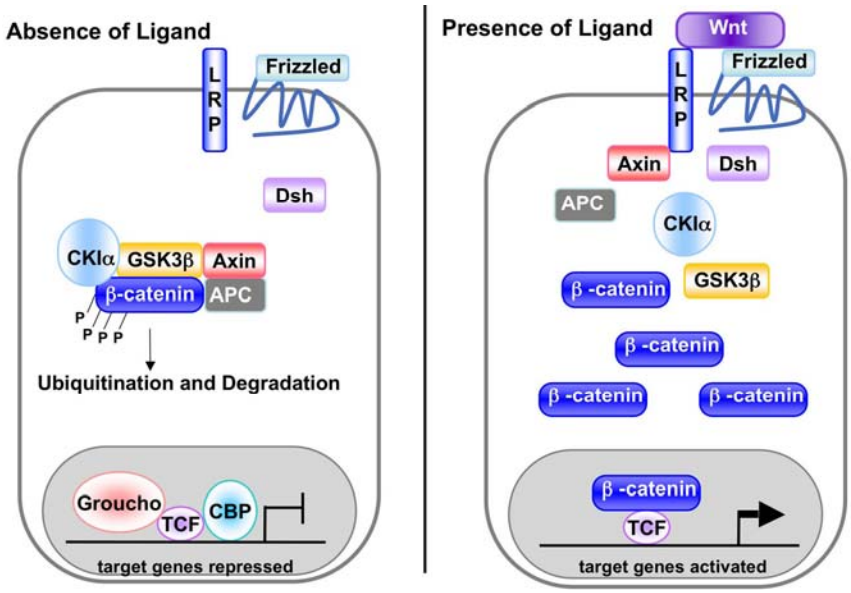

Fig.2. ***Schematic reprensentation of a canonical Wnt signaling pathway.***

In the absence of signal, action of the destruction complex (CKIα, GSK3β, APC, Axin) creates a hyperphosphorylated β-catenin, which is a target for ubiqitination and degradation by the proteosome. Binding of Wnt ligand to a Frizzled/LRP-5/6 receptor complex leads to stabilization of hypophosphorylated β-catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription. In a canonical pathway, CKIα, GSK3β, APC, and Axin act as negative regulators and all other components act positively (Adopted from Eisenmann 2005).

In the absence of β-catenin, gene transcription can be repressed either by dominant-negative variants of TCFs or by TCFs assosiated with transcriptional repressors of Groucho family (Cavallo *et al.*, 1998, Roose *et al.*, 1998). In tumours, either overexpression of Wnts or impaired degradation of β-catenin, leads to stabilization of β-catenin and unwanted activation of target genes. Mutations occur predominantly in the tumour supressor gene APC (in 80% of colorectal carcinomas), at the N-terminal part of β-catenin (in some colorectal carcinomas and in variety of other tumors), and in axin and conductin (in small subsets of liver and colon tumors and medulloblastomas) (Lustig and Behrens, 2003).

Wnt signals are modulated extracellularly by diverse secreted proteins. They bind to Wnt and thereby antagonize its function, e.g. Wnt-inhibitory factor-1 (WIF-1), secreted

frizzled-related proteins (Sfrps) (Lin *et al.*, 1997, Rattner *et al.*, 1997, Miller, 2002) and Cerberus (Piccolo *et al.*, 1999). Cerberus is a multifunctional inhibitor of Bmp, Nodal and Wnt signals (Piccolo *et al.*, 1999). The *Dickkopf* (Dkk) family of secreted cysteine rich proteins represent another class of Wnt inhibitors that regulate Wnt pathways by interacting with Wnt co-receptor LRP5/6 and with Kremen class of transmembrane proteins (Mao *et al.*, 2001, Mao and Niehrs, 2003). Dkk1 is required for embryonic head induction and limb morphogenesis (Glinka *et al.*, 1998, Mukhopadhyay *et al.*, 2001). Dkk2 controls the integrity of cornea and fate determinantion of ocular surface epithelium (Mukhopadhyay *et al.*, 2006).

Many vertebrate Wnts are expressed in the embryonic central nervous system (Parr *et al.*, 1993, Hollyday *et al.*, 1995). In mice, by E9.5 several Wnts are expressed in the presumptive brain and spinal cord (Salinas and Nusse, 1992, Parr *et al.*, 1993). Inactivation of Wnt-1 results in failure of midbrain and rostral hindbrain development (McMahon *et al.*, 1992, Mastick *et al.*, 1996, Serbedzija *et al.*, 1996). Specific inactivation of β-catenin in the domain of Wnt-1 expression leads to brain malformations and failure of craniofacial development (Brault *et al.*, 2001).

There are several publications indicating the essential role of Wnt/β-catenin signalling during mouse eye development. Wnt-3, -5a, -5b and -7b are expresssed in the neural retina and there is a dynamic pattern of Wnt receptor and Wnt antagonists, Sfrps in the embryonic and perinatal neural retina (Liu *et al.*, 2003, Liu *et al.*, 2007). Wnt pathway is suggested to inhibit an early lens development and to restrict the region of lens competent surface ectoderm (Smith *et al.*, 2005). This is in consistence with the expression of multiple *Frizzled* and *Sfrp* genes during lens morphogenesis and differentiation (Chen *et al.*, 2004). Moreover loss of Wnt signaling in the surface ectoderm results in the formation of ectopic lentoid bodies (Smith *et al.*, 2005). Defects in mouse deficient for *Lrp6*, co-receptor of Wnt/β-catenin pathway, also suggest that Wnt signaling is required for lens epithelial integrity and differentiation during later lens development (Stump *et al.*, 2003). Moreover *Lrp6* mutant eyes revealed ocular coloboma and dorsoventral neuroretinal patterning defects (Zhou *et al.*, 2008).

Recent studies have shown that Wnt/β-catenin signaling is essential for the ciliary margin development in mice (Kubo *et al.*, 2003, Liu *et al.*, 2007).

It was also shown that neural crest derived ocular mesenchyme (OM), which separates the presumptive lens and retinal epithelia, is Wnt pathway responsive (Song *et al.*, 2007).

II.4. Visual apparatus of Tripedalia cystophora

Vision is one of the most important animal senses. In animal phyla the repertoire of the eyes differs from the simplest minimal eye which is composed just of a single photoreceptor cell and a cell expressing dark shielding pigment to a complex organ such as the vertebrate eye.

Cnidaria, the likely sister group to the Bilateria, constitute the earliest branching phylum containing a well developed visual system. In general among Cnidarians, it is the class Cubozoa (known as 'box jellyfish' due to their cuboidal shape) that have lens-containing eyes (Piatigorsky and Kozmik, 2004). The cubozoan *Tripedalia cystophora* studyed in our lab, has four equally spaced sensory structures called rhopalia which hang from a bell on stalks and are situated within sensory niche surrounding the bell (Fig.3A,B). On each rhopalium there is a large and a small complex camera-type eye with a cellular lens and two pairs of more simple „eyes" (one pair of pit-shaped ocelli and one pair of slit-shaped ocelli) (Fig.3B). This makes four rhopalia per medusa, six eyes per rhopalium and results in a total of twenty-four eyes per one animal.

The behavioral role of the eyes of *Tripedalia cystophora* is under investigation (Coates, 2003). Nevertheless it is known that *Tripedalia* is an active swimmer and ommits obstacles when swimming in the water. Their natural habitat is the shallow water near the mangrove roots on Portorico. The medusae are collecting small crustaceans in the bright shafts of light. Moreover the vision is important for their sexual reproduction since the adult female and male medusae have to find each other in order to produce new generation. Tripedalia, as other jellyfish, undergo an alternation of generations between sessile, non-sexual polyps and swimming, sexually dimorphic medusae. Their life cycle and successful cultivation have been described (Werner *et al.*, 1971, Kostrouch *et al.*, 1998). Fertilization is internal and the planulae larvae develop in the gastral pocket of the females; the swimming larvae are released within 2 days into the sea water, settle on the bottom and become polyps. Full-grown polyps metamorphose into swimming ephydrae (immature medusae) within 4 or 5 days.

The complex eyes of *Tripedalia cystophora* show striking similarities in overall structure with the camera-type eye of vertebrates even though they differ in numerous anatomical details (Fig.3C-F). The complex (lensed) eyes are covered with one-layered cornea, possess a cellular lens, retina and pigmented epithelium. In addition, adult large and small complex jellyfish eyes have ciliated vertebrate type photoreceptors, rather than the

rhabdomeric (microvillar) photoreceptors generally described in invertebrates (Eakin and Westfall, 1962, Yamasu and Yoshida, 1976, Piatigorsky and Kozmik, 2004).

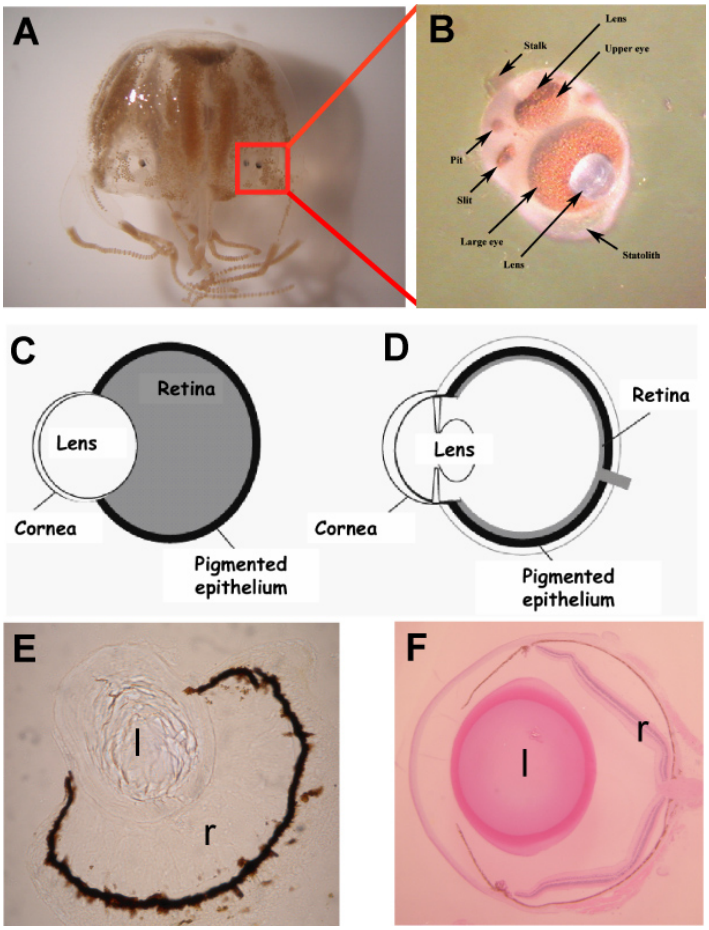

Fig. 3. ***(A)*** *Adult Tripedalia cystophora medusae with rhopalia (marked with red box).* ***(B)*** *Higher magnification of rhopalium with two lens-containing camera-type eyes, a slit and a pit eye. Another set of slit and pit eyes is symmetrically located on the other side of the rhopalium.* ***(C, D)*** *Schematic drawing of vertebrate eye* ***(C)*** *versus lensed-eye of Tripedalia cystophora* ***(D).*** *Histological section throught adult mouse eye* ***(E)*** *and lensed-eye of Tripedalia cystophora* ***(F)****. Abbreviations: l, lens; r, retina.*

II.4.1. Lenses and crystallins of Tripedalia cystophora

Recently it has been shown that *Tripedalia* lenses contain a refractive index gradient allowing transmitted light to produce a nearly aberration-free image (Nilsson *et al.*, 2005). It has been proposed that this ability of *Tripedalia* lens to focus light is due to the presence of lens crystallins (Nilsson *et al.*, 2005).

Tripedalia lenses contain three novel crystallins (J1-, J2- and J3-crystallin) (Piatigorsky *et al.*, 1989). J1-crystallins comprise three distinct polypeptides (J1A, J1B and J1C), each encoded by a different although extremely similar gene (Piatigorsky *et al.*, 1993). The J1-crystallin polypeptides show sequence similarity to ADP-ribosylglycohydrolases and belong to the broad category of enzyme-crystallins (Piatigorsky and Kozmik, 2004, Castellano *et al.*, 2005). On the contrary, J3-crystallin is encoded by single gene and shows homology to vertebrate saposins, multifunctional proteins that bridge lysosomal hydrolases to lipids and activate enzymatic activity (Piatigorsky *et al.*, 2001). *In situ* hybridization showed that jellyfish crystallins are also expressed outside of the lens consistent with their non-optical functions (Piatigorsky *et al.*, 2001, Kozmik *et al.*, 2003).

II.4.2. Pax transcription factors of Tripedalia cystophora

Crystallin gene expression is regulated by a complex set of transcription factors, among those Pax6 is one of the main interest.

Pax proteins are a family of transcriptional factors characterized by the presence of an evolutionarily conserved paired domain, which is additional DNA-binding domain of these homeodomain containing proteins (Bopp *et al.*, 1986, Treisman *et al.*, 1991). Pax genes are involved in many developmental processes in higher eucaryotes. In particular, the role of *Pax* genes during eye development has been well characterized (Gehring and Ikeo, 1999, Kozmik, 2005). Pax6 has been shown to be implicated in the regulation of α-, β- and γ-crystallins, as well as the taxon-specific δ1-crystallin (chicken) and the ζ-crystallin (guinea pig) (Cvekl and Piatigorsky, 1996, Duncan *et al.*, 1998, Kralova *et al.*, 2002).

In *Tripedalia cystophora* the only *Pax* gene (*PaxB*) was so far identified (Kozmik *et al.*, 2003). *PaxB* gene is expressed both in the larva and the adult jellyfish (retina, lens, and statocyst). *PaxB* encodes a paired domain, an octapeptide, and a homeodomain. In contrast to Pax6-like homeodomain, three amino acids (Q,R,H) responsible for DNA binding specificities of the *PaxB* paired domain are identical to those in the paired domain of Pax2/5/8. PaxB was shown to activate *Drosophila* rhodopsin *rh6* promoters and induce small ectopic eyes in *Drosophila* (Kozmik *et al.*, 2003). Moreover PaxB activates J1- and J3-crystallin promoters and binds to J3-crystallin promoter (Kozmik *et al.*, 2003).

PaxB also has other Pax2 trait. Besides the activation (paired) domain it possesses inhibitory domain typical of the Pax2/5/8 subfamily of proteins and it rescues the *Drosophila*

Pax2 eye mutant (Kozmik et al., 2003). In summary *PaxB* of jellyfish *Tripedalia cystophora* can carry on the functions of both *Pax6* and *Pax2* in higher metazoans (Kozmik et al., 2003).

II.5. Classification of opsins and photoreceptors

The function of photoreceptors (PRCs) is to convert light (stream of photons) into intracellular signaling. The photoreceptor cells of animals can be classified into two types: rhabdomeric and ciliary. One of the main differences between these types relates to the morphology of the enlarged apical membrane of photoreceptor, which serves for the storage of the photopigment (opsin). The rhabdomeric photopigment cells fold the apical cell surface, on contrary the ciliary photoreceptor cells extend membranes from a modified cilium (Eakin and Brandenburger, 1968, Eakin, 1982).

The rhabdomeric PRCs are characteristic invertebrate eyes; whereas the ciliary PRCs are typical of vertebrate eyes (Fernald, 2006). In both ciliary and rhabdomeric PRCs, the seven-transmembrane receptor (opsin) associates with retinal to constitute a functional photosensitive pigment. After absorption of a photon 11-cis retinal undergoes isomerisation to all-trans retinal. During isomerisation the shape of retinal is changed which in turn leads to a conformational change of opsin and activation of the alpha subunit of a G-protein. Afterward each photoreceptor type uses a separate phototransduction cascade (Fig.4). In ciliary PRCs, the G-protein activates a phosphodiesterase that hydrolyses cyclic GMP in the cytoplasm, leading to the closure of Na+ ion channels in the plasma membrane and hyperpolarization of the membrane. Rhabdomeric photoreceptors use phospholipase C cascade and the whole photoactivation results in depolarization. „Shutting off‘ in both types is mediated by phosphorylation of the opsin followed by the binding of an inhibitory protein, arrestin.

The two opsins groups, ciliary (c-) and rhabdomeric (r-), can be distinguished according to their sequence. The three amino acids critical for the interraction with G-alpha subunit of the trimeric G-proteins of the downstream cascade are NR/KQ for the ciliary opsin (c-opsin) and HPK in rhabdomeric opsin (r-opsin).

In summary rhabdomeric photoreceptors use r-opsins and a phospholipase C cascade, whereas ciliary photoreceptors employ c-opsins and a phosphodiesterase (PDE) cascade (Fig.4) (Arendt and Wittbrodt, 2001, Arendt, 2003).

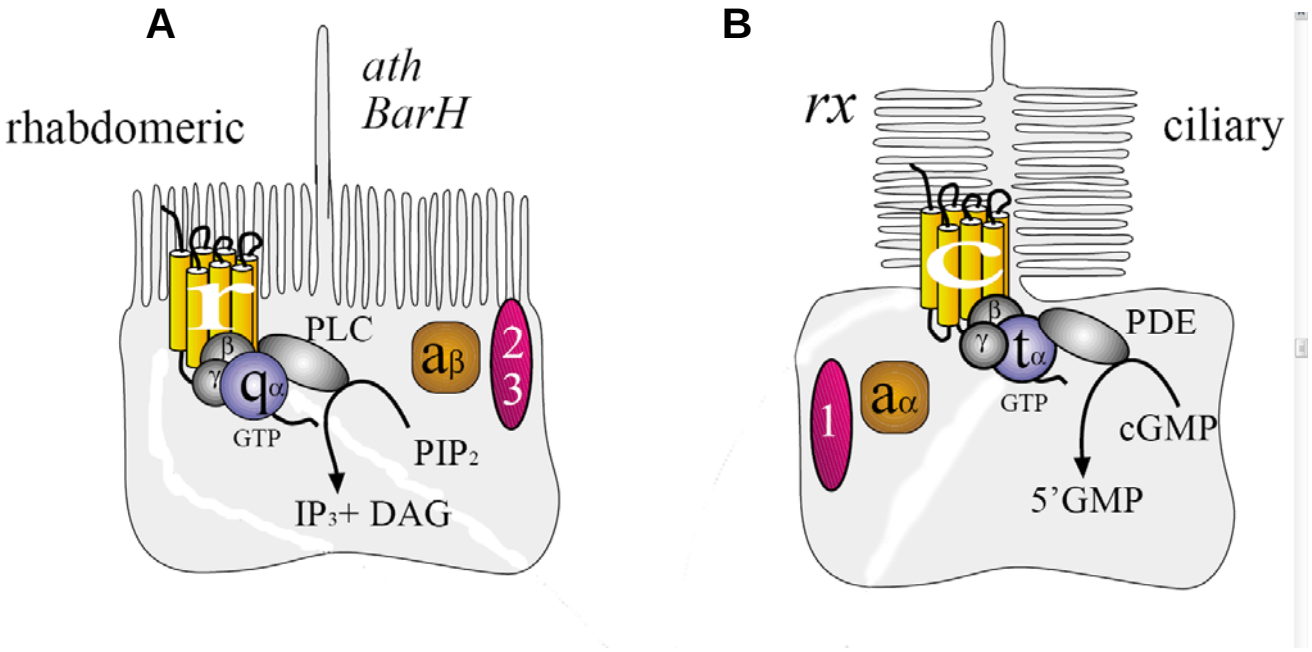

Fig.4. ***Schematic reprensentation of rhabdomeric (A) and ciliary (B) photoreceptor cells with relevant components of their respective pototransduction cascades.***

Rhabdomeric (r, orange) and ciliary (c, orange) opsins, G-α subunits (blue), arrestin α and β (brown) and rhosopsin kinases (purple). Abbreviations: cGMP, cyclic guanosylmonophosphate; DAG, diacylglycerol; GTP, Guanosytrisphosphate; IP3, inositol-1,3,5-trisphosphate; PDE, phosphodiesterase; PIP2, Phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C (Adopted from Arendt 2003).

Even though ciliary and rhabdomeric photoreceptive visual systems coexist throughout the animal taxa (Fernald, 2006), the present evidence suggests that their evolutionary histories differ. For photodetection, all invertebrate PRCs examined employ Go/*r-opsin*, and all vertebrate PRCs employ *c-opsin* (Arendt and Wittbrodt, 2001, Arendt, 2003). Importantly, *c-opsin* is expressed in the ciliary PRCs in the brain of the polychaete worm, *Platynereis dumerili*, whereas *r-opsin* is expressed in rhabdomeric PRCs in the eyes (Arendt *et al.*, 2004). Based on this result, it was proposed that early metazoans possessed a single type of PRC with an ancestral opsin for light detection that later diversified into two distinct PRC and opsin types (Arendt *et al.*, 2004). In *Protostomes* the rhabdomeric PRCs (with r-opsin) were used in the eyes for photoreception, whereas the ciliary PRCs (with c-opsin) were incorporated into the evolving brain. These findings are consistent with vertebrates confining r-opsin to retinal ganglion cells apparently for photoperiodicity and using ciliary PRCs containing c-opsins exclusively for visual photoreception (rods and cones). Taken together, the data suggest that the ganglion cells of the vertebrate retina are

the evolutionary descendents of rhabdomeric PRCs (Arendt, 2003, Arendt *et al.*, 2004). Moreover, because no *opsin* gene identified so far in cnidarians (Plachetzki *et al.*, 2007) contains a typical r-opsin fingerprint tripeptide HPK critical for coupling to the downstream phototransduction cascade, it was proposed that r-opsins are a bilaterian innovation that originated after the separation of the cnidarian and bilaterian lineages (Plachetzki *et al.*, 2007).

II.6. Shielding pigment of eyes

All eyes have shielding pigment typically found in cells adjacent to the photoreceptors. In general, the dark pigment reduces photon scatter and restricts the direction of incoming light. The biochemical nature of the dark pigment appears more diverse than the phototransduction cascades used by the PRCs. Vertebrate eyes use melanin as their exclusive dark pigment. However, among invertebrates, pterins constitute the eye pigment in the polychaete *Platynereis dumerilii* (Visconti.M *et al.*, 1970), pterins and ommochromes are accumulated in eyes of *Drosophila* (Shoup, 1966), and melanin is found rarely such as in the inverse cup-like eyes of the planarian, *Dugesia* (Hase *et al.*, 2006).

III. COMMENTS ON PRESENTED PUBLICATIONS

III.1.

Kozmik Z., Swamynathan S.K., **Ruzickova J.**, Jonasova K., Paces V., Vlcek C., Piatigorsky J.: *Cubozoan crystallins: evidence for convergent evolution of pax regulatory sequences.* **Evolution & Development 10:1, 52–61 (2008).**

Cubozoan jellyfish and vertebrates both use a cellular lens to increase visual sensitivity and produce a sharp image in the desired plane of focus. The optical properties of cellular lenses are caused by the high-level expression of proteins collectively called crystallins. This work describes novel lens crystallin of jellyfish *Tripedalia cystophora*. Moreover it deals with the question which regulatory sequences and transcription factors are directing tissue specific expression of crystallins. In addition the usage of Pax transcription factors in evolutionary consequences is discussed.

First, we have characterized J2-crystallin in *Tripedalia cystophora*. We show that *J2-crystallin* gene is composed of a single exon and encodes a 157-amino acid cytoplasmic protein with no detectable homology to known proteins from other species and is preferentially expressed in the jellyfish eye lens. The non-lens expression of J2-crystallin suggests non-optical as well as crystallin functions consistent with the gene sharing strategy that has been used during evolution of lens crystallins in other invertebrates and vertebrates. The *J2-crystallin* promoter is activated by the jellyfish paired domain transcription factor PaxB in co-transfection tests via binding to three paired domain sites. PaxB paired domain binding sites were also identified in the PaxB-regulated promoters of the *J1A* and *J1B*-crystallin genes, which are not homologous to the J2-crystallin gene. These results argue for an independent co-option of Pax binding sites within the regulatory regions of cubozoan crystallin genes.

Taken together with previous studies on the regulation of the diverse crystallin genes by Pax6, the present report strongly supports the idea that convergent changes in the promoter activity of nonhomologous genes within and between species, as well as within

gene families, (involving Pax family transcription factors) have driven the recruitment of lens crystallins throughout evolution.

Author contribution: I did the expression analysis of *J2-crystallin* and jellyfish collection and culture.

III.2.

Kozmik Z., **Ruzickova J.**, Jonasova K., Matsumoto Y., Vopalensky P., Kozmikova I., Strnad H., Kawamura S., Piatigorsky J., Paces V., Vlcek C.: *Assembly of the cnidarian camera-type eye from vertebrate-like components.*

Proc Natl Acad Sci U S A. 2008 Jul 1;105(26):8989-93

Animal eyes are morphologically diverse. Their assembly, however, always relies on the same basic principle, i.e., photoreceptors located in the vicinity of a cell expressing dark shielding pigment. Thus the simplest eye involves just a single photoreceptor cell situated close of a cell with shielding pigment. Both these cells are characterized by a unique set of proteins and transcription factors directing its cell fate. The photoreceptor cells (PRCs) are classified into two distinct types: rhabdomeric, characteristic of vision in invertebrate eyes; and ciliary, characteristic of vision in vertebrate eyes. In both ciliary and rhabdomeric PRCs, the seven-transmembrane receptor (opsin) associates with retinal to constitute a functional photosensitive pigment. Each photoreceptor type uses a separate phototransduction cascade. Rhabdomeric photoreceptors employ r-opsins and phospholipase C cascade, whereas ciliary photoreceptors use c-opsins and phosphodiesterase (PDE) cascade. Concerning the shielding pigment, vertebrate eyes use melanin as their exclusive dark pigment, in contrast to invertebrates‘ eyes, where several different pigments have been described (ie. pterins, ommochromes and rarely also melanin). Cnidaria as the likely sister group to the Bilateria are the earliest branching phylum with a well developed visual system. The cubozoan jellyfish, *Tripedalia cystophora* has camera-type eyes with cornea, lens, retina and pigmented layer. Unexpectedly, the cubozoan retina has ciliated PRCs that are typical for vertebrate eyes.

In the present study, we screened an expressed sequence tag (EST) library derived from rhopalia of *Tripedalia* to identify the genes that are involved in vision; orthologues of

other invertebrates and vertebrates were identified by phylogenetic analysis. The *Tripedalia* opsin EST clustered with the c-opsins. The c-opsin was localized by immunohistochemistry in the retinal ciliated PRCs of both complex eyes of adult jellyfish. Next we tested photochemical properties of *Tripedalia* c-opsin and we concluded that it is functional and it was most sensitive to blue-green region of the spectrum. Next we identified catalytic subunit of *pde* which phylogenetically clusters with the group of GAF domain containing PDEs including vertebrate rod- and cone-specific *PDE6.* Furthermore, we identified other components of the ciliary-type cascade associated with deactivation or adaptation of phototransduction, such as the inhibitory subunit of phosphodiesterase (*PDE6D*), *phosducin* and *guanylate cyclase.* All the above mentioned data suggested that the camera-type eye of *Tripedalia* uses a ciliary-type phototransduction cascade similar to that of vertebrates. Than we analyzed conspicuous ring of dark shielding pigment which surrounds the area of c-opsin expression. The biochemical nature of the *Tripedalia* pigment was suggested by the identification of an orthologue of the vertebrate oculocutaneous albinism type 2 (*Oca2*) gene in our EST library. *Oca2* (also known as *pink-eyed dilution*) is an essential gene for melanin biosynthesis. To adress the expression of *oca2* we performed *In situ* hybridization analysis and it revealed that *Tripedalia oca* is expressed near the pigmented retina layer of PRCs. In addition the results of a direct chemical assay (Fontana–Masson) were consistent with melanin being the pigment in the *Tripedalia* retina. Moreover we have cloned and analyzed the expression pattern of a *mitf* (microophthalmia-associated transcription factor) orthologue*,* a gene necessary for development of melanin-producing cells and specification of retinal pigment cells. Our data shows that the shielding pigment of *Tripedalia* eyes is melanin, the exclusive pigment of vertebrate eyes.

In conclusion, we showed that camera-type eyes of the cubozoan jellyfish, *Tripedalia cystophora*, use genetic building blocks typical of vertebrate eyes, namely, a ciliary phototransduction cascade and melanogenic pathway. Our findings indicative of parallelism provide an insight into eye evolution. Combined, the available data favor the possibility that vertebrate and cubozoan eyes arose by independent recruitment of orthologous genes during evolution.

Author contribution: I did the cloning of *Oca2* gene, immunohistochemistry of Opsins and J1-crystallin and Oca2 RNA *in situ* hybridisation, Fontana-Masson method, melanin bleach procedure and jellyfish collection and culture.

III.3.

Kreslova J., Machon O., **Ruzickova J.**, Lachova J., Wawrousek E.F., Keller R., Krauss S., Piatigorsky J., Kozmik Z.: *Abnormal lens morphogenesis and ectopic lens formation in the absence of β-catenin function.*

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β-Catenin plays a key role in cadherin-mediated cell adhesion as well as in canonical Wnt signaling. Since the *β-catenin* knockout mice can die early in the embryonic development (Huelsken *et al.*, 2000), it is necessary to study the function of β-catenin in the tissue of interest using conditional gene targeting. In the current study, we have established a new lens- and retina-specific mouse Cre-line, which enables us to investigate the role of β-catenin during early eye development.

We have used three tandem copies of the Pax6-derived EE regulatory element (Williams *et al.*, 1998, Kammandel *et al.*, 1999, Xu *et al.*, 1999), fused to the minimal Pax6 P0 promoter, to drive expression of Cre recombinase in the presumptive lens ectoderm of LR-Cre mice. We reasoned that multiple copies of the EE element would make expression more robust, and independent of positional effects. We have observed Cre recombinase activity not only in the surface ectoderm, but also in developing mouse retina. We believe that retina may represent an abberant expression domain of the 3xEE regulatory module. Such interpretation is consistent with the observation that weak expression of Le-Cre transgene (Ashery-Padan et al., 2000) is present in the retina.

Than we used LR-Cre mice for inactivation of β-catenin in future lens and retina and revealed that it does not suppress lens fate, but instead results in abnormal morphogenesis of the lens. Using BAT-gal reporter mice, we show that β-catenin-mediated Wnt signaling is notably absent from lens and neuroretina throughout eye development. The observed defect is therefore likely due to the cytoskeletal role of β-catenin, and is accompanied by impaired epithelial cell adhesion. In contrast, inactivation of β-catenin in the nasal ectoderm, an area with active Wnt signaling, results in formation of crystallin-positive ectopic lentoid bodies. These data suggest that, outside of the normal lens, β-catenin functions as a coactivator of canonical Wnt signaling to suppress lens fate.

In conclusion, we established LR-Cre mice as a tool for Cre-mediated recombination in lens and retina. Moreover our data suggest that β-catenin plays a dual role in lens

development. As a cytoskeletal component, β-catenin affects lens morphogenesis while it suppresses lens fate in nasal ectoderm as part of canonical Wnt signaling.

Author contribution: I did all the experiments with BAT-gal mice.

III.4.

Ruzickova J., Piatigorsky J., Kozmik Z.: *Eye-specific expression of an ancestral jellyfish PaxB gene interferes with Pax6 function despite its conserved Pax6/Pax2 characteristics.* accepted in **Int.J.Dev.Biol. 2009**

Eye development in invertebrates and vertebrates depends upon the regulated expression of *Pax6* and *Pax2*. However, the cubozoan jellyfish *T. cystophora*, which has *PaxB* instead of *Pax2* or *Pax6*, has sophisticated lens-containing eyes that share numerous characteristics with vertebrate eyes (see the previous paper Kozmik et al. 2008). Jellyfish PaxB has a Pax2-like paired domain and octapeptide and a Pax6-like homeodomain potentially giving it functional properties of both Pax2 and Pax6. In mice, functionally distinct Pax6 and Pax2 proteins have unique as well as redundant roles in eye morphogenesis. The main goal of this study was to further investigate the functional properties of jellyfish *PaxB* by expressing it in the developing lens and retina of transgenic mice.

We used the LR (lens, retina)-module to drive the expression of PaxB and Cre cDNAs connected via an internal ribosomal entry site (IRES) (the same "LR-module" was used in the paper Kreslova et al., 2007). We demonstrated that the expression of *PaxB* strongly influences normal development of the lens, cornea and retina; adult mice appeared microphthalmic. The lens defects in *PaxB* transgenic mice prompted us to examine the expression of several lens specific markers and transcription factors. At E10.5 expression of *FoxE3*, *Mab21l, Prox1*and *crystallins* was markerdly reduced, which was in agreement with delayed and disrupted lens development. To further explore the molecular basis for lens defects in *PaxB* transgenic embryos, we investigated the expression of Six3, Meis1 and Meis2 proteins, known transcription factors directing the lens development upstream of *Pax6*. The expression of all these transcription factors did not change. Our data indicated that *PaxB* expression downregulates genes encoding transcription factors downstream of the

Pax6 regulatory network for lens development and disrupts lens development but does not affect genes upstream of Pax6 within the pathway. Our data indicated that *PaxB* expression downregulates genes encoding transcription factors downstream of the Pax6 regulatory network for lens development and disrupts lens development but does not affect genes upstream of Pax6 within the pathway. So we decided to test if *PaxB* expression directly influences the expression of an endogenous *Pax6*. By using different approaches (immunohistochemistry, mRNA *In situ* hybridization and *in vivo* testing in transgenic mouse *EE-EGFP*) we revealed that endogenous Pax6 is downregulated. In addition to the lens phenotype, there is also a phenotype in the neuroretina of the *PaxB* transgenic mice. We have again observed the downregulation of Pax6 immunoreactivity in the cells expressing PaxB. As a result no amacrine cells and reduced number of retinal ganglion cells were detected in adult retina of transgenic mice. Moreover we showed that PaxB binds to *Pax6* autoregulatory binding site within the lens specific ectoderm enhancer (EE) and retinal specific α-enhancer and is able to displace Pax6 protein from it.

The present data suggest that PaxB, due to its Pax2-like paired domain and Pax-6 like homeodomain, disturbs the transcriptional network regulated by Pax6 in the developing lens and retina. Taken together, our data suggest that molecular properties of individual mouse Pax2 and Pax6 proteins are essential determinants of mouse eye development and cannot be substituted for by jellyfish PaxB which possesses elements of vertebrate Pax2 and Pax6.

Author contribution: I did all the experiments, except for EMSA.

IV. CONCLUSIONS

The main results of my PhD thesis can be summarized as follows:

We have described a new crystallin gene, J2-crystallin, in jelyfish *Tripedalia cystophora*. We showed that J2-crystallin is encoded within a single exon, has no detectable homology to any known protein, and is preferentially (but not exclusively) expressed in the jellyfish eye lens. Moreover we have demonstrated that transcription factor PaxB binds the J1A-, J1B-, and J2-crystallin promoters and activates the J2-crystallin promoter. These data indicate that similar transcription factors including those of the *Pax* gene family have been independently recruited for the regulation of nonhomologous crystallin genes in *Tripedalia* and vertebrates to achieve a gradient of refractive index within their transparent lenses.

Animal eyes are morphologically diverse. Their assembly, however, always relies on the same basic principle, i.e., photoreceptors located in the vicinity of dark shielding pigment. Cnidaria as the likely sister group to the Bilateria are the earliest branching phylum with a well developed visual system. We have characterized genes required for the assembly of camera-type eyes in *Tripedalia.* We show that the genetic building blocks typical of vertebrate eyes, namely ciliary opsin and the melanogenic pathway, are used by the cubozoan eyes. Although our findings of unsuspected parallelism are consistent with either an independent origin or common ancestry of cubozoan and vertebrate eyes, we believe the present data favour the former alternative.

We have investigated the role of β-catenin during early eye development by using the conditional CRE/lox system in mouse. For that purpose we established a new LR-Cre mice line which express *Cre-recombinase* in lens and retina. We showed that loss-of-function of β-catenin did not prevent formation of lens tissue from the presumptive lens ectoderm. However, morphogenesis of the lens was grossly affected due to the defect in cell adhesion. In contrast, loss-of-function of β-catenin in the nasal ectoderm lead to the formation of ectopic lentoid bodies, expressing crystallin proteins. The appearance of ectopic lentoid bodies in β-catenin loss-of-function mutant embryos coincided with downregulation of the BAT-gal Wnt reporter gene in the region nasal to the eye. Our data thus suggest that β-catenin plays a dual role in lens development. As a cytoskeletal component, β-catenin affects

lens morphogenesis while it suppresses lens fate in nasal ectoderm as part of canonical Wnt signaling.

Further we have investigated the functional properties of jellyfish *PaxB* by expressing it in the developing lens and retina of transgenic mice. We have observed severe eye defects in PaxB transgenic mice, ie. micropthalmia, reduced lens, loss of amacrine and retinal ganglion cells. The reason was that the expression of jellyfish *PaxB* have downregulated expression of endogenous *Pax6*, disturbed the transcriptional network regulated normally by Pax6, and yet it was unable to take over the developmental functions of Pax6 in these eye tissues. Taken together, our data suggest that, unlike the situation in *Drosophila* (Kozmik et al., 2003), in mice the molecular properties of Pax2 and Pax6 are essential determinants of mouse eye development and cannot be substituted for the chimeric PaxB protein of jellyfish.

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VI. PRESENTED PUBLICATIONS

VI.1. Kozmik Z., Swamynathan S.K., **Ruzickova J.**, Jonasova K., Paces V., Vlcek C., Piatigorsky J.: *Cubozoan crystallins: evidence for convergent evolution of pax regulatory sequences.* **Evolution & Development 10:1, 52–61 (2008).**

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Cubozoan crystallins: evidence for convergent evolution of pax regulatory sequences

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SUMMARY Cnidaria is the earliest-branching metazoan phylum containing a well-developed, lens-containing visual system located on specialized sensory structures called rhopalia. Each rhopalium in a cubozoan jellyfish *Tripedalia cystophora* has a large and a small complex, camera-type eye with a cellular lens containing distinct families of crystallins. Here, we have characterized J2-crystallin and its gene in *T. cystophora*. The *J2-crystallin* gene is composed of a single exon and encodes a 157-amino acid cytoplasmic protein with no apparent homology to known proteins from other species. The non-lens expression of J2-crystallin suggests nonoptical as well as crystallin functions consistent with the gene-sharing strategy that has been used during evolution of lens crystallins in other invertebrates and vertebrates. Although nonfunctional in transfected mammalian lens cells, the *J2-crystallin* promoter is activated by the jellyfish paired domain transcription factor PaxB in co-transfection tests via binding to three paired domain sites. PaxB paired domain-binding sites were also identified in the PaxB-regulated promoters of the *J1A*- and *J1B*-crystallin genes, which are not homologous to the J2-crystallin gene. Taken together with previous studies on the regulation of the diverse crystallin genes, the present report strongly supports the idea that crystallin recruitment of multifunctional proteins was driven by convergent changes involving Pax (as well as other transcription factors) in the promoters of nonhomologous genes within and between species as well as within gene families.

INTRODUCTION

The abundant, water-soluble proteins responsible for the optical properties of the transparent lens are called crystallins. In contrast to the conservation of visual pigments (opsins) in the retina, the lens crystallins are diverse, multifunctional, and taxon-specific, that is they are often used selectively in different species (Wistow and Piatigorsky 1988; de Jong et al. 1989; Piatigorsky and Wistow 1989). Many of the crystallins are identical or related to common, ubiquitously expressed metabolic enzymes or physiological stress proteins. For instance, of the crystallins present in all vertebrate lenses, the α-crystallins are small heat shock proteins (Ingolia and Craig 1982; de Jong et al. 1993) and the β/γ-crystallins are related to microbial stress proteins (Wistow 1990; D'Alessio 2002). This hallmark of being recruited from diverse multifunctional proteins led to the idea that crystallins are unified more by their high, lens-preferred expression than by their structure per se (Piatigorsky and Wistow 1991; Piatigorsky et al. 1993; Carosa et al. 2002). In addition to their role as crystallins, most if not all crystallins also serve nonlens functions. We have called the dual use of a single protein encoded in one gene "gene sharing" (Piatigorsky et al. 1988; Piatigorsky and Wistow 1989; Piatigorsky 2007). An important implication of gene sharing illustrated by the lens crystallins is that a protein can evolve a new role, without losing its original function, by a change in gene expression in the presence or in the absence of gene duplication (Piatigorsky and Wistow 1991; Piatigorsky 2003, 2007).

Cnidaria is the likely sister group to Bilateria and thus constitutes the earliest-branching phylum containing a well-developed visual system. In general, among Cnidarians, it is the Cubozoa (known as "box jellyfish" because of their cuboidal shape) that have lens-containing eyes (Piatigorsky and Kozmik 2004). The cubozoan that we have been investigating, *Tripedalia cystophora* (Fig. 1A), has four equally

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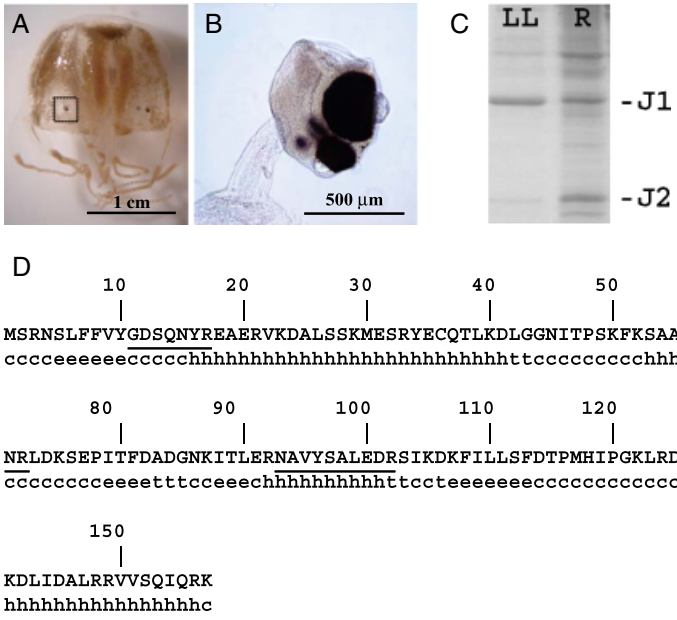

Fig. 1. Cloning of *J2-crystallin* from *Tripedalia*. *Tripedalia* medusae (A) and rhopalium (B, inset in A). (C) Sodium dodecyl sulfate-polyacrylamide gel with a rhopalium sample. The position of J1- and J2-crystallin in samples of large eye lens (LL) and rhopalia (R) is indicated. (D) Amino acid sequence of J2-crystallin derived from cDNA. The sequence of peptides identified by direct protein sequencing is underlined. The predicted secondary structure is shown below the peptide sequence; h, α-helix; c; random coil; e, extended strand; t, β-turn.

spaced sensory structures called rhopalia (Fig. 1B) dangling from a stalk and situated within the sensory niche surrounding the bell. Each rhopalium has two complex, lens-containing eyes, one larger than the other, situated at right angles to each other. It was shown recently by Nilsson et al. (2005) that *Tripedalia* lenses contain a refractive index gradient allowing transmitted light to produce a nearly aberration-free image. This demonstrates that even simple animals have been able to evolve the sophisticated visual optics previously known only from a few advanced bilaterian phyla. It has been proposed that this ability of *Tripedalia* lens to focus light is due to the presence of lens crystallins (Nilsson et al. 2005).

Tripedalia lenses contain three novel crystallins (J1-, J2-, and J3-crystallin) (Piatigorsky et al. 1989). J1-crystallins in turn comprise three distinct polypeptides (J1A, J1B, and J1C), each encoded by a different although extremely similar gene (Piatigorsky et al. 1993). The J1 crystallin polypeptides show sequence similarity to ADP-ribosylglycohydrolases and fall under the broad category of enzyme crystallins (Piatigorsky and Kozmik 2004; Castellano et al. 2005). By contrast, J3-crystallin is encoded in a single gene and shows homology to vertebrate saposins, multifunctional proteins that bridge lysosomal hydrolases to lipids and activate enzyme activity (Piatigorsky et al. 2001). Initial in situ hybridization showed that jellyfish crystallins are also expressed outside of the lens, consistent with having nonoptical functions (Piatigorsky et al. 2001; Kozmik et al. 2003).

Pax proteins are a family of transcriptional regulators characterized by the presence of an evolutionarily conserved DNA-binding domain, the paired domain (Bopp et al. 1986; Treisman et al. 1991). Pax genes are involved in many developmental processes in all higher eucaryotes. In particular, the role of *Pax* genes during eye development has been characterized extensively [for reviews, see (Gehring and Ikeo 1999; Kozmik 2005)]. We have previously identified the *Tripedalia PaxB* gene that encodes a paired domain, an octapeptide, and a full-length homeodomain. *Tripedalia PaxB* is expressed in swimming larvae as well as in the eyes of adult jellyfish and clusters with the *Pax2/5/8* subfamily in a phylogenetic tree analysis (Kozmik et al. 2003). This cluster most likely represents an ancient group of genes within the *Pax* family because its members are found in sponges (Hoshiyama et al. 1998) and Placozoa (Hadrys et al. 2005).

In the present study, we characterize J2-crystallin in *Tripedalia*. We show that J2-crystallin is encoded within a single exon, has no detectable homology to any known protein, and is preferentially (but not exclusively) expressed in the jellyfish eye lens. Our previous investigations showed that PaxB activates the J1- and J3-crystallin promoters and binds the J3-crystallin promoter (Kozmik et al. 2003). Here we demonstrate that PaxB binds the J1A-, J1B-, and J2-crystallin promoters and activates the J2-crystallin promoter. Taken together with the earlier demonstrated regulation of diverse crystallin genes by Pax6 [for reviews, see (Cvekl et al. 1995, 2004)], our results indicate that convergent, functional changes in gene regulation, involving PaxB in *Tripedalia*, played a key role in recruiting the nonhomologous crystallin genes in different species and within gene families.

EXPERIMENTAL PROCEDURES

Jellyfish collection and culture

Adult *T. cystophora* were collected in mangroves of La Parguerra, Puerto Rico. The laboratory culture was established

using settling larvae and artificial sea water. Settled larvae metamorphosed into young polyps. Young polyps were transformed into budding (asexually reproducing) polyps by feeding them with *Artemia* once a week. Spontaneously metamorphosed polyps (new born medusae) were fixed for ISH. Polyps as well as young medusae were maintained under laboratory conditions at a temperature of 26°C.

Identification and cloning of J2 crystallin cDNA

The dissected rhopalia of adult *T. cystophora* were homogenized in a buffer containing 63 mM Tris-HCl pH 7.4, 5% β–mercaptoethanol, and 10% glycerol, centrifuged at 10,000 *g* at 4°C for 10 min, and the supernatant was applied on 12% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) . The band corresponding to J2-crystallin was isolated and subjected to protein sequencing (Harvard MicroChem). The peptide sequences identified (GDSQNYR, CSEDFKN, and QDDI/LFMF) were used to design degenerate primers. Reverse transcription (RT)-PCR using various primer combinations produced specific bands that were cloned into pCR-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced. The complete open reading frame (ORF) was isolated using SMART RACE (BD Biosciences, San Jose, CA, USA). To isolate the genomic region encoding the ORF of J2, PCR was performed on *T. cystophora* genomic DNA using primers AAGCAC GAGCCGAATTAAAGC (787A) and CATCTTAGCCAG TTAAGGAAG (787B), covering the region of a start codon and a stop codon. The PCR product was cloned into pCR-TOPO and sequenced.

Subcellular localization

Monkey kidney COS7 cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum on glass coverslips were transfected with EGFP or DsRed fusion constructs encoding J2-crystallin ORF or Pax6 paired domain using Fugene6 reagent (Roche, Nutley, NJ, USA). After a 48-h incubation, cells were washed with phosphate-buffered saline (PBS) and were fixed with 4% paraformaldehyde at room temperature (RT) for 10 min. Cells were viewed in a Nikon DIAPHOT 300 inverted microscope (Nikon, Melville, NY, USA) equipped with fluorescence optics.

Promoter characterization

The 0.9 kb promoter of the *J2* crystallin gene was cloned using the Universal Genome Walker kit (BD Biosciences). The gene-specific primers were ATGCAGCAGACTTGAACTT GGAAGATG (769B) and TTCATATCTGCTCTCCATCT TGCTTGA (769C). The 0.9 kb promoter and its nested deletions were inserted into luciferase reporter vector pGL3-basic (Promega, Madison, WI, USA). Mutagenesis of individual PaxB binding sites was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Human kidney HEK293 cells, monkey kidney COS7 cells, mouse embryonal carcinoma P19 cells, rabbit lens epithelial N/N1003 cells, and mouse lens epithelial α – TN4 cells were cultured in DMEM containing 10% fetal bovine serum. All cell lines were transfected with promoter constructs using Fugene6 reagent (Roche). In some experiments, an expression vector encoding paired domain transcription factor PaxB was co-transfected as described previously (Kozmik et al. 2003). The pRL-control vector was used to normalize the transfection efficiency. After 2 days, cells were washed with cold PBS and lysed with passive lysis buffer (Promega). The lysate was analyzed using a dual-luciferase assay kit (Promega) and a Tropix TR717 microplate luminometer (Applied Biosystems, Foster City, CA, USA).

Electrophoretic mobility shift assay (EMSA)

Paired domain and homeodomain sequences were cloned into the expression vector pET42, expressed in BL21(DE3)RIPL cells, and purified under native conditions using immobilized glutathione agarose (Pierce, Rockford, IL, USA). EMSA with the FLAG-tagged paired domain and full-length PaxB was performed as described previously (Kozmik et al. 2003) using double-stranded oligonucleotides comprising binding sites. The FLAG antibody was used for supershift. For competition experiment, the Pax consensus sequence (Kozmik et al. 2003) was used.

Expression analysis

Jellyfish were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose overnight at 4°C, embedded, and frozen in OCT (Tissue Tek, Sakura Finetek, Torrance, CA, USA). Frozen sections were performed at 6–8 μm thickness. The cryosections were washed three times in PBS and subsequently hybridized with an RNA anti-sense probe. In situ hybridization on cryosections was carried out as follows: plasmid carrying J2 cDNA was linearized with an appropriate restriction enzyme and an anti-sense riboprobe was synthesized using the DIG RNA labeling kit (Roche). The cryosections were refixed in 4% paraformaldehyde for 20 min and thereafter washed twice with PBS. Samples were then hybridized at 68°C with preheated hybridization buffer containing a digoxigenin-labeled probe (approximately 1 μg/ml) overnight. The hybridization buffer was composed of 50% formamide, 20% dextran sulfate, 10 mg/ml yeast tRNA, 0.02% (w/v) each Ficoll, acetylated bovine serum albumin (BSA), polyvinylpyrrolidone (PVP-90), and 1 × salt solution (0.2 M NaCl, 10 mM Tris, 5 mM $NaH_2PO_4 \cdot 2H_2O$, 5 mM $Na_2HPO_4 \cdot 12H_2O$, 5 mM EDTA). After the hybridization, unbound riboprobe was washed three times (30 min each) in prewarmed (68°C) 1 × SSC, 50% formamide, 0.1% Tween-20. Then, the sam-

ples were incubated twice (30 min each) at RT in MABT pH 7.5 (100 mM maleic acid, 150 mM NaCl, 0.1% Tween-20). Blocking was performed in 20% heat-inactivated sheep serum in MABT for 1.5 h at RT. After that, the samples were incubated overnight with anti-digoxigenin-AP Fab fragments (Roche, 1:1000) in 20% heat-inactivated sheep serum in MABT at RT. Unbound anti-digoxigenin Fab fragments were removed by five washes in MABT (20 min each) at RT. Then the sections were rinsed twice for 10 min in alkaline phosphatase staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween-20). Staining was performed with 10 µl NBT/BCIP Stock Solution (Roche) in 500 µl staining buffer in the dark and at RT. Staining was stopped with several washes of 1 mM EDTA in PBT (0.1% Tween-20 in PBS). RT-PCR was carried out as described previously (Kozmik et al. 2003).

RESULTS AND DISCUSSION

J2-crystallin is a novel cytoplasmic protein

The 20-kDa polypeptide band (J2-crystallin) was isolated from jellyfish rhopalia by elution from an SDS-PAGE gel, and several peptides separated by HPLC were sequenced (Fig. 1C). Degenerate oligonucleotides corresponding to the peptide sequences were designed and used to generate products by RT-PCR using rhopalial RNAs as templates. Several primer combinations produced PCR products that were cloned and sequenced. Conceptual translation of one of the PCR products revealed the presence of an internal peptide identical to one of the peptides obtained by direct protein sequencing. The 5′ and 3′ ends of the J2-crystallin cDNA were extended by RACE. The complete J2-crystallin cDNA sequence was deposited into GenBank (Accession No. EF202826).

The J2-crystallin cDNA encodes a 157-amino acid protein with a large portion of the protein (42%) being predicted to possess an α-helical secondary structure using a self-optimized method for protein secondary structure prediction (SOPM algorithm) (Geourjon and Deleage 1994) (Fig. 1D). The J2-crystallin has a deduced molecular weight of 18.2 kD and a theoretical pI of 9.25. Computer-assisted analysis (http://www.ncbi.nlm.nih.gov/BLAST/) indicated that the deduced amino acid sequence of J2-crystallin shows no significant homology to any known protein. This is in contrast to either J1-crystallin that bears homology to ADP-ribosylglycohydrolases (Piatigorsky and Kozmik 2004; Castellano et al. 2005) or J3-crystallin that is homologous to saposins (Piatigorsky et al. 2001).

Lens crystallins are generally cytoplasmic proteins. Given the fact that J2-crystallin is a novel protein, we wanted to examine its subcellular localization. To this end, we fused the complete ORF of J2-crystallin to the ORF of either EGFP or DsRed fluorescent proteins. The expression vectors encoding the respective fusion proteins were transfected into cultured mammalian cells and the fused proteins were identified by fluorescence microscopy. As shown in Fig. 2, J2-crystallin was localized predominantly in the cytoplasm as expected for a lens crystallin.

The abundance of crystallins is critical for the optical properties of the lens (collectively 80–90% of the water-soluble protein within the lens). Although many crystallins are related or identical to stress proteins or metabolic enzymes, many different proteins could potentially function as crystallins when expressed at high levels in the lens, a possibility supported by the diversity of crystallins throughout animals (Wistow and Piatigorsky 1988; Tomarev and Piatigorsky 1996). J2-crystallin in the cubozoan *Tripedalia* represents still another protein that has been recruited as a member of the diverse, taxon-specific lens crystallins.

Expression of the *J2-crystallin* gene in *Tripedalia*

Tripedalia, as other jellyfish, undergo an alternation of generations between sessile, nonsexual polyps and swimming,

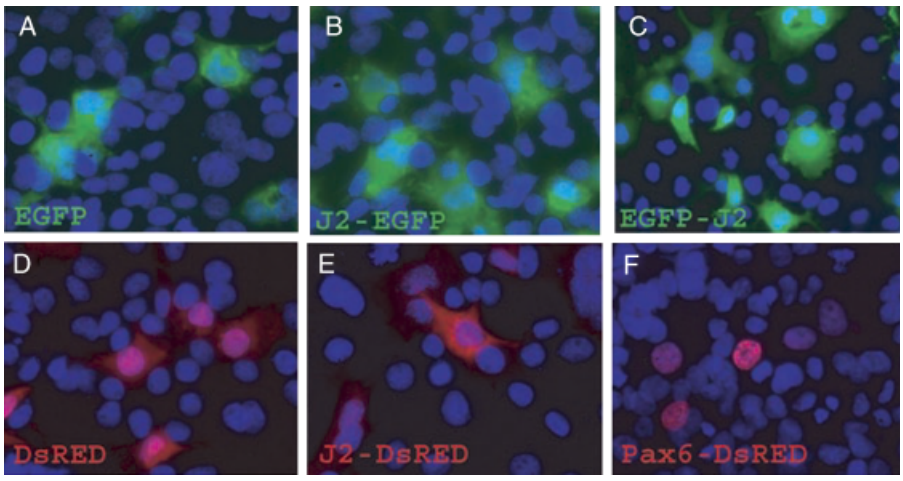

Fig. 2. J2-crystallin is a cytoplasmic protein. COS7 cells were transfected with an EGFP control vector (A), an N-terminal J2-EGFP fusion construct (B), a C-terminal EGFP-J2 fusion construct (C), a DsRed control vector (D), an N-terminal J2-DsRed fusion construct (E), and a Pax6-DsRed fusion construct (F), respectively. The localization of the fluorescent protein was monitored 48 h later.

sexually dimorphic medusae (Werner et al. 1971). Fertilization is internal and the planulae larvae develop in the gastral pocket of the females. The swimming larvae are released into seawater, and settle on the bottom to become polyps. Each full-grown polyp metamorphoses into a swimming immature medusa. Here, we investigated the expression of *J2-crystallin* throughout the life cycle of *Tripedalia*.

The expression pattern of the *J2-crystallin* gene was explored by in situ hybridization using an anti-sense riboprobe (Fig. 3, A, C, and D). The *J2-crystallin* mRNA is expressed in the lens of a newly metamorphosed medusa (Fig. 3C) and in the lenses of the large and small eyes of an adult jellyfish (Fig. 3, A and D). Control tests with the J2-crystallin sense riboprobe were negative (Fig. 3B). The results of RT-PCR tests were consistent with the in situ hybridization data and indicated, in addition, that *J2-crystallin* mRNA is expressed only in the adult medusa stage, within and outside of the rhopalia (Fig. 3F).

It is noteworthy that earlier electrophoretic tests indicated that J2- and J3-crystallin proteins are confined to the lens of the larger *Tripedalia* eyes (Piatigorsky et al. 1989), whereas our present in situ hybridization experiments indicate that J2-crystallin mRNA is present in the lenses of both the large and the small eyes. Whether this discrepancy reflects inefficient J2-crystallin mRNA translation in the lenses of the small eye or technical difficulties requires further experimentation.

Extraocular expression of *J2-crystallin* was seen at the bottom of the gastrovascular cavity of the rhopalia (Fig. 3D, red arrowheads) and in the stalk canal (Fig. 3D, red arrows). A gastrovascular cavity is situated in the center of each rhopalium (see histology staining in Fig. 3E) and together with the stalk canal comprises the jellyfish gastrovascular system (Garm et al. 2006). Our data suggest that the in situ hybridization signal for J2-crystallin mRNA matches the gastrodermal lining of the cavity and its elongation (canal), both of which are of endodermal origin and have nutritive function. However, at present it is not possible to exclude some additional staining of epidermal and gastrodermal nerves that are located in the stalk (Garm et al. 2006). The precise physiological role of *J2-crystallin* in the jellyfish gastrovascular system is unknown. It is likely that the function of J2-crystallin in the gastrovascular system, where no lens-like or photorecep-

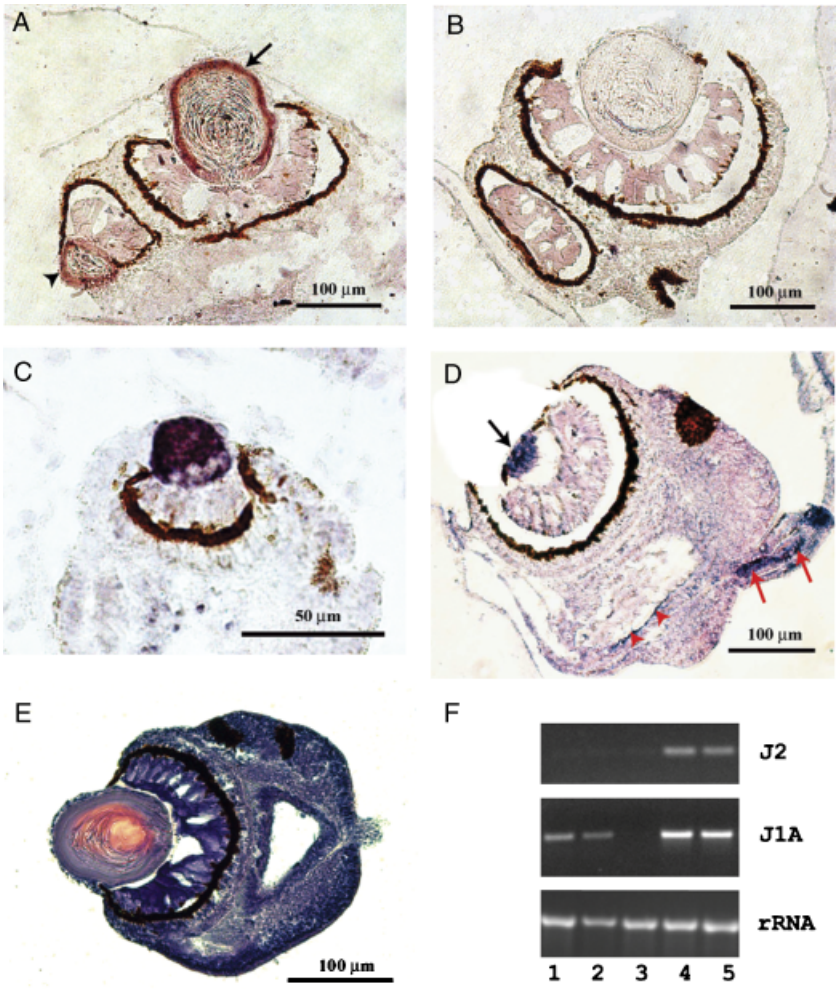

Fig. 3. Expression of the *J2-crystallin* gene in *Tripedalia*. Expression of *J2-crystallin* was determined by in situ hybridization on cryosections (A–D) and reverse transcription-PCR (F). An anti-sense *J2-crystallin* probe was used in hybridizations shown in panels A, C, and D; panel B represents a control hybridization using a sense probe. The cryosection through rhopalium stained with hematoxylin and eosin is shown in panel E. *J2-crystallin* is expressed in the lenses of both small and large eyes of an adult jellyfish (A, D, black arrow), in the lens of newly metamorphosed medusae (C), at the bottom of the gastrovascular cavity (red arrowheads), and in its protrusion in the stalk (red arrows) of an adult jellyfish (D). (F) Reverse transcription-PCR analysis of *J2-* and *J1A-crystallin* expression in nonpigmented larva (lane1), pigmented larva (lane 2), polyp (lane 3), adult rhopalium (lane 4), and adult medusa with experimentally removed rhopalia (lane 5).

tive structures are seen, differs from its refractive function in the lens where the protein is very abundant for its optical role (Garm et al. 2006).

Together, these data are fully consistent with the notion that J2-crystallin, like the many other crystallins that are expressed within and outside of the lens (Wistow and Piatigorsky 1988; de Jong et al. 1989), has acquired optical and nonoptical roles via a gene-sharing process (Piatigorsky et al. 1988; Piatigorsky and Wistow 1989; Piatigorsky 2007). The extension of this strategy to the lens of the complex eye of the cubomedusan jellyfish that is evolutionarily distant to Bilaterians is consistent with the idea that gene sharing applies to the evolutionary history of lens crystallins throughout the animal kingdom.

J2-crystallin gene is composed of a single exon

We have examined the structure of the *J2-crystallin* gene by PCR using *Tripedalia* genomic DNA. PCR products of the same size were obtained on cDNA and genomic DNA using primers covering the *J2-crystallin* ORF. Sequencing of the genomic PCR product revealed complete co-linearity with the *J2-crsytallin* cDNA (data not shown). We conclude that the entire coding region is covered by a single exon. This is also the situation for J1-crystallin polypeptides that are encoded in three intronless genes, *J1A*, *J1B*, and *J1C*, respectively (Piatigorsky et al. 1993).

J2-crystallin gene promoter is not active in cultured mammalian lens cells

J2-crystallin is strongly expressed in the *Tripedalia* lenses. Remarkably, high lens expression of crystallin genes of vertebrates and apparently of other invertebrates is governed by a similar set of conserved transcription factors despite different embryological origins of their eyes and lenses (Cvekl and Piatigorsky 1996; Carosa et al. 2002). We therefore examined the possibility that *J2-crystallin* gene promoter can function as a regulatory element in cultured mammalian cells. A 0.93-kb-promoter region of the *J2-crystallin* gene (GenBank Accession No. EF202827) was isolated and the transcriptional start site for this gene was determined by 5′ RACE (Fig. 4A, arrow). This promoter fragment or several deletions thereof were fused to the *luciferase* reporter gene and the resulting constructs were transfected into kidney cells (HEK293, COS7), embryonal carcinoma cells (P19), and lens epithelial cells (N/N1003, αTN4). Despite its high and preferential expression in the jellyfish lens, the isolated *J2-crystallin* promoter fragment showed little if any activity above the promoterless control vector used in the cultured mammalian cells (Fig. 4B, data not shown). The most likely explanation for the inability of *J2-crystallin* promoter to function in the nonhomologous cells is the large evolutionary distance separating jellyfish and mammals.

J2-crystallin gene promoter is activated by the jellyfish paired domain transcription factor PaxB

We have previously shown that *J1A-, J1B-,* and *J3-crystallin* gene promoters are activated by the jellyfish paired domain transcription factor PaxB in transiently transfected cells (Kozmik et al. 2003). In order to investigate whether the low basal level of *J2-crystallin* promoter activity in the transfected mammalian cells can also be upregulated by PaxB, we have co-transfected the *J2-crystallin* promoter: reporter gene construct with the PaxB expression vector into human embryonic kidney cells HEK293 and COS7 cells (Fig. 5A, data not shown). Co-transfection of wild-type PaxB activated *J2-crystallin* promoter:*luciferase* reporter gene expression, whereas the DNA-binding mutant PaxB(I84R) affecting the paired domain did not. In addition, PaxB(IQN) containing a paired domain mutation conferring Pax6 rather than Pax2-binding capability to PaxB (Kozmik et al. 2003) did not activate the reporter gene. Based on the present and earlier results (Kozmik et al. 2003), we conclude that the promoters of all three *Tridpedalia* crystallin genes (*J1-*, *J2-*, and *J3-crystallin*) can be activated by jellyfish PaxB.

Deletion analysis of the J2-*crystallin* promoter led to the identification of a minimal construct ($-148/+10$) that is activated by PaxB (Fig. 5A). Three putative PaxB-binding sites are present within the $-148/+10$ fragment (Fig. 5B). PaxB binding to all three sites was experimentally verified by EMSAs; the affinity of PaxB toward site C was lower than that to sites A and B (Fig. 5C). Using the longer probe encompassing all three binding sites in EMSAs, we were able to detect three retarded complexes, suggesting that sites A, B, and C can be occupied simultaneously (Fig. 5D). Mutations were introduced into each of the PaxB-binding sites (Fig. 5B) and tested by EMSAs. As shown in Fig. 5E, mutations Am1, Bm1, and Cm1 in binding sites A, B, and C, respectively, disrupted binding of the PaxB paired domain. Inspection of site B reveals two TAAT consensus binding sequences for homeodomain-containing proteins that are separated by four nucleotides. Three mutations within site B were introduced into one or both of the TAAT motifs. As shown in Fig. 5F, each mutation reduced the binding of full-length PaxB as well as the binding of isolated paired domain and homeodomain fragments. These data support the idea that both the paired domain and the homeodomains recognize site B. However, a negligible activation by the paired domain mutants PaxB(IQN) and PaxB(I84R) (Fig. 5A) suggests that the contribution of the homeodomain to the *J2-crystallin* activation is small at best.

Finally, to determine whether the three identified PaxB-binding sites are responsible for the activation of the *J2-crystallin* promoter fragment, we generated reporter constructs in which site A, B, or C was individually mutated (Fig. 5G). Mutation in each of the three sites reduced the level of

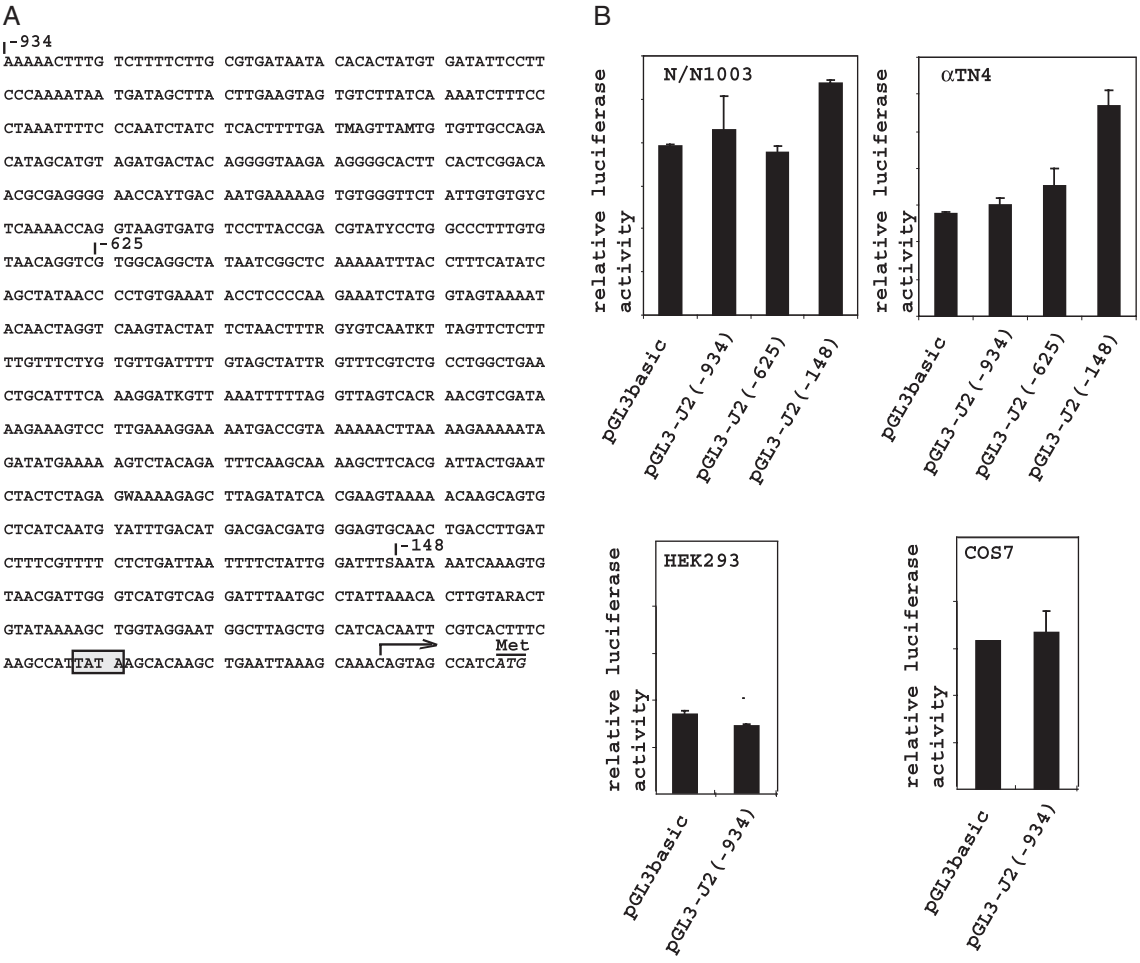

Fig. 4. Analysis of the *J2-crystallin* gene promoter. (A) The sequence of a 0.93 kb *J2-crystallin* gene promoter fragment including polymorphisms found among 10 sequenced clones. The full-length promoter sequence (−934/+10) or its deletions (−625/+10, −148/+10) were used to generate luciferase reporter constructs. The start of transcription as determined by a 5′ RACE is indicated by an arrow. Met marks the beginning of J2-crystallin ORF. The position of a putative TATA box is boxed. (B) *J2-crystallin* gene promoter analysis in cultured mammalian lens (N/N1003, αTN4) and nonlens (HEK293, COS7) cell lines.

activation; the greatest reduction for PaxB-mediated activation of the *J2-crystallin* promoter fragment was in sites B and C (Fig. 5H). Based on these results, we conclude that PaxB binds to three sites within the proximal *J2-crystallin* promoter and is able to activate its expression in transfected mammalian cells.

Convergent evolution of Pax regulatory sequences within cubozoan crystallin promoters

J2-crystallin, a novel taxon-specific lens crystallin gene that appears to be multifunctional as judged by its high lens and nonlens expression, belongs to the growing number of diverse crystallin genes regulated by a Pax family transcription factor (Cvekl and Piatigorsky 1996; Carosa et al. 2002; Cvekl et al. 2004). We have now cloned the three taxon-specific crystallins of *Tridpedalia,* allowing detailed studies of their coordinated and complex expression patterns within the developing and adult jellyfish (Piatigorsky et al. 1993, 2001). Furthermore, we have shown that PaxB activates the J1A-, J1B-, J2-, and J3-crystallin promoters (Kozmik et al. 2003). These results argue for an independent co-option of Pax-binding sites within the regulatory regions of cubozoan crystallin genes. In order to test this hypothesis further, we searched for Pax-binding sites within the promoter regions of two members of the J1-crystallin gene family. This search is particularly interesting because there is a high degree of identity within the coding sequence of the J1A- and J1B-crystallin genes, but there is no apparent homology between their corresponding flanking regulatory regions (Fig. 6A). As expected, we found two paired domain-binding sites in the proximal promoters of J1A- and J1B-crystallin genes that conform very well to the consensus motif and that bind the PaxB paired domain

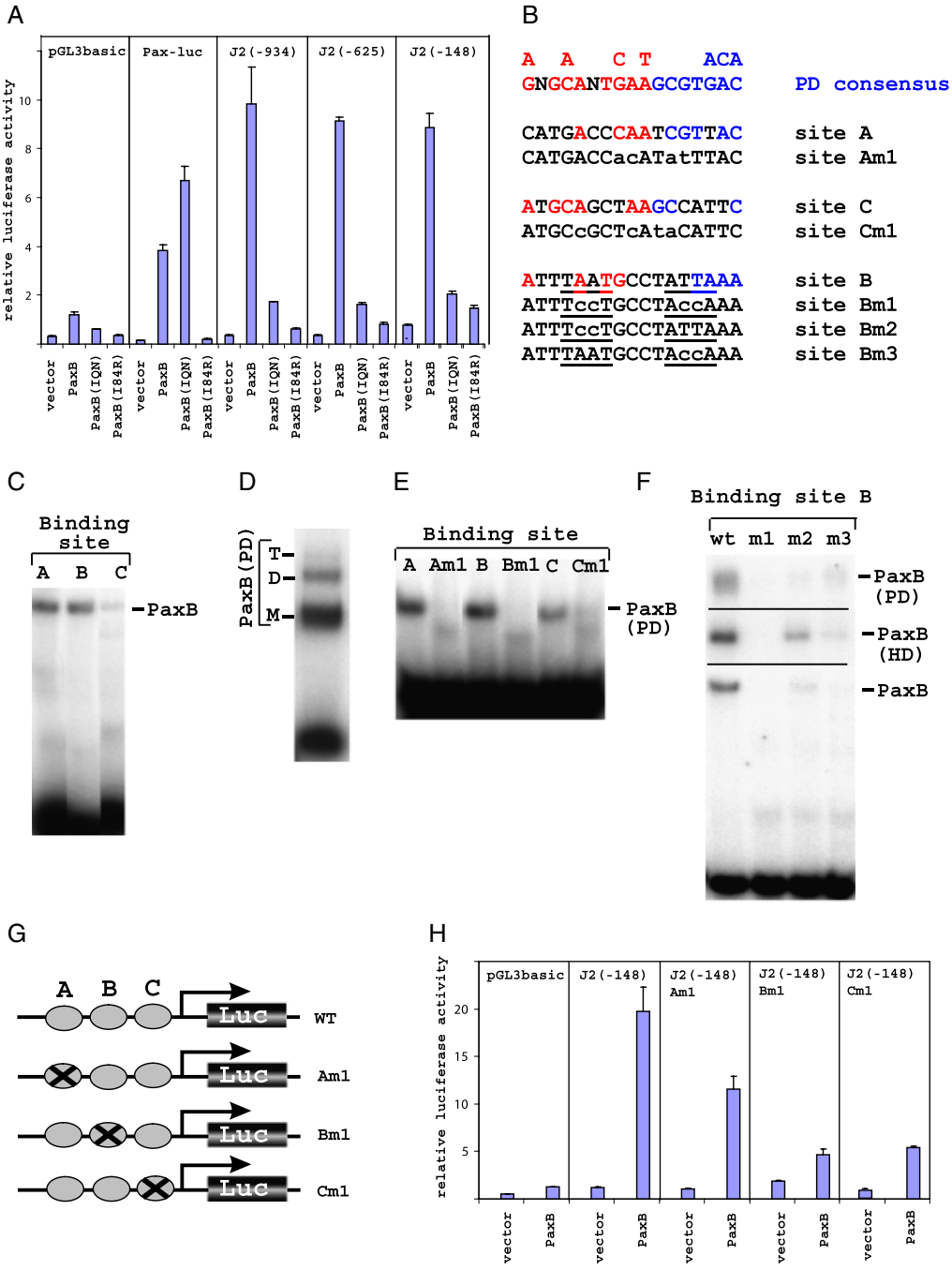

Fig. 5. Paired domain and homeodomain transcription factor PaxB binds to and activates *J2-crystallin* promoter. (A) PaxB activates the *J2-crystallin* gene promoter fragment in transiently transfected HEK293 cells. The full-length promoter sequence (−934/+10) or its deletions (−625/+10, −148/+10) were co-transfected with an empty expression vector or an expression vector encoding PaxB, Pax(IQN), and PaxB(I84R). Luciferase control vector pGL3basic served as a negative control whereas Pax-luc (−353Glu-luc) served as a positive Pax2/Pax6-responsive reporter construct. (B) The sequence of three putative binding sites A, B, and C present within the −148/+10 deletion construct. Mutations designed within each binding site are indicated. Homeodomain-like recognition sequences within site B are underlined. Paired domain consensus is shown on top, with the PAI recognition sequence in blue and the RED recognition sequence in red. (C) The three putative Pax-binding sites are recognized by full-length PaxB. (D) Promoter fragment corresponding to deletion construct −148/+10 was used in electrophoretic mobility shift assay with a purified paired domain. Slowly migrating complexes were detected representing simultaneous occupancy of one (monomer, M), two (dimer, D), or three (trimer, T) binding sites. (E) Mutations Am1, Bm1, and Cm1 disrupt binding of the PaxB paired domain. (F) Mutations within site B affect binding of both the paired domain and the homeodomain. (G) Schematic view of J2 (−148/+10) reporter constructs with individually mutated PaxB-binding sites. (H) All three PaxB-binding sites (A, B, C) contribute to the activation of the *J2-crystallin* reporter gene by PaxB in transiently transfected HEK293 cells.

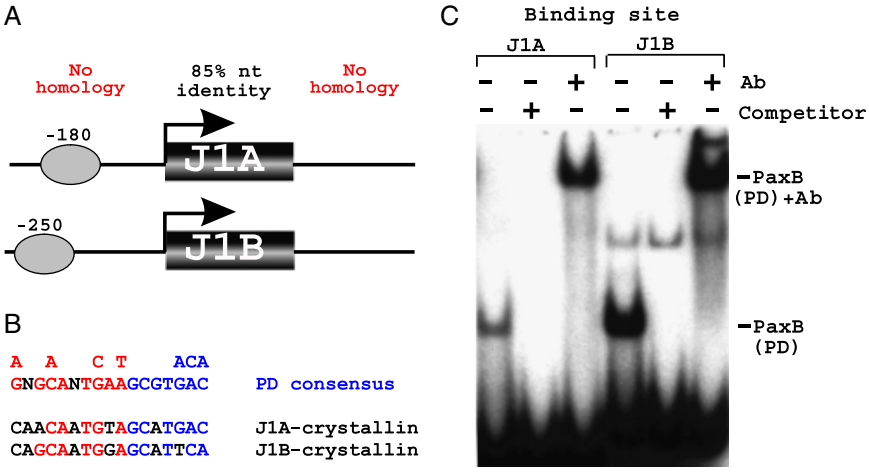

Fig. 6. Pax paired domain-binding sites are present in J1A- and J1B-crystallin gene promoters. (A) Schematic view of J1A- and J1B-crystallin genes. Despite the high degree of nucleotide identity between the coding sequences of J1A- and J1B-crystallin, the 5′ or 3′ flanking regions are dissimilar. The positions of co-opted Pax-binding sites are indicated (shaded ovals). (B) The sequences of Pax paired domain binding sites present in the J1A- and J1B-crystallin promoters are aligned with the paired domain consensus motif shown on top. The PAI recognition sequence is highlighted in blue and the RED recognition sequence in red. (C) Electrophoretic mobility shift assays showing the binding of a FLAG-tagged PaxB paired domain to double-stranded oligonucleotides comprising the binding sites shown in (A). Anti-FLAG antibody was used for the supershift tests. The competition was performed using the Pax paired domain consensus-binding site.

(Fig. 6B). Together, these data provide convincing evidence that convergent changes in the promoter activity of nonhomologous genes within and between species, as well as within gene families, involving Pax family transcription factors have driven the recruitment of lens crystallins throughout evolution (Piatigorsky 2007).

Acknowledgments

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VI.2. Kozmik Z., **Ruzickova J.**, Jonasova K., Matsumoto Y., Vopalensky P., Kozmikova I., Strnad H., Kawamura S., Piatigorsky J., Paces V., Vlcek C.: *Assembly of the cnidarian camera-type eye from vertebrate-like components.* **Proc Natl Acad Sci U S A. Jul1; 105(26):8989-93 (2008).**

Assembly of the cnidarian camera-type eye from vertebrate-like components

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Animal eyes are morphologically diverse. Their assembly, however, always relies on the same basic principle, i.e., photoreceptors located in the vicinity of dark shielding pigment. Cnidaria as the likely sister group to the Bilateria are the earliest branching phylum with a well developed visual system. Here, we show that camera-type eyes of the cubozoan jellyfish, *Tripedalia cystophora*, use genetic building blocks typical of vertebrate eyes, namely, a ciliary phototransduction cascade and melanogenic pathway. Our findings indicative of parallelism provide an insight into eye evolution. Combined, the available data favor the possibility that vertebrate and cubozoan eyes arose by independent recruitment of orthologous genes during evolution.

evolution | gene | opsin | photoreceptor | cnidaria

The assembly of diverse animal eyes requires two fundamental building blocks, photoreceptors and dark shielding pigment. The function of photoreceptors is to convert light (stream of photons) into intracellular signaling. The photoreceptor cells (PRCs) are classified into two distinct types: rhabdomeric, characteristic of vision in invertebrate eyes; and ciliary, characteristic of vision in vertebrate eyes (1). In both ciliary and rhabdomeric PRCs, the seven-transmembrane receptor (opsin) associates with retinal to constitute a functional photosensitive pigment. Each photoreceptor type uses a separate phototransduction cascade. Rhabdomeric photoreceptors employ r-opsins and a phospholipase C cascade, whereas ciliary photoreceptors use c-opsins and a phosphodiesterase (PDE) cascade (2, 3). In general, the dark pigment reduces photon scatter and orients the direction optimally sensitive to light. The biochemical nature of the dark pigment appears more diverse than the phototransduction cascades used by the PRCs. Vertebrate eyes use melanin as their exclusive dark pigment. However, among invertebrates, pterins constitute the eye pigment in the polychaete *Platynereis dumerilii* (4), pterins and ommochromes are accumulated in eyes of *Drosophila* (5), and melanin is found rarely such as in the inverse cup-like eyes of the planarian, *Dugesia* (6).

Cnidaria, the likely sister group to the Bilateria, constitute the earliest branching phylum containing a well developed visual system. For example, Cubozoa (known as "box jellyfish") have camera-type eyes with cornea, lens, and retina; unexpectedly, the cubozoan retina has ciliated PRCs that are typical for vertebrate eyes (7–9). Cubomedusae are active swimmers that are able to make directional changes in response to visual stimuli (10). The cubozoan jellyfish, *Tripedalia cystophora* (Fig. 1*A*), has four sensory structures called rhopalia that are equally spaced around the bell. In addition to two camera-type lens containing eyes at right angles to one another, each rhopalium has two pit-shaped and two slit-shaped pigment cup eyes (Fig. 1*B*). Thus, with six eyes located on each rhopalium, *Tripedalia* has 24 eyes altogether. Because the visual fields of individual eyes of the rhopalium partly overlap, *Tripedalia* (like other Cubomedusae) has an almost complete view of its surroundings. The lens-containing *Tripedalia* eyes have sophisticated visual optics as do advanced bilaterian phyla (11).

In the present work, we characterize genes required for the assembly of camera-type eyes in *Tripedalia*. We show that the genetic building blocks typical of vertebrate eyes, namely ciliary opsin and the melanogenic pathway, are used by the cubozoan eyes. Although our findings of unsuspected parallelism are consistent with either an independent origin or common ancestry of cubozoan and vertebrate eyes, we believe the present data favor the former alternative.

Results

Ciliary Opsin Is Expressed in Camera-Type Eyes of *Tripedalia*. We screened an expressed sequence tag (EST) library derived from rhopalia of *Tripedalia* to identify the jellyfish genes that are involved in vision; orthologues of other invertebrates and vertebrates were identified by phylogenetic analysis. Of the four opsin types present at the base of the bilaterians [rhabdomeric (r-opsins), ciliary (c-opsin), G_o-opsins, and peropsin/RGR (12–14)], the *Tripedalia* opsin EST clustered with the c-opsins, an orthology consistent with the conservation of the characteristic stretch of deduced amino acids between the transmembrane domain VII and cytoplasmic tail [supporting information (SI) Fig. S1]. This region includes the c-opsin fingerprint tripeptide NR/KQ (NRS in *Tripedalia*) that is critical for coupling to the downstream phototransduction cascade through interaction with a GTP-binding protein subunit $G\alpha_t$ in the vertebrate rods and cones (15). An antibody generated against *Tripedalia* c-opsin recognized a single electrophoretic band in protein extracts prepared from rhopalia and COS-7 cells transfected with c-opsin cDNA (Fig. 1*C*). Camera-type eyes of adult jellyfish (Fig. 1*D*) were immunostained with an anti-c-opsin antibody. The c-opsin localized in the retinal ciliated PRCs of both complex eyes (Fig. 1 *E* and *F*) in a pattern resembling that by staining with anti-acetylated tubulin antibody (Fig. 1*G*), which specifically labels stabilized microtubules in axons and cilia (13).

Spectral Sensitivity of *Tripedalia* c-Opsin. To address the question of whether the identified *Tripedalia* c-opsin can function as a true

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The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. EU310498 (*c-opsin*), EU310502 (*oca*), EU310499 (*mitf*), EU310500 (catalytic *pde*), EU310501 (inhibitory *pde6d*) and EU310503 (*guanylate cyclase*)].

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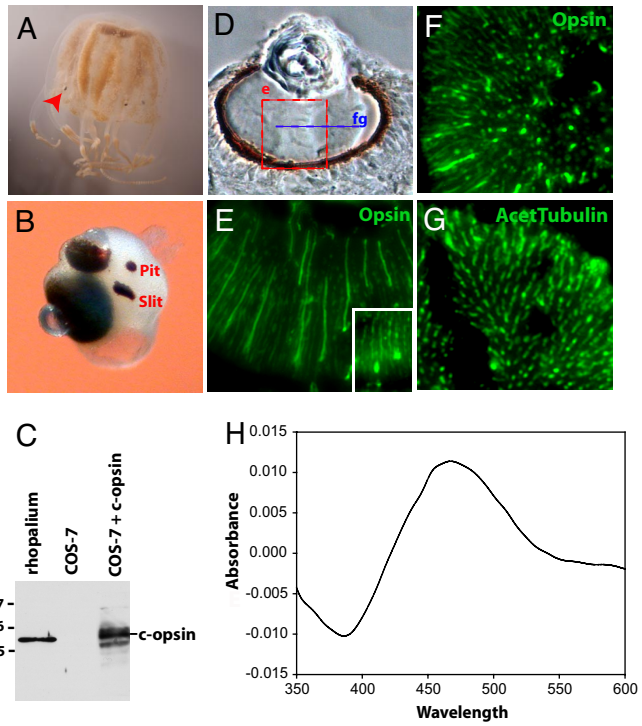

Fig. 1. Ciliary opsin is the functional photopigment of *Tripedalia* camera-type eyes. (*A*) *Tripedalia* medusae with rhopalia (arrowhead). (*B*) Rhopalium with two lens-containing camera-type eyes, a slit and a pit eye. Another set of slit and pit eyes is symmetrically located on the other side of the rhopalium. (*C*) Specificity of an anti-*Tripedalia* c-opsin antibody tested by Western blotting with protein extracts from rhopalia and COS-7 cells transiently transfected with *Tripedalia* c-opsin cDNA. (*D*) Bright-field section through the large camera-type eye. The area shown in *E* is boxed. The plane of sectioning in *F* and *G* is indicated (fg). (*E–G*) Immunohistochemical staining (green) of *Tripedalia* retina using antibodies to c-opsin (*E* and *F*) or acetylated tubulin (*G*). (*E Inset*) Immunohistochemical staining for c-opsin in the small camera-type eye. (*H*) Dark–light difference absorption spectrum of the reconstituted *Tripedalia* c-opsin photopigment. The photopigment reconstituted with 11-*cis*-retinal forms a functional photopigment most sensitive to blue–green light.

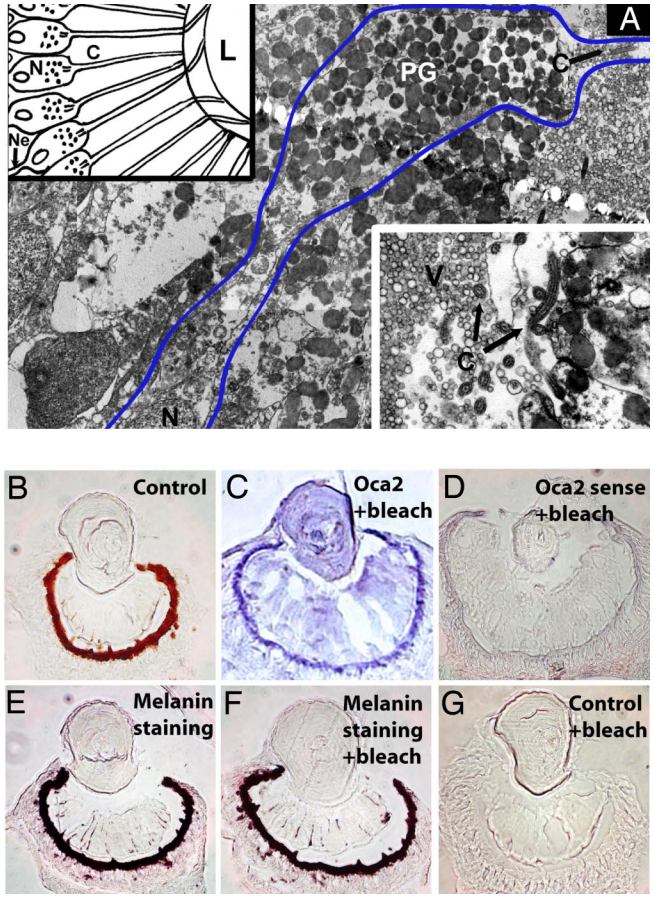

Fig. 2. Melanin is the shielding pigment of *Tripedalia* camera-type eyes. (*A*) Electron micrograph of camera-type eye PRCs. Blue line borders one PRC containing shielding pigment layer (PG) as well as photosensitive cilium (C). (*Upper Left Inset*) Diagram of PRCs with their cilia protruding to the lens capsule. Note that all PRC nuclei are located behind the shielding pigment. (*Lower Right Inset*) Apical end of a PRC with cilium. C, cilium; L, lens; N, nucleus; Ne, neurite; PG, pigment granules; V, vitreous body. (*B*) Bright-field cryosection of camera-type eye. (*C* and *D*) *In situ* hybridization (blue) using *oca2* antisense (*C*) or control sense (*D*) probes. (*E–G*) Melanin detection by Fontana–Masson staining. Tissue sections shown in *C*, *D*, *F*, and *G* were bleached to remove melanin.

visual opsin, we tested its photochemical properties. *Tripedalia* c-opsin was expressed in COS-1 cells and reconstituted as a functional photosensitive pigment with 11-*cis*- retinal. The reconstituted c-opsin was most sensitive to the blue–green region of the spectrum with a peak absorbance (λ_{max}) at 465–470 nm (Fig. 1*H*), in agreement with the spectral sensitivity of the *Tripedalia* electroretinogram (16). We conclude that *Tripedalia* c-opsin is a functional, vertebrate-like photopigment expressed in the PRCs of the camera-type cubozoan eye.

***Tripedalia* Orthologues of Vertebrate-Like Phototransduction Genes.** In vertebrates, activated heterotrimeric G proteins use cGMP PDE for signal transduction. In accordance with our identification of a vertebrate-like *c-opsin* in *Tripedalia*, we found that the catalytic subunit of *pde* expressed in the *Tripedalia* rhopalium phylogenetically clusters with the group of GAF domain-containing *PDEs* including vertebrate rod- and cone-specific *PDE6* (Fig. S2*A*). Furthermore, we identified other components of the ciliary-type cascade associated with deactivation or adaptation of phototransduction, such as the inhibitory subunit of phosphodiesterase (*PDE6D*), *phosducin* and *guanylate cyclase* (Fig. S2 *B–D*). Thus, the nature of the genes expressed in the rhopalia (detected by RT-PCR; Fig. S3) suggests that the camera-type eye of *Tripedalia* uses a ciliary-type phototransduction cascade similar to that of vertebrates.

Melanin Granules in *Tripedalia* PRCs. A conspicuous ring of dark shielding pigment surrounds the area of c-opsin expression (compare Fig. 1 *D* and *E*). Most if not all PRCs in the *Tripedalia* retina contain pigment granules (Fig. 2*A*). These PRCs thus resemble what one might imagine a prototypical ancestral cell combining photoreceptor and pigment functions to look like (17). The biochemical nature of the *Tripedalia* pigment was suggested by the identification of an orthologue of the vertebrate ocular and cutaneous albinism-2 (*Oca2*) gene in our EST library (Fig. S4*A*). *Oca2* (also known as *pink-eyed dilution*) is an essential gene for melanin biosynthesis. It is the most commonly mutated gene in cases of human albinism (18). In addition, mutations in *Oca2* are responsible for pigmentation defects in mouse (19), medaka (20), and independently arisen populations of the cave fish, *Astyanax* (21). *In situ* hybridization analysis revealed that *Tripedalia oca* is conspicuously expressed near the pigmented retina layer of PRCs (Fig. 2*C*). Control staining with an *oca* sense probe (Fig. 2*D*) did not yield a signal. The results of a direct chemical assay (Fontana–Masson) were consistent with melanin being the pigment in the *Tripedalia* retina (Fig. 2 *E–G*). In vertebrates, development of melanin-producing cells and specification of retinal pigment cells require the conserved

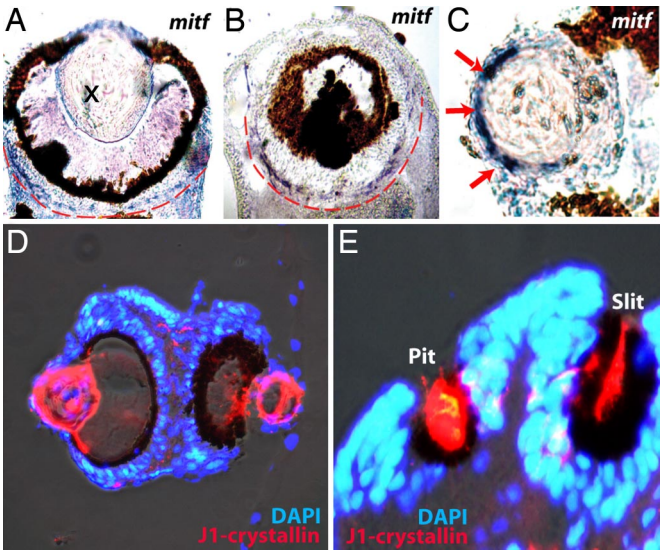

Fig. 3. Expression of *mitf* and *J1-crystallin* in *Tripedalia* eyes. (*A–C*) *In situ* hybridization (blue) detects *mitf* expression in the circle around pigment deposits (*A* and *B*) and in the lens (*C*, arrows). (*D* and *E*) Immunohistochemistry staining using an antibody to the major cubozoan lens crystallin, J1 (red). Nuclei of cells are visualized by DAPI staining (blue). J1-crystallin expression is localized to lenses of camera-type eyes (*D*) as well as to the slit and pit eyes (*E*).

microophthalmia-associated transcription factor, Mitf (22–24). Mitf regulates expression of *tyrosinase* and *tyrosinase-related protein-1* and *-2*, which are necessary for melanin biosynthesis (for review, see ref. 25). Here, we have cloned a *mitf* orthologue from *Tripedalia* (Fig. S4*B*) expressed in a ring-like pattern just outside of the melanin deposits (Fig. 3 *A* and *B*); this area contains the PRC nuclei (Figs. 2*A* and 3*D*). Thus, *mitf* is a conserved transcription factor with shared expression patterns in the complex eyes of *Tripedalia* and vertebrates.

***Mitf* Expression in Lens and Crystallin Expression in Pigmented PRCs of Nonlens Eyes of *Tripedalia*.** In addition to expression in the pigmented PRCs of camera-type eyes, *mitf* mRNA was detected in the outermost cells of the *Tripedalia* lens (Fig. 3*C*). Consequently, to investigate a possible relationship between the pigmented PRCs and the cellular lens we examined whether J1-crystallin, the major protein of the *Tripedalia* lens (26), is expressed in PRCs. The J1-crystallin antibody immunostained the slit and pit eyes as well as the cellular lens (Fig. 3 *D* and *E*). The presence of J1-crystallin in the slit and pit eyes of *Tripedalia* was unexpected because these cup-like eyes lack cellular lenses. They do, however, have pigmented PRCs, suggesting a relationship between the PRCs and cellular lens that warrants further study.

Discussion

The present work reveals surprising similarities in the genetic components used for visual system development in vertebrates and cubozoan jellyfish. If Cubozoa and vertebrates express orthologous c-opsins in their PRCs and make use of the same pigmentation pathway including the key transcription factor Mitf, does this represent a parallel evolution or conservation of an ancestral "eye" program between those evolutionarily distant animal phyla (Fig. 4)? Although our data are formally consistent with both evolutionary scenarios, we believe that they favor the former.

Even though ciliary and rhabdomeric photoreceptive systems coexist throughout the animal taxa (1), the present evidence suggests that their evolutionary histories differ. For photodetection, all invertebrate PRCs examined employ G_o/*r-opsin*, and all vertebrate PRCs employ c-opsin (2, 3). Importantly, *c-opsin* is expressed in the ciliary PRCs in the brain of the polychaete worm, *P. dumerili*, whereas *r-opsin* is expressed in rhabdomeric PRCs in the eyes (13). Based on this result, Arendt *et al.* (13) have proposed that early metazoans possessed a single type of PRC with an ancestral opsin for light detection that later diversified into two distinct PRC and opsin types. The rhabdomeric PRCs (with r-opsin) were used in the eyes for photoreception, whereas the ciliary PRCs (with c-opsin) were incorporated into the evolving brain. These findings are consistent with vertebrates confining r-opsin to retinal ganglion cells apparently for photoperiodicity and using ciliary PRCs containing c-opsins exclusively for photoreception (rods and cones). Taken together, the data suggest that the ganglion cells of the vertebrate retina are the evolutionary descendents of rhabdomeric PRCs (3, 13). Moreover, because no identified *opsin* gene in cnidarians (27) contains a typical r-opsin fingerprint tripeptide HPK critical for coupling to the downstream phototransduction cascade, it was proposed that r-opsins are a bilaterian innovation that originated

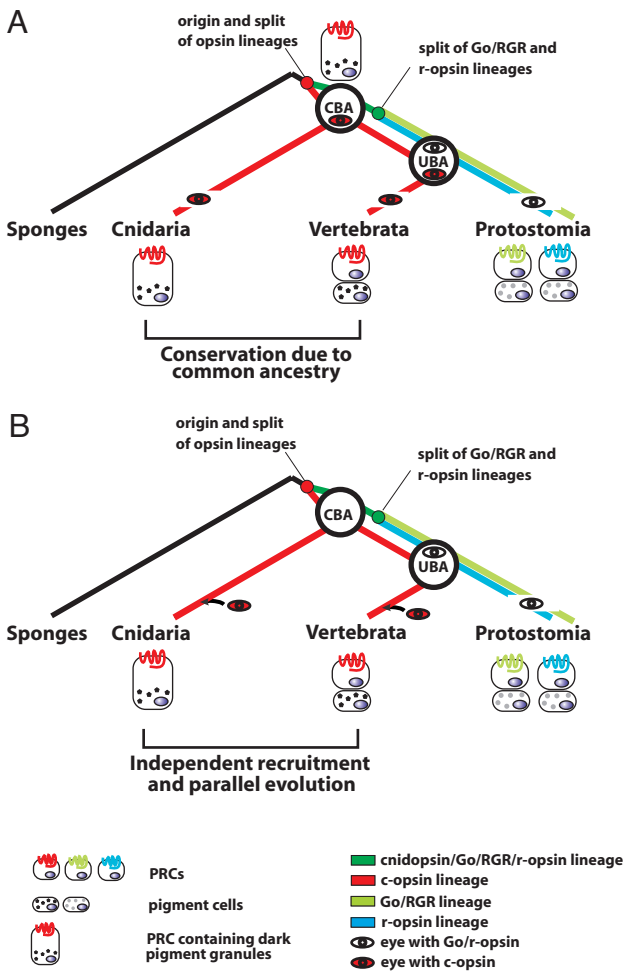

Fig. 4. Two scenarios for the use of ciliary phototransduction and melanogenic pathway in eye evolutionary history. A simplified view of the two evolutionary scenarios is compatible with the data in the present work. The use of similar genetic components in vertebrate and cubozoan eyes is either due to common ancestry (*A*) or independent parallel recruitments in cnidarian and vertebrate lineages (*B*). The *c-opsins* and G_o/*r-opsins* arose by duplication and diversification of an ancestral opsin in the early metazoans (27). In the schemes, only the visual (i.e., the eye-specific) PRCs and opsins are considered. Different shading of pigment granules indicates possible distinct chemical composition. CBA, cnidarian–bilaterian ancestor; UBA, urbilaterian ancestor.

after the separation of the cnidarian and bilaterian lineages (Fig. 4) (27).

All eyes have shielding pigment typically found in cells adjacent to the PRCs. Melanin, the dark pigment of *Tripedalia* eyes, presumably performs the same function in vertebrate eyes as in the simple cup-like eyes of a basal lophotrochozoan, *Dugesia* (6). Interestingly, *Dugesia* uses another pigment, an ommochrome, as the body pigment (2, 6). Pterins constitute the dark eye pigment of the polychaete *P. dumerilii* (4), and pterins and ommochromes are the pigments in eyes of *Drosophila* (5). Thus, as with the opsins, *Tripedalia* shares the same dark pigment in the eye with vertebrates.

Unlike in the *Dugesia* eye, the camera-type *Tripedalia* eye combines the photoreceptor and pigment functions in the same cell consistent with an ancestral (basal) condition (Fig. 4*A*). However, the medusae stage of cubozoans may well be a derived rather than ancestral condition for Cnidaria, complicating discussions about the basal state of the cubozoan visual system (Anthozoans, for example, do not have eyes). Nevertheless, it remains possible that the pigmented PRCs in *Tripedalia* are descendants of one of the postulated ancient prototypical photosensitive cells diversified by natural selection (17, 28). However, this does not require a common origin for the eyes. It was estimated through computer-based modeling (29) that fewer than a half-million generations would be required under selective pressure to proceed from a cluster of light-sensitive cells to a sophisticated camera-type eye. In theory, this relatively short time interval would allow sophisticated eyes to have originated *de novo* several times during evolution (polyphyletic eye origin).

For the common-ancestry model to be true, the cnidarian-bilaterian ancestor (CBA) must have had the same genetic determinants as its descendants. The common-ancestry scenario for cubozoan and vertebrate eyes requires, however, that animals in many bilaterian phyla lost their eyes that were initially assembled by using the same building blocks as in present-day vertebrates and Cubozoa (*c-opsins*, *melanin*) to explain the exclusive occurrence of rhabdomeric PRCs in invertebrate eyes. There is no obvious explanation for such a specific selection against ciliary PRCs to be used for visual purposes. Eyes in general provide a freely moving animal with a tremendous advantage, and as such there should be a constant selection for eye maintenance, except in, for example, cave or underground animals.

Although not definitive, there are at least two additional complications to the common-ancestry model that arise if one invokes the developmental argument that similar transcription factor cascades may direct development of vertebrate and cubozoan eyes. The first is that PaxB, a Pax2/6/8-related transcription factor, is used in *Tripedalia* (30) instead of *Pax6* as in vertebrates (31) as well as flies (32, 33) and other species (34). The second is the apparent evolutionary "promiscuity" of developmental cascades in general; entire regulatory circuits can be co-opted for development of different cell types, tissues, or organs. For example, the *Pax–Six–Eya–Dach* gene regulatory network has a fundamental role in *Drosophila* visual system development but is also used for specification of muscle cells or placodes in vertebrates (35). Co-opting orthologous suites of genes for similar functions could be a possible explanation for independent or parallel evolution of cubozoan and vertebrate eyes with ciliary-type PRCs (Fig. 4*B*) (1, 36, 37). Independent derivation of *Tripedalia* and vertebrate eyes would also fit conceptually with the early idea that PRCs originated multiple times (38), although it does not address how many times PRCs themselves may have originated. That vertebrates and Cnidaria share many more genes than anticipated (39, 40), including *pax*, *mitf*, *c-opsin*, *pde*'s, *phosducin*, *guanylate cyclase*, and *oca2* (ref. 30 and this work), supports the notion that both animal groups use similar sets of genes to generate significantly different body plans. It follows that changes in gene regulation, rather than "new" genes, may drive novelties such as eyes during evolution. Finally, ectopic eye formation by misexpression of Pax6 provides an astounding example of how an eye might arise *de novo* in a foreign tissue environment (33, 34). The fact that ectopic eyes can be generated experimentally suggests that the same gene, *Pax*, used by various eyes of present-day animals could have been instrumental in creating eyes independently numerous times during evolution.

In addition to sharing the same genetic building blocks in their PRCs (ciliary phototransduction, melanogenic pathway), cubozoans and vertebrates both use a cellular lens to increase visual sensitivity and produce a sharp image in the desired plane of focus. The optical properties of cellular lenses are caused by the high-level expression of proteins collectively called crystallins (ref. 41 and this work). In striking contrast to the conservation of opsins as the visual pigments in the PRCs, the lens crystallins are diverse proteins that are often taxon-specific, i.e., entirely different proteins function as crystallins in different species. Similar transcription factors including those of the *Pax* gene family have been independently recruited for the regulation of nonhomologous crystallin genes in *Tripedalia* and vertebrates (30, 42, 43) to achieve a gradient of refractive index within their transparent lenses. The independent recruitment of lens crystallins is consistent with parallel evolution of cubozoan and vertebrate eyes and provides a striking example of the role of convergence in eye evolution.

Finally, the present findings of *mitf* in the lens and J1-crystallin in the pigmented slit and pit ocelli of *Tripedalia* support the idea that the cellular cubozoan lens arose from a pigmented cell ancestor. It is known that pigment cells may acquire the capacity to secrete lens-forming material (44). Combined, our data on J1-crystallin and *mitf* expression suggest that the cellular cubozoan lens with its remarkable ability to refract light without spherical aberration (11) originated from a pigment cell ancestor and that the primitive cup-like eyes located on the cubozoan rhopalia might be evolutionary forerunners of camera-type eyes.

In conclusion, the present study uncovers a surprising molecular parallelism in the eye design of vertebrates and cubozoan jellyfish. Although the current data do not distinguish unambiguously between the common-ancestry and independent-recruitment scenarios, we propose that they lean in the direction of the latter, favoring multiple independent reorganizations of common elements and independent recruitments of similar suites of genes during evolution of the diverse eyes.

Materials and Methods

Jellyfish Collection and Culture. *T. cystophora* was collected and cultured as described in ref. 43.

Isolation of Rhopalium-Expressed Genes and Phylogenetic Analysis. An EST cDNA library was generated from rhopalia mRNA, and 2,433 individual clones from the library were sequenced by using an ABI capillary sequencer. The accession numbers for the clones are as follows: *c-opsin* (EU310498), *oca* (EU310502), *mitf* (EU310499), catalytic *pde* (EU310500), inhibitory *pde6d* (EU310501) and *guanylate cyclase* (EU310503). Details on phylogenetic analysis including the accession numbers of individual sequences are described in *SI Materials and Methods*.

RNA *in Situ* Hybridization. Jellyfish were fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose overnight at 4°C, and embedded and frozen in OCT (Tissue Tek). RNA *in situ* hybridization was performed as described in ref. 43.

Immunohistochemistry. The cryosections were refixed in 4% PFA for 10 min, washed three times with PBS, permeabilized with PBT (PBS + 0.1% Tween 20) for 15 min, and blocked in 10% BSA in PBT for 30 min. The primary antibodies were diluted in 1% BSA in PBT, incubated overnight at room temperature, washed three times with PBS, and incubated with secondary antibodies in 1% BSA in PBT. The sections were counterstained with DAPI and mounted. Primary antibodies used were: anti-*Tripedalia* c-opsin, anti-*Tripedalia* J1-crystallin, and anti-acetylated tubulin (Sigma). The following secondary anti-

bodies were used: Alexa Fluor 488- or 594-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes).

Generation of Antibodies, COS-7 Cell Transfection, and Western Blotting. Antibodies directed against *Tripedalia* c-opsin and J1-crystallin were prepared by immunization of rabbits as follows. The C-terminal region of *c-opsin* cDNA corresponding to amino acids 274–329 was cloned into the expression vector pET42, expressed in BL21(DE3)RIPL cells (Stratagene), and purified by using His_6 tag chromatography. The N-terminal peptide of J1-crystallin AAIVGSLVADAATQPVHK was attached to KLH via the C-terminal lysine and used for immunization. Monkey kidney COS-7 cells were transfected with CMV-c-opsin (amino acids 1–329) expression vector by using FuGENE6 reagent (Roche). Total extracts were prepared from c-opsin-transfected cells, mock-transfected cells, and rhopalia and were analyzed by Western blotting by using anti-c-opsin rabbit serum and chemiluminescent detection kit (Pierce). To avoid formation of multimeric opsin complexes, protein extracts from transfected cells were diluted and heated at low temperature (37°C) before SDS/PAGE.

Fontana–Masson Method. The cryosections were hydrated in distilled water and then incubated with Fontana silver nitrate working solution (2.5% silver nitrate) at 56°C for 1–2 h. After three washes in distilled water, sections were treated in 0.2% gold chloride at room temperature for 2 min, rinsed once in distilled water, placed in 5% sodium thiosulfate at room temperature for 1 min, washed again in water, and mounted.

Melanin Bleach Procedure. Bleaching was performed either after Fontana–Masson staining or RNA *in situ* hybridization. The sections were hydrated in distilled water and exposed to 0.25% potassium permanganate for 30 min at room temperature. The sections were treated with 5% oxalic acid for 5 min, washed with water, and mounted.

Transmission Electron Microscopy. Rhopalia excised from juvenile medusae were treated with Karnovsky fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in cacodylate buffer) for 24 h at 4°C. Fixed tissue was washed 12 h in 0.1% cacodylate buffer at 4°C. Karnovsky-fixed juvenile rhopalia and PFA-fixed adult rhopalia were postfixed in 2% OsO_4 for 2 h at 4°C and then washed in water. Samples were dehydrated in series of ethanol solutions, transferred to pure acetone, and embedded in Poly/Bed 812/Araldite 502 resin. Ultrathin sections (600–800 nm) were cut on Ultracut E (Reichert–Jung), placed on copper grids, and treated with 2.5% uranyl acetate for 1 h followed by lead citrate for 15 min. The material was examined by transmission electron microscopy (Jeol-1011), and images were taken with a MEGAview III Soft imaging system.

Expression, Reconstitution, and Spectroscopic Analysis of *Tripedalia* c-opsin. *Tripedalia* c-opsin cDNA was expressed in transfected COS-1 cells. Transfected cells were resuspended with 5 μM 11-*cis*-retinal, solubilized with 1% dodecyl maltoside, and the resulting c-opsin photopigment was purified by using immobilized 1D4 (Cell Culture Center, Minneapolis, MN). The UV-visible absorption spectrum was recorded for the c-opsin photopigment from 250 to 650 nm at 0.5-nm intervals by using the Hitachi U3010 dual-beam spectrometer at 20°C. Five replicates were performed in the dark and five more after 3 min of light exposure (with a $<$440-nm cut-off filter). The λ_{max} value was taken from the dark–light difference spectrum.

For additional details, see *SI Materials and Methods*.

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Supporting Information

Kozmik *et al*. 10.1073/pnas.0800388105

Materials and Methods

Jellyfish Collection and Culture. Adult *Tripedalia cystophora* were collected in mangroves of La Parguerra, Puerto Rico. Laboratory cultures were established using settling larvae and artificial sea water. Settled larvae metamorphosed into young polyps. Young polyps were transformed into budding (asexually reproducing) polyps by feeding with artemia once a week. Spontaneously metamorphosed polyps (newborn medusae) were fixed for *in situ* hybridization. Polyps as well as young medusae were maintained at 26°C.

Isolation of Rhopalium-Expressed Genes. The expressed sequence tag (EST) cDNA library was generated from rhopalia mRNA by using pBluescript II. Individual clones from the library were sequenced using an ABI capillary sequencer, and full-length cDNAs were obtained by SMART RACE (BD Biosciences). A fragment of *Tripedalia* Mitf cDNA was isolated by reverse transcription-PCR of rhopalia mRNA by using degenerate primers zk665A 5′-AARAARGAYAAYCAYAA-3′ and zk665F 5′-TTDATNCKRTCRTTDATRTT-3′. The resulting partial Mitf cDNA was extended by RACE. The accession numbers for the clones are as follows: *c-opsin* (EU310498), *oca* (EU310502), *mitf* (EU310499), catalytic *pde* (EU310500), inhibitory *pde6d* (EU310501), and guanylate cyclase (EU310503).

Phylogenetic Analysis. Amino acid alignment created by MUSCLE software (11) with default settings was edited manually, and highly divergent stretches were excluded. The phylogenetic trees were constructed using the Phylip 3.6 package. Bootstrap sample set was generated by SEQBOOT (1,000 replicates), protein distances were estimated using PROTDIST (PAM matrix, 1,000 datasets), and the NJ tree was constructed by NEIGHBOR (1,000 replicates, random input order) and CONSENSE programs. Maximum-likelihood trees were constructed by PROML (JTT matrix, random input order, 500 replicates) and final consensus tree by CONSENSE. The numbers above each node represent the percentage of bootstrap probability based on 1,000 replicates. *Tripedalia* protein sequences clustered consistently in trees inferred by the maximum-likelihood method. Accession numbers of sequences used in the trees are as follows.

Opsin tree. The numbers of *Strongylocentrotus purpuratus* genes represent the gene ID in public assembly of Sea Urchin Genome Project (hgsc.bcm.tmc.edu/projects/seaurchin), Gallus adenosine receptor NP_990418.1, *Mus* serotonine receptor NP_766400.1, *Branchiostoma belcheri* opsin 6 BAC76024.1, *Drosophila* Rh6 NP_524368.3, *Drosophila* Rh2 NP_524398.1, *Drosophila* Rh3 NP_524411.1, *Apis* blue rhodopsin NP_001011606.1, *Octopus* opsin P09241, *Sepia* rhodopsin AAC26329.1, *Mizuhopecten* Gq O15973, *Platynereis* r-opsin CAC86665.1, *B. belcheri* Mop Q4R1I4, *Xenopus* melanopsin AAC41235.1, *Danio* melanopsin NP_840074.1, *Homo* melanopsin NP_150598.1, *Danio* LW NP_571250.1, *Homo* MW NP_000504.1, *Gallus* LW NP_990740.1, *Homo* rhodopsin NP_000530.1, *Danio* extraocular NP_571287.1, *Danio* MW4 NP_571329.1, *Latimeria* Rh2 AAD30520.1, *Danio* SW opsin NP_571394.1, *Xenopus* violet P51473, *Xenopus* green AAO38746.1 *Gallus* blue NP_990848.1, *Salmo* VAL opsin O13018, *Danio* VAL opsin NP_571661.1, *Ciona* opsin NP_001027727.1, *Xenopus* parapinopsin NP_998830.1, *Uta* parietopsin AAZ79904.1, *Xenopus* parietopsin NP_001039256.1, *Mus* encephalopsin NP_034228.1, *Homo* encephalopsin NP_055137.2, *Platynereis* c-opsin AAV63834.1, *Takifugu* TMT NP_001027778.1, *B. belcheri* opsin 4 BAC76021.1, *B. belcheri* opsin 5 BAC76022.1, *Apis* pteropsin NP_001035057.1, *Aedes* opsin EAT43163.1, *Anopheles* GPRop11 XP_312503.3, *Anopheles* GPRop12 XP_312502.2, *Strongylocentrotus* Sp1 GLEAN3_05569, *Mizuhopecten* G_o O15974, *B. belcheri* opsin 2 BAC76020.1, *B. belcheri* opsin 1 BAC76019.1, *Strongylocentrotus* Sp3.2 GLEAN3_27633, *Strongylocentrotus* Sp3.1 GLEAN3_27634, *Rattus* Opn5 NP_861437.1, *Homo* Opn5 NP_859528.1, *Homo* peropsin NP_006574.1, *Mus* peropsin AAC53344.1, *B. belcheri* opsin 3 BAC76023.1, *Gallus* RGR NP_001026387.1, *Mus* RGR NP_067315.1, *Todarodes retinochrome* CAA40422.1.

MITF/TFE tree. *Saccharomyces cerevisiae* RTG3 NP_009447.1, *Caenorhabditis briggsae* XP_001671854.1, *Caenorhabditis elegans* NP_500461.1, *Nematostella vectensis* XP_001636474.1, *Ciona intestinalis* NP_001087207.1, *Apis mellifera* XP_394278.2, *Drosophila melanogaster* AAQ01726.1, *Mus musculus* TfeB NP_035679.2, *Homo sapiens* TfeB NP_009093.1, *M. musculus* Tfe3 NP_766060.2, *H. sapiens* Tfe3 NP_006512.2, *Danio rerio* Tfe3 CAE30419.1, *D. rerio* TfeC CAH68937.1, *H. sapiens* TfeC NP_036384.1, *M. musculus* TfeC NP_112475.1, *D. rerio* MiTF NP_570998.1, *M. musculus* MiTF AAI08978.1, *Canis lupu familiaris* MiTF NP_001003337.1, *H. sapiens* MiTF NP_006713.1, *Xenopus tropicalis* MiTF NP_001093747.1, *Gallus gallus* NP_990360.1.

Catalytic PDE tree. *H. sapiens* PDE1A P54750, *Strongylocentrotous purpuratus* PDE5 NP_001029121.1, *H. sapiens* PDE5A (NP_001074), *D. melanogaster* PDE6 (CG8279-PA) AAF55066, *H. sapiens* PDE1A NP_005010.2; *S. purpuratus* PDE1 NP_001091918.1, *H. sapiens* PDE2A NP_002590.1, *S. cerevisiae* PDE2-like NP_015005.1, *S. cerevisiae* PDE1 CAA64139.1, *H. sapiens* PDE3A NP_000912.3, *Rattus norvegicus* PDE3 (predicted) XP_574187.2, *H. sapiens* PDE4 ANP_006193.1, *D. melanogaster* PDE4 (CG32498) AAF45865.2, *Nematostella vectensis* PDE5 (predicted) XP_001631585.1, *H. sapiens* PDE5A1 NP_001074.2, *D. melanogaster* PDE6 (CG8279-PA) NP_650369.2, *H. sapiens* PDE6A P16499, *H. sapiens* PDE6B P35913, *H. sapiens* PDE6C P51160, *N. vectensis* PDE6-like XP_001636689.1, *H. sapiens* PDE7A NP_002594.1, *M. musculus* PDE7 NP_032828.1, *D. melanogaster* PDE8A AAM68263.1, *H. sapiens* PDE8B NP_003710.1, *D. melanogaster* PDE9 (CG32648-PA) NP_727644.1, *H. sapiens* PDE9A AF048837, *H. sapiens* PDE11A1 NP_001070664.1, *H. sapiens* PDE11A2 NO00107826, *D. melanogaster* PDE11 (CG10231-PA) NP_609885.1, *D. melanogaster* PDE11 XP_001356584.1, *Trichomonas vaginalis* GAF-containing protein (GAF PDE outgroup) XP_001324213.1.

PDE6D alignment. *Nemastostella* PDE6pred XP_001629547.1, *Homo* PDE6D NP_002592.1, *Mus* PDE6D 032827.1, *Bos* PDE6D NP_776845.1, *Canis* PDE6D NP_001003156.1, *Ciona* PDE6D_NP_001027639.1, *Apis* PDE6delta_XP_394004.2, *Strongylocenter* PDE6delta_XP_001177685.1, *Caenorhabditis* PDE6delta_NP_495490.1, *Aedes* PDE6delta XP_320754.1, *Drosophila* GA21678-PA_XP_001355815.1, *Tetrahymena* GMPPDE_XP_001007775.1.

Oca2 tree. *H. sapiens* OCA2 NP_000266.2, *Sus scrofa* OCA2 NP_999259.2; *Oryzias latipes* OCA2 NP_001098262, *M. musculus* OCA2 NP_068679, *G. gallus* OCA2 XP_425579, *Nematostella vectensis* _OCA2XP_001627452, *D. melanogaster* RE09889 (P protein) AAN71295.1, *Aedes aegypti* Tyr transp (hoepl-like) XP_001658764.1, *D. melanogaster* Tyr_transp (hoepl1) NP_608876.1hoepel1, *Clostridium botulinum* Ars pump YP_001253041.1, *Carboxydothermus hydrogenoformans* Ars transp YP_360838.1, *Thermococcus kodakarensis* Ars pump.

Membrane-associated guanylate cyclase tree. *Strognylocentrotus* GC NP_999705.1, *Asterias* GC BAB85468.1, *Xenopus* GC 2C NP_001079334.1, *Mus* GC 2C NP_659504.1, *Homo* GC 2C NP_004954.1, *Xenopus* GC 2B NP_001084176.1, *Anguilla* GC 2B P55202.1, *Homo* GC 2B P20594.1, *Mus* GC 2A P18293.2, *Aedes* retinal -type GC XP_001658332.1, *Mus* GC 2E NP_032218.2, *Homo* GC 2D NP_000171.1, *Bos* GC 2D NP_776973.1, *Canis* GC 2D NP_001003207.1, *Gallus* GC 2E AAC24500.1, *Mus* GC 2D XP_001474460.1, *Oryzias* Olgc-R1 NP_001098133.1, *Danio* GC 2F XP_689630.1, *Oryzias* OlGC5 NP_001098551.1, *Oryzias* OlGC-R2 BAA76301.1, *Danio* GC 2 NP_001103165.1, *Bos* GC 2F NP_776974.1, *Homo* GC 2F NP_001513.2, *Rattus* GC 2F NP_446283.1, *Mus* GC 2F NP_001007577.1. The last 80 amino acids of protein kinase-like domain of guanylate cyclases were aligned. Membrane guanylate cyclase of *Strongylocentrotus* and *Asterias* were used as an outgroup.

Phosducin tree. *Homo* PhLP3 NP_076970.1, *Rattus* PhLP3 NP_001020880.1, *Danio* PhLP3 46391102; *Nematostella* (protein ID) 119091, *Drosophila* AAF49974.2, *Mus* PhLP2 NP_075997.1, *Homo* PhLP2 NP_689614.2, *Bos* PhLP2 NP_001035641.1, *Schizosaccharomyces* CAB39851.2, *Mus* PhL AAH06578.1, *Bos* PhL NP_001035641.1, *Homo* PhL NP_005379.3, *Danio* phosducin AAH60908.1, *Mus* phosducin Q9QW08.1, *Homo* phosducin NP_002588.3, *Canis* phosducin NP_001003076.1. Amino acid sequences spanning from helix 5 up to the end of helix 7 of phosducin protein family were aligned.

Analysis by RT-PCR. Total RNA was isolated from *Tripedalia cystophora* rhopalia using TRIzol reagent (Invitrogen). The RT reaction was performed using random oligonucleotide hexamers in the presence or absence (negative control) of the PowerScript enzyme (Clontech). The specific primers for the phototransduction genes were as follows: *guanylate cyclase*, 5′-GGATGTCTACAGCTATGGCATCAT-3′ (forward) and 5′-CGTTGATTCTTTTCATGACTTTCA-3′ (reverse); inhibitory *PDE6D*, 5′-TCTACCGTTAACGAAATGCAGCAC-3′ (forward) and 5′-GTCTCGTATCAATCTGTACTTTGC-3′ (reverse); catalytic *PDE*, 5′-GAGGACTATTCTCTGCATGCCCAT-3′ (forward) and 5′-GTTCTTCCTGACGGATAAGATCCA-3′ (reverse); *phosducin*, 5′-CCAGCAATATCCACAGATCAA-3′ (forward) and 5′-TCGAATCGGTTTCGCTATTCC-3′ (reverse).

Expression, Reconstitution, and Spectroscopic Analysis of *Tripedalia* c-Opsin. The entire coding region was amplified from the *Tripedalia* c-opsin cDNA clone by primer pairs designed within the 5′ and 3′ edges of the coding regions with necessary sequences for cloning and translation purposes as in ref. 12: 5′-AAAAAA-GAATTCCACCATGGCAGATCACGGAAGGAATAC-3′ (the forward primer with an EcoRI site underlined) and 5′-TTCTAGGTCGACTCCGGCTCAACA-GAATTTCCACAGAG-3′ (the reverse primer with a SalI site underlined). Via the restriction sites set in the primers, the amplified cDNA fragment was cloned into the pMT5 expression vector, which contains the last 15 amino acids of the bovine rod opsin necessary for immunoaffinity purification by 1D4 monoclonal antibody (13). Cultured COS-1 cells (RIKEN Cell Bank) were transfected with the pMT5-cDNA clone, incubated with 5 μM 11-*cis*-retinal (Storm Eye Institute, Medical University of South Carolina, Charleston, SC), and solubilized with 1% dodecyl maltoside. The c-opsin photopigment was purified using immobilized 1D4 (Cell Culture Center, Minneapolis, MN). The UV-visible absorption spectrum was recorded for the c-opsin photopigment from 250 to 650 nm at 0.5-nm intervals using the Hitachi U3010 dual beam spectrometer at 20°C. Five replicates were performed in the dark and five more after 3 min of light exposure (with a $<$440 nm cut-off filter) as described in ref. 13. The Savitzky–Golay least-squares smoothing method was carried out for the absorbance curve, with 100 repetitions to eliminate spurious spikes. The λ_{max} value was taken from the dark–light difference spectrum.

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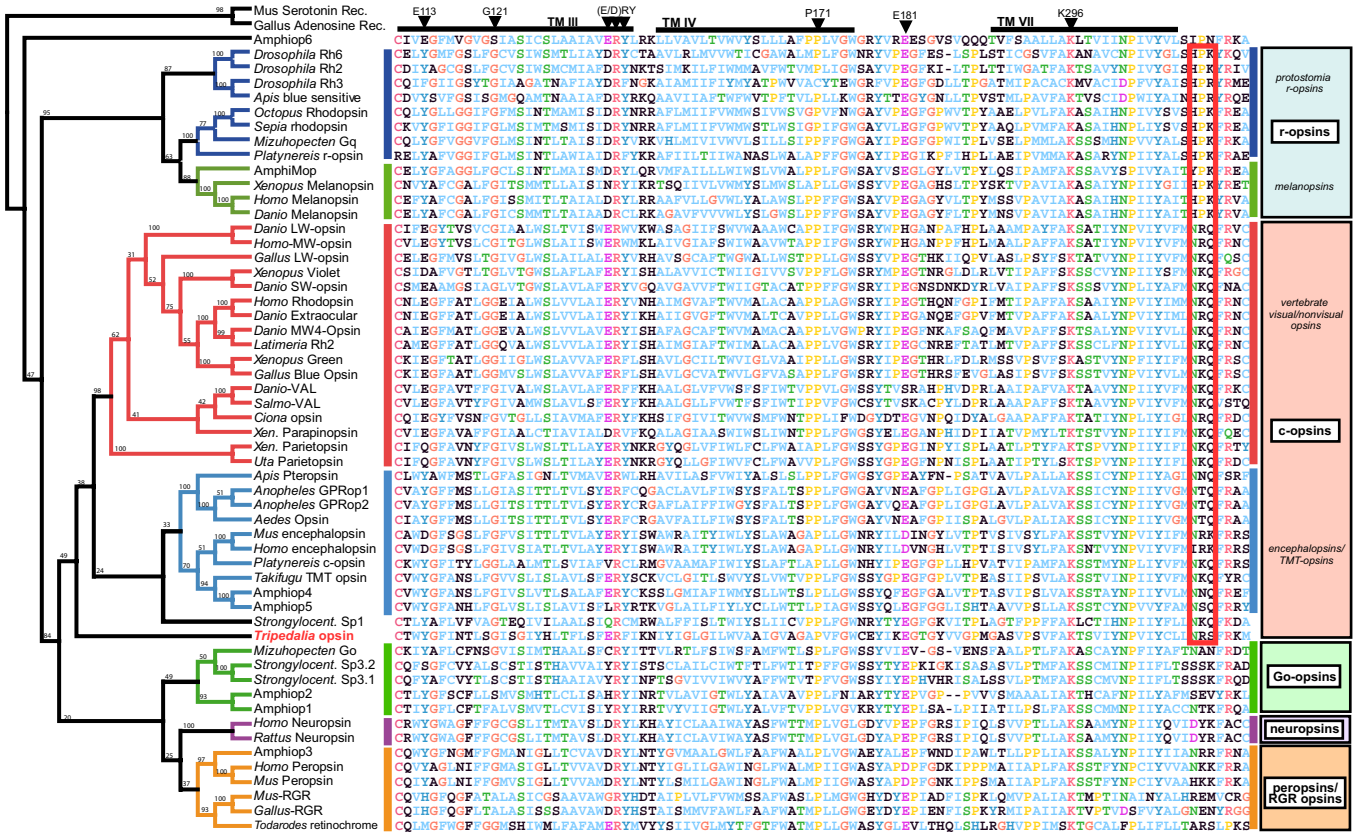


Fig. S1. Alignment and phylogenetic tree of opsins. The phylogenetic tree was inferred by the neighbor-joining method using murine adenosine and chicken serotonin receptors as outgroup sequences. Nonhomologous stretches were excluded from the analysis. The black lines above the alignment represent the extent of transmembrane helices III, IV, and VII. The color lines on both sides of the alignment demarcate opsin subfamilies. Critical amino acids allowing opsin classification are indicated by the black arrowheads and numbered according to the bovine rhodopsin protein sequence. The lysine residue K296 is critical for covalent binding of retinal via Schiff base linkage, which is stabilized by counterion E113 or E181 (1). The (E/D)RY triade, which is highly conserved among G protein-coupled receptors (GPCRs), is important for G protein interaction (2). G121, P171, and W175 are evolutionary trace residues typical for the opsin family but not for the GPCRs in general (3). The positions of the HPK and the NR/KQ motifs conserved among rhabdomeric and ciliary opsins, respectively (4), are boxed in red. The classification of opsin families is given in the colored boxes on the right.

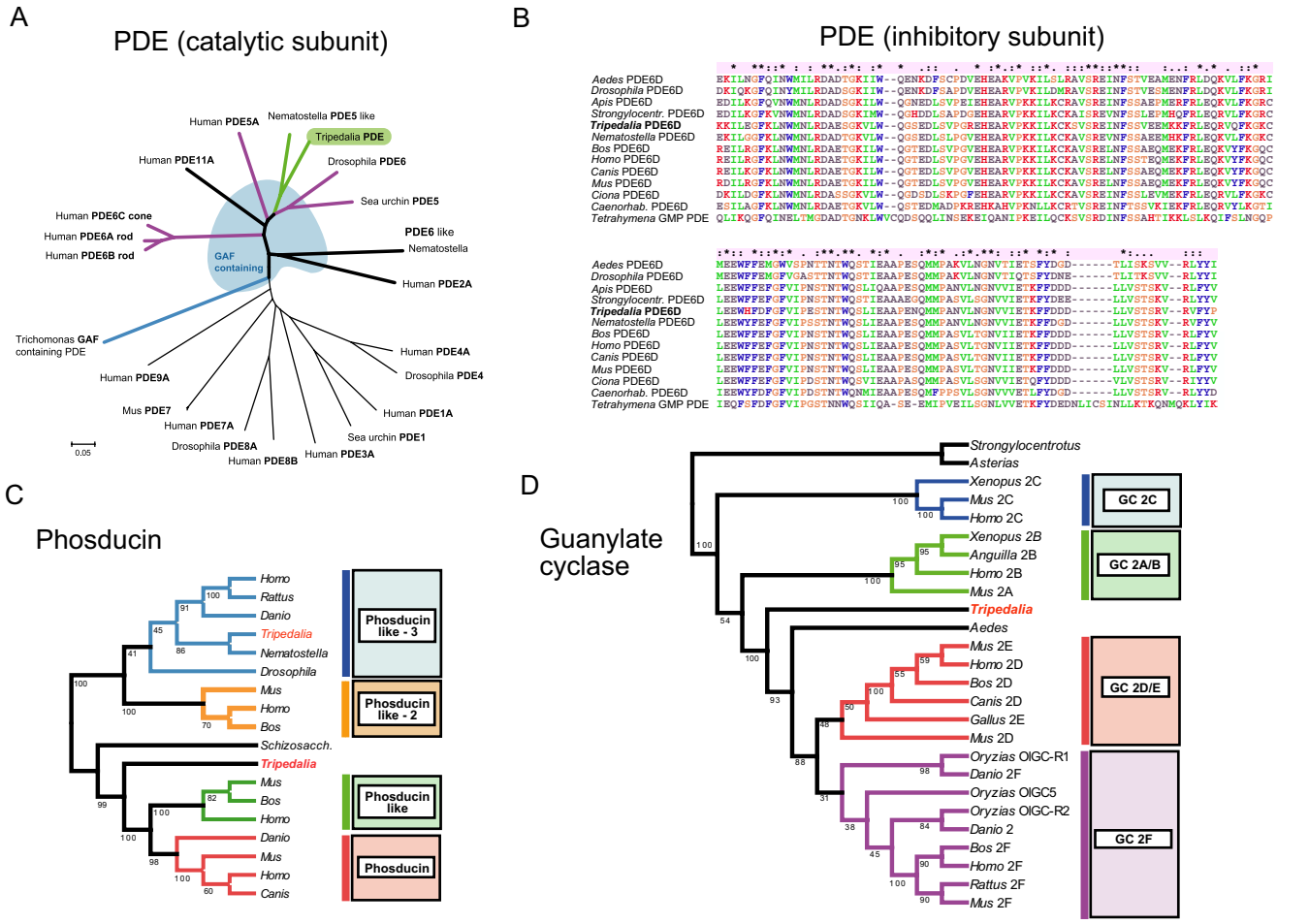


Fig. S2. Phylogenetic analysis of *Tripedalia* components of the ciliary phototransduction cascade. (*A*) Phylogenetic tree of the catalytic subunit of the *phosphodiestrase* (*PDE*) gene family. *Tripedalia* PDE contains two GAF domains and a phosphodiesterase catalytic domain, PDase I. *Tripedalia* PDE groups with cGMP-specific PDEs used in phototransduction and is phylogenetically close to PDE5 and PDE6. (*B*) Alignment of the inhibitory subunit of *phosphodiesterase PDE6D*. A δ subunit of PDE (PDE6D) in the rod outer segment can bind the PDE$\alpha\beta\gamma_2$ complex and solubilize it, disrupting its normally close association with the disk membrane (5). Among bilaterians, the highest degree of sequence identity/similarity of *Tripedalia* PDE6D is with PDE6D from various vertebrates. The percentage of identity/similarity between *Tripedalia* and other species is as follows: *Homo* (82/94%), *Bos* (82/94%), *Canis* (82/95%), *Mus* (81/94%), *Ciona* (80/91%), *Strongylocentrotus* (74/92%), *Caenorhabditis* (69/89%), *Aedes* (62/84%), and *Drosophila* (59/82%). (*C*) Phylogenetic tree of the *phosducin* gene family. Phosducin modulates phototransduction by interacting with the $\beta\gamma$ subunits of G protein transducin (6, 7). *Phosducin* and *phosducin-like* genes are expressed in *Tripedalia*, the former clustering with the vertebrate retina-specific *phosducins*. (*D*) Phylogenetic tree of the *guanylate cyclase* (*GC*) gene family. *Tripedalia* guanylate cyclase clusters with membrane-associated retina-specific GC2E (also known as retGC-1) and GC2F (also known as retGC-2), both of which are expressed in vertebrate rods and cones (8, 9). These specialized photoreceptor guanylate cyclases in outer segments of rods and cones resynthesize cGMP that is rapidly depleted through activated PDE upon light stimulus.

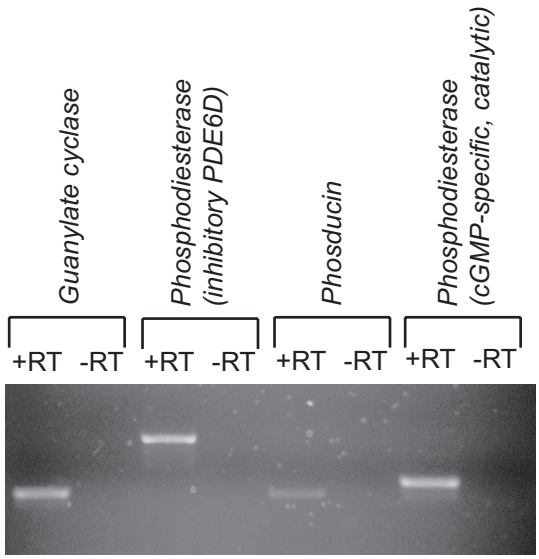
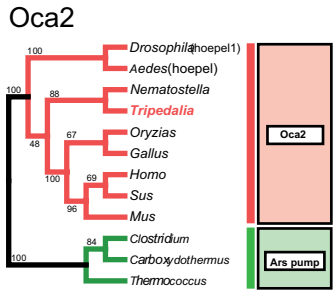


Fig. S3. Expression analysis of ciliary phototransduction cascade genes. RT-PCR analysis of *guanylate cyclase*, *phosphodiesterases*, and *phosducin* gene expression in rhopalia of *T. cystophora*. In all cases, no PCR product was obtained in the absence of reverse transcriptase (−RT).

A



B

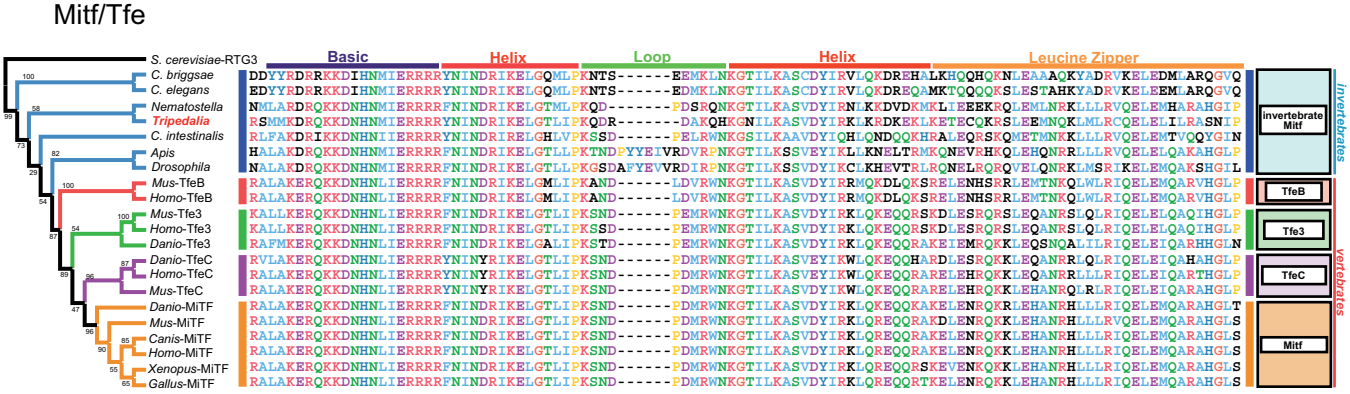


Fig. S4. Phylogenetic analysis of melanogenic pathway-specific genes. (*A*) Phylogenetic tree of the *Oca2* gene family. Oca2 proteins (also known as P protein, P permease, or hoepel) from different organisms and protein sequences of three arsenic pumps (closest homologues to Oca2 family) were aligned, and conserved transmembrane helices were used for phylogenetic analysis. The tree was inferred by the neighbor-joining method using arsenic pumps as outgroup sequences. The numbers above and under the branches indicate bootstrap support (1,000 replicates). (*B*) Alignment and phylogenetic tree of the *Mitf/TFE* gene family. The phylogenetic tree was inferred by the neighbor-joining method using *Saccharomyces cerevisiae* RTG3 as an outgroup sequence. Colored bars above the alignment demarcate the secondary structure motifs (according to ref. 10). *Caenorhabditis* sequences form an outgroup probably because of the high level of diversification.

VI.3. Kreslova J., Machon O., **Ruzickova J.**, Lachova J., Wawrousek E.F., Keller R., Krauss S., Piatigorsky J., Kozmik Z.: *Abnormal lens morphogenesis and ectopic lens formation in the absence of β-catenin function.* **Genesis, Apr;45(4):157-168 (2007).**

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ARTICLE

Abnormal Lens Morphogenesis and Ectopic Lens Formation in the Absence of β-Catenin Function

Jana Kreslova,[1] Ondrej Machon,[1] Jana Ruzickova,[1] Jitka Lachova,[1] Eric F. Wawrousek,[2] Rolf Kemler,[3] Stefan Krauss,[4] Joram Piatigorsky,[2] and Zbynek Kozmik[1]*

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Summary: β-Catenin plays a key role in cadherin-mediated cell adhesion as well as in canonical Wnt signaling. To study the role of β-catenin during eye development, we used conditional Cre/loxP system in mouse to inactivate β-catenin in developing lens and retina. Inactivation of β-catenin does not suppress lens fate, but instead results in abnormal morphogenesis of the lens. Using BAT-gal reporter mice, we show that β-catenin-mediated Wnt signaling is notably absent from lens and neuroretina throughout eye development. The observed defect is therefore likely due to the cytoskeletal role of β-catenin, and is accompanied by impaired epithelial cell adhesion. In contrast, inactivation of β-catenin in the nasal ectoderm, an area with active Wnt signaling, results in formation of crystallin-positive ectopic lentoid bodies. These data suggest that, outside of the normal lens, β-catenin functions as a coactivator of canonical Wnt signaling to suppress lens fate. genesis 45:157–168, 2007.
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Key words: Wnt signaling; β-catenin; eye; lens; retina

INTRODUCTION

The development of the vertebrate eye requires mutually inductive interactions between lens and retina tissue. The eye lens forms from head surface ectoderm, whereas retina forms from the neural tube (Chow and Lang, 2001). Lens has long served as a simple model system to study basic developmental principles such as tissue induction. Lens development begins with the lens placode, a thickening of the surface ectoderm that comes into contact with the optic vesicle. Formation of a morphologically apparent lens placode coincides with the expression of specialized tissue-preferred proteins, the so called crystallins (Wistow and Piatigorsky, 1988). Crystallins are expressed at high levels in the lens and are required for generating and maintaining lens transparency (Wistow and Piatigorsky, 1988). Coordinated invagination of the lens placode and the optic vesicle results in the formation of a lens vesicle and a double-layered optic cup. The inner layer of the optic cup (facing the lens) forms the neural retina, while the outer layer of the optic cup gives rise to the retinal pigmented epithelium (RPE) (Chow and Lang, 2001). As the lens placode detaches from the surface ectoderm to form the lens vesicle, the surface ectoderm above the lens vesicle forms the future corneal epithelium. The lens vesicle is almost spherical and has a large central cavity which becomes filled by the primary lens fibre cells elongating from the posterior part of the lens vesicle. The cells located at the anterior of the lens vesicle remain as epithelial cells. Mitotically active cells in the central region of the lens epithelium move into the equatorial region, where they elongate and differentiate into secondary lens fibres. Finally, the terminally differentiated lens fibre cells lose their organelles to allow lens transparency (Graw, 2003).

Several signaling molecules, in particular, the members of the FGF and BMP families of growth factors, have been implicated in lens induction and differentiation (Lovicu and McAvoy, 2005). More recent studies implicated Wnt signaling in the lens epithelium (Stump *et al.*, 2003) and lens fiber cell differentiation (Lyu and Joo, 2004). A number of Wnt signaling molecules, their Frizzled (Fz) receptors, as well as Wnt antagonists such as secreted frizzled-related proteins (Sfrps) and Dikkopfs (Dkks), were shown to be expressed during mammalian lens development (Ang *et al.*, 2004; Chen

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et al., 2004; Liu *et al.*, 2003; Stump *et al.*, 2003). β-Catenin has a dual intracellular role: it plays a key role in cadherin-mediated cell adhesion, as well as in canonical Wnt signaling. β-Catenin is a central component of the cadherin–catenin adhesion complex, linking cadherins to α-catenin, which anchors the adhesion complex to the actin-based cytoskeletal network (Aberle *et al.*, 1996). As a mediator of canonical Wnt signaling, β-catenin functions as a transcriptional coactivator in the nucleus by binding to the members of the TCF/LEF family of DNA-binding proteins (Huelsken and Behrens, 2002). Mouse embryos lacking β-catenin exhibit early gastrulation patterning defects (Haegel *et al.*, 1995; Huelsken *et al.*, 2000).

In the current study, we have investigated the role of β-catenin during early eye development by using the conditional CRE/lox system in mouse. We show here that loss-of-function of β-catenin does not prevent formation of lens tissue from the presumptive lens ectoderm. However, morphogenesis of the lens is grossly affected due to the defect in cell adhesion. In contrast, loss-of-function of β-catenin in the nasal ectoderm leads to the formation of ectopic lentoid bodies, expressing crystallin proteins. The appearance of ectopic lentoid bodies in β-catenin loss-of-function mutant embryos coincides with downregulation of the BAT-gal Wnt reporter gene in the region nasal to the eye. Our data thus suggest that β-catenin plays a dual role in lens development. As a cytoskeletal component, β-catenin affects lens morphogenesis while it suppresses lens fate in nasal ectoderm as part of canonical Wnt signaling.

RESULTS

Conditional Inactivation of β-Catenin in Developing Mouse Eye

We used conditional gene targeting to inactivate β-catenin in the developing mouse eye. We took advantage of the conditional loss-of-function β-catenin allele $Catnb^{lox(ex2-6)}$ (Brault *et al.*, 2001) and LR-Cre transgenic mice (Fig. 1a). In the LR-CRE transgenic construct, three copies of a lens-specific element (designated EE for ectoderm enhancer), derived from Pax6 gene (Williams *et al.*, 1998), were cloned upstream of the Pax6 P0 minimal promoter. The EE regulatory element is known to drive expression in the presumptive lens ectoderm as well as the surrounding head ectoderm (Kammandel *et al.*, 1999; Williams *et al.*, 1998; Xu *et al.*, 1999). We have used the 3xEE-P0 regulatory module to drive expression of Cre and EGFP cDNAs connected via an internal ribosomal entry site. Although we have detected weak EGFP fluorescence in live embryos, this fluorescence was rapidly lost upon even very short fixation. Therefore, we were unable to use the EGFP as a tracer of expression. To spatially and temporally define the region in which LR-Cre is active, we have used a Rosa26R reporter line (Soriano, 1999), in which Cre-mediated recombination results in the activation of β-galactosidase activity. As expected, the LR-Cre directs expression of Cre recombinase to the presumptive lens and surrounding head surface ectoderm starting from embryonic day E9.5 (Fig. 1b). In addition, we have observed LR-Cre recombinase activity in the developing retina, starting from the optic vesicle stage at E9.5, albeit at a lower frequency, and with frequent appearance of mosaic recombination (J.K. and Z.K., 2004, unpublished data). By E10.5, LR-Cre activity is detected in the lens vesicle, presumptive retina, and corneal epithelium (Fig. 1b). At later stages of development, LR-Cre activity persists in developing lens, retina, and future cornea (J.K. and Z.K., unpublished data). In summary, LR-Cre represents a new tool to study ocular development.

To achieve the conditional inactivation of β-catenin, the LR-Cre transgene was introduced into homozygous floxed β-catenin embryos ($Catnb^{lox(ex2-6)/lox(ex2-6)}$). The genotype of each embryo used in the analysis is shown in the corresponding figure panel. However, for the sake of simplicity we refer throughout the text to LR-Cre/$Catnb^{lox(ex2-6)/lox(ex2-6)}$ as mutant and LR-Cre/$Catnb^{lox(ex2-6)/+}$ or $Catnb^{lox(ex2-6)/lox(ex2-6)}$ as control animals, respectively. First the effectiveness of β-catenin deletion was assessed using the immunohistochemistry. By E10.5, β-catenin immunoreactivity in the presumptive lens pit was eliminated (boxed area in Fig. 1c). Although we have observed small patches of β-catenin-deficient cells in the presumptive retina by E10.5, most cells in the retina do express β-catenin even after several days of LR-Cre activity (J.K. and Z.K., 2004, unpublished data). Therefore, we have focused our analysis on the role of β-catenin in the developing lens, where deletion of β-catenin appears complete.

Adult mice deficient for β-catenin in the lens appear anophthalmic (Fig. 1e), with irregularly formed eyelids and RPE manifested at E17.5 (Fig. 1d). The onset and the phenotypic consequences of β-catenin inactivation in the lens were first investigated at the histological level throughout embryonic development starting from day E10 (Fig. 1f). By E10, the lens placode starts to invaginate normally in both control and mutant embryos. The first manifestation of abnormal eye development was observed at E10.5. Whereas in the control embryos a regularly shaped lens pit is formed by the invagination of the lens placode and such invagination is not detected in the mutant embryos. As a result of this all the subsequent stages of lens development in the central ocular region were morphologically abnormal in the mutant embryos. The underlying optic cup becomes misshapen at E10.5 (Fig. 1f). By E15.5, a highly convoluted presumptive retina tissue is found in the central ocular region. By sectioning later stages of mutant embryos we have often detected lens-like opalescent structures located periocularly (labeled as lens mass [lm] in mutant embryos at E17.5 in Fig. 1f; see also Fig. 3b). Combined, our data show that LR-Cre-mediated deletion of β-catenin in the presumptive lens, surrounding head ectoderm, and in the optic cup results in abnormal eye morphogenesis.

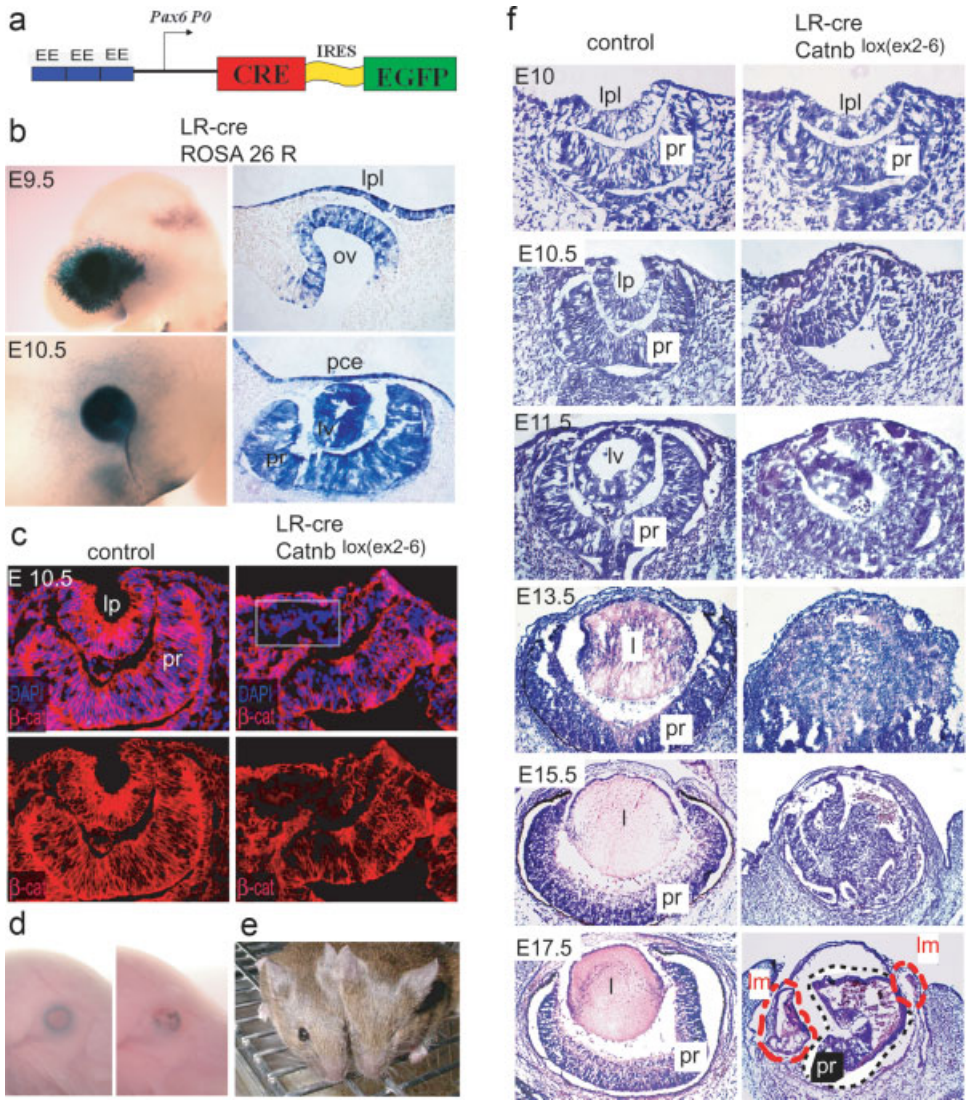

FIG. 1. Lens- and retina-specific conditional inactivation of the β-catenin gene affects eye development. (**a**) Schematic diagram of the LR-Cre transgenic construct. Three copies of the lens-specific ectoderm enhancer (EE) derived from the Pax6 gene (Williams *et al.*, 1998) were cloned upstream of the Pax6 P0 minimal promoter. (**b**) Detection of Cre recombinase activity in LR-Cre mice using ROSA26R reporter mice. (**c**) The expression of β-catenin (red) is abolished in the developing lens pit (boxed area), while it is still detectable in presumptive retina. Nuclei of individual cells are stained with DAPI (blue). (c–e) Abnormal eye development upon deletion of exons 2–6 of the β-catenin gene, as determined at E10.5 (c), E17.5 (**d**), and in the adult mice (**e**, left control mice, right mutant mice). (**f**) Defects in lens and retina development in β-catenin mutant mice. Cryosections at the indicated embryonic stages, from the control and mutant mice, were stained with hematoxylin and eosin. Dotted lines mark the areas of the convoluted presumptive retina (black) and the lens mass (red), respectively. Abbreviations used in this, and in subsequent figures are as follows: lpl, lens placode; ov, optic vesicle; lv, lens vesicle; l, lens; lm, lens mass; lp, lens pit; pce, presumptive corneal epithelium; pr, presumptive retina.

β-Catenin Is Required for Lens Cell Adhesion

β-Catenin plays a key role in cadherin-mediated cell adhesion, as it is a central component of the cadherin–catenin adhesion complex, linking cadherins to α-catenin, which then anchors the adhesion complex to the actin-based cytoskeletal network (Aberle *et al.*, 1996). We have therefore analyzed cryosections of the invaginating lens placode at E10 using phalloidin staining. Phalloidin binds F-actin, and it can thus be used to visualize cytoskeletal complexes. As shown in Figure 2a, phalloidin labeling is detected in a continuous layer in lens epithelial cells of control animals. In contrast, in the absence of β-catenin protein phalloidin labeling appears as a discontinuous layer on the inner face of the invaginating lens placode (Fig. 2a, red arrowheads). These results suggest that β-catenin is required for lens cell adhesion. Discontinuity of the lens epithelium in the mutant embryos could very well explain the aberrant lens placode invagination leading to abnormal lens morphogenesis (Fig. 1f). This is likely to be the result of the functional role of β-catenin in stabilizing actin filaments in the F-actin–cadherin complex. As a result of this lens adhesion pheno-

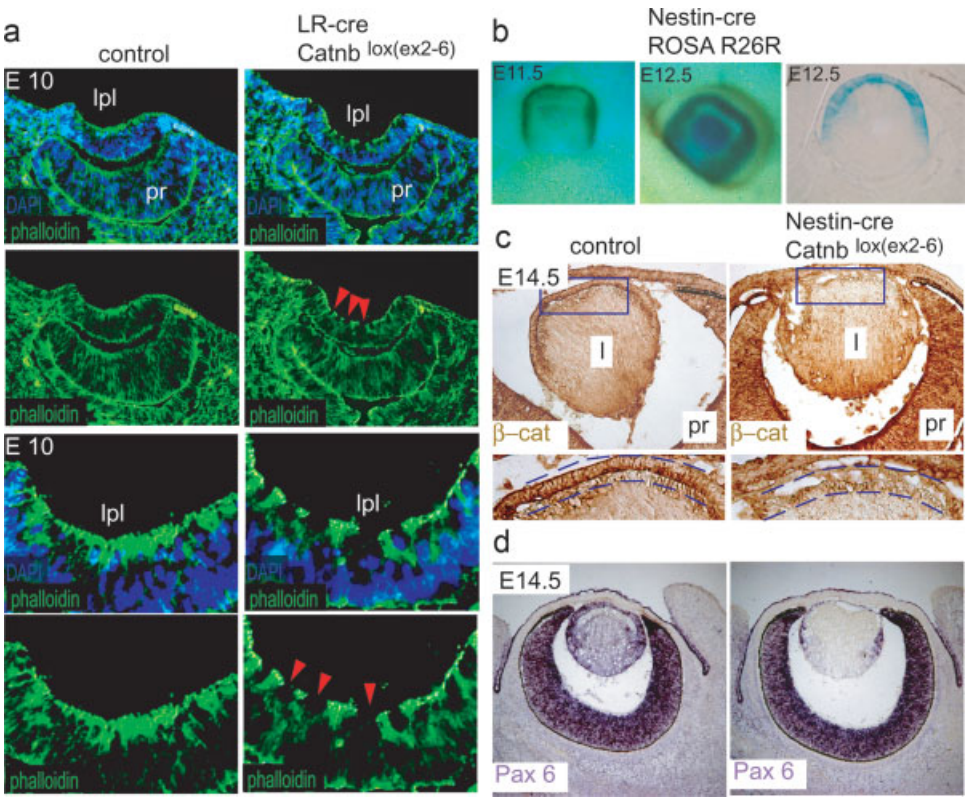

FIG. 2. β-catenin is required for cell adhesion in lens epithelial cells. (**a**) Cryosections from control and mutant mice from stage E10 were stained with phalloidin (green) and DAPI (blue). Red arrowheads point to discontinuity of phalloidin staining in the lens epithelial cells of mutant mice. (**b**) The onset of activity of Nestin-Cre in the lens at E12.5 as detected by the ROSA26R reporter line. Please note the expression of Nestin-Cre in the lens epithelium (right panel). (**c,d**) Defects in lens epithelial cell adhesion in Nestin-Cre/Catnb$^{lox(ex2–6)}$ mice at E14.5 when compared with control mice. Cryosections were stained for β-catenin using immunohistochemistry (c) or for Pax6 expression using RNA in situ hybridization (d). The boxed area in each upper panel in (c) is magnified below to document the disrupted lens epithelium.

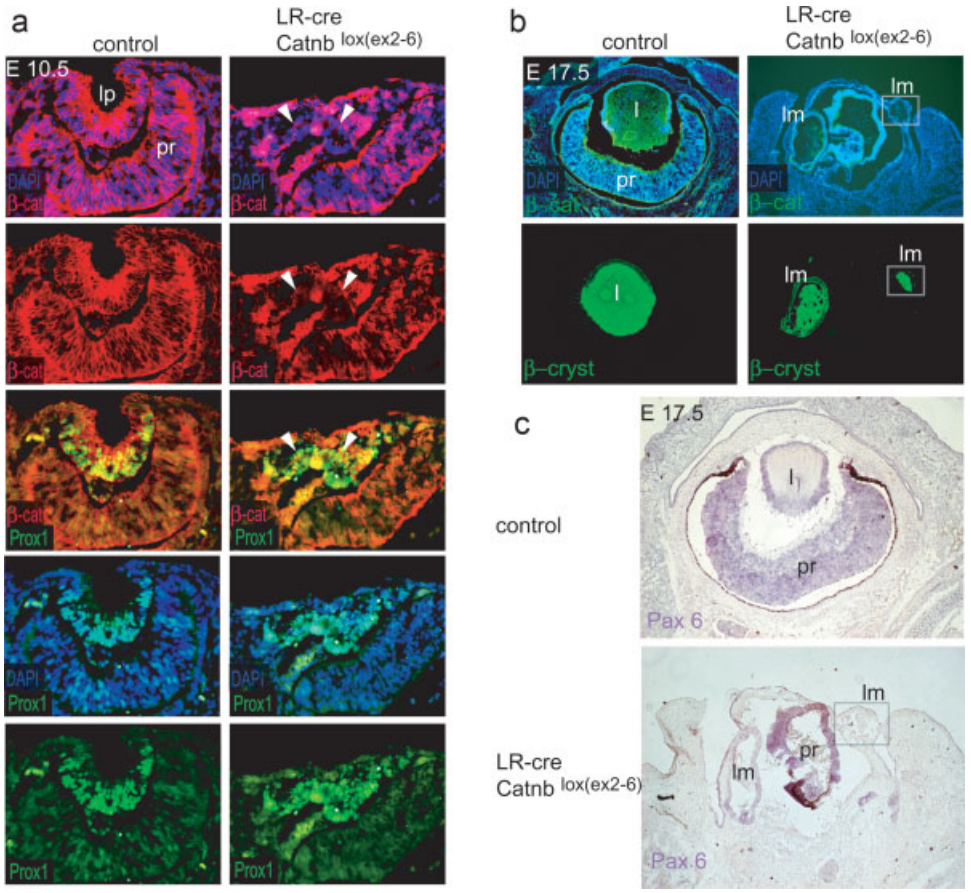

FIG. 3. Lens cells can develop in the absence of β-catenin. (**a**) Cryosections from control and mutant embryos at E10.5 were stained for β-catenin (red), early lens marker Prox1 (green), and DAPI (blue). (**b**) Cryosections from eyes of control and mutant embryos at E17.5 were stained for β-catenin (upper panels) and the lens-specific marker β-crystallin (lower panels). Immunoreactivity for β-crystallin is found in cells lacking detectable β-catenin expression (boxed area). (**c**) Expression of Pax6 is detected in the retina of mutant mice at E17.5, but not in the lens masses in the periocular region (boxed area).

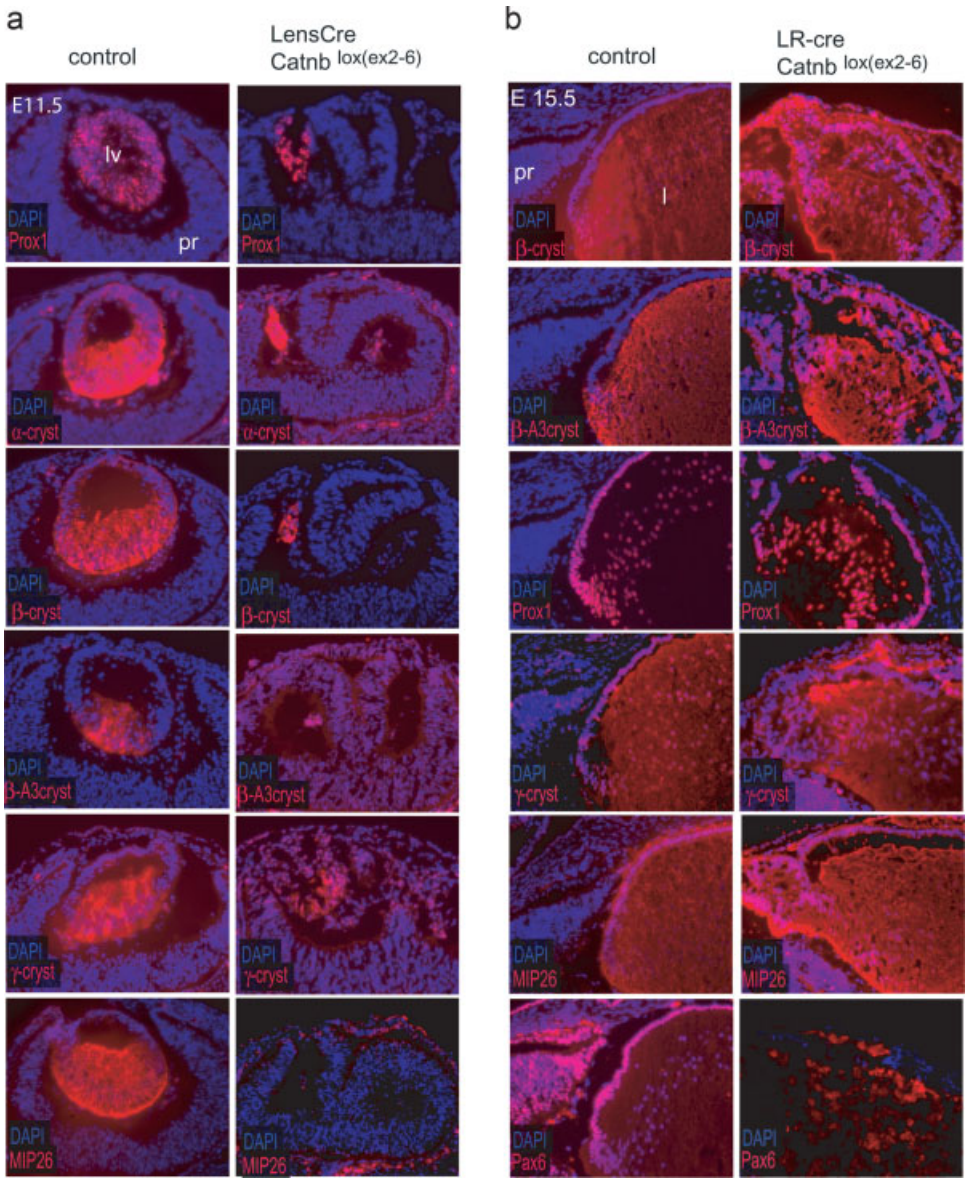

FIG. 4. Lens differentiation proceeds normally in the absence of β-catenin. Expression of lens-specific markers was examined at E11.5 (**a**) and E15.5 (**b**) in the control and mutant lenses.

type, the misshapen lens-like bodies (lens masses) are found within or outside of the retinal cup at E17.5 (Fig. 3b). We then asked if the cell adhesion role of β-catenin is also required in the later stages of lens morphogenesis. We made use of a Nestin-Cre transgenic line (Tronche *et al.*, 1999) in which Cre recombinase is active in lens epithelial cells beginning at E12.5 (Fig. 2b). By E14.5, strong β-catenin immunoreactivity is detected in the cuboidal/columnar epithelial cells found at the anterior pole of the developing lens of control animals (Fig. 2c, left panels). In contrast, we were unable to detect such characteristically localized β-catenin in the lens epithelium of the mutant embryos. As a consequence, the lens epithelial cell layer that is continuous in control animals appears disrupted in the mutants and displaced from the underlying fiber cells (Fig. 2c, right panels). Furthermore, staining for Pax6 expression in the lens epithelium indicates that the epithelial layer is thinner in the mutants as opposed to the control animals (Fig. 2d). Our data, using conditional deletion at two stages of lens development, suggest that β-catenin is required for lens epithelial cell adhesion, and its deficiency results in abnormal lens morphology.

β-Catenin Is Not Required for Lens Fate Determination

As shown earlier, β-catenin deficiency has a profound effect on cell adhesion. In order to see if the loss-of-function of β-catenin also has an impact on lens fate, we have analyzed the expression of lens-specific markers in β-catenin-deficient cells. The homeobox gene Prox1, which is essential for elongation of lens fiber cells (Wigle *et al.*, 1999), is also one of the earliest known lens

markers (Duncan *et al.*, 2002). We have therefore analyzed expression of Prox1 in the β-catenin-deficient lens cells at E10.5. As shown in Figure 3a, we have detected Prox1-positive/β-catenin-negative cells in the aberrantly invaginating lens placode (white arrowheads). Several days later, by E17.5, amorphous lens masses are found within or outside of the ocular region that contains β-catenin-negative cells, which also stain strongly for the lens-specific differentiation marker β-crystallin (Fig. 3b). These crystallin-positive cells do not seem to express high levels of Pax6 (Fig. 3c, please compare expression Pax6 in the lens epithelium in the top panel with the lack of Pax6 expression in the lens mass in the inset of the bottom panel).

To further clarify the character and differentiation status of β-catenin-deficient lenses, expression of various lens markers was examined at E11.5 (Fig. 4a) and E15.5 (Fig. 4b). At E11.5, α/β-crystallins and Prox1 are expressed by all cells of the lens, whereas βA3-crystallin, γ-crystallin, and MIP26 are expressed predominantly by developing fiber cells. As shown in Figure 4a, positive immunoreactivity was detected for each of the lens markers at E11.5 in the mutant lens although the markers specific for fibers cells were expressed significantly less well when compared with normal lens. At E15.5, all tested lens markers (Fig. 4b) were expressed by the mutant lens, suggesting that the differentiation of the lens has proceeded normally. Combined, these data suggest that β-catenin is not required for lens fate determination or lens cell differentiation but rather for lens morphogenesis, and thus indirectly for proper folding of the retinal cup.

Activity of Canonical Wnt Signaling in the Developing Mouse Embryonic Lens

To map the spatial and temporal activity of canonical Wnt signaling throughout mouse eye development, we took advantage of the BAT-gal reporter line that expresses lacZ under the control of multimerized TCF/LEF binding sites (Maretto *et al.*, 2003). Upon activation of canonical Wnt signaling, β-catenin protein is stabilized, and accumulates in the cell nucleus where it binds to the transcription factors of LEF1/TCF family, which results in the activation of BAT-gal reporter gene. We have analyzed BAT-gal reporter gene activity in the presumptive eye region from E9.5 to E15.5 (Fig. 5a; J.R. and Z.K., data not shown). At E9.5, no reporter activity is detected either in the presumptive lens placode or in the presumptive retina (Fig. 5a). Only few scattered lacZ-positive cells are detected in the dorsal aspect of the developing eye. At E10.5, strong BAT-gal activity is detected in the dorsal optic vesicle in the region corresponding to the presumptive RPE layer. No BAT-gal reporter activity is detected in the invaginating lens pit at E10.5, lens vesicle at E11.5, lens at E13.5 (Fig. 5a) or at E15.5 (data not shown). Within the eye, reporter gene expression is found in the presumptive RPE at E11.5 and E13.5, as well as in developing eyelids and conjunctiva at E13.5 (Fig. 5a; data not shown). In contrast to the eye,

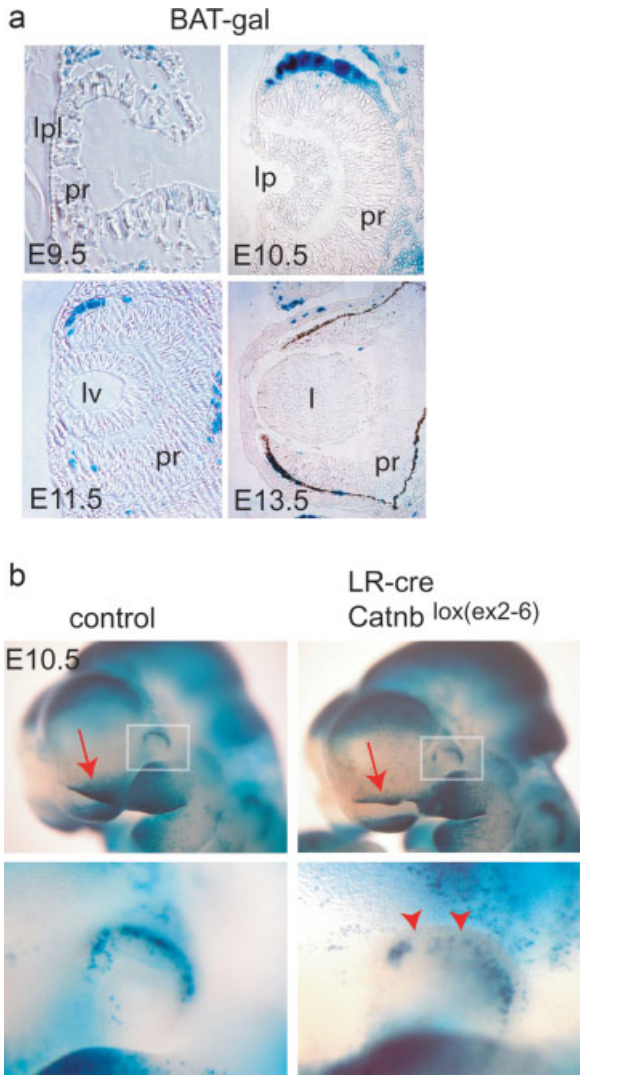

FIG. 5. Wnt reporter activity in control and β-catenin-deficient mice. (**a**) Cryosections from embryos of Wnt reporter line (BAT-gal) at E9.5, E10.5, E11.5, and E13.5 were stained for β-galactosidase activity. X-gal staining is absent from the lens tissue at all stages of development, most notably at the onset of lens induction (E9.5; inset). (**b**) Whole-mount staining for β-galactosidase activity in the Wnt reporter line BAT-gal in control and mutant embryos at E10.5. The red arrows in the upper panels point to differential expression of β-galactosidase in the frontonasal area. The red arrowheads in the lower right panel indicate the loss of β-galactosidase activity in the dorsal periocular region.

where canonical Wnt signaling is mostly absent (Fig. 5b, inset), strong BAT-gal activity is detected nasal to the eye at E10.5 (Fig. 5b, left panel, red arrow). This activity is markedly reduced in LR-Cre/Catnb$^{lox(ex2-6)/lox(ex2-6)}$ mutant animals (Fig. 5b, right panel, red arrow), as well as in a few cells within the dorsal optic vesicle (Fig. 5b, inset) at E10.5. Combined, our data indicate that canonical Wnt signaling appears absent from lens at all stages of embryonic development. Furthermore, we show that LR-Cre-mediated deletion of β-catenin results in the abrogation of canonical Wnt signaling in the frontonasal area.

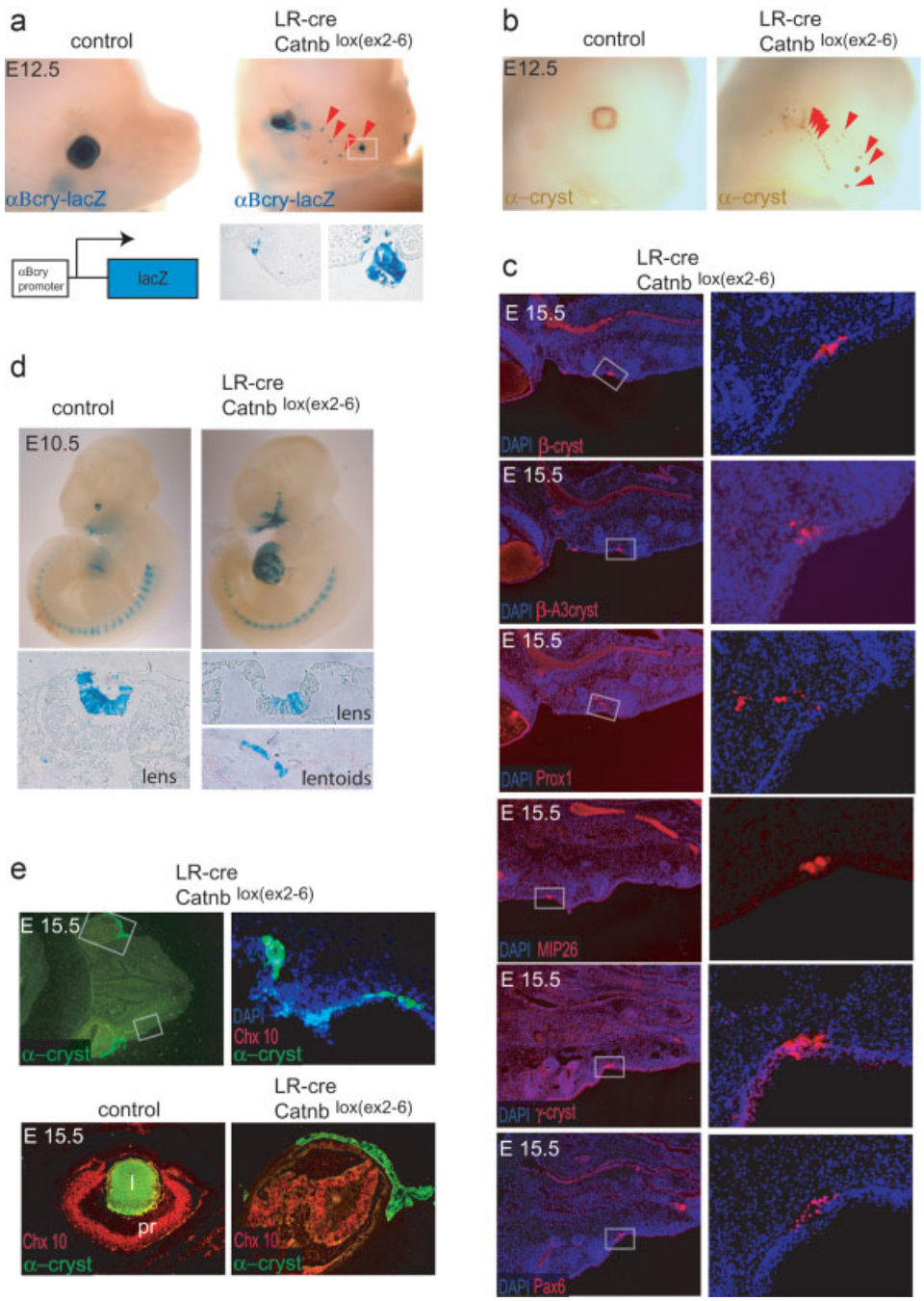

FIG. 6. Loss-of-function of β-catenin in the surface ectoderm results in the formation of ectopic lentoid bodies. (**a**) Whole-mount staining for β-galactosidase activity in the αB-crystallin transgenic reporter line in control and mutant embryos at E12.5. A schematic diagram of the transgenic construct is shown in the lower left panel. The β-galactosidase-positive cell clusters can be detected in the frontonasal area (upper right panel, red arrowheads). Sections through the small (arrowheads) and large (inset) ectopic lentoids from the mutant embryo are shown below. (**b**) Whole-mount immunohistochemistry using α-crystallin antibody detects immunoreactivity in a streak of cell clusters nasal to the eye (red arrowheads). (**c**) Expression of a panel of lens markers (red) in ectopic lentoid bodies at E15.5. Nuclei of individual cells are labeled with DAPI (blue). Right panels show magnification of the area within the insets. (**d**) Whole-mount staining for β-galactosidase activity in the αB-crystallin transgenic reporter line in control and mutant embryos at E10.5 (upper panels). Sections through the areas of a developing lens and developing lentoid bodies are shown below. (**e**) Cryosections of control and mutant embryos at E15.5 stained for α-crystallin (green), Chx10 (red), and DAPI (blue). α-Crystallin-immunoreactive cells are detected in the lens mass in the periocular region (large inset; lower right panel) and in the ectoderm nasal to the eye (small inset; upper right panel). Retina tissue (Chx10 immunoreactivity) is adjacent to the lens mass (α-crystallin-immunoreactivity) in the central ocular region (lower right panel). No Chx10 immunoreactive cells are closely associated with lentoid bodies in the frontonasal region (upper right panel).

Loss-of-Function of β-Catenin Results in the Formation of Ectopic Lentoid Bodies in the Frontonasal Area

Staining for lens-specific crystallin expression in mutants between E12.5 and E17.5 has often shown immunoreactivity well outside of the original lens position (Fig. 3b; J.K., data not shown). In order to visualize the position of crystallin-positive cells within the embryo, we have made use of a transgenic line (αBcry-lacZ) in which the promoter of αB-crystallin is linked to the lacZ gene (Haynes *et al.*, 1996). At E12.5, the activity of αBcry-lacZ in the head region is limited to the lens in control animals (Fig. 6a, left panel). To our surprise, in the mutant embryos, a large number of β-galactosidase-positive cell clusters is found in the ectoderm nasal to the eye (Fig. 6a, right panel, red arrowheads) and likely represent ectopic lentoid bodies. Most of the cell clusters are limited to a few cells, although some larger lentoids were also found (Fig. 6a, inset). Although the αBcry-lacZ transgenic reporter proved to be very sensitive, its expression could only represent an aberrant behavior of the promoter used. We have therefore independently confirmed the presence of α-crystallin-positive lentoid bodies by whole mount in situ immunohistochemistry using α-crystallin antibody (Fig. 6b, red arrowheads). To provide further evidence of the lens-like character of ectopic lentoid bodies, we took advantage of a large panel of lens specific markers. Cell clusters located in the frontonasal region stained positive for lens markers Prox1, Pax6, β-crystallin, βA3-crystallin, γ-crystallin, and MIP26 (Fig. 6c), consistent with the idea that these structures represent bona fide lentoid bodies.

We have then investigated the onset of the lentoid formation with respect to the onset of normal lens development and crystallin expression (E9.5–E10.5). In order to visualize lentoids within the embryo, we have made use of a transgenic line (αBcry-lacZ) in which the promoter of αB-crystallin is linked to the lacZ gene (Haynes *et al.*, 1996). As shown in Figure 6d, ectopic lentoids in the frontonasal area are detected as early as E10.5, corresponding with the normal events of lens induction.

Under normal circumstances, lens and retina develop upon close contact, and due to mutual inductive events. We have therefore investigated the possibility that the ectopic lentoids in the frontonasal area are associated with adjacent retina tissue. Homeobox gene *Chx10* is the earliest known gene to be expressed specifically in the presumptive neuroretina, beginning at E9.5 (Liu *et al.*, 1994). Later on, *Chx10* remains expressed in the proliferating progenitor cells in the neuroretina as well as in bipolar cells (Burmeister *et al.*, 1996). Members of the FGF family secreted from the surface ectoderm are known to signal through Chx10 to organize the neuroretina adjacent to the future lens (Horsford *et al.*, 2005). As shown in Figure 6e, Chx10 immunoreactivity in mutant embryos is found adjacent to the abnormally shaped lens in the central ocular region (large inset). In contrast, we have never observed Chx10 immunoreactivity near the crystallin-positive ectopic lentoid bodies. Combined, our data indicate that β-catenin is required for lens fate suppression in certain areas of the surface ectoderm nasal to the eye. This is most likely due to abrogation of its transactivation function, which in turn leads to the inactivation of canonical Wnt signaling.

DISCUSSION

LR-Cre Mice as a Tool for Cre-Mediated Recombination in Lens and Retina

A lens lineage-specific Cre (designated lens-cre) was previously developed using 6.5 kb of the Pax6 upstream regulatory region which contains the lens-specific EE element (Ashery-Padan *et al.*, 2000). Lens-Cre promotes effective recombination in a large area of surface ectoderm in the prospective eye region beginning at E9.0. Later on, Cre activity is maintained in the developing lens and future cornea, as well as in the pancreas (Ashery-Padan *et al.*, 2000). More recently, Pax6(Lens)-Cre was made, in which a single copy of the EE element was fused to the minimal Pax6 P0 promoter, thus avoiding Cre recombinase activity in the pancreas (Yoshimoto *et al.*, 2005). Cre recombinase activity in developing retina was detected in neither lens-cre mice (Ashery-Padan *et al.*, 2000) nor Pax6(Lens)-Cre mice (Yoshimoto *et al.*, 2005).

We have used three tandem copies of the Pax6-derived EE regulatory element (Kammandel *et al.*, 1999; Williams *et al.*, 1998; Xu *et al.*, 1999), fused to the minimal Pax6 P0 promoter, to drive expression of Cre recombinase in the presumptive lens ectoderm. We reasoned that multiple copies of the EE element would make expression more robust, and independent of positional effects. We have generated several independent LR-Cre transgenic mouse lines using this construct. In each case, we have observed Cre recombinase activity not only in the surface ectoderm, but also in the underlying optic vesicle (J.K. and Z.K., 2004, unpublished data). This is in agreement with several other transgenic mouse lines that we have recently generated using the same regulatory sequences (Z.K., 2004, unpublished data). At present, we do not know the reason for ectopic, but consistent, expression of the 3xEE regulatory element in developing mouse retina.

The Cytoskeletal Function of β-Catenin Is Required for Lens (and Retina) Morphogenesis

In the current study, we have investigated the role of β-catenin in the early stages of lens development. A similar study has recently been done by others (Smith *et al.*, 2005), who used Lens-Cre (Ashery-Padan *et al.*, 2000) for tissue specific deletion of β-catenin using the conditional loss-of-function allele $Catnb^{lox(ex2-6)}$ (Brault *et al.*, 2001). β-Catenin has a dual intracellular role: it plays a key role in cadherin-mediated cell adhesion, and in canonical Wnt signaling. On one hand, β-catenin is a central component of the cadherin–catenin adhesion complex, linking cadherins to α-catenin, which anchors the

adhesion complex to the actin-based cytoskeletal network (Aberle *et al.*, 1996). At the same time, β-catenin functions as a transcriptional coactivator in the canonical Wnt signaling pathway (Huelsken and Behrens, 2002). Our data suggest that tissue-specific deletion of β-catenin in a large area of head surface ectoderm (and to some extent in the underlying optic vesicle) does not influence lens induction or the appearance of lens fate markers Prox1 and crystallins. However, lens morphogenesis is severely disturbed. This is in complete agreement with the recently published results of Smith *et al.* (2005). Based on our data, as well as those of Smith *et al.* (2005), we hypothesize that the transcriptional role of β-catenin is most likely not required for proper lens morphogenesis (see later). Instead the abnormally shaped lenses in LR-Cre/Catnb$^{lox(ex2-6)/lox(ex2-6)}$ mutant embryos result from the absence of the cytoskeletal role of β-catenin, leading to uncoordinated epithelial cell morphogenesis. The normally continuous staining of F-actin in invaginating wild-type lens pit becomes discontinuous in mutant animals at E10.5 (Smith *et al.*, 2005; this study). A similar defect in epithelial cell adhesion and morphogenesis was observed in Nestin-Cre/Catnb$^{lox(ex2-6)/lox(ex2-6)}$ mutant lenses, in which deletion of β-catenin begins only at E12.5, thus allowing the initial stages of lens development to proceed normally.

Lens and retina develop in close proximity, and multiple mutual induction events have been postulated (Chow and Lang, 2001). It is thus not surprising that abnormal lens development at E10.5 influences the nearby optic cup, leading to a misshapen retina (Smith *et al.*, 2005). However, the retinal defects in LR-Cre/Catnb$^{lox(ex2-6)/lox(ex2-6)}$ mutant mice are much more prominent than the defects in Lens-Cre/Catnb$^{lox(ex2-6)/lox(ex2-6)}$ mutant mice (Smith *et al.*, 2005). The most obvious explanation for such differences lies in weak, but consistent, LR-Cre activity in developing retina. Although the deletion of β-catenin within the retina of mutant mice is by far not complete (Figs. 1c and 3b), we attribute the observed phenotype (multilayered, severely misfolded retina), at least in part, to the β-catenin deficiency within the retina itself. It was shown recently that β-catenin is essential for the proper lamination of retina (Fu *et al.*, 2006).

Activity of Canonical Wnt Signaling During the Mouse Lens Development

In order to find out if the observed defect is due to the transcriptional or cytoskeletal role of β-catenin, we took advantage of the Wnt reporter transgenic line BAT-gal (Maretto *et al.*, 2003), which allows integration of the input from large numbers of Wnts, their Fz receptors, and Wnt inhibitors expressed during mammalian lens development (Ang *et al.*, 2004; Chen *et al.*, 2004; Liu *et al.*, 2003; Stump *et al.*, 2003). By analyzing the expression of the Wnt reporter gene BAT-gal, we have found that canonical Wnt signaling appears inactive during all stages of mouse embryonic lens development. A similar conclusion has been reached by Smith *et al.* (2005), who used a different Wnt transgenic reporter line, TOPGAL (DasGupta and Fuchs, 1999). Yet another Wnt transgenic reporter line (TCF/Lef-LacZ) has been analyzed for its activity in the developing mouse eye and found to be active in anterior lens epithelial cells at E13.5 (Liu *et al.*, 2003). However, in agreement with the results obtained using BAT-gal (this study) and TOPGAL (Smith *et al.*, 2005), we and others have not detected prominent nuclear accumulation of β-catenin in the anterior lens epithelium (Lyu and Joo, 2004; Smith *et al.*, 2005), which is a hallmark of Wnt activation. Wnt signaling has been shown, primarily in explant culture studies, to promote lens fiber cell differentiation (Lyu and Joo, 2004). In agreement with this, nuclear β-catenin, as well as Ser9-phosporylated (inactive) GSK3β, has been found in the differentiating fiber cells near the lens equator at P4 (Lyu and Joo, 2004).

Conflicting reports have been published regarding the role of LDL-related protein coreceptor 6 (LRP6) during mouse lens development. LRP6 is a key component of the receptor complex used by Wnt signaling molecules (Pinson *et al.*, 2000). Stump *et al.* (2003) have postulated a rather late (E12.5–E18.5) role for LRP6 in lens epithelial cell differentiation, although considerable variation in the severity of the LRP6−/− phenotype was noticed. In contrast, Smith *et al.* (Smith *et al.*, 2005) was unable to recover any LRP6−/− embryos past E10.5. The reason for this discrepancy is presently unknown. Nevertheless, even in the complete absence of LRP6 function in the mouse embryo, the normal induction of the early lens markers Pax6 and Sox2 was detected (Smith *et al.*, 2005). This suggests, once again, that the activity of canonical Wnt signaling is not required to induce the lens fate.

Canonical Wnt Signaling Suppresses Lens Fate Decision in the Frontonasal Area

We and others (Smith *et al.*, 2005) have observed ectopic lentoid bodies forming in β-catenin-deficient, Pax6-expressing surface ectoderm. The Pax6 transcription factor is a well-known regulator that is both necessary (Ashery-Padan *et al.*, 2000; Collinson *et al.*, 2000) and, at least in some circumstances, sufficient (Altmann *et al.*, 1997) for lens induction. The lentoid bodies are made from clusters of cells positive for expression of multiple lens-specific markers, and are found over a large area nasal to the eye. No Chx10-expressing retina tissue has been found in the vicinity of these ectopic lentoids. Interestingly, a consistent and relatively regular pattern of lentoid formation has been observed. Most lentoids form in the narrow streak running nasally from the ocular region to the tip of the nose, an area which corresponds to the region where LR-Cre-mediated deletion of β-catenin results in the abrogation of canonical Wnt signaling as determined by BAT-gal reporter activity (Fig. 5b). Smith *et al.* (2005) were unable to detect TOPGAL activity in most of the frontonasal region, where many small ectopic lentoids were formed. This is most

likely due to the lower inducibility of TOPGAL compared with the BAT-gal reporter gene. The inability to detect canonical Wnt signaling using TOPGAL in areas where ectopic lentoids were formed represented a difficulty in the explanation of the observed lentoid distribution (Smith *et al.*, 2005). Moreover, due to the difference in Cre drivers used in the two studies, the area affected by β-catenin deletion might be slightly different. In the earlier study (Smith *et al.*, 2005) most of the large lentoid bodies were detected in the periocular region, whereas in our hands many large lentoids form as far as the tip of the nose (Fig. 6a; J.K., data not shown). Previous study (Smith *et al.*, 2005) did not address the onset of lentoid formation, their differentiation potential, or what happens with ectopic lentoids at later stages. Using the crystallin reporter line αBcry-lacZ we were able to show that the onset of lentoid formation (E10.5 or earlier) coincides with the development of the endogenous lens. By examining lentoids at E15.5 we found expression of lens markers typical of lens epithelium, primary fiber cells, as well as mature fiber cells (such as MIP26 or γ-crystallin), suggesting that lens differentiation can proceed in these ectopic lentoids. The fate of the lentoids is less clear. We have been able to detect some ectopic lentoids in the newborn mice although at a smaller frequency when compared with E12.5 (J.K. and Z.K., data not shown).

Our data and data of Smith *et al.* suggests that canonical Wnt signaling suppresses lens fate in certain areas outside of the lens-fated surface ectoderm such as in the frontonasal region. Consistent with this idea is the fact that ectopic activation of Wnt signaling in the lens surface ectoderm, through stabilization of β-catenin, leads to complete arrest of lens development (Smith *et al.*, 2005; J.K. and Z.K., 2005, unpublished data). It follows that the transcriptional, rather than cytoskeletal, role of β-catenin is required in the frontonasal region to suppress lens fate. We cannot rule out the possibility that some of the ectopic lentoids formed from cells that have migrated nasally from the central ocular region, although we find this relatively unlikely. The current studies have been limited by the use of regionally restricted Cre drivers (Lens-Cre, LR-Cre). It remains to be seen how large an area of the head facial ectoderm is competent to become a lens in the absence of activated canonical Wnt signaling. Recent work of Bailey *et al.* (Bailey *et al.*, 2006) suggests a broad capacity of the pre-placodal region to adopt the lens fate. Finally, the molecular basis of Wnt-mediated lens fate suppression in the frontonasal region is currently unknown, but it represents an intriguing topic for future studies.

EXPERIMENTAL

Mouse Lines

LR-Cre was constructed by ligating three copies of the Pax6 lens enhancer (Williams *et al.*, 1998) to the P0 minimal promoter of the *Pax6* gene. LR-Cre transgenic lines were generated by microinjection and maintained in an FVB/N inbred background. Transgenic mice were identified by PCR analysis of genomic DNA from the tail. Primers: forward, CAACCAATGAGGGCATTGCTGGCG; reverse, CGTTGCATCGACCGGTAATGCA (ZK297/ZK377A). Analysis of the Cre-mediated recombination pattern in LR-Cre line was performed by mating to the ROSA26R reporter line, as described previously (Soriano, 1999). The ROSA26R mice were purchased from Jackson Lab (Stock no. 003309). Nes11-Cre (Tronche *et al.*, 1999) were purchased from Jackson Lab (Bar Harbor, ME) (Stock no. 003771). To determine the canonical Wnt pathway during eye development, we used the BAT-gal reporter line (Maretto *et al.*, 2003). In order to investigate lens fate in the periocular region, we used a reporter line αB-crystallin-lacZ generated previously in our laboratory (Haynes *et al.*, 1996). The $Catnb^{lox(ex2-6)}$ mice (Brault *et al.*, 2001), with a conditional "floxed" allele of β-catenin, were used to inactivate the gene for β-catenin.

Tissue Collections and Histology

Mouse embryos were obtained from timed pregnancy mating, with noon of the date that the vaginal plug was observed defined as embryonic day 0.5 (E0.5). Embryos were harvested in cold PBS, fixed in either 4% paraformaldehyde for various times ranging from 1 h to overnight (hematoxylin–eosin staining, immunohistochemistry) or 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, and 5 mM EGTA for 1 h (for X-gal staining). Fixed embryos were cryoprotected in 30% sucrose overnight at 4°C, embedded and frozen in OCT (Tissue Tek; Sakura Finetek, Zoeterwoude, The Netherlands). Horizontal frozen sections were done at 6–8 μ thickness. The cryosections were washed three times in PBS and subsequently stained with an antibody or hybridized with RNA antisense probes.

X-Gal Staining

The β-galactosidase assay was carried out as described by Hogan (1994). After the fixation, the cryosections were directly stained with the staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris, pH 7.3, and 1 mg/ml X-gal). For whole-mount staining, fixed embryos were washed three times in rinse buffer (0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 20 mM Tris, pH 7.3, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40), and incubated overnight at 37°C in staining solution.

Immunohistochemistry

The cryosections were refixed for 10 min in 4% paraformaldehyde, washed with PBS, and permeabilized with PBS/0.1% Tween 20 (PBT) for 15 min prior to blocking. Sections were blocked for 30 min in 10% BSA/PBT, incubated overnight with primary antibodies at room temperature, washed three times with PBS, incubated 1 h at room temperature with secondary antibodies, washed three times with PBS, and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Primary

antibodies used were: anti-α-crystallin (a gift of Sam Ziegler, NEI), anti-β-crystallin (a gift of Sam Ziegler, NEI), anti-γ-crystallin (a gift of Hisato Kondoh), anti-βA3-crystallin (a gift of Hisato Kondoh), anti-MIP26 (a gift of Joe Horwitz), anti-β-catenin (Transduction Laboratories, Franklin Lakes, NJ), anti-β-catenin (Cell Signaling Technology, Beverly, MA), anti-β-tubulin TUJ-1 (R&D Systems, Minneapolis, MN), anti-Chx10 (Exalpha Biologicals, Maynard, MA), anti-Pax6 (Covance, Berkeley, CA), anti-Prox1 (Chemicon, Temecula, CA). To detect F-actin, Alexa-488 labeled phalloidin was used (Molecular Probes, Carlsbad, CA). The following secondary antibodies were used: Alexa-488- or 594-conjugated goat anti-mouse or anti-rabbit IgG, Alexa-594-conjugated donkey anti-sheep IgG (Molecular Probes).

Whole-Mount Immunohistochemistry

Whole-mount immunohistochemistry was carried out as described (Hogan, 1994). In brief, embryos were fixed in methanol:DMSO (4:1) at 4°C overnight, incubated in methanol:DMSO:H_2O_2 (4:1:1), and then stored in 100% methanol at −20°C. Samples were rehydrated in 50% methanol, washed in PBS, blocked in PBS/2% milk/0.5% Triton X-100 (PBSMT), incubated overnight with primary antibodies at 4°C, washed five times in PBSMT, incubated overnight with secondary antibodies at 4°C, and washed five times with PBSMT. After several washes in PBS/0.2% BSA/0.5% Triton X-100, peroxidase staining was done using FAST DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma, St. Louis, MO) for 20 min at room temperature, followed by PBS washes.

In Situ Hybridization

In situ hybridization on cryosections was carried out as described previously (Machon *et al.*, 2002). Plasmid carrying mouse Pax6 cDNA (provided by P. Gruss) was linearized with appropriate restriction enzyme, and antisense riboprobe was synthesized using the DIG RNA labeling kit (Roche, Mannheim, Germany).

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Eye-specific expression of an ancestral jellyfish *PaxB* gene interferes with *Pax6* function despite its conserved Pax6/Pax2 characteristics

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ABSTRACT Pax transcription factors are evolutionarily conserved regulators of eye development and can be distinguished on the basis of three functional domains: two DNA-binding domains (the paired domain and the paired-type homeodomain), and the octapeptide motif. PaxB of the eyed cubozoan jellyfish, *Tripedalia cystophora*, is characterized by a Pax2-like paired domain and octapeptide, and a Pax6-like homeodomain. In mice, functionally distinct Pax6 and Pax2 proteins have unique as well as redundant roles in eye morphogenesis. Here, we show that expression of the jellyfish *PaxB* gene in mouse embryonic eye tissues impairs normal development of lens and retina. Our data show that *PaxB* misexpression leads to a downregulation of endogenous Pax6 protein in the prospective lens and in subsets of cells within the inner nuclear layer of transgenic retina. In addition to *Pax6* downregulation, the expression of *PaxB* leads to an almost complete loss of amacrine cells in the adult transgenic retina, a phenotype that differs from a loss-of-function of the *Pax6* gene. The present data suggest that PaxB, due to its Pax2-like paired domain and Pax-6 like homeodomain, disturbs the transcriptional network regulated by Pax6 in the developing lens and retina. Taken together, our data suggest that molecular properties of individual mouse Pax2 and Pax6 proteins are essential determinants of mouse eye development and cannot be substituted for by jellyfish PaxB which possesses elements of vertebrate Pax2 and Pax6.

KEY WORDS: *Pax, eye, lens, retina*

Introduction

Pax genes encode transcription factors critical for metazoan development. Members of the Pax protein family can be classified based on the presence and characterictic features of their three evolutionarily conserved domains: a paired domain, a paired-type homeodomain and an octapeptide. The paired domain and the paired-type homeodomain encode two independent DNA-binding domains, while the octapeptide plays an important role in protein-protein interactions. Pax proteins interact with similar DNA sequences although differences in specificity exist. In particular, three amino acids (at positions 42, 44, and 47) within the N-terminal half of paired domain are responsible for the difference in the DNA-binding specificities between Pax6 and Pax2/5/8 subfamilies (Czerny and Busslinger, 1995). The amino acids IQN at these positions specify Pax6, whereas amino acids QRH determine Pax2/5/8 specificity (Czerny and Busslinger, 1995). Pax2 of the Pax2/5/*8* subfamily has a DNA binding paired domain, and an octapeptide, but only a truncated homeodomain, and has been implicated in eye development both in mice and *Drosophila.* The *Drosophila Pax2* orthologue has a key role in the development of ommatidial cone and and pigment cells (Fu and Noll, 1997). In mice, *Pax2* deficiency results in eye, kidney and inner ear defects (Favor *et al.*, 1996; Torres *et al.*, 1996; Gehring and Ikeo, 1999). Pax6 is an evolutionarily highly conserved transcription factor that has been considered as a key regulator of the eye

Abbreviations used in this paper: EE, ectoderm enhancer; HD, homeodomain; PD, paired domain; RGC, retinal ganglion cell; RPC, retinal progenitor cell; RPE, retinal pigmented epithelium.

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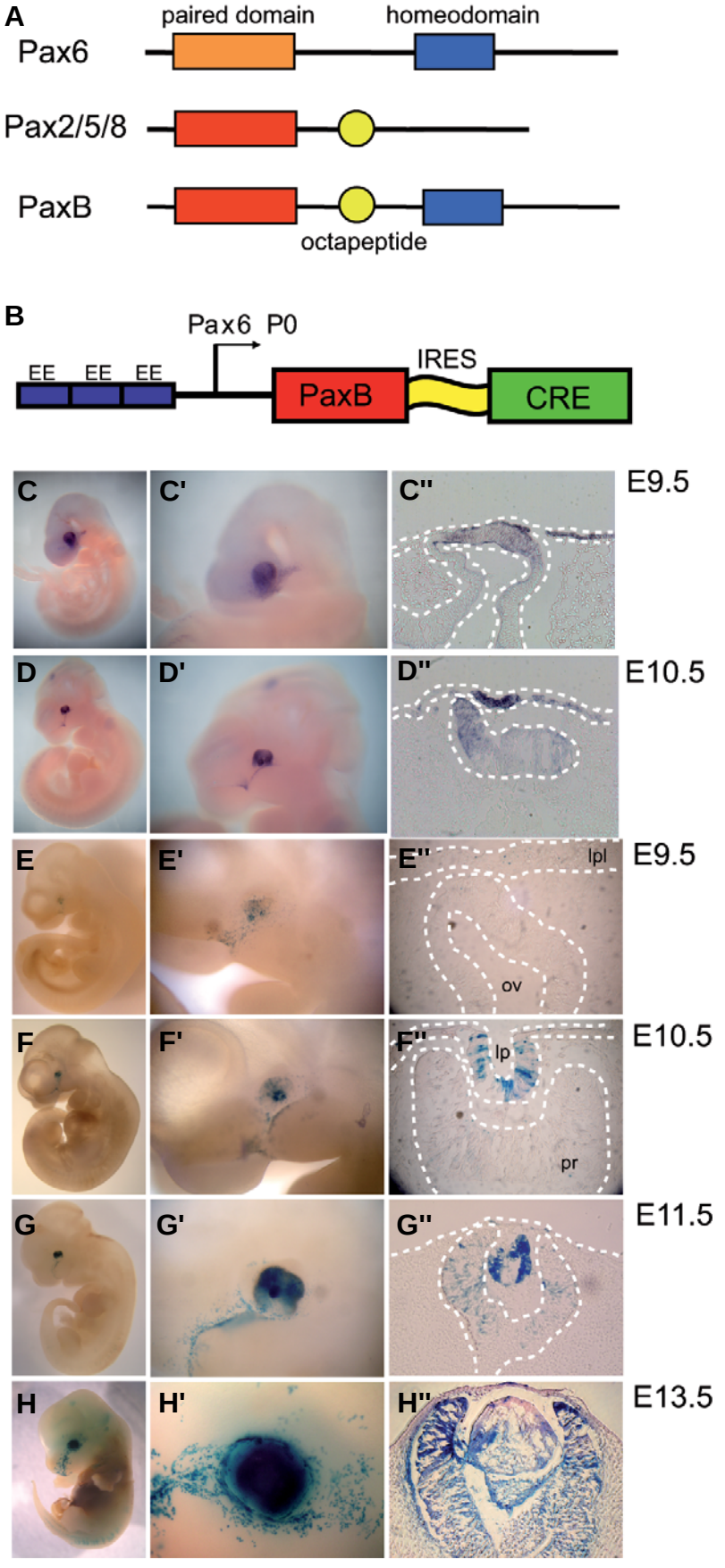


development among metazoans (Favor *et al.*, 1996; Torres *et al.*, 1996; Gehring and Ikeo, 1999; Gehring, 2002; Gehring, 2005; Kozmik, 2005). *Pax6* mutations are associated with aniridia in humans and the Small eye (Sey) phenotype in mice (Hanson and van Heyningen, 1995). *Drosophila* possess two *Pax6* homologs, *eyeless* (*ey*) and *twin of eyeless* (*toy*), both of which are able to induce ectopic eyes (Quiring *et al.*, 1994; Halder *et al.*, 1995; Czerny *et al.*, 1999). Pax6 directly activates rhodopsin genes in *Drosophila* (Sheng *et al.*, 1997; Papatsenko *et al.*, 2001) and lens crystallins of vertebrates (Cvekl and Piatigorsky, 1996; Duncan *et al.*, 2004). Pax6 contains, in addition to the paired domain, a second DNA-binding domain, the homeodomain (HD) but it lacks the octapeptide, which is responsible for repression abilities of Pax2/5/8 proteins mediated through interaction with Groucho co-repressors (Eberhard *et al.*, 2000; Kozmik *et al.*, 2003).

The *Pax6* gene encodes three isoforms of Pax6 protein, and these may play different roles in eye development. The so-called canonical Pax6 protein contains the paired domain (PD) and a homeodomain (HD). Alternative RNA splicing of the Pax6 primary transcript in mice and humans leads to a second Pax6 isoform, Pax6(5a), where a peptide encoded in an additional exon is inserted within the paired domain (Epstein *et al.*, 1994). Both isoforms mentioned above are encoded by transcripts that initiate from P_1- and P_0- *Pax6* promoters (Kammandel *et al.*, 1999; Xu *et al.*, 1999). Finally, an isoform of Pax6 that lacks the paired domain and is encoded by the transcript starting from the P_{alpha} promoter has also been described (Kammandel *et al.*, 1999; Kleinjan *et al.*, 2004; Kim and Lauderdale, 2006).

Paired-less Pax6 is likely involved in the cell fate

Fig. 1. Construction and expression of the *PaxB* transgene. *Structure of Pax6, Pax2 and PaxB proteins; PaxB has a Pax2-like paired domain (red rectangle), a conserved octapeptide motif that is also present in Pax2 (yellow circle), and a Pax6-like homeodomain (blue rectangle)* **(A)**. *Schematic diagram of the* PaxB *transgene Three copies of the lens-specific ectoderm enhancer (EE) derived from the* Pax6 *gene were cloned upstream of the Pax6 P0 minimal promoter (LR-module; Kreslova* et al., *2007) to drive the expression of PaxB and Cre cDNAs connected via internal ribosomal entry site (IRES)* **(B)**. *The onset of* PaxB *expression analyzed by whole mount RNA* in situ *hybridization at E9.5* **(C,C',C'')** *and E10.5* **(D,D',D'')** *embryos. PaxB mRNA was observed in the presumptive lens ectoderm and neuroectoderm* **(C'',D'')** *as early at E9.5. Cre recombinase activity in* PaxB *transgenic mice (detected using ROSA26R reporter mice) was delayed compared to PaxB mRNA expression* **(E-H'')**. *A mosaic expression of LacZ staining was detected at E9.5* **(E,E',E')**. *At E10.5 there was strong staining for PaxB mRNA in the presumptive lens* **(F,F',F'')**, *and at E13.5 high expression levels of Cre were observed in the distal retina* **(H,H',H'')**. *Please note that the lacZ-positive material in the forming vitreous cavity in (H'') represents a histology artifact. Abbreviations used in this, and in subsequent figures are as follows: el, eye lids; l, lens; lp, lens pit; lpl, lens placode; ov, optic vesicle; pce, presumptive corneal epithelium; pr, presumptive retina.*

decision leading to the generation of amacrine cells since its immunoreactivity can be detected in the subset of GABAergic amacrine cells (Lakowski *et al.*, 2007). Overexpression of Pax6ΔPD in the distal retina results in a microphthalmic phenotype (Kim and Lauderdale, 2006). In general, *Pax6* function depends on a proper expression level; any change in the dosage of Pax6 protein disrupts eye development (Schedl *et al.*, 1996; Collinson *et al.*, 2000; van Raamsdonk and Tilghman, 2000; Davis-Silberman *et al.*, 2005; Kim and Lauderdale, 2006). The expression of *Pax6* in lens is driven by an ectoderm enhancer (EE) (Williams *et al.*, 1998; Kammandel *et al.*, 1999). Targeted deletion of EE is accompanied by distinctive defects at every stage of lens development (Dimanlig *et al.*, 2001). In addition the exact dosage of Pax6 protein is required for lens placode formation (Collinson *et al.*, 2000; van Raamsdonk and Tilghman, 2000; Davis-Silberman *et al.*, 2005). Moreover *Pax6* activity in the lens primordium is necessary for correct placement of the retina in the eye (Ashery-Padan *et al.*, 2000). *Pax6* expression is maintained throughout retina development, from early optic vesicle formation to specification of neuroretina and differentiation and timing of distinct retinal cell types. Pax6 protein is abundant in proliferative zones of the neuroretina and subsequently in three types of retinal neurons: RGC, amacrine and horizontal cells. Null mutations in *Pax6* arrest optic vesicle formation early in eye development (Hogan *et al.*, 1986; Grindley *et al.*, 1995). Nevertheless retinal progenitor cells (RPCs) differentiate in the optic vesicle of *Pax6* mutant mice, earlier than in wild-type, although exhibiting reduced proliferation (Philips *et al.*, 2005). *Pax6* is known to control the proliferation rate of neuroepithelial progenitors (Duparc *et al.*, 2007) and retinal stem cells in the mouse optic vesicle (Xu *et al.*, 2007). Conditional inactivation of *Pax6* in RPCs of the distal retina prior to initiating retinogenesis resulted in reduced RPCs proliferation and exclusive differentiation of amacrine neurons (Marquardt *et al.*, 2001). By contrast, differentiating neurons were identified in the absence of amacrine cells in *Pax6* mutant optic vesicles of Sey^{1Neu} mice (Philips *et al.*, 2005).

It is apparent that in mice, *Pax6* and *Pax2* play distinct as well

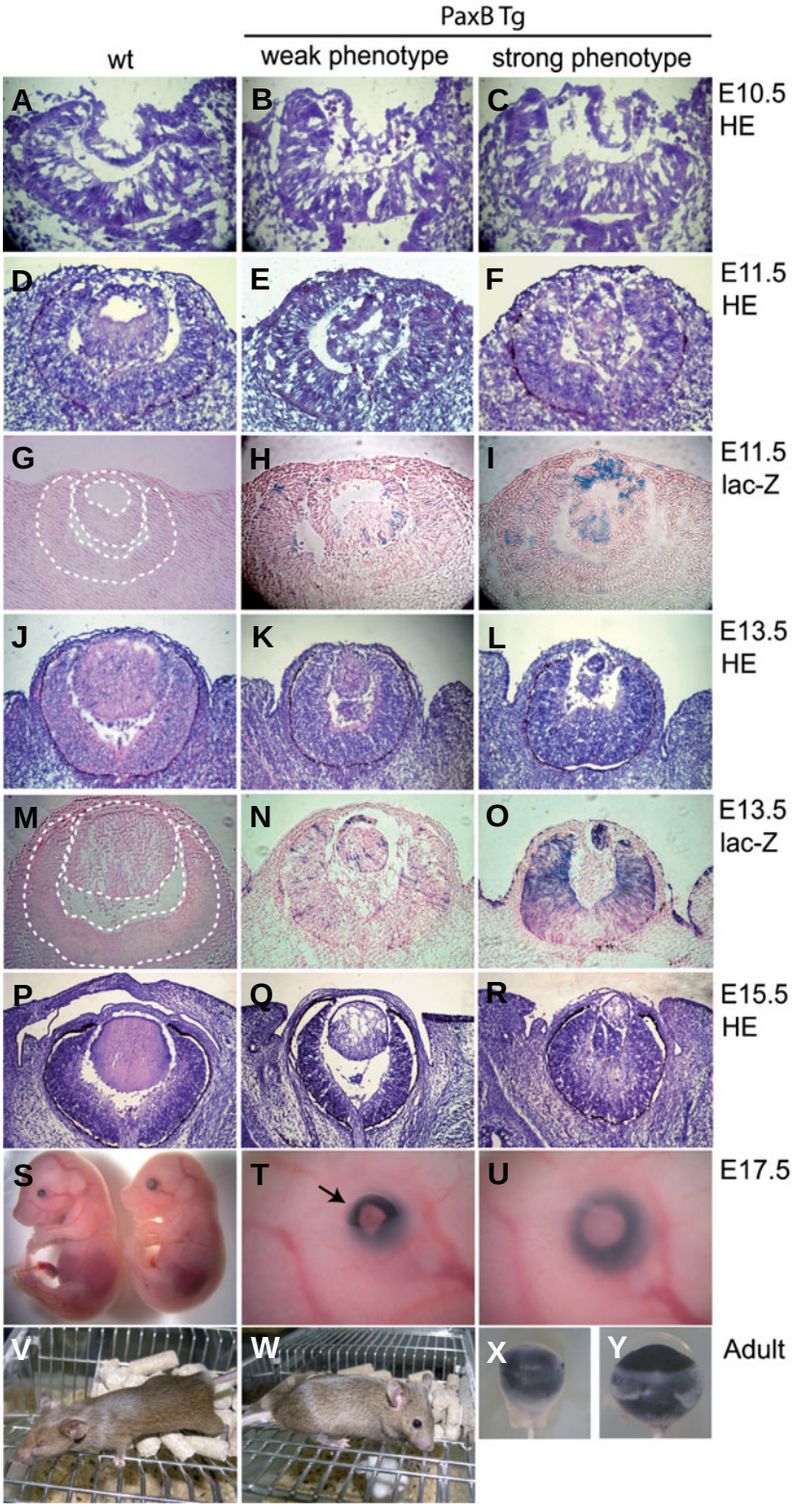


Fig. 2. Phenotypes of *PaxB* transgenic mice. *Cryosections at the indicated embryonic stages from the wild-type and* PaxB *transgenic mice were stained either with hematoxylin and eosin* **(A,B,C,D,E,F,J,K,L,P,Q,R)** *or lac-Z* **(G,H,I,M,N,O)**. *We sorted* PaxB *transgenic mice with weak and strong phenotypes based on the observed intensity of transgene expression* **(H,I,N,O)** *and lens defects. At E10.5 the transgenic lens pit* **(B,C)** *appeared similar to that in the wild-type mice* **(A)**. *At E11.5 and thereafter formation of the lens vesicle was delayed in* PaxB *mice, resulting in a smaller lens* **(F,I,L,O,R)**. *At E13.5 mosaic lacZ staining was detected both in the lens and retina* **(N)** *of the transgenic mice with weak phenotypes; by contrast, the transgenic mice with strong phenotypes stained mainly in the lens vesicle* **(I)** *at E11.5, however, at E13.5 strong staining was observed also in the distal retina and surface ectoderm* **(O)**. *A severe micropthalmic phenotype was revealed in the transgenic embryos* **(left in S;T,V,X)** *compared to the wild-type embryos* **(right in S;U,W,Y)** *at E17.5* **(S,T,U)** *and in adults* **(V,W,X,Y)**. *Note the open eye lids in the transgenic mice (marked with black arrow in T).*

as redundant roles during the eye development. *Pax6* is expressed in the developing mouse eye in the prospective lens, retina and pigmented epithelium and *Pax6* mutations cause several eye defects. *Pax2* is initially co-expressed with *Pax6* within the optic vesicle, but soon after invagination of the optic cup *Pax2* expression becomes restricted to the optic stalk (Nornes *et al.*, 1990; Torres *et al.*, 1996). In contrast to Pax6, the Pax2 protein can not be found in lens at any time during eye development. However, redundant activities of Pax2 and Pax6 specify the optic cup/optic stalk boundary (Schwarz *et al.*, 2000) and determinate the retinal pigmented epithelium (RPE) in the mouse eye (Baumer *et al.*, 2003). There are Pax2- and Pax6-binding sites on the retina enhancer of the *Pax6* gene and on the *Pax2* upstream control region (Schwarz *et al.*, 2000). The Pax2 protein cooperates with Vax transcription factors to repress *Pax6* expression in the ventral region of the optic vesicle and the optic stalk (Mui *et al.*, 2005).

Cnidaria are the most basal organisms which possess camera-type eyes and *Pax* genes (Kozmik *et al.*, 2003). PaxB protein of cubosoan jellyfish, *Tripedalia cystophora*, has *a Pax2*-like paired domain, and a Pax6-like homeodomain, and an octapeptide. Three amino acids (Q,R,H) responsible for DNA binding specificities of the *PaxB* paired domain are identical to those in the paired domain of Pax2/5/8, however the Pax6-like homeodomain of jellyfish PaxB allows it to activate the *Drosophila* rhodopsin promoter. Like Pax6, PaxB can induce small ectopic eyes in *Drosophila* (Kozmik *et al.*, 2003). PaxB also has Pax2 traits. It has the activation and inhibitory domains typical of the Pax2/5/8 subfamily of proteins and it rescues the *Drosophila* Pax2 eye mutant (Kozmik *et al.*, 2003). Taken together *PaxB* of jellyfish *Tripedalia cystophora* can carry on functions of both *Pax6* and *Pax2* in higher metazoans (Kozmik *et al.*, 2003). Here we investigate further the functional properties of jellyfish *PaxB* by expressing it in the developing lens and retina of transgenic mice.

Results

Generation of PaxB *transgenic mice*

To construct the *PaxB* transgene utilized here, three copies of mouse *Pax6* lens-specific element (also known as ectoderm enhacer EE) (Williams *et al.*, 1998) were cloned upstream of the *Pax6*P0 minimal promoter as described for LR-Cre mice (Kreslova *et al.*, 2007). We used this LR (lens, retina)-module to drive the expression of PaxB and Cre cDNAs connected via an internal ribosomal entry site (IRES) (Fig. 1B). Thus we were able to obtain mRNA encoding both PaxB and Cre recombinase in the same cell. To spatially and temporally define the region in which the transgene is active, we employed the Rosa26R reporter line (Soriano, 1999), in which Cre-mediated recombination results in the activation of β-galactosidase activity. In order to determine more precisely when the *PaxB* transcription starts we conducted whole-mount RNA *in situ* hybridisation tests with a *PaxB* riboprobe. A strong staining was observed as early as E9.5 in the presumtive lens ectoderm and neuroectoderm of the transgenic embryos (Fig. 1 C,C',C''). At E10.5 strong *PaxB* expression was observed in the lens pit and weaker staining in the prospective neuroretina (Fig. 1 D,D',D''). Moreover at this stage lens invagination was delayed in the transgenic embryos compared to the wild-type embryos (data not shown). β-galactosidase staining resulting from the Cre activity of the transgene was first observed in the area of the presumptive lens and surrounding head surface ectoderm of E10.5 transgenic embryos (Fig. 1 F,F'). At this stage the strongest expression was detected in the lens vesicle; weaker signal was visible in the presumptive retina and surface ectoderm (Fig. 1F''). At E13.5 both the maturing lens and the

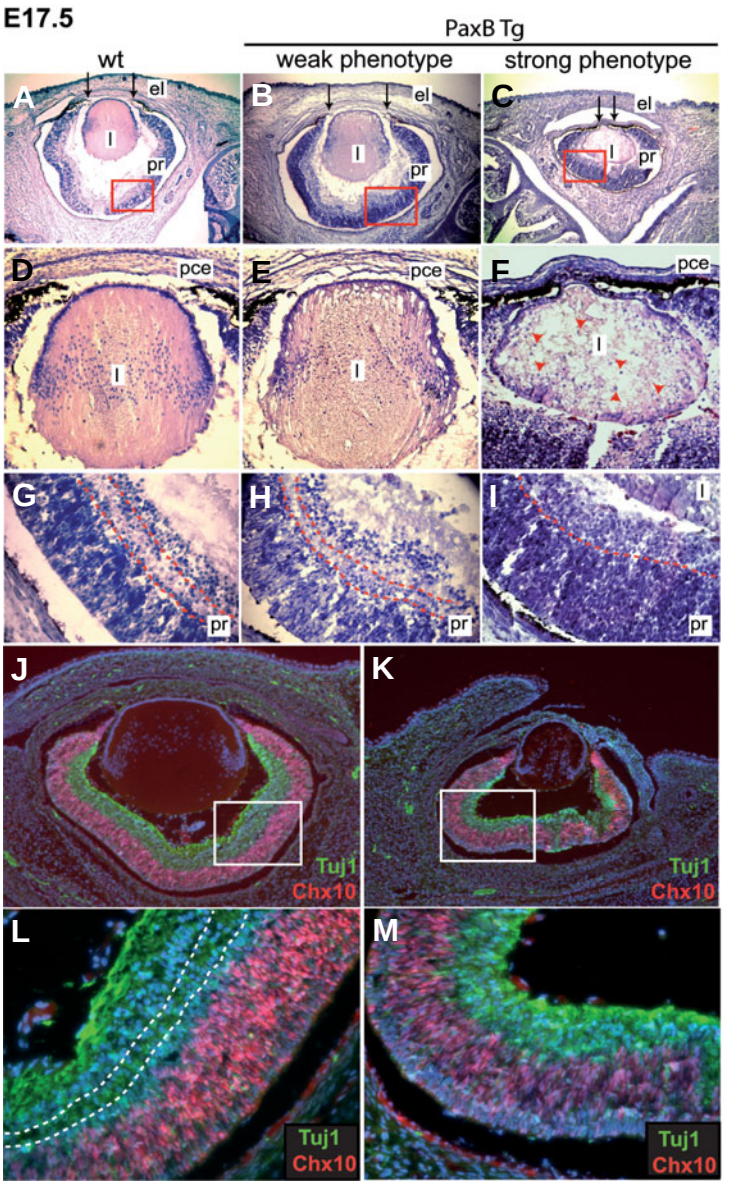


Fig. 3. Lens and retina defects detected at E17.5 in *PaxB* transgenic mice. *Cryosections of transgenic and wild-type embryos at E17.5 were stained with hematoxylin and eosin* **(A-I)**. *In addition to a smaller lens and whole eye size, there were several nuclei and vacuoles visible throughout the lens of the "strong" phenotypes* **(F)** *(marked with red arrowheads), and the diameter of the pupil was reduced in the transgenic mice* **(C)** *(marked with black arrows). The immunoreactivity of Vsx2 (Chx10) (red) and β-tubulin (Tuj1, green) was compared in the developing retina of wild-type* **(J,L)** *and transgenic mice with strong phenotypes* **(K,M)**. *Cell nuclei were stained with DAPI (blue). Higher magnification of the red* **(G,H,I)** *and white* **(L,M)** *boxed areas reveal the difference in lamination and and thickness of the presumptive retina.*

presumptive retina revealed intensive β-galactosidase staining and mosaic expression was detected in the presumptive RPE and cornea (Fig. 1 H,H',H'').

Phenotype of PaxB *transgenic mice*

The onset and the phenotypic consequences of *PaxB* expression in the eye were investigated at the histological level throughout embryonic development starting from day E10.5 (Fig. 2 A,B,C). At E10.5 the lens pit appeared normal both in wild-type and *PaxB* transgenic embryos. The first manifestation of abnormal eye development was observed at E11.5. The transgenic lens vesicle was smaller and the elongation of lens fibers was delayed (Fig. 2 E,F) compared to control embryos (Fig. 2D). We observed a phenotypic variability even within a single line of transgenic mice. This phenomenon is most likely a result of small stochastic differences in the transgene expression among individual transgenic embryos and an extreme dosage sensitivity of eye tissue to the level of *Pax6* gene (see bellow for explanation). Interestingly, the stronger the expression of the transgene (Fig. 2 H,I,N,O), the more affected was eye development (Fig. 2 E,F,K,L). Based on these observations we sorted the transgenic mice into weak and strong phenotypes. All the subsequent stages of lens development were morphologically abnormal in the transgenic embryos (Fig. 2 Q,R). At E17.5, the transgenic mice had smaller eyes with disrupted lenses and open eyelids (Fig. 2 S,T,U). Adult mice appeared microphthalmic (Fig. 2V) compared to wild-type littermates (Fig. 2W). Dissected eyes were smaller and revealed several abnormalities (Fig. 2 X,Y); furthermore eyes from one individual differed side-by-side (data not shown). Eyes of E17.5 wild-type and *PaxB* transgenic mice were also analyzed at the histological level (Fig. 3 A,B,C). The strong phenotypes had a much smaller, misshapen lens connected with the presumptive cornea (Fig. 3 C,F). Moreover there were many nuclei and vacuoles visible throughout the transgenic lens (Fig. 3F) compared to the wild-type lens which had nuclei confined to the equatorial layer (Fig. 3D). The diameter of the pupil was reduced in the severely affected transgenic mice (Fig. 3C, marked with black arrows). There were also differences in lamination and distribution of retinal progenitor cells in the transgenic retinas (Fig. 3 I,K,M) compare to the wild-type retinas (Fig. 3 J,L). No phenotype was observed in the negative control transgenic line in which the same LR regulatory module was used to drive Cre expression (Supplementary Fig. 1). Together, these observations demonstrate that the expression of *PaxB* strongly influences normal development of the lens, cornea and retina.

Impaired regulation of early lens development in PaxB *transgenic mice*

The lens defects in *PaxB* transgenic mice prompted us to examine the expression of *FoxE3* and *Mab21l1*, since mice with mutated *FoxE3* or *Mab21l1* have phenotypes that are similar to those of the *PaxB* transgenic mice, namely, a rudimentary lens and persistent connection between lens and cornea (Blixt *et al.*, 2000; Yamada *et al.*, 2003; Medina-Martinez *et al.*, 2005). Moreover, it is known that the early expression of *FoxE3* and *Mab21l1* is downregulated in Sey mice and therefore dependent on *Pax6* gene dosage (Yamada *et al.*, 2003; Blixt *et al.*, 2007).

FoxE3 expression was observed in the lens placode and in the brain of wild-type embryos (Fig. 4A). By contrast, *FoxE3* mRNA was not detected in the area of the invaginating lens in *PaxB* transgenic mice (Fig. 4A'). However, *FoxE3* expression in the *PaxB* transgenic brain was indistinguishable from the control littermate. At E11.5 there was notably weaker *FoxE3* staining in the lens remnant of the *PaxB* transgenic mice (Fig. 4B'), compared to the wild-type embryo (Fig. 4B). Similar reductions were observed for *Mab21l1* mRNA levels (Fig. 4 C,C').

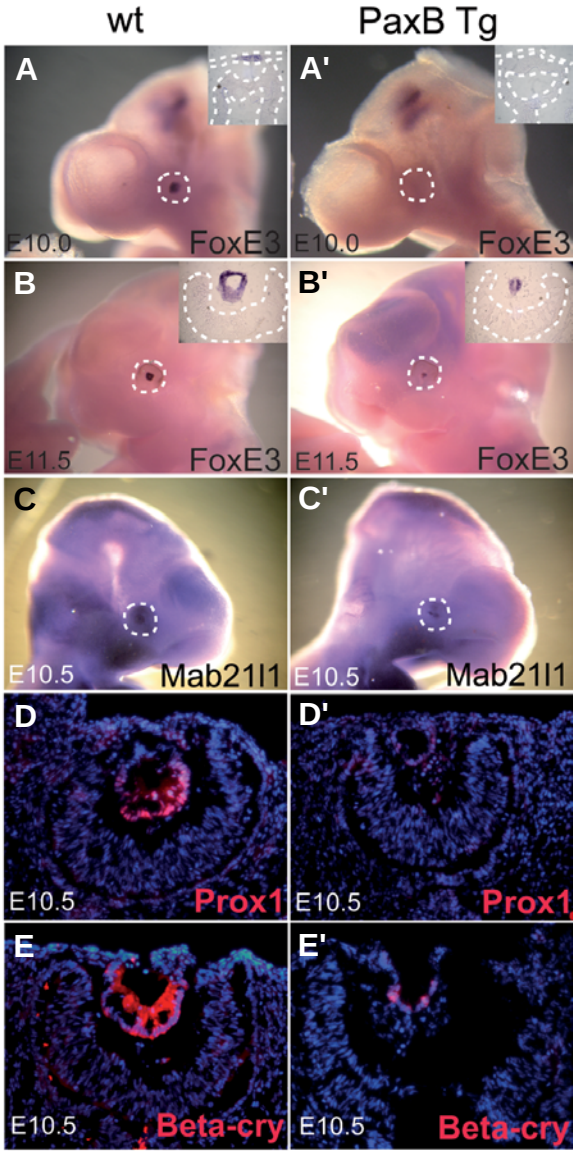
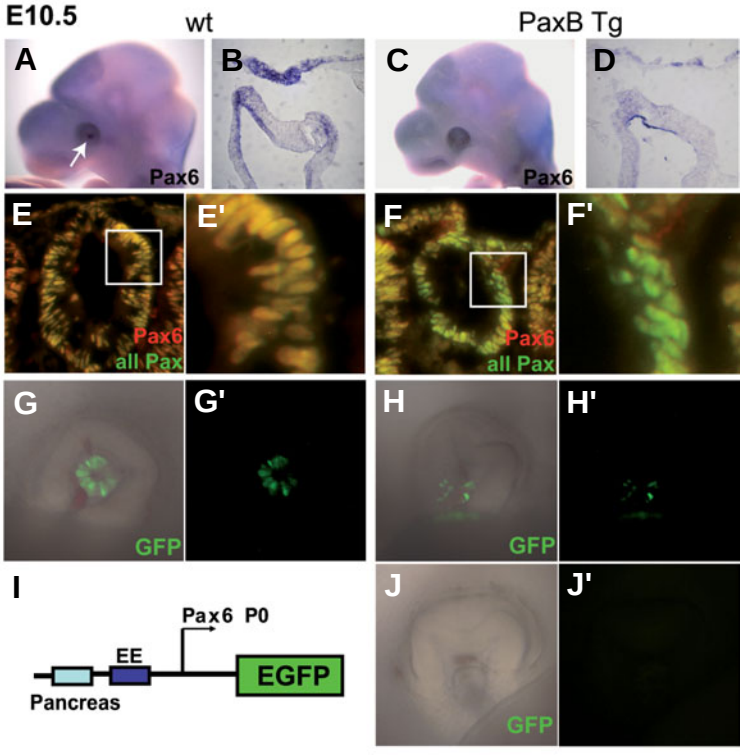


Fig. 4. Downregulation of lens specific markers in *PaxB* transgenic mice. *Whole mount RNA* in situ *hybridization* **(A–C')** *and RNA hybridization in sections (insets in A-B') at E10.0* **(A,A')**, *E10.5* **(C,C')** *and E11.5* **(B,B')** *in wild-type* **(A,B,C)** *and* PaxB *transgenic* **(A',B',C')** *embryos with probes for* FoxE3 **(A–B')** *and* Mab21l1 **(C,C')**. *Immunohistochemistry using anti-Prox1* **(D,D')** *and anti-β-crystallin* **(E,E')** *antibodies on sections from E10.5 embryos. Sections were counterstained with DAPI* **(D-E')**. *At E10.0* FoxE3 *was expressed in the presumptive lens ectoderm and in a restricted area of the brain* **(A)**. FoxE3 *mRNA was not detected in the ocular surface ectoderm of* PaxB *transgenic mice, whereas staining in the brain was unaffected* **(A')**. *At E11.5 low expression of* FoxE3 *was seen in the lens remnant of* PaxB *transgenic mice* **(B')**. *A similar reduction was observed for* Mab2111 *mRNA* **(C')** *and for immunoreactivity for Prox1* **(D')** *and β-crystallin* **(E')** *in the transgenic mice. Note the delayed formation of the lens pit in the* PaxB *transgenic mice* **(D',E')**.

To investigate if the decrease in *FoxE3* and *Mab21l1* levels reflected a general delay in activation of lens markers, we examined the expression of *Prox1*, which is essential for lens fiber differentiation and cell cycle exit (Wigle *et al.*, 1999), and of *β-crystallin*, which is a marker of lens fibers differentiation. At E10.5 the lenses of wild-type mice showed high immunoreactivity of Prox1 and β-crystallin (Fig. 4 D,E) whereas the *PaxB* transgenic mice had negligible staining for those markers (Fig.4 D',E'). Other lens markers were tested by immunohistochemistry at E11.5 (Supplementary Fig. 2) to further clarify the character and differentiation status of the lens of *PaxB* transgenic mice. Both Prox1 and c-Maf were abundant in the lens of control mice whereas their expression in the *PaxB* transgenic mice was much weaker (Supplementary Fig. 2 A,A',B,B'). Positive immunoreactivity in *PaxB* transgenic mice was detected for α-, β- and γ- crystallin proteins (Supplementary Fig. 2 C'-F') and MIP26 (Supplementary Fig. 2G') although in strikingly lower amount compared to wild-type mice (Supplementary Fig.2 C-G).



Effect of PaxB is independent of Six3 and Meis homeoproteins

PaxB transgenic mice were tested for endogenous *Pax6* and *Pax2* expression because these genes are necessary for spatial specification of eye territories (Schwarz *et al.*, 2000). E10.5 *PaxB* transgenic mice revealed no difference in either Pax6 or Pax2 staining (Supplementary Fig. 3B,D) compared to wild-type mice (Supplementary Fig. 3 A,C) consistent with a normal optic cup/optic stalk boundary. Next, we examined expression of the homeobox gene *Vsx2 (Chx10)* (Liu *et al.,* 1994), the earliest known marker specifically expressed in the proliferating retinal progenitor cells and subsequently in the bipolar cells (Burmeister *et al.*, 1996). Control and *PaxB* transgenic neuroretina did not show any difference in Vsx2 (Chx10) pattern (Supplementary Fig. 3 E,F).

To further explore the molecular basis for lens defects in *PaxB* transgenic embryos, we investigated the expression of Six3, Meis1 and Meis2 proteins, known transcription factors which direct lens development. Conditional knockout of *Six3* in the presumptive lens ectoderm disrupts lens formation and is accompanied by downregulation of *Pax6* (Liu *et al.*, 2006). Meis1 and Meis2 are direct regulators of Pax6 expression in prospective lens ectoderm (Zhang *et al.*, 2002). The expression of all these transcription factors did not change in the E10.5 *PaxB* transgenic eyes (Supplementary Fig. 3 H,J,L), compared to wild-type eyes (Supplementary Fig. 3 G,I,K).

PaxB *downregulates endogenous* Pax6 *expression in developing lens*

Our data indicated that *PaxB* expression downregulates genes encoding transcription factors downstream of the Pax6 regulatory network for lens development and disrupts lens development but does not affect genes upstream of

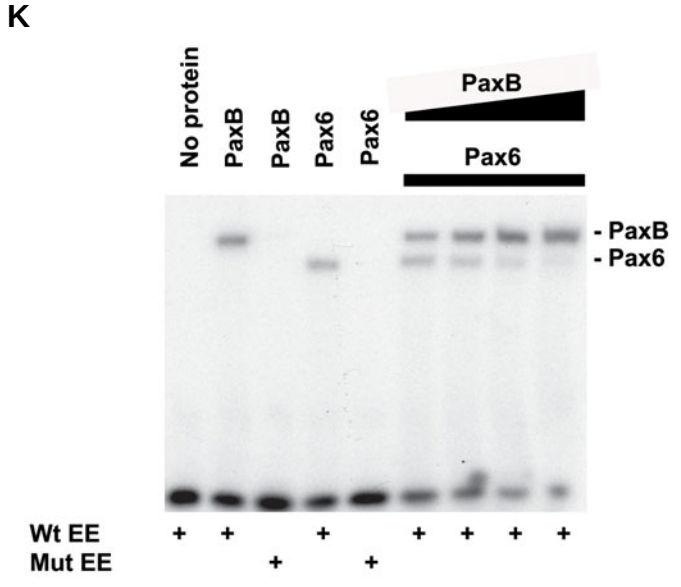


Fig. 5. Decreased levels of endogenous Pax6 in the *PaxB* transgenic lens. *All stainings were performed on E10.5 wild-type* **(A,B,E,E',G,G')** *and* PaxB *transgenic* **(C,D,F,F',H,H',J,J')** *embryos. Whole mount Pax6 RNA* in situ *hybridization* **(A-D)** *and RNA hybridizations were conducted using sections of developing eyes* **(B,D)**. *Lower levels of* Pax6 *mRNA were found in the presumtive lens ectoderm in* PaxB *transgenic mice* **(C,D)**. *Immunohistochemistry was performed on cryosections* **(E-F')** *using Pax6 (Covance; red) and anti-paired domain (staining all Pax genes; green) antibodies (E,F; white boxed areas was magnified in in E',F'). The lens vesicle and surface ectoderm of* PaxB *transgenic mice* **(F,F')** *revealed cells stained more in green than in red, which could be due to lower levels of endogenous* Pax6 *expression [compare with wild-type lens vesicle* **(E,E')***]. In order to test if PaxB repressed expression of* Pax6 *we employed transgenic mice* EE-EGFP *(MD63) in which EGFP was driven by the lens specific ectoderm enhancer (EE) and pancreas specific Pax6 enhancer* **(I)**. *EGFP fluorescence readily observed in wild-type embryos* **(G,G')** *was decreased* **(H,H')** *or absent* **(J,J')** *in* PaxB *transgenic embryos despite the presence of the lens tissue. Binding of mouse Pax6 and* Tripedalia *PaxB proteins to the wild-type but not mutated Pax6 autoregulatory element located within EE. Increasing amounts of PaxB protein are able to displace Pax6 from the autoregulatory element* **(K)**.

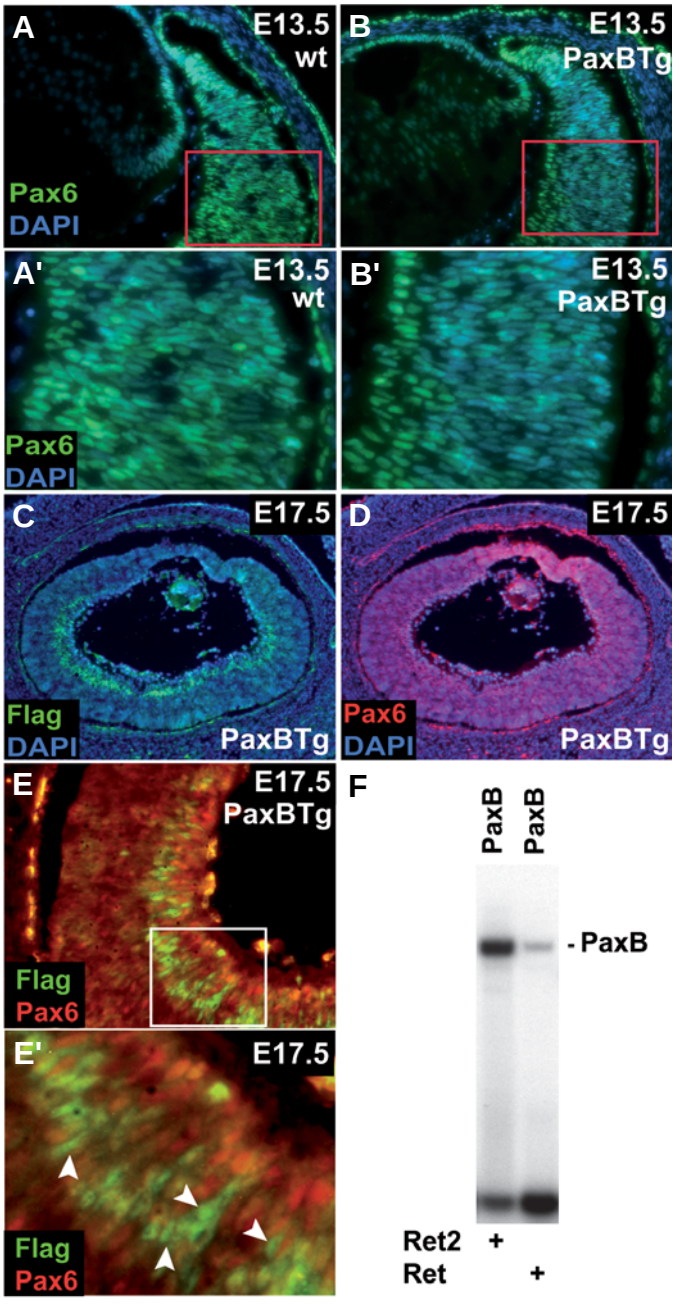


Fig. 6. Pax6 is repressed in the *PaxB* expressing cells. *The immunohistochemistry on tissue sections using anti-Pax6 antibody (green) at E13.5* **(A,B)** *(area in red rectangle was magnified in A',B'). All sections were counterstained with DAPI.* Pax6 *expression in the retina of* PaxB *transgenic animals* **(B,B')** *was diminished (visible as a weaker green staining (Pax6) as compared to wild-type retinas* **(A,A')***). Cryosections from* PaxB *transgenic mice from stage E17.5* **(C-E')** *were stained with Flag (PaxB; green), Pax6 (DSHB; red) and DAPI (blue). A transgenic eye section at E17.5 revealed strong Flag (PaxB) immunoreactivity in the inner nuclear layer of both the central and distal retina* **(C)** *relative to Pax6 expression* **(D)***. The area within the white rectangle was magnified in* **(E,E')***. White arrowheads point to cells expressing Flag (PaxB, green)* **(E')***; red color (Pax6) was absent in these cells, i.e. Pax6 staining was diminished in PaxB positive cells.* **(F)** *Binding of* Tripedalia *PaxB protein to Pax6 recognition sites Ret and Ret2 located within the mouse Pax6 retinal-specific α-enhancer.*

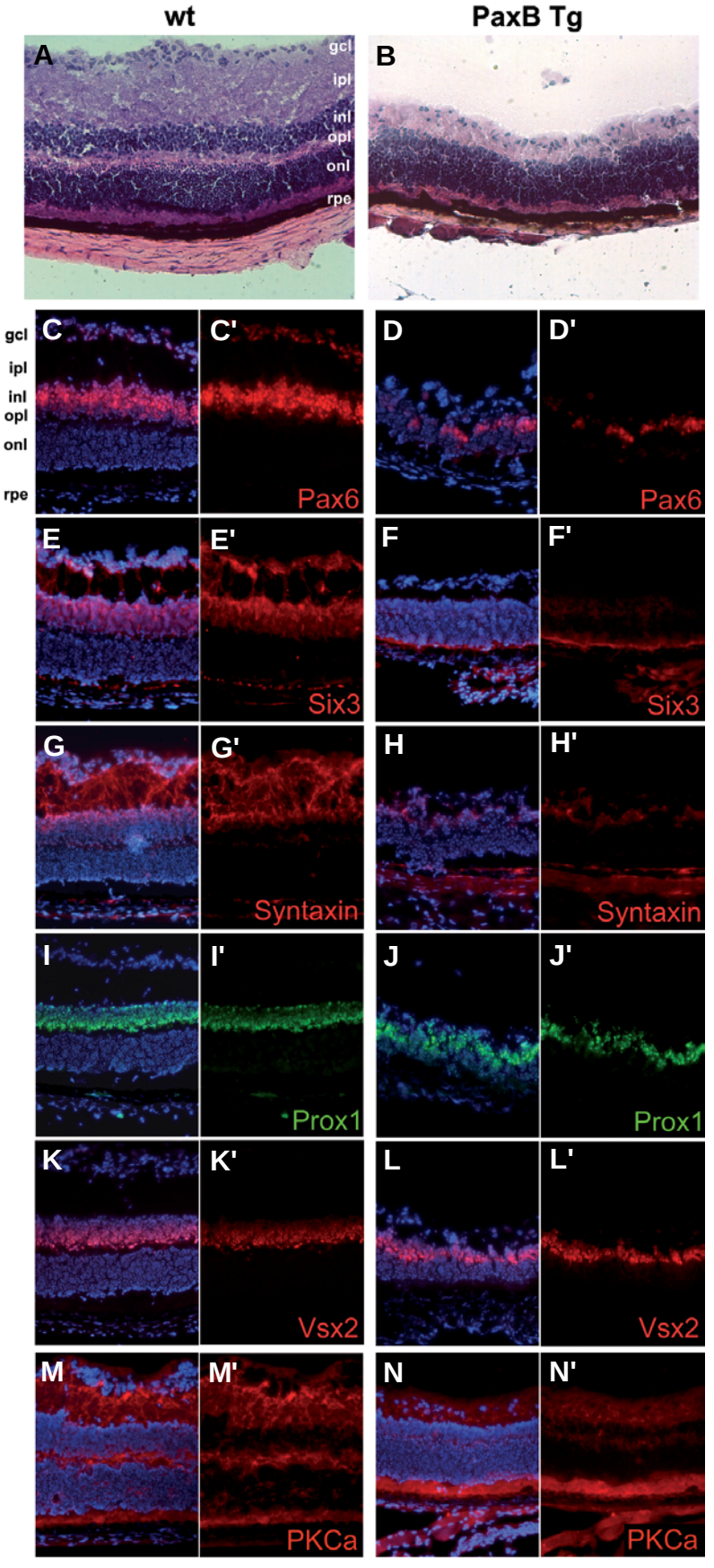
Pax6 within the pathway. We tested if *PaxB* expression directly influences the expression of an endogenous *Pax6*. First we examined *Pax6* expression by RNA *in situ* hybridization at E10.5. The staining for *Pax6* mRNA in the lens placode was markedly reduced in the *PaxB* transgenic embryos (Fig. 5 C,D) compared to wild-type embryos (Fig. 5 A,B). We next stained for Pax6 imunohistochemically using Pax6 specific and paired domain (PD) specific antibodies. The latter enabled us to detect all members of Pax family proteins (including PaxB, Pax6 and Pax2). At E10.5, *Pax6* is the only *Pax* gene expressed in the wild-type lens vesicle; *Pax2* at this timepoint is expressed exclusively at the optic vesicle/optic stalk boundary (Schwarz *et al.*, 2000). As expected, all the cells within the wild-type lens vesicle were stained by both anti-Pax6 (red) and anti-PD (green), which resulted in yelow staining (Fig. 5 E,E'). In contrast, we only detected PaxB together with Pax6 in the *PaxB* transgenic lens using anti-PD antibody (green) (Fig. 5 F,F'). We observed several green cells among yellow cells in the *PaxB* transgenic lens vesicle (Fig. 5 F,F'), indicating a decline of endogenous *Pax6* expression in the cells expressing PaxB protein. Combined, our data suggest that endogenous *Pax6* expression is downregulated in the *PaxB*-expressing cells of the lens vesicle.

Pax6 expression during lens development is regulated by the ectoderm enhancer (EE) (Williams *et al.*, 1998; Kammandel *et al.*, 1999; Xu *et al.*, 1999). To test if PaxB could downregulate *Pax6* via this element, we employed another transgenic mouse *EE-EGFP* (MD63), where the EE element of *Pax6* was used to drive EGFP to the developing lens (Fig. 5I). After crosssing the *EE-EGFP* (MD63) and *PaxB* transgenic mice, we assessed EGFP levels at E10.5. EGFP intensity was decreased or completely abrogated in the lens of PaxB transgenic mice (Fig. 5H,H',J,J') as compared to the control mice (Fig. 5G,G'). These results support the idea that PaxB downregulates *Pax6* gene expression and suggest that this suppression acts through the ectoderm enhancer (EE) of *Pax6*.

Since *Pax6* expression in the lens placode involves an autoregulatory mechanism (Aota *et al.*, 2003) we tested if PaxB is able to bind to the *Pax6* autoregulatory binding site within the EE. As shown in Fig. 5K, PaxB binds to this autoregulatory site and is able to displace Pax6 protein from it. Our data thus suggest that in lens PaxB suppresses *Pax6* expression presumably through a direct competition for the *Pax6* autoregulatory element within EE.

PaxB influences the development of neuroretina in the PaxB *transgenic mice*

In addition to the lens phenotype, there is also a phenotype in the neuroretina of the *PaxB* transgenic mice. As mentioned above *PaxB* expression driven by three copies of EE of the *Pax6* gene was detected in the presumptive retina as early as E9.5 (Fig. 1C") and later, at E13.5, in the distal retina (Fig. 2O). At E13.5 reduced *Pax6* expression was detected in *PaxB* transgenic retina (Fig. 6 A,B). At perinatal stages the expression of *PaxB* transgene was detected in the inner nuclear layer of both the central and distal retina (Fig. 6C). We believe that this region may represent an abberant expression domain of the 3xEE regulatory module. Such interpretation is consistent with the observation that weak expression of Le-Cre transgene (Ashery-Padan *et al.*, 2000) is present in the retina exemplified by the fact that in Le-Cre; Z/AP double transgenic mice the recombination was detected in the



vitreal side of the P4 retina (Davis-Silberman *et al.*, 2005). At E17.5 histology and immunoreactivity of the *PaxB* transgenic retina (Fig. 3 I,K,M) were markedly different from the retina of wild-type animals (Fig. 3 G,J,L). To further characterize *PaxB* expressing cells we took advantage of a FLAG epitope present in the *PaxB* transgene. Strong staining with anti-FLAG antibody was observed at E17.5 in subsets of cells within the inner nuclear layer (INL), both in the distal and central retina of the *PaxB* transgenic mice (Fig. 6C). Moreover double-staining with anti-Pax6 antibody (red) showed a decrease in endogenous *Pax6* expression in the cells expressing the *PaxB* transgene (green) (Fig. 6E,E'). Pax6 is known to regulate its own expression through binding to retina-specific α-enhancer located in intron 4 of *Pax6* gene (Schwarz *et al.*, 2000). We tested if PaxB is able to bind to Ret and Ret2 binding sites described previously (Schwarz *et al.*, 2000). As shown in Fig. 6F, PaxB binds to both of these elements suggesting that PaxB suppresses *Pax6* expression presumably through a direct competition.

We next compared the histology of adult wild-type and *PaxB* transgenic retinas. Compared to the well-organized wild-type retina (Fig. 7A), the retina of *PaxB* transgenic mice was thinner with no proper lamination and the outer plexiform layer (OPL) was missing (Fig. 7B). To investigate the expression of cell-type specific markers we performed several immunohistochemistry stainings on one month old *PaxB* transgenic and control retinas. First we investigated expression of Pax6, which is a marker of both amacrine and retinal ganglion cells (RGCs) and some horizontal cells (Marquardt *et al.*, 2001). The number of Pax6 possitive cells was markedly decreased in the transgenic retina (Fig. 7 D,D'); few cells were stained in the fused remnant of inner- (INL) and outer nuclear layer (ONL) and no staining occured in the GCL (compared to wild-type retina Fig. 7 C,C'). Six3, a homeobox transcription factor specifying amacrine cells in the INL and displaced amacrines in the GCL (Oliver *et al.*, 1995), was absent in the transgenic (Fig. 7 F,F') but not the wild-type retina (Fig.7E,E'). The immunoreactivity of another marker for all amacrine cells, syntaxin (HPC-1), was undetect-

Fig. 7. Characterization of adult retina in *PaxB* transgenic mice. *Cryosections of adult retina from the wild-type (left panels) and PaxB transgenic mice (right panels) were stained either with hematoxylin and eosin* **(A,B)** *or with antibodies against the following proteins: Pax6 (Covance,* **C-D'***), Six3* **(E-F')***, syntaxin* **(G-H')***, Prox1* **(I-J')***, Vsx2 (Chx10)* **(K-L')** *and PKCα* **(M-N')***. The nuclei were counterstained with DAPI. The* PaxB *transgenic retina was notably thinner with no proper lamination* **(B)***; outer (onl) and inner (inl) nuclear layers were not separated. Immunohistochemistry showed reduced staining of Pax6* **(D,D')***, Six3* **(F,F')** *and syntaxin* **(H,H')** *indicating the absence of amacrine cells. The number of bipolar and horizontal cells did not appear to be affected in the* PaxB *transgenic retina as revealed by detection of Vsx2 (Chx10), Prox1 and PKCα. Abbreviations for distinct retinal layers described in this and subsequent figures are as follows: gcl-ganglion cell layer, ipl-inner plexiform layer, inl-inner nuclear layer, opl-outer plexiform layer, onl-outer nuclear layer, rpe-retinal pigment epithelium.*

able in the transgenic retina (Fig. 7 H,H'). The levels of Prox1, normally found in horizontal, bipolar and some amacrine cells (Dyer *et al.*, 2003), and bipolar cell markers Vsx2 (Chx10) (Burmeister *et al.*, 1996) as well as PKCα, were comparable between transgenic and control mice (Fig. 7 I-N'). In addition to Pax6, three other markers were used for staining of RGCs; Brn3b (Xiang *et al.*, 1995), OLF-1 (Davis and Reed, 1996) and β-tubulin (TUJ1). No immunoreactivity was observed for any of these proteins in the *PaxB* transgenic mice (Fig. 8 A-F'). The presence of photoreceptors was examined by using several markers: CRX (cone-rod homeobox containing gene) (Zhu and Craft, 2000), NR2e3 (protoreceptor specific nuclear receptor), M-opsin (M-cone photoreceptors) (Zhu *et al.*, 2003), S-opsin (S-cone photoreceptors) (Zhu *et al.*, 2003), rhodopsin (rod-photoreceptors), arrestin (rod-photoreceptors), GRK1 (rhodopsin kinase) (Results are summarized in Supplementary Fig. 4). Of all of these tested markers only rhodopsin was reduced in the *PaxB* transgenic retina (Supplementary Fig. 4 K-L').

Taken together, our data suggest that amacrine cells and RGC's were missing in *PaxB* expressing retinas. To test whether the absence of amacrine cells was due to reduced cell numbers in the INL of the transgenic retina, we sought the presence of amacrine cells using the specific markers Meis2, Calretinin, GAD65/67 (glutamic acid decarboxylase) and GlyT-1 (glycine transporter 1). Expression of homeodomain-containing transcription factor Meis2 was recently described in a subpopulation of GABAergic amacrine cells (Bumsted-O'Brien *et al.*, 2007). GABAergic amacrine cells also express GAD65/67 (Haverkamp and Wassle, 2000). Calretinin (calcium-binding protein) selectively binds A2 cells which is the most common subtype of amacrines (MacNeil and Masland, 1998). Glycinergic amacrine cells were detected using Glyt-1 antibody. None of these markers were observed in the *PaxB* transgenic retina (Fig. 8 G-N').

In summary, we provide evidence that endogenous expression of *Pax6* is suppressed by PaxB in the optic vesicle of the transgenic mice, affecting development of the retina and resulting in the absence of amacrine cells and decrease of RGC's.

Discussion

Eye development in invertebrates and vertebrates depends upon the regulated expression of *Pax6* and *Pax2* (see Introduction). However, the cubozoan jellyfish *T. cystophora*, which has *PaxB* instead of *Pax2* or *Pax6*, has sophisticated lens-containing eyes that share numerous

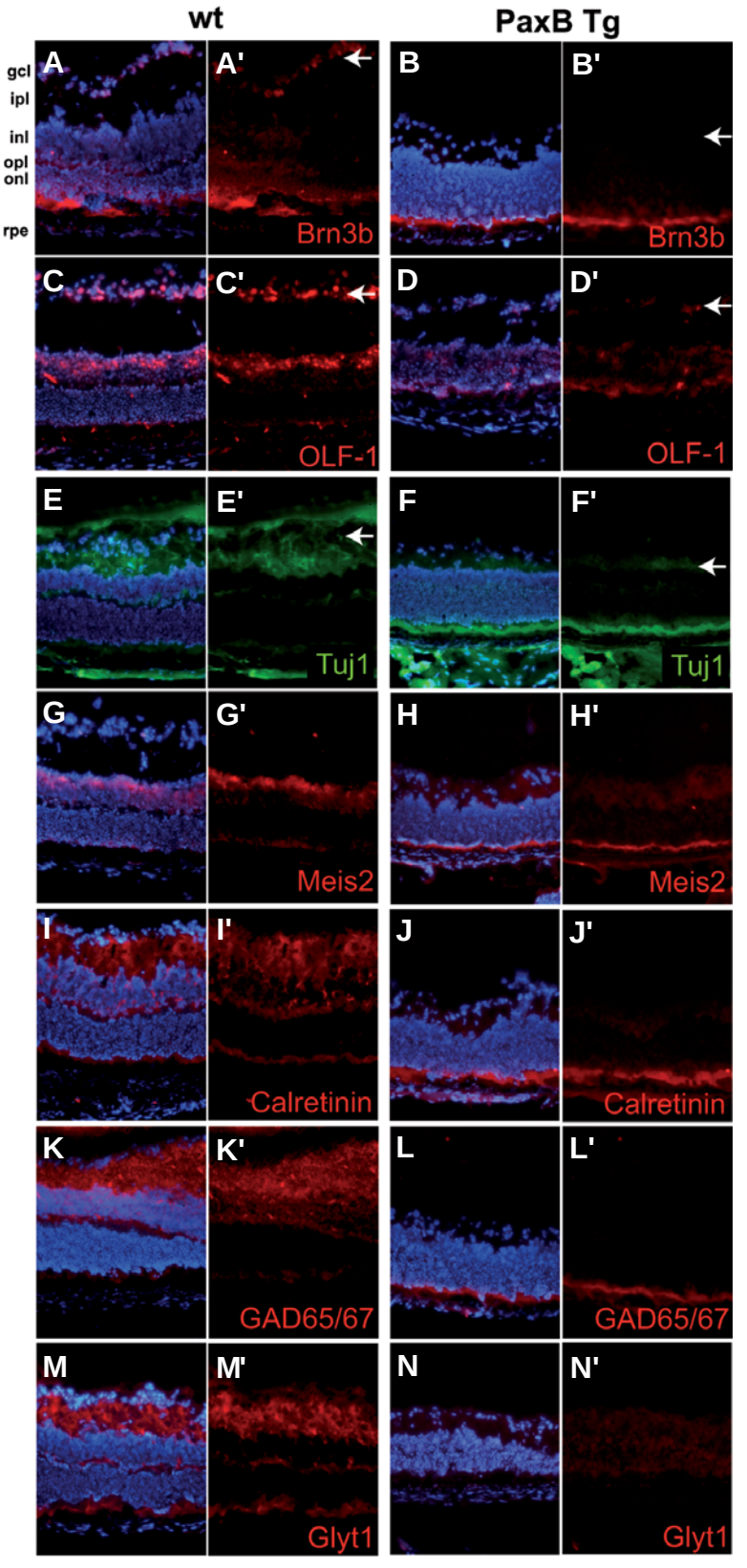


Fig. 8. *PaxB* expression supresses amacrine cell fate in adult retina. *Cryosections of adult retina from the wild-type (left panels) and PaxB transgenic mice (right panels) were stained using antibodies against the following proteins: Brn3b* **(A-B')**, *OLF-1* **(C-D')**, *Tuj1 (β-tubulin,* **E-F'***), Meis2* **(G-H')**, *calretinin* **(I-J')**, *GAD65/67 (glutamic acid decarboxylase,* **K-L'***), Glyt1 (glycine transporter 1,* **M-N'***). The nuclei were counterstained with DAPI. The immunoreactivity of retinal ganglion cells was diminished in* PaxB *transgenic retinas (Brn3b, OLF-1 and Tuj1; gcl is marked by white arrow), although some cells remained within the ganglion cell layer (visible with DAPI). The lack of amacrine cells in the* PaxB *transgenic retina was confirmed by staining with the following amacrine-subpopulation markers: Meis2 and GAD65/67 (GABAergic), Glyt1 (Glycineric) and calretinin (A2 amacrine cells).*

characteristics with vertebrate eyes (Kozmik *et al.*, 2008). Jellyfish PaxB has a Pax2-like paired domain and octapeptide and a Pax6-like homeodomain potentially giving it functional properties of both Pax2 and Pax6. Indeed, it has been shown that jellyfish *PaxB* can induce ectopic eyes, like *Pax6*, and can substitute for *Pax2* in *Drosophila* (Kozmik *et al.*, 2003); moreover, *PaxB* is expressed in the jellyfish eye (Kozmik *et al.*, 2003). In the present study we found that unlike its combined Pax2 and Pax6-like properties in *Drosophila*, PaxB neither directs eye development nor substitutes for Pax6 for eye development in transgenic mice. Indeed, *PaxB* expression in transgenic mice creates microphthalmic eyes with abnormal lens and retinal phenotypes. In the *PaxB* transgenic mice PaxB is co-expressed with the Cre recombinase in the lens and retina. It is highly unlikely that any observed phenotype is due to Cre expression since none of these defects was detected in the LR-Cre mice (Kreslova *et al.*, 2007) (Supplementary Fig. 1).

Our data indicate that one of the reasons why *PaxB* disrupts mouse eye development is that it suppresses *Pax6* gene expression. *PaxB* suppression of *Pax6* expression is consistent with the presence of a Pax6 autoregulatory site in the Pax6 EE enhancer required for lens expression (Kammandel *et al.*, 1999; Aota *et al.*, 2003). Moreover, the retinal α-enhancer of *Pax6* contains autostimulatory Pax6 sites that drive *Pax6* expression in the retina (Kammandel *et al.*, 1999; Schwarz *et al.*, 2000). Pax2 cooperates with homeodomain containing proteins Vax1 and Vax2 to repress the *Pax6* α-enhancer (Mui *et al.*, 2005). We propose that the Pax2-like and Pax6-like structural properties of PaxB enable it to interact with the autoregulatory enhancers of the endogenous *Pax6*, preventing normal expression of this critical gene for eye development. Lowered levels of Pax6 are known to prevent the normal development of mouse eyes (Collinson *et al.*, 2000; van Raamsdonk and Tilghman, 2000; Dimanlig *et al.*, 2001). In addition, heterozygous *Pax6* mutations are responsible for the Small eye (Sey) phenotype in mice and for aniridia and Peters' anomaly in humans (Hill *et al.*, 1991; Ton *et al.*, 1991; Glaser *et al.*, 1992; Hanson *et al.*, 1994). Likewise, increased levels of Pax6 were shown to result in various eye abnormalities (Schedl *et al.*, 1996; Kim and Lauderdale, 2006; Kim and Lauderdale, 2008; Manuel *et al.*, 2008). However, none of the phenotypes described previously is identical to the phenotype generated by misexpression of *PaxB*.

The phenotypes in the abnormal eyes of the *PaxB* transgenic mice support the idea that PaxB suppresses endogenous *Pax6* expression in lens. First, the present findings that the genes acting downstream of Pax6 during lens development encoding Mab2111, FoxE3, Prox1 and crystallins are reduced, while expression of *Six3*, *Meis1* and *Meis2*, all genes upstream of Pax6, are unaffected in the lenses of the *PaxB* transgenic mice. Second, targeted disruption of *FoxE3*, whose expression critically depends on Pax6 (Blixt *et al.*, 2007) results in lens defects resembling the phenotype of *PaxB* transgenic mice (Blixt *et al.*, 2000; Medina-Martinez *et al.*, 2005). Moreover delayed lens development in *PaxB* transgenic mice resembled the smaller lens pit or vesicle of Pax6 $^{Sey/+}$ heterozygote and Pax6 $^{\Delta EE/\Delta EE}$ mice (van Raamsdonk and Tilghman, 2000; Dimanlig *et al.*, 2001). Finally, EE contains a Pax6 autoregulatory site (Aota *et al.*, 2003) which can be supressed either by direct binding of PaxB or by lower levels of Pax6. These data suggest that *PaxB* suppresses the expression of *Pax6* in the developing lens, making *Pax6* dosage insufficient for normal lens proliferation and induction of downstream transcription factors (FoxE3, Mab21l1 and Prox1) and crystallin genes. Lens degeneration due to low *Pax6* levels has been shown using different mouse models (Collinson *et al.*, 2000; van Raamsdonk and Tilghman, 2000; Dimanlig *et al.*, 2001).

Pax6 is the only Pax protein known to be expressed in the mouse lens. The situation is different in the retina. At early stages of eye development *Pax2* is co-expressed with *Pax6* in the optic vesicle, and during optic nerve formation *Pax2* becomes resticted to the ventral neuroretina surrounding the closing optic fissure (Nornes *et al.*, 1990; Baumer *et al.*, 2003). The presence of *Pax2*-binding sites on the retina enhancer of the *Pax6* gene and co-transfection experiments revealed reciprocal inhibition of *Pax6* enhancer activity by Pax2 (Schwarz *et al.*, 2000). It is unlikely, however, that the retina phenotype observed in *PaxB* transgenic mice is solely due to the downregulation of *Pax6*. Conditional deletion of the *Pax6* gene using α-Cre results in the exclusive generation of amacrine cells (Marquardt *et al.*, 2001) while *PaxB* transgenic mice exhibit a complete depletion of amacrine cell population in retina (see below). Therefore it appears that PaxB is able to elicit a dominant phenotype in the retina by means of its modified DNA-binding specificity and/or altered transactivation potential.

It follows that the distinct molecular properties of Pax6 are required for normal mouse eye development and this role of Pax6 can not be substituted by an ancestral PaxB protein. A comparison of properties between Pax6 and PaxB transcription factors gives a partial hint towards the molecular mechanism underlying the observed phenotype. Both PaxB and Pax6 have two independent DNA-binding domains, the paired domain and the paired-type homeodomain, respectively. Pax6 and PaxB paired domains interact with very similar DNA sequences although some differences in specificity exist. In particular, three amino acids (at positions 42, 44, and 47) within the N-terminal half of paired domain are responsible for the difference in the DNA-binding specificities between Pax6 and PaxB/Pax2/5/8 subfamilies (Czerny and Busslinger, 1995; Kozmik *et al.*, 2003). However, so far these amino acid differences have been shown to restrict the DNA-binding specificity of Pax6 rather than expand it (Czerny and Busslinger, 1995; Kozmik *et al.*, 2003). This opens up a formal possibility that in the PaxB transgenic mice PaxB inappropriately regulates retinal genes not normally regulated by Pax6. Unlike the situation with paired domain, the DNA-binding specificity of paired-type homeodomain of Pax6 and PaxB is likely shared as it is defined as a palindromic sequence composed of two inverted TAAT motifs. Both Pax6 and PaxB act as transcriptional activators when tested in the cell culture system. Pax6 contains a C-terminal transactivation domain composed of short regions that act in synergy with each other (Tang *et al.*, 1998). PaxB includes in its C terminus adjacent activation and inhibitory domains, a characteristic of Pax2/5/8 (Dorfler and Busslinger, 1996; Lechner and Dressler, 1996; Kozmik *et al.*, 2003). The nature of co-factors interacting with the inhibitory region is unknown. However, the inhibitory region of Pax2/5/8 does not seem to be an autonomous unit as it is unable to impose an inhibition on a heterologous activation domain (Dorfler and Busslinger, 1996) suggesting that it might function through

intramolecular interaction within an intact Pax2/5/8.

The only well-recognizable structural element outside of the DNA binding domains is the octapeptide motif which mediates efficient interaction of PaxB and Pax2/5/8 with groucho co-repressors (Eberhard *et al.*, 2000; Kozmik *et al.*, 2003). By this mechanism Pax2/5/8 proteins are converted from transcriptional activators to transcriptionl repressors (Eberhard *et al.*, 2000). Combined, our data suggest that groucho-mediated repression by PaxB at sites normally bound by wild type Pax6 may be responsible, at least in part, for the observed retinal phenotype (loss of amacrine cells). A new transgenic line expressing a modified Pax6 (including an artificially fused octapeptide motif) will be necessary to provide a more definitive evidence for such a hypothesis.

The present investigation provides evidence that amacrine and retinal ganglion cells (RGCs) were absent and/or reduced in the adult retina of *PaxB* transgenic mice. This was consistent with downregulation of endogenous *Pax6* in the cells expressing *PaxB* in the inner layer of the optic cup at E17.5. Moreover PaxB immunoreactivity in the developing neuroretina was observed as early at E9.5 (Fig. 1 C-D''), consistent with the retina being affected early in development. It is known that *Pax6* is required for the multipotent state of retinal progenitor cells (RPCs) and that upon *Pax6* inactivation in the distal retina only amacrine interneurons are generated from RPCs at the expense of other cell types (Marquardt *et al.*, 2001). It is also known Pax6 is required for cell proliferation in the optic vesicle and for RPCs to adopt specific retinal cell fates (Philips *et al.*, 2005; Duparc *et al.*, 2007). Amacrine cells do not differentiate in *Pax6*-null optic vesicles, where the proneural gene *Mash1* is expressed instead of *NeuroD1* (Philips *et al.*, 2005). For the differentiation of each retinal cell type specific transcription factors have to be co-expressed in the RPCs. As retinal neurons differentiate, they exit the cell cycle and migrate towards the inner (vitreal) side of the optic cup. Distinct members of the basic helix-loop-helix (bHLH) family of transcription factors specify the cell fate within a developing mouse retina (Livesey and Cepko, 2001). With respect to the present study it is noteworthy that Pax6 directly regulates genes encoding Ngn2, Mash1 and Math5 (Marquardt *et al.*, 2001; Hufnagel *et al.*, 2007). For RGC formation both *Math5* and *Pax6* are required (Brown *et al.*, 2001; Marquardt *et al.*, 2001; Wang *et al.*, 2001; Hutcheson *et al.*, 2005; Mu *et al.*, 2005). RGCs are formed at late E12.5/ early E13.0 stage when they appear within a central domain of the retina and are marked by strong expression of Pax6. The early progenitor cells that co-express *Math3* and *NeuroD1* (Inoue *et al.*, 2002) together with *Pax6/Six3/Prox1* (Dyer *et al.*, 2003) or *Pax6/Six3/Lim1* (Liu *et al.*, 2000) adopt the amacrine or horizontal cell fate. Pax6 is normally expressed by both amacrine cells and RGCs in postnatal retinas (Davis and Reed, 1996). In *PaxB* transgenic mice we were not able to detect several markers of amacrine cells: syntaxin, calretinin, GAD65/67, which was accompanied by lower levels of *Pax6*, *Six3* and *Meis2* (Figs. 7, 8). Furthermore, RGCs (visualised with Brn3b and OLF-1 antibodies), which normally express Pax6, were reduced. Together, these data suggest that in *PaxB* expressing mice differentiation of amacrine and retinal ganglion cells was impaired due to downregulation of endogenous *Pax6* in the cells within the central domain of neuroretina where the differentiation of RPCs takes place.

Gene swapping experiments have demonstrated functional redundancy between transcription factor family members and have indicated that spatial and temporal differences in expression of related transcription factors are often more crucial than differences in biochemical activities of the corresponding proteins. For example, in mid-hindbrain development En1 mutant phenotype was rescued by replacing En1 with closely related En2 (Hanks *et al.*, 1995). Likewise, the *Drosophila* orthologue engrailed substituted for mouse En1 function in mid-hindbrain, but not in limb development (Hanks *et al.*, 1998). In the retina, the closely related transcription factors Pou4f1 and Pou4f2 appear interchangeable in their ability to to regulate RGC differentiation if they are expressed from the *Pou4f2* locus (Pan *et al.*, 2005). An extensive functional equivalency was demonstrated for Pax2 and Pax5 during mouse development. The *Pax5* minigene, when expresed from the *Pax2* locus, was able to substitute for Pax2 function in the midbrain, cerebellum, inner ear and genital tracts (Bouchard *et al.*, 2000). In addition, *Pax5* was able to rescue most but not all *Pax2* mutant defects in the developing eye and kidney, that are highly sensitive to Pax2 protein dosage. Despite this redundancy between transcription factor family members, the present study shows that jellyfish *PaxB* interferes with the function of mouse *Pax6*. Expression of jellyfish *PaxB* in the lens and retinal of transgenic mice downregulated expression of endogenous *Pax6*, disturbed the transcriptional network regulated normally by Pax6, and yet it was unable to take over the developmental functions of Pax6 in these eye tissues. Taken together, our data suggest that, unlike the situation in *Drosophila* (Kozmik *et al.*, 2003), in mice the molecular properties of Pax2 and Pax6 are essential determinants of mouse eye development and cannot be substituted for the chimeric PaxB protein of jellyfish.

Materials & Methods

Mouse lines

PaxB transgenic mouse was constructed by ligating three copies of the Pax6 EE (Williams *et al.*, 1998) to the P0 minimal promoter of the *Pax6* gene. This regulatory element (3xEE-P0) was fused to a cassete containing FLAG-tagged PaxB and Cre linked via IRES to generate *PaxB* transgenic construct. *PaxB* transgenic mice were generated by microinjection of linearized DNA and were maintained in a C57/Bl6 inbred background. Transgenic mice were identified by PCR analysis of genomic DNA from the tail. Primers used were as follows:
forward, CAACCAATGAGGGCATTGCTGGCG;
reverse, CGTTGCATCGACCGGTAATGCA (349A/349B).
Three founder mice exhibited a microphthalmic phenotype. One of them (MB05-8) was used to establish a *PaxB* transgenic line described here. Analysis of the Cre-mediated recombination pattern in *PaxB* trangenic line was performed by mating to the ROSA26R reporter line as described (Soriano, 1999). The ROSA26R mice were purchased from Jackson Lab (stock #003309). LR-Cre line was described previously (Kreslova *et al.*, 2007). *Pax6* reporter line *EE-EGFP* (MD63) was constructed by ligating the 5kb upstream region of Pax6 including the EE (Williams *et al.*, 1998) to the EGFP cassete including SV40 intron/polyA sequences. *EE-EGFP* (MD63) transgenic mice were generated by microinjection of linearized DNA and were maintained in a C57/Bl6 inbred background. Transgenic mice were identified by PCR analysis of genomic DNA from the tail using EGFP-specific primers:

forward, ACGTAAACGGCCACAAGTTC;
reverse, AAGTCGTGCTGCTTCATGTG (785A/785B).

Tissue collections and histology

Mouse embryos were obtained from timed pregnancy matings, with noon of the date that the vaginal plug was observed defined as embryonic day 0.5 (E0.5). Embryos were harvested in cold PBS, fixed in either 4% paraformaldehyde for various times ranging from 1h to overnight (hematoxylin-eosin staining, immunohistochemistry) or 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$ and 5 mM EGTA for 1h (for X-gal staining). Fixed embryos were cryoprotected in 30% sucrose overnight at 4°C, embedded and frozen in OCT (Tissue Freezing Medium, Jung). Horizontal frozen sections were done at 6-8 μm thickness. The cryosections were washed three times in PBS and subsequently stained with an antibody or hybridized with RNA antisense probes.

X-gal staining

The β-galactosidase assay was carried out as described by (Hogan *et al.*, 1986). After the fixation, the cryosections were directly stained with the staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.3, and 1 mg/ml X-gal). For whole-mount stainings, fixed embryos were washed three times in rinse buffer (0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 20 mM Tris pH7.3, 0.01% sodium deoxycholate and 0.02% Nonidet P-40), and incubated overnight at 37°C in staining solution.

Immunohistochemistry

The cryosections were re-fixed for 10 min. in 4% paraformaldehyde, washed with PBS, permeabilized with PBS/0.1%Tween20 (PBT) for 15 min. prior to blocking. Sections were blocked for 30 min. in 10%BSA/PBT, incubated overnight with primary antibodies at room temperature or 4°C, washed three times with PBS, incubated 40 minutes at room temperature with secondary antibodies, washed three times with PBS, exposed 5 minutes to DAPI/PBS and mounted in glycerol. Primary antibodies and sera used were: anti-α-crystallin (a gift of Sam Ziegler, NEI), anti-β-crystallin (a gift of Sam Ziegler, NEI), anti-γ-crystallin (a gift of Hisato Kondoh), anti-βA3-crystallin (a gift of Hisato Kondoh), anti-MIP26 (a gift of Joe Horwitz), anti-β-tubulin (TUJ-1, R&D Systems), anti-Chx10 (Vsx2) (Exalpha Biologicals), anti-Pax6 (Covance), anti-Pax6 (DSHB), anti-Prox1 (Chemicon), anti-PD808 (Kozmik *et al.*, 2003), anti-Pax2 (Zymed), anti-Six3 (a gift of Paola Bovolenta), anti-Meis1 (a gift of Arthur Buchberg), anti-Meis2 (a gift of Arthur Buchberg), anti-Flag (OctA, Santa Cruz), anti-syntaxin (Sigma), anti-PKCα (Sigma), anti-Brn3b (Santa Cruz), anti-OLF-1 (a gift of Randall Reed), anti-calretinin (Sigma), anti-GAD65/67 (Sigma), anti-Glyt1 (Chemicon), anti-cMaf (Santa Cruz), anti-CRX (a gift of Cheryl Craft) (Zhu and Craft, 2000), anti-NR2e3 (p183, a gift of Shiming Chen), anti-M-opsin (a gift of Cheryl Craft) (Zhu *et al.*, 2003), anti-S-opsin (a gift of Cheryl Craft) (Zhu *et al.*, 2003), anti-arrestin (a gift of Dale Gregerson), anti-rhodopsin (Sigma), anti-GRK1 (Sigma). The following secondary antibodies were used: Alexa-488- or 594-conjugated goat anti-mouse or anti-rabbit IgG, Alexa-594-conjugated donkey anti-goat IgG (Molecular Probes).

Whole-mount* In situ *hybridization

Plasmid was linearized with appropriate restriction enzyme and antisense riboprobe was synthesized using the DIG RNA labeling kit (Roche).

Fixed embryos were washed three times in PBS/0.1%Tween20 (PBT) and thereafter bleached with methanol: 30% H_2O_2 (4:1) for 20 min at RT. After three times washing in PBT, Proteinase K (0.05 U/ml PBT) was applied for 15 min at RT. The reaction was stopped with two washes (5 min each) of glycin solution (2 mg/ml PBT). Glycin was washed out with PBT and embryos were refixed with 0.2% glutaraldehyde/4% paraformaldehyde for 20min at RT. The embryos were then rinsed two times in PBT and were prehybridized for 90 min at 70°C in prehybridization solution (50% formamide, 5×SSC pH 4.5, 5 mM EDTA, 0.1%Tweeen20 with 0,05 mg/ml Heparin). After that half of the prehybridization solution was replaced with prewarmed hybridization solution (50% formamide, 5×SSC pH 4.5, 5 mM EDTA, 0.1%Tweeen20, 50 μg/ml Heparin, 50 μg/ml tRNA and 50 μg/ml herring sperm DNA). Meanwhile digoxigenin-labeled riboprobe in 100 μl hybridization solution was denaturated for 10 min at 75-80°C. The denatured probe was added to the samples (final concentration 0.5-1 μg/ml) and incubated overnight at 70°C. After the hybridization unbound riboprobe was washed two times (30min each) in prewarmed (70°C) solution I (50% formamide, 4×SSC, 1% SDS). Then the samples were equilibrated in solution I/II (1:1) for 10 min at 70°C. Afterwards samples were washed three times (5 min each) at 70°C in solution II (0.5 M NaCl, 10mM Tris-Cl pH 7.5, 0.1% Tween20) and were treated with RNaseI in the solution II twice per 30min at RT. After that embryos were washed twice (30min each) in prewarmed (70°C) solution III (50% formamide, 2xSSC). Following steps were three washes in TBST (130 mM NaCl, 2.7mM KCl, 25mM Tris-Cl pH 7.5, 0.1% Tween20) with 2 mM Levamisole. Blocking was performed in 10% heat inactivated sheep serum in TBST for 2hours at RT. After that embryos were incubated overnight with anti-digoxigenin-AP Fab fragments (Roche, 1:1000) in 10% heat inactivated sheep serum in TBST at RT. Unbound anti-digoxigenin Fab fragments were removed by extensive washing in TBST with 2 mM Levamisole. After that embryos were rinsed twice for 20 min in alkaline phosphatase staining buffer (100 mM NaCl, 100 mM Tris-Cl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween20). Staining was performed with 20 μl NBT/BCIP Stock Solution (Roche) in 10 ml staining buffer at dark and RT. Reaction was stopped with several washes 1 mM EDTA in PBT. Plasmid carrying following cDNAs were used: mouse Pax6 (provided by Peter Gruss), mouse FoxE3 (provided by Peter Carlsson) (Blixt *et al.*, 2000), mouse Mab21l1 (provided by Giacomo Consalez), and *Tripedalia cystophora* PaxB (Kozmik *et al.*, 2003).

Electroforetic mobility shift assay (EMSA)

EMSA with the full-length FLAG-tagged PaxB and Pax6 was performed as previously described (Kozmik *et al.*, 2003) using double-stranded oligonucleotides comprising autoregulatory Pax6 binding site from lens EE or retina-specific α-enhancer binding sites Ret and Ret2 (Schwarz *et al.*, 2000).

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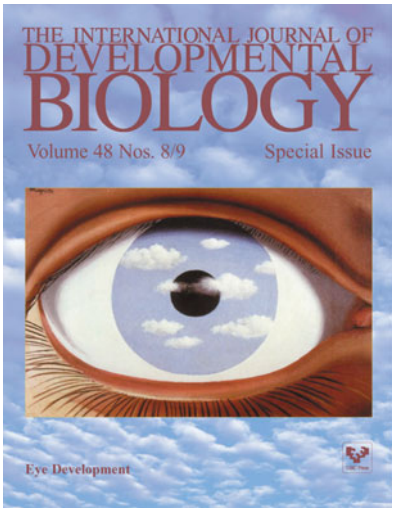

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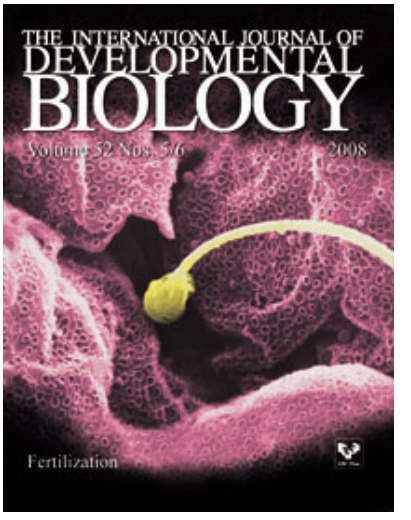

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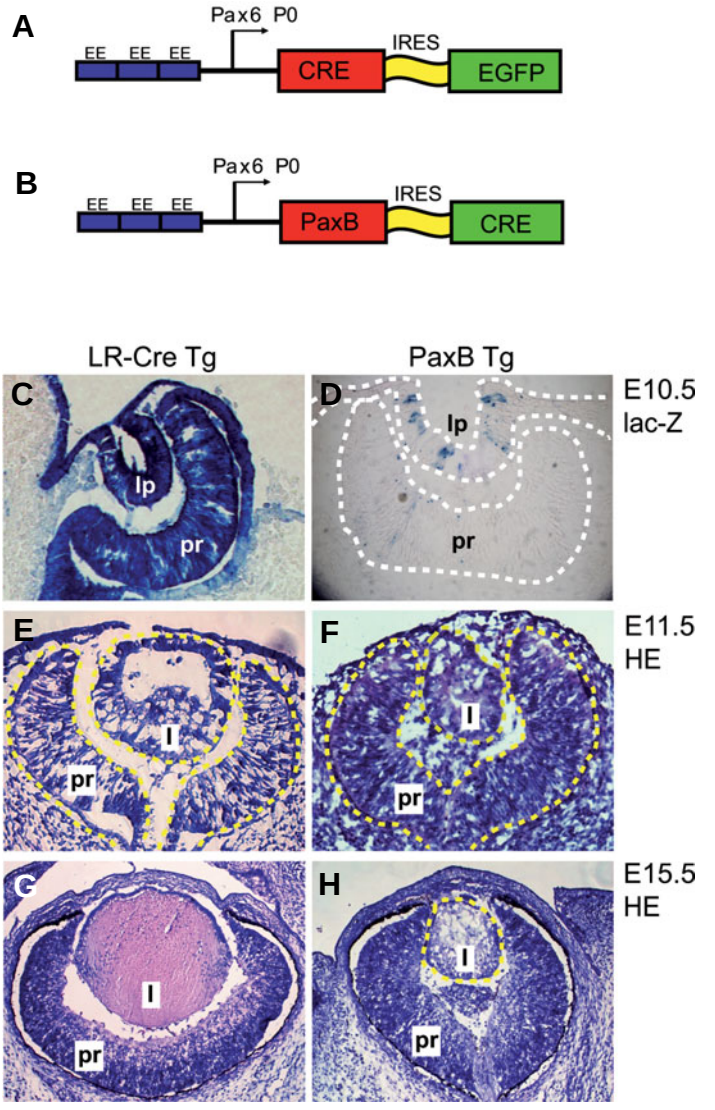
SUPPLEMENTARY MATERIAL

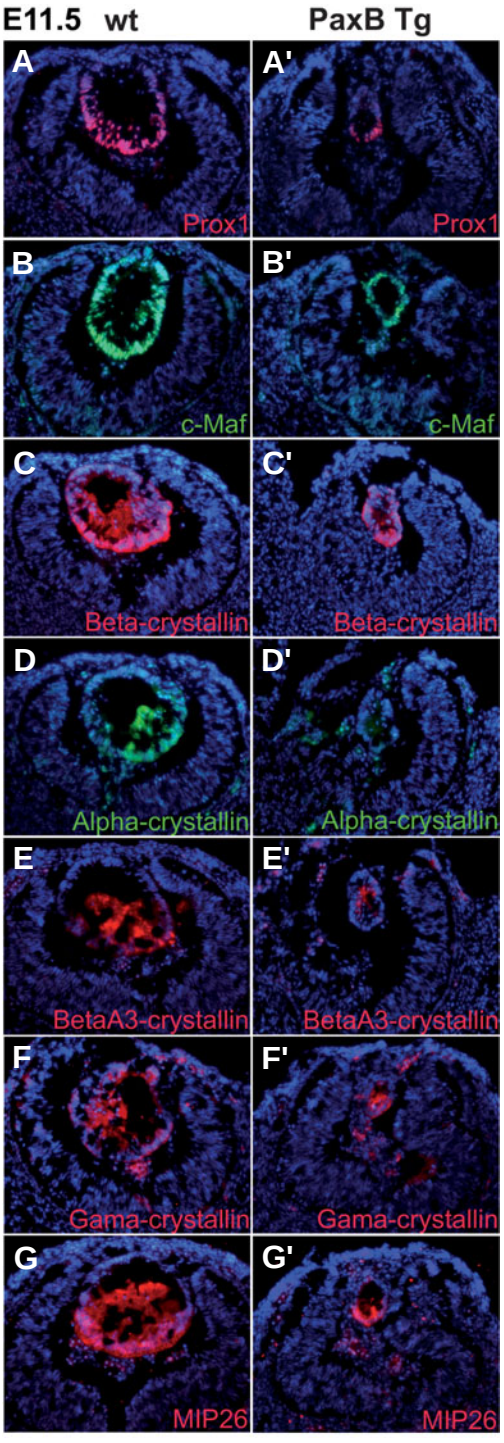
corresponding to:

Eye-specific expression of an ancestral jellyfish *PaxB* gene interferes with *Pax6* function despite its conserved Pax6/Pax2 characteristics

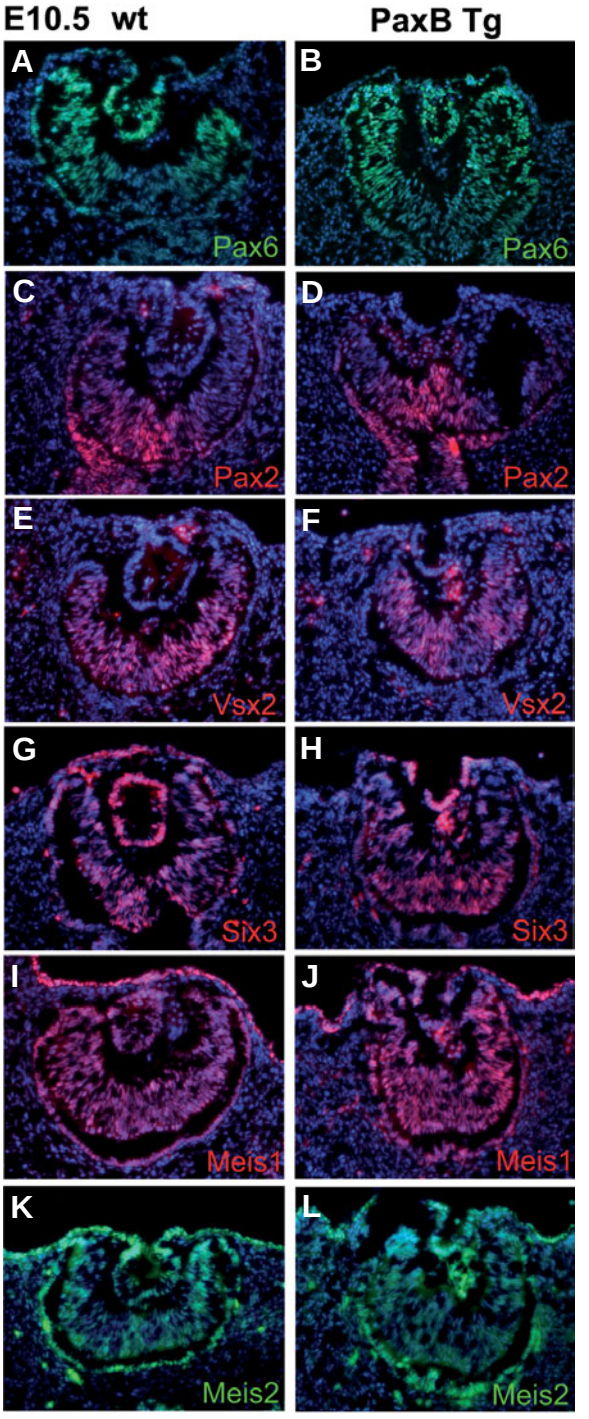
JANA RUZICKOVA, JORAM PIATIGORSKY and ZBYNEK KOZMIK



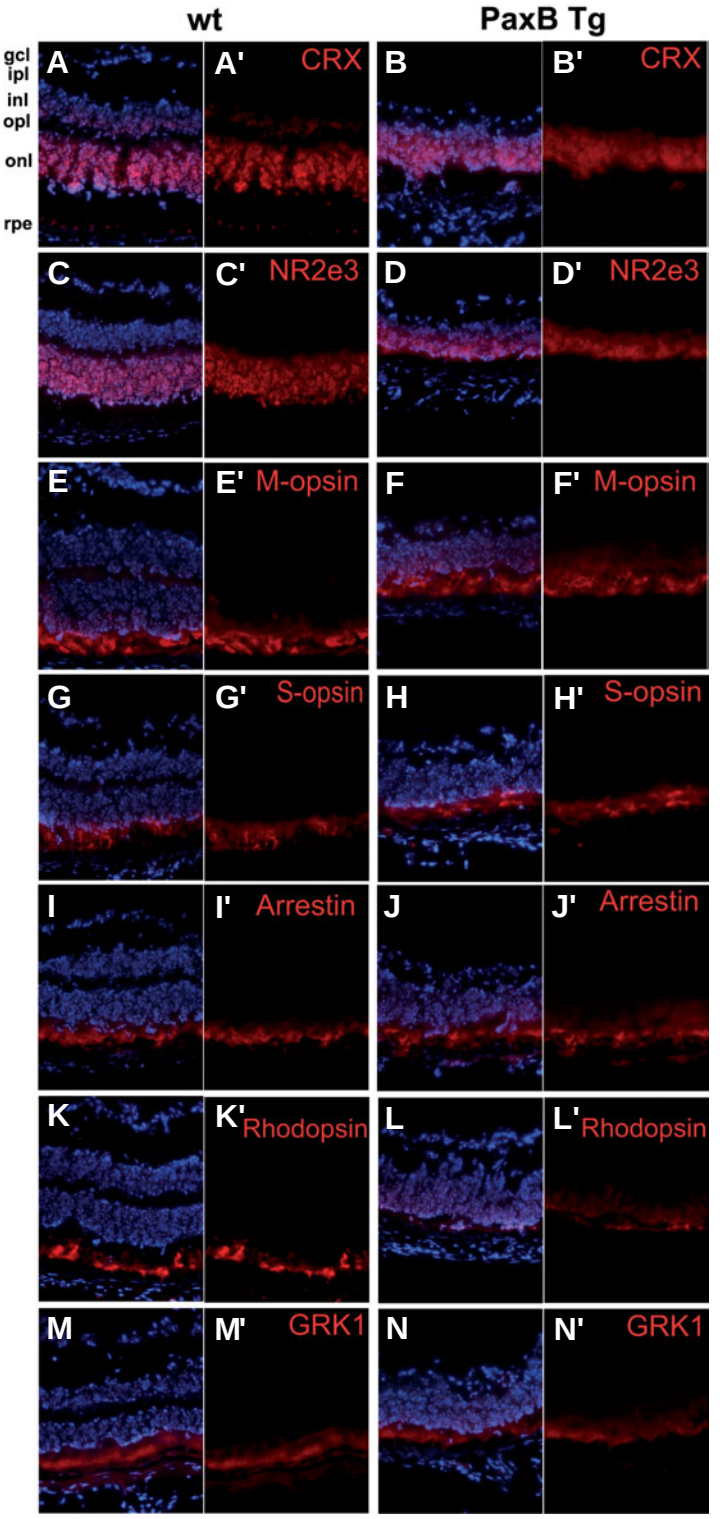
Supplementary Fig. 1. Normal gross morphology of the control LR-Cre transgenic eyes. *Schematic diagram of LR-Cre* **(A)** *and PaxB* **(B)** *transgenic constructs. Three copies of the lens-specific ectoderm enhancer (EE) derived from the* Pax6 *gene were cloned upstream of the Pax6 P0 minimal promoter to drive the expression of Cre and EGFP* **(A; LR-Cre Tg)** *or the expression of PaxB and Cre cDNAs* **(B; PaxB Tg)** *in bicistronic constructs via internal ribosomal entry site (IRES). Cre recombinase activity in LR-Cre* **(C)** *and* PaxB *transgenic* **(D)** *mice (detected using ROSA26R reporter mice) at E10.5. In comparison to* PaxB *transgenic mice the Cre recombinase activity in LR-Cre transgenic mice is much stronger in all developing eye tissues. Note the intensive staining in the presumptive retina of LR-Cre transgenic mice. Cryosections at the indicated embryonic stages from the LR-Cre* **(E,G)** *and* PaxB *transgenic* **(F,H)** *mice stained with hematoxylin and eosin. The morfology of E11.5 and E15.5 LR-Cre transgenic eyes appears normal as compared to* PaxB *transgenic mice despite stronger expression of Cre. Abbreviations used in this are as follows: l, lens; lp, lens pit; pr, presumptive retina.*



Supplementary Fig. 2. Lens specific transcription factors and crystallins are reduced in *PaxB* transgenic mice. *Cryosections of wild-type* **(A,B,C,D, E,F,G)** *and* PaxB *transgenic* **(A',B',C',D', E',F',G')** *embryos at E11.5 stained for Prox1* **(A,A')**, *cMaf* **(B,B')**, *β-crystallin* **(C,C')**, *α-crystallin* **(D,D')**, *βA3-crystallin* **(E,E')**, *γ-crystallin* **(F,F')** *and MIP26* **(G,G')**, *and counterstained with DAPI (blue). Lower immunoreactivity of all above mentioned markers and smaller lens was observed in the* PaxB *transgenic mice.*



Supplementary Fig. 3. Transcription factors important for the eye development were expressed normally in *PaxB* transgenic mice. *Cryosections of wild-type* **(A,C,E,G,I,K)** *and PaxB transgenic* **(B,D,F,H,J,L)** *embryos at E10.5 were stained for Pax6 (Covance,* **A,B***), Pax2* **(C,D)***, Vsx2 (Chx10)* **(E,F)***, Six3* **(G,H)***, Meis1* **(I,J)** *and Meis2* **(K,L)***, counterstained with DAPI (blue). Pax6, Pax2 and Vsx2 (Chx10) staining appeared normal in the* PaxB *transgenic mice. No difference was observed in the expression Six3, Meis1 and Meis2, transcription factors acting upstream of Pax6.*



Supplementary Fig. 4. The photoreceptor cells were not affected in the *PaxB* transgenic retina. *Immunostaining of the* PaxB *transgenic* **(right panels)** *and wild-type* **(left panels)** *adult retinal sections. Applied antibodies were as follows: CRX (cone-rod homeobox containing gene,* **A-B'***), NR2e3 (protoreceptor specific nuclear receptor,* **C-D'***), M-opsin (M-cone photoreceptors,* **E-F'***), S-opsin (S-cone photoreceptors,* **G-H'***), arrestin (rod-photoreceptors,* **I-J'***), rhodopsin (rod-photoreceptors,* **K-L'***), GRK1 (rhodopsin kinase,* **M-N'***). The nuclei were counterstained with DAPI. No difference was apparent in the photoreceptor cells using a variety of photoreceptor markers between wild-type and* PaxB *transgenic retinas. Abbreviations used in this figure are as follows: gcl, ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; onl, outer nuclear layer; opl, outer plexiform layer; rpe, retinal pigment epithelium.*