Int. J. Dev. Biol. 53: 469-482 (2009)
doi: 10.1387/ijdb.082793jr

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

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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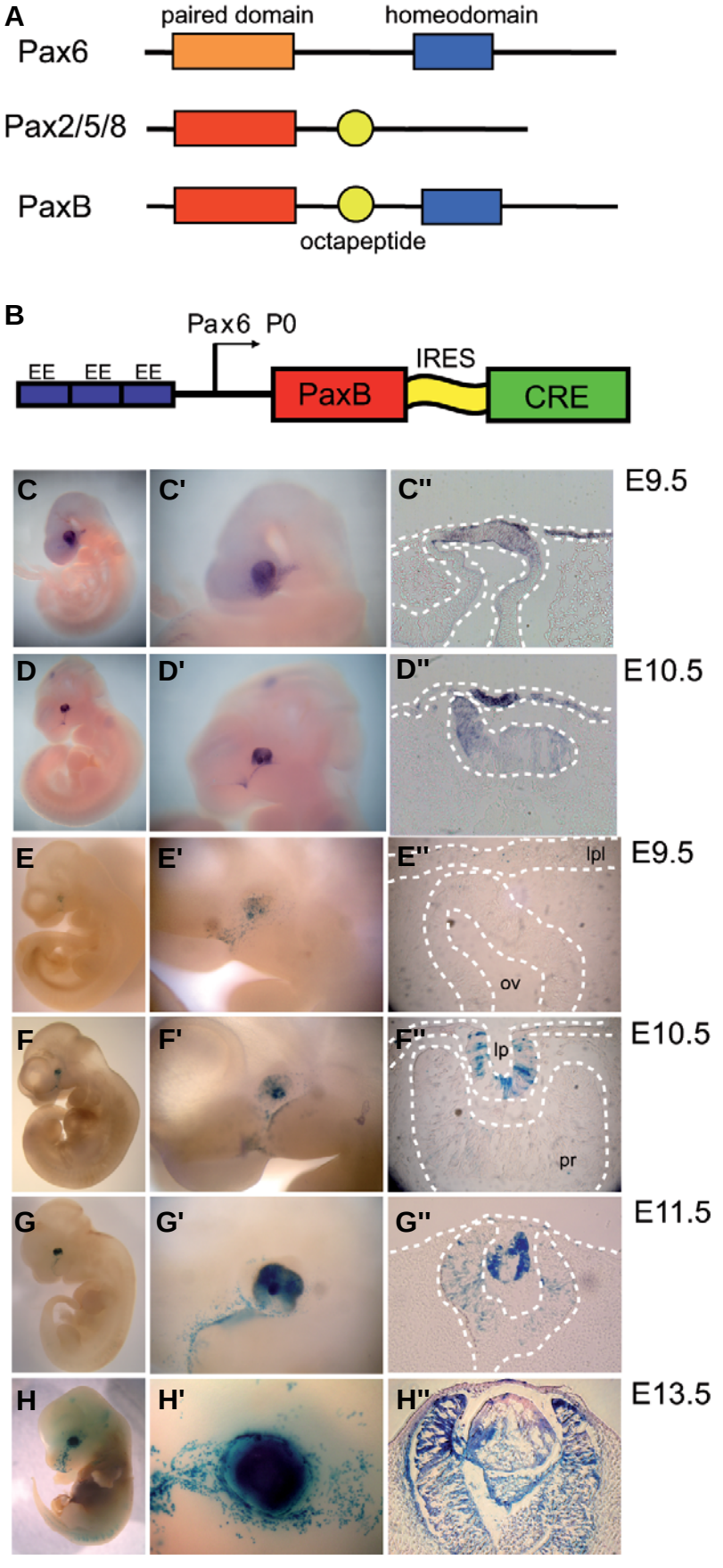
Supplementary Material (four additional figures) for this paper is available at: http://dx.doi.org/10.1387/ijdb.082793jr

Accepted: 15 January 2009. *Published online:* 27 February 2009.

ISSN: Online 1696-3547, Print 0214-6282



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

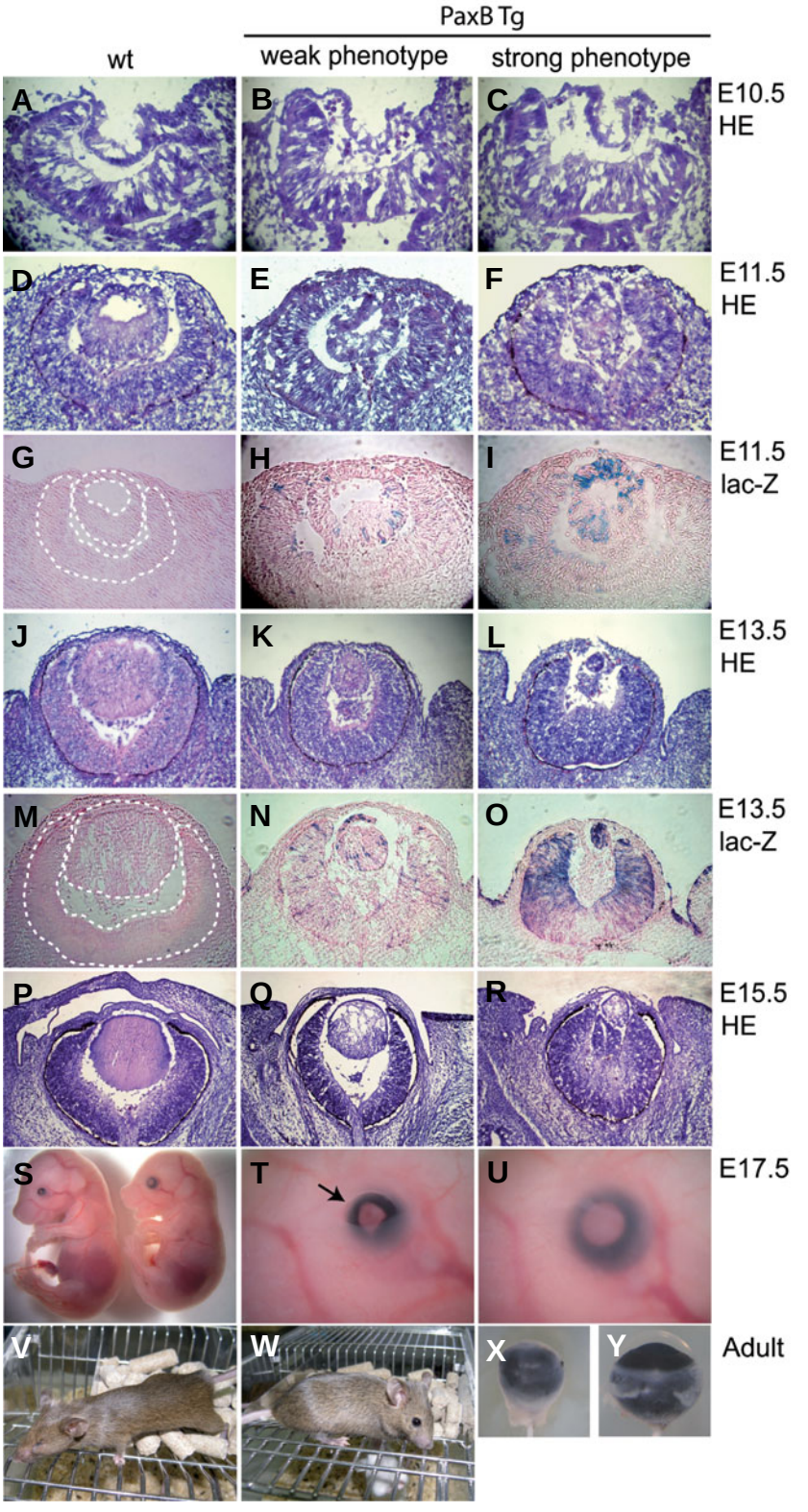


Fig. 2. Phenotypes of *PaxB* transgenic mice. *Cryosections at the indicated embryonic stages from the wild-type and* PaxB *transgenic mice were stained either with hematoxylin and eosin* **(A,B,C,D,E,F,J,K,L,P,Q,R)** *or lac-Z* **(G,H,I,M,N,O)**. *We sorted* PaxB *transgenic mice with weak and strong 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

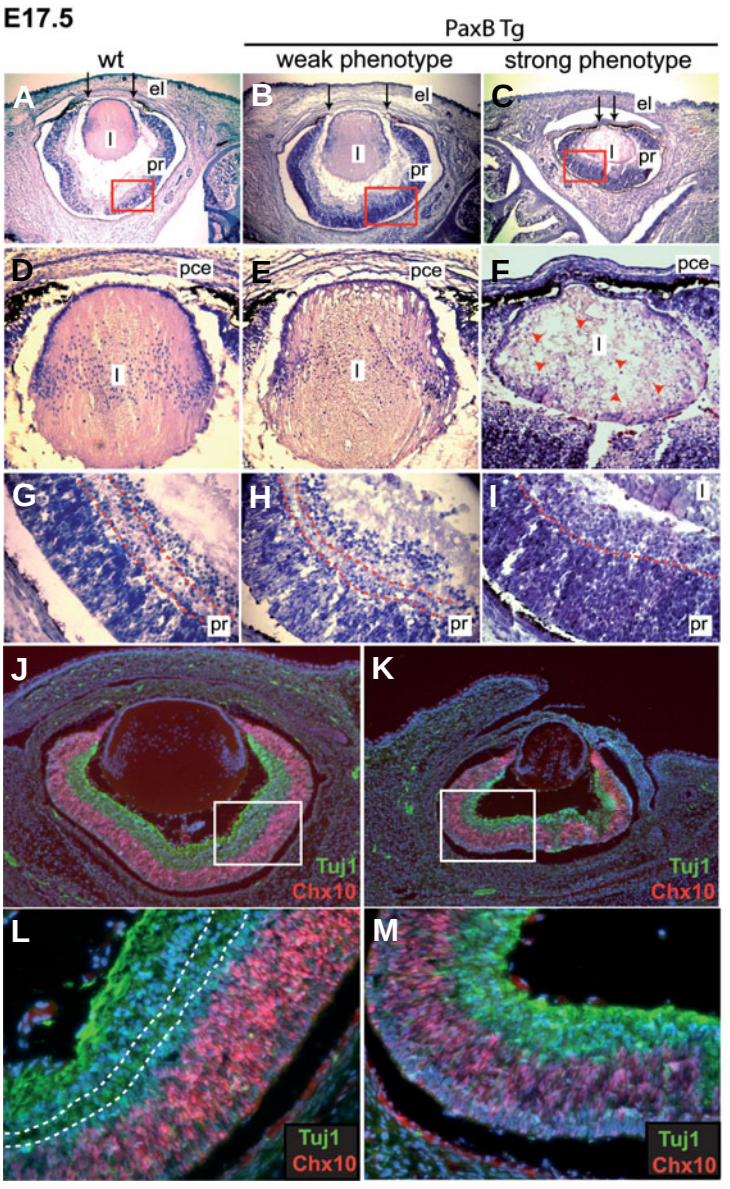


Fig. 3. Lens and retina defects detected at E17.5 in *PaxB* transgenic mice. *Cryosections of transgenic and wild-type embryos at E17.5 were stained with hematoxylin and eosin* **(A-I)**. *In addition to a smaller lens and whole eye size, there were several nuclei and vacuoles visible throughout the 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').

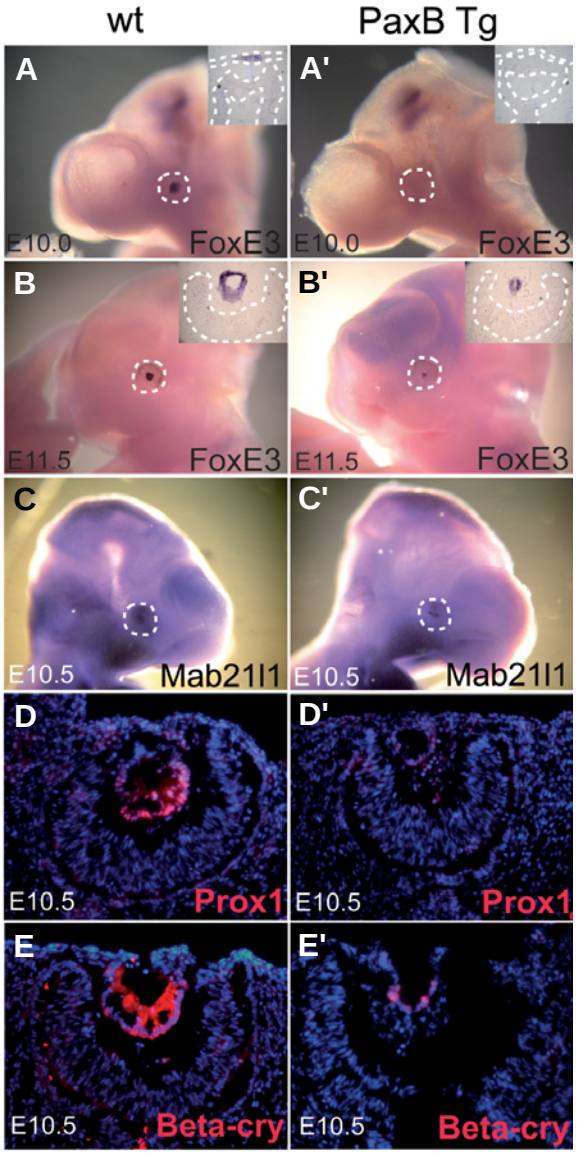


Fig. 4. Downregulation of lens specific markers in *PaxB* transgenic mice. *Whole mount RNA* in situ *hybridization* **(A–C')** *and RNA hybridization in sections (insets in A-B') at E10.0* **(A,A')**, *E10.5* **(C,C')** *and E11.5* **(B,B')** *in wild-type* **(A,B,C)** *and* PaxB *transgenic* **(A',B',C')** *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

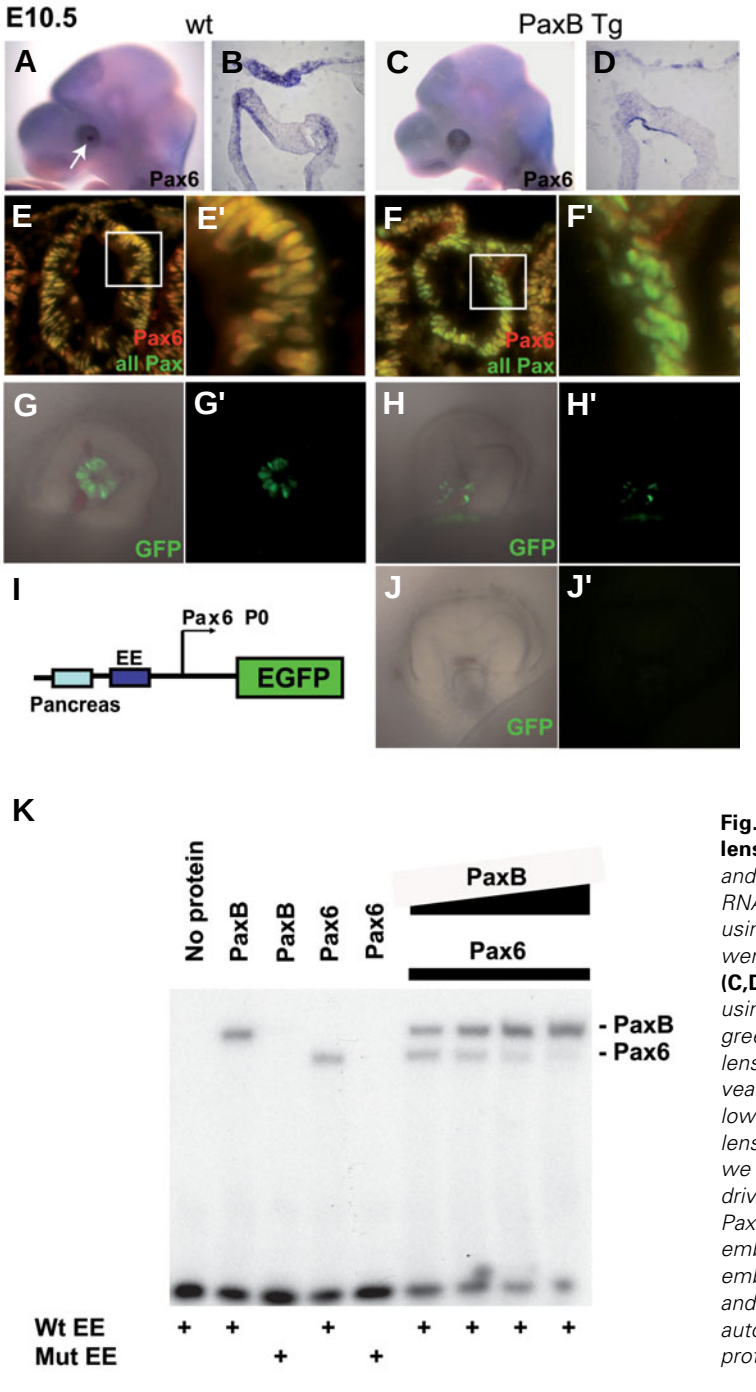


Fig. 5. Decreased levels of endogenous Pax6 in the *PaxB* transgenic lens. *All stainings were performed on E10.5 wild-type* **(A,B,E,E',G,G')** *and* PaxB *transgenic* **(C,D,F,F',H,H',J,J')** *embryos. Whole mount Pax6 RNA* in situ *hybridization* **(A-D)** *and RNA hybridizations were conducted using 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)**.

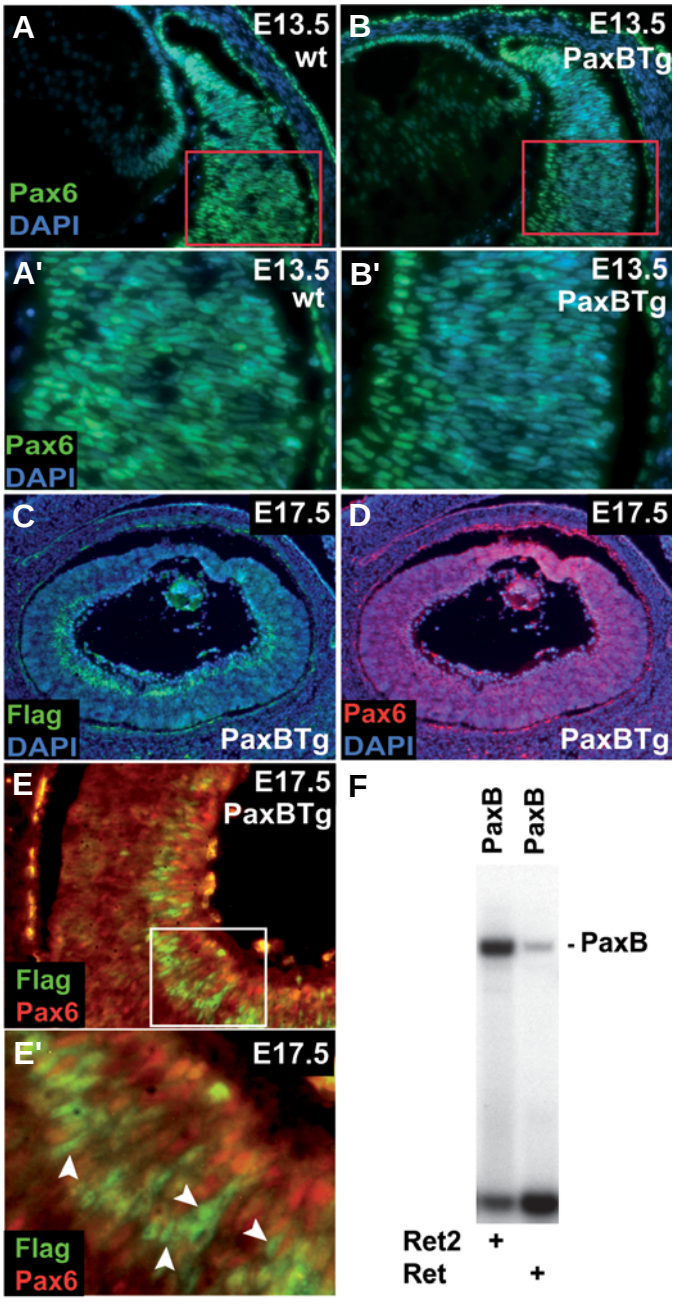


Fig. 6. Pax6 is repressed in the *PaxB* expressing cells. *The immunohistochemistry on tissue sections using anti-Pax6 antibody (green) at E13.5* **(A,B)** *(area in red rectangle was magnified in A',B'). All sections were counterstained with DAPI.* Pax6 *expression in the retina of* PaxB *transgenic 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.*

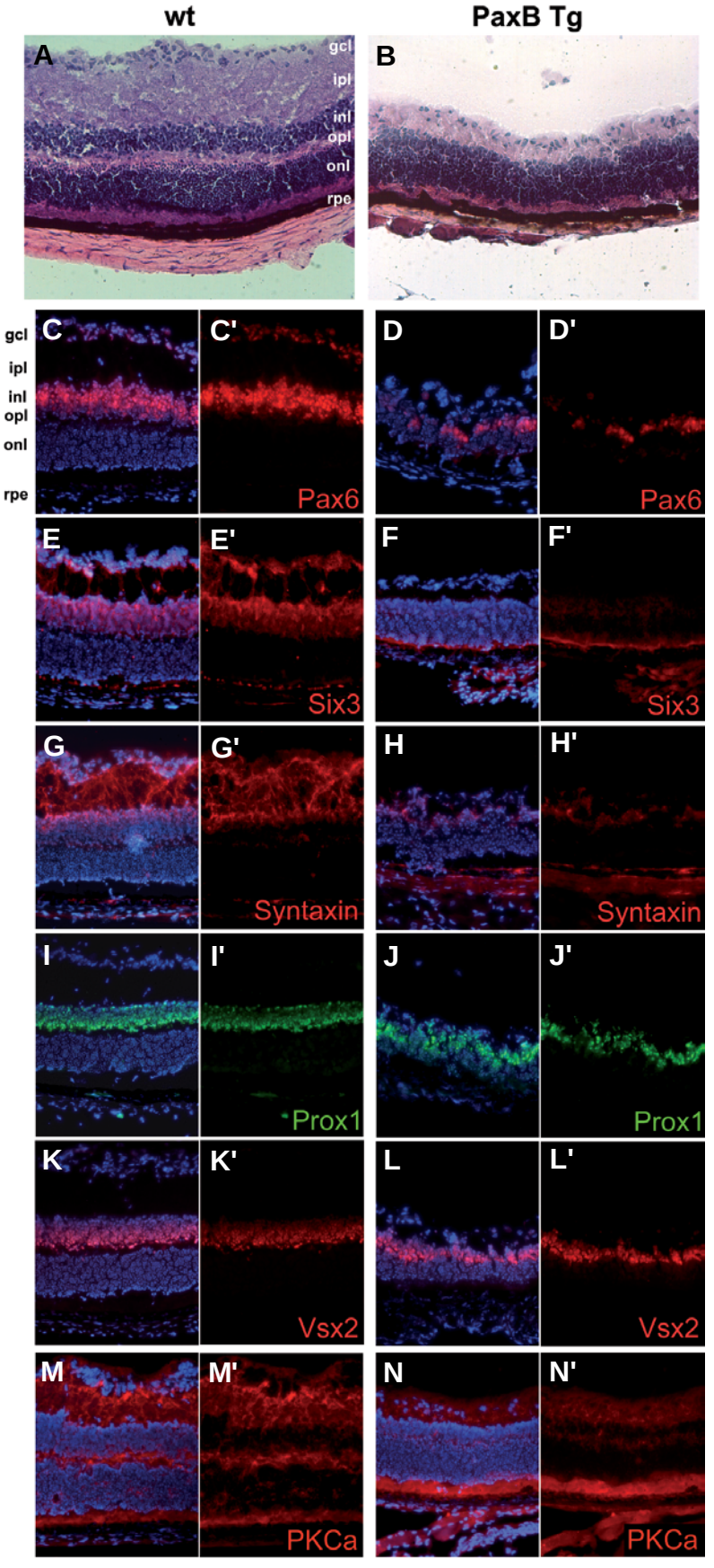
Pax6 within the pathway. We tested if *PaxB* expression directly influences the expression of an endogenous *Pax6*. First we examined *Pax6* expression by RNA *in situ* hybridization at E10.5. The staining for *Pax6* mRNA in the lens placode was markedly reduced in the *PaxB* transgenic embryos (Fig. 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

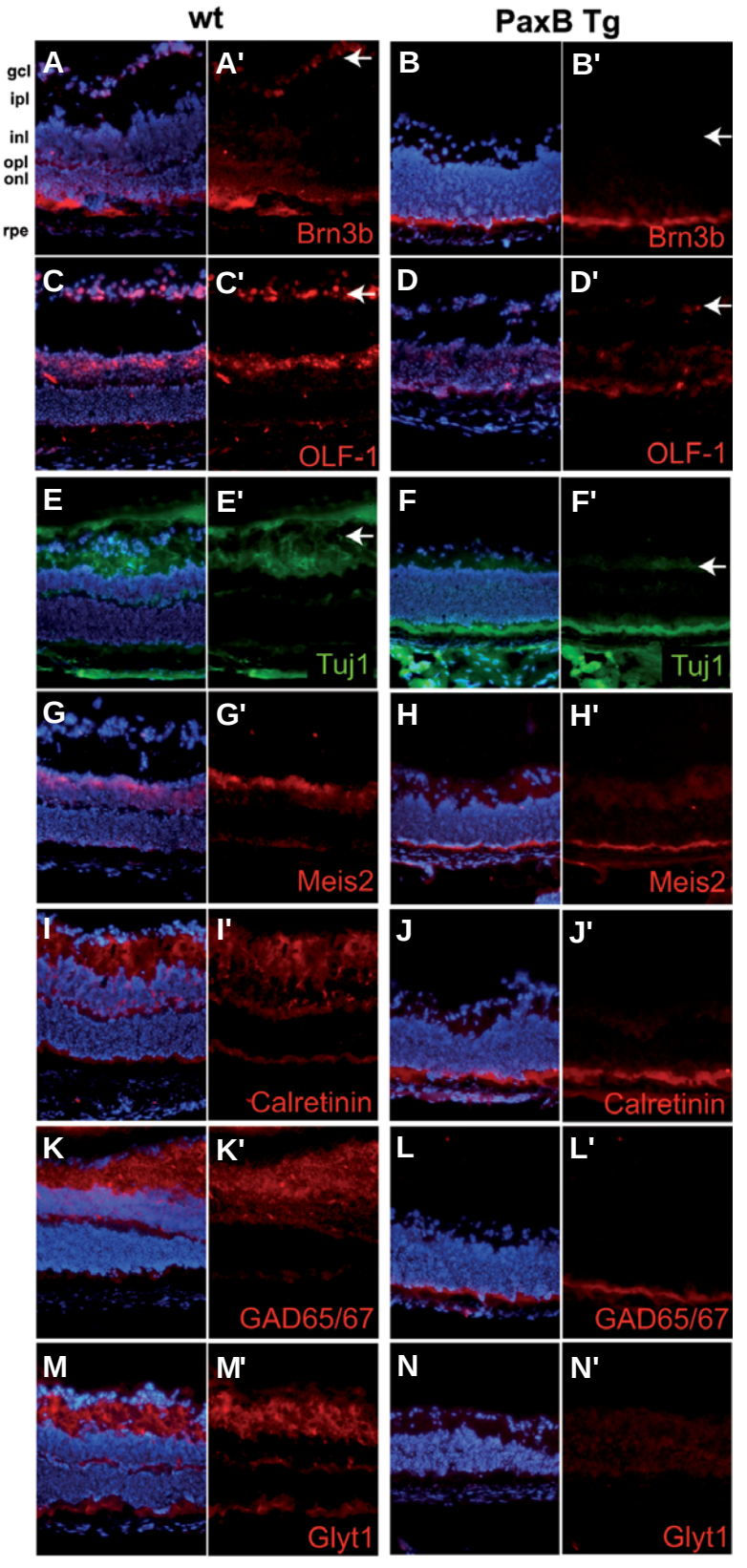


Fig. 8. *PaxB* expression supresses amacrine cell fate in adult retina. *Cryosections of adult retina from the wild-type (left panels) and PaxB transgenic mice (right panels) were stained using antibodies against the following proteins: Brn3b* **(A-B')**, *OLF-1* **(C-D')**, *Tuj1 (β-tubulin,* **E-F'**), *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).

Acknowledgements

We would like to thank Mrs. Veronika Noskova for excellent technical assistance. We thank Ales Cvekl and Romana Kucerova for critical reading of the manuscript and helpful discussions. We wish to thank Cheryl Craft from Mary D. Allen Laboratory for Vison Research, Doheny Eye Institute for CRX, S- and M-opsin antibodies. We are grateful to J. Samuel Zigler Jr. and Hisato Kondoh for anti-crystallin antibodies, Joe Horwitz for MIP26 antibody, Paola Bovolenta for Six3 antibody, Arthur Buchberg for Meis1 and Meis2 antibodies, Randall Reed for OLF-1 antibody, Shiming Chen for NR2e3 antibody, Dale Gregerson for arrestin antibody, Peter Carlsson for FoxE3, Giacomo Consalez for Mab21l1 and Peter Gruss for Pax6 riboprobe plasmid. The Pax6 monoclonal antibody developed by A. Kawakami was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported in part by grants no. AV0Z50520514 and IAA500520908 from the Academy of Sciences of the Czech Republic, by Center for Applied Genomics grant no. 1M6837805002 awarded by Ministry of Education, Youths and Sports of the Czech Republic and grant no. 204/08/1618 from the Grant Agency of Czech Republic.

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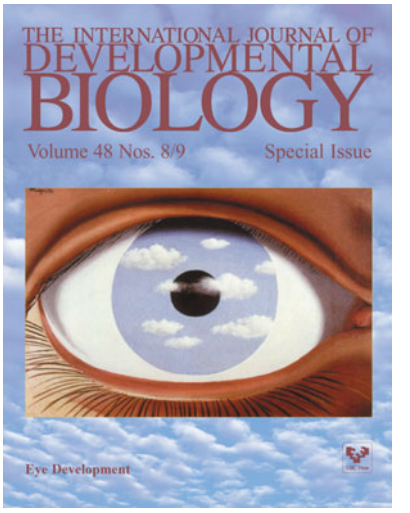

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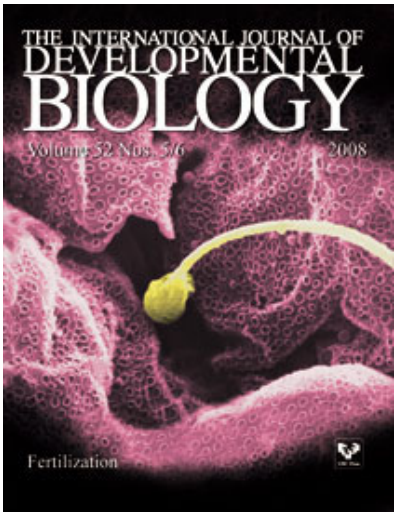

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