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Eye formation in the absence of retina

Eric C. Swindell[a,b], **Chaomei Liu**[a,b], **Rina Shah**[a,b], **April N. Smith**[c,d], **Richard A. Lang**[c,d], and **Milan Jamrich**[a,b,*]

[a] Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA

[b] Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, USA

[c] Division of Pediatric Ophthalmology, Children's Hospital Research Foundation and Department of Ophthalmology, University of Cincinnati, Cincinnati, OH 45229, USA

[d] Division of Developmental Biology, Children's Hospital Research Foundation and Department of Ophthalmology, University of Cincinnati, Cincinnati, OH 45229, USA

Abstract

Eye development is a complex process that involves the formation of the retina and the lens, collectively called the eyeball, as well as the formation of auxiliary eye structures such as the eyelid, lacrimal gland, cornea and conjunctiva. The developmental requirements for the formation of each individual structure are only partially understood. We have shown previously that the homeobox-containing gene *Rx* is a key component in eye formation, as retinal structures do not develop and retina-specific gene expression is not observed in *Rx*-deficient mice. In addition, *Rx*−/− embryos do not develop any lens structure, despite the fact that *Rx* is not expressed in the lens. This demonstrates that during normal mammalian development, retina-specific gene expression is necessary for lens formation. In this paper we show that lens formation can be restored in *Rx*-deficient embryos experimentally, by the elimination of β-catenin expression in the head surface ectoderm. This suggests that β-catenin is involved in lens specification either through *Wnt* signaling or through its function in cell adhesion. In contrast to lens formation, we demonstrate that the development of auxiliary eye structures does not depend on retina-specific gene expression or retinal morphogenesis. These results point to the existence of two separate developmental processes involved in the formation of the eye and its associated structures. One involved in the formation of the eyeball and the second involved in the formation of the auxiliary eye structures.

Keywords

β-catenin; Conjunctiva; Crystallin; Development; Eyelid; *Foxe3*; Lacrimal gland; Lens; *Pax6*; Periocular mesenchyme; *RAX*; Retina; *Rx*; *Wnt*

Introduction

The development of the vertebrate eye and its auxiliary structures has fascinated researches for more than a century. However, in spite of steady progress, a comprehensive understanding of the formation of the eye and its auxiliary structures is still missing.

*Corresponding author. Department of Molecular and Cellular Biology, N620, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. Fax: +1 713 798 3017, E-mail address: jamrich@bcm.tmc.edu (M. Jamrich).

Eye formation is a complicated process, as the different components of the eye, such as the retina, lens, cornea, conjunctiva, eyelids, eyelid muscles and lacrimal glands, are formed from different tissues. The retina is formed from the neuroectoderm, the lens from the surface ectoderm and the auxiliary tissues are formed from the head surface ectoderm, neural crest cells and the head mesoderm. How these processes are coordinated is a key question of the developmental biology of the eye.

For historical reasons, the retina and the lens together are called the eyeball. The cornea, eyelids, eyelid muscles, conjunctiva, lacrimal glands and other eye structures not derived from the retina or the lens are called the auxiliary eye structures.

Formation of the retina begins with the specification of retinal cells in the anterior neuroectoderm. The first morphological sign of this specification is the formation of two lateral grooves in the anterior neuroectoderm called the optic sulci. The cells of the optic sulci evaginate and form the optic vesicle. The distal portion of the optic vesicle will form the retina and the proximal will develop into the optic stalk. The homeobox-containing gene *Rx* is a key component in formation of retinal structures, as mice lacking *Rx* function do not form optic sulci or optic vesicles and do not display retina-specific gene expression (Mathers et al., 1997; Zhang et al., 2000). Studies in human, medaka, zebrafish and *Xenopus* suggest that *Rx* genes are required for the formation of the vertebrate retina in general (Bailey et al., 2004; Casarosa et al., 1997; Chuang et al., 1999; Furukawa et al., 1997; Kennedy et al., 2004; Loosli et al., 2003; Ohuchi et al., 1999; Voronina et al., 2004).

First morphological signs of lens formation are visible when the evaginating optic vesicle contacts the head surface ectoderm. This head surface ectoderm begins to thicken and forms a lens placode. In species like mouse and chick, the lens placode invaginates forming a lens vesicle. In other species, like zebrafish and *Xenopus*, the lens delaminates from the overlaying ectoderm (Ishibashi and Yasuda, 2001; Soules and Link, 2005). The distal part of the optic vesicle invaginates and forms a cup around the developing lens. Whether the formation of the lens depends on the formation of the retina is one of the oldest questions in the field of eye development. Hans Spemann concluded in 1901 (Spemann, 1901) that in the frog *Rana fusca*, lens induction depends on the optic cup, as upon its removal, the lens does not form. Several investigators came to the same conclusions, but others voiced dissenting views, as they found “free lenses” in several amphibian species upon experimental manipulation (for review see Mangold, 1931). Occasionally, contradictory conclusions were reached when the same species was used for experimental purposes (for review see von Woellwarth, 1961). Interestingly, it was found that the formation of “free lenses” greatly depended on the temperature at which the animals were raised prior to experimental manipulation (Ten Cate, 1956). For example, in *Rana esculenta*, lens formation was dependent on the optic cup if the animals were raised at 12 °C (54 °F), but appeared to be independent of the optic cup if the animals were raised at 25 °C (77 °F). These experiments explained why different investigators reached different conclusions using very similar experimental designs. This also demonstrated the difficulties with interpretation of results obtained from manipulated embryos. While a comprehensive understanding of lens formation in different amphibian species will require a detailed reinvestigation using molecular markers, in mice there is genetic evidence that formation of the lens depends on retinal formation. In *Rx*-deficient mouse embryos, which do not form any retinal structure and do not display any retina-specific gene expression, the lens placode, and consequently, the mature lens do not develop, demonstrating that in this species, retinal cells are necessary for lens formation (Brownell et al., 2000; Mathers et al., 1997; Swindell et al., 2006). It is not certain at this point whether the presumptive retinal cells exert their influence on lens formation through gene expression or through the formation of the optic cup. There is good evidence that signaling from the optic vesicle is essential for the activation of the lens-

specific gene network and the formation of the lens placode (Faber et al., 2002; Furuta and Hogan, 1998; Kamachi et al., 1998; Wawersik et al., 1999). However, several investigators presented evidence indicating that it is the mechanical protection of the optic cup against the neural crest cells that is the critical for lens formation (Bailey et al., 2006; Sullivan et al., 2004; von Woellwarth, 1961).

Formation of the auxiliary eye structures takes place upon the invagination/delamination of the lens. The corneal epithelium forms where head surface ectoderm is overlying the lens. The ectoderm surrounding the eye proliferates and folds over the developing cornea, forming a conjunctival sack. The ectoderm of this sack will form the ectoderm of the cornea, conjunctiva, lacrimal gland and eyelid. To what degree the development of these auxiliary eye structures depends on the development of the eyeball, or its individual components, i.e. the retina and the lens, is an interesting question that was intensely studied in the first half of the last century. Is the formation of auxiliary eye structures a consequence of gene expression in the developing retina and lens, or is the formation of these structures a craniofacial process, in which the development of the retina and the lens play only a limited role?

Otto Mangold attempted to address this question at the beginning of last century by the ablation of the eye rudiment in amphibians (Mangold, 1931). Since he found that the ablation of the eye rudiment did not prevent eyelid formation, he proposed that the specification of auxiliary eye structures either takes place at very early stages of development, before the experimental ablation of the eye is possible, or that the eyeball does not control the development of the auxiliary eye structures. Others attempted to answer this question by analyzing accidental and hereditary anophthalmia. However, no definitive conclusions were reached, as it was impossible to determine when the ablation of the eyeball took place and whether it was complete (Recordon and Griffiths, 1938; Rogalski, 1926; Voronina et al., 2004; Woolard, 1926). A good example demonstrating the nature of this problem is present in the anophthalmic individual lacking normal *RAX (RX)* function identified by Voronina et al. (2004). In this study, an anophthalmic child is presented that is missing a wild type copy of the *RAX* gene. This child has apparently normal eyelids. However, a detailed analysis shows that the mutated RAX genes of this child encode partially functional proteins. The activity of these proteins might be sufficient to initiate the development of a rudimentary eye and lead to normal development of auxiliary eye structures. Therefore, no firm conclusions can be made about the relationship between the formation of the eyeball and the formation of auxiliary eye structures in this case.

In this study, we took advantage of the *Rx*-deficient embryos to investigate the dependence of the formation of the auxiliary eye structures on the development of the eyeball and its components, the retina and the lens. *Rx*-deficient embryos do not develop eyeballs, and importantly, while the extent of brain defects varies in these embryos, they never develop any retinal or lens structure and they do not display any retina or lens-specific gene expression.

Materials and methods

Mouse lines

P6 5.0 LacZ reporter mice were used and genotyped as previously reported (Williams et al., 1998; Makarenkova et al., 2000). *Rx*−/− mice were used and genotyped as previously reported (Mathers et al.,1997).

LacZ reporter staining and histology

Embryos were fixed in 4% paraformaldehyde, washed in PBS and then stained for lacZ activity using X-gal. Stained embryos were then dehydrated, embedded in paraffin and sectioned. Sections were dewaxed and counterstained with eosin.

In situ hybridization

In situ hybridizations using *Pitx2*, *Foxl2* and *keratocan* riboprobes were performed using standard protocols (Wilkinson, 1992).

Skeletal staining

Newborn pups were fixed in 95% ethanol and then stained with Alcian blue and Alizarin red to visualize cartilage and bone.

Results

Formation of auxiliary eye structures

In order to directly address the question of whether the development of auxiliary eye structures depends on the formation of the retina and the lens, we analyzed the formation of auxiliary eye structures in *Rx*-deficient embryos. *Rx*-deficient embryos do not form any retina or lens structure and consequently do not form an eyeball (Mathers et al., 1997; Zhang et al., 2000). This phenotype is 100% penetrant. Furthermore, *Rx*-deficient embryos do not display any retina or lens-specific gene expression (Brownell et al., 2000; Zhang et al., 2000). Serial sections of *Rx*−/− newborn pups indicated the presence of eyelids and conjunctival sacks (Fig. 1A). The *Pax6-lacZ* reporter (Williams et al., 1998) was crossed into the *Rx*−/− strain because in wild type E16.5 embryos, this reporter is expressed in the lens, as well as in the ectoderm of the conjunctival sack, which includes the ectoderm of the cornea, conjunctiva and eyelid (Figs. 1B, C, F, G) (Kammandel et al., 1999; Makarenkova et al., 2000). This reporter is also expressed in the lacrimal gland that forms from the conjunctival sack by evagination and multiple branching (Fig. 1C) (Johnston et al., 1979; Kammandel et al., 1999; Makarenkova et al., 2000). *Pax6-lacZ* reporter expression in E16.5 *Rx*-deficient embryos shows that the lacrimal gland is present in these embryos (Figs. 1D, E). The conjunctival sack is also present, although not stretched by the eyeball as in the wild type embryos. The conjunctival ectoderm, as well as the eyelid ectoderm, is clearly visualized by the expression of this reporter (Figs. 1H, I). In wild type embryos, expression of the *Pax6-lacZ* reporter begins at E8.75 in the lateral head surface ectoderm. This ectoderm is frequently called the presumptive lens ectoderm (PLE) (Dimanlig et al., 2001; Williams et al., 1998). By E10, the center of this area forms the lens placode and is strongly positive for the expression of the *Pax6-lacZ* reporter (Fig. 2A). In *Rx*-deficient embryos, *lacZ* activity is present in the lateral head surface ectoderm — just like in wild type embryos (Fig. 2B). However, there is no upregulation of expression of this reporter in the center of the expression domain that is typical of lens development. The lack of upregulation in the central area of the PLE is not necessarily surprising, as we have demonstrated previously that the lens does not form in *Rx*−/− embryos (Brownell et al., 2000; Mathers et al., 1997; Medina-Martinez and Jamrich, 2007; Zhang et al., 2000). However, the expression of the *Pax6-lacZ* reporter in the lateral head surface ectoderm of *Rx*−/− embryos is unexpected, as it indicates that this expression signifies the development of auxiliary eye structures rather than the development of lens ectoderm. Furthermore it shows that retinal cells are not involved in the induction of this expression. While for historical reasons this expression area is called the presumptive lens ectoderm (PLE), it is clear the default fate of the cells expressing this reporter is the ectoderm of the conjunctiva, eyelid and lacrimal gland. Only

in the presence of the retina will some cells of this ectoderm will be diverted to form a lens. Lens in turn might divert some cells of the presumptive conjunctiva into corneal fate.

The development of auxiliary eye structures can be demonstrated in *Rx*-deficient embryos by in situ hybridization using probes specific for genes known to play a role in the formation of auxiliary eye structures. A key component in the development of several auxiliary eye structures is the periocular mesenchyme. This is a mixture of cells of neural crest origin and cells of the head mesoderm. The homeobox-containing gene *Pitx2* is expressed in the periocular mesenchyme of wild type embryos and is required for the development of auxiliary eye tissues (Gage and Camper, 1997). Mice lacking *Pitx2* function display abnormal development of the anterior ocular segment and do not develop extra-ocular muscles (Evans and Gage, 2005; Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). In E10 wild type embryos, *Pitx2* expression can be visualized as a ring of cells surrounding the developing eyeball (Fig. 2C). In *Rx*−/− embryos, *Pitx2* is expressed in the mesenchyme surrounding the area where the eyeball would normally form, demonstrating the presence of the periocular mesenchyme (Fig. 2 D).

The Fox gene, *Foxl2,* is expressed in the neural crest cells of the periocular mesenchyme that form the eyelid muscles (Crisponi et al., 2001 and our unpublished observations). In humans, mutations in this gene cause blepharophimosis ptosis epicanthus inversus syndrome that manifests itself in part by drooping eyelids, presumably because of the weak eyelid muscles (Crisponi et al., 2001). Fig. 2E shows the expression of this gene in E12.5 wild type embryos in the developing eyelids. In *Rx*−/− embryos, *Foxl2* is also expressed in the developing eyelids (Fig. 2F). Additional evidence demonstrating that the craniofacial development of the eye region is independent of retinal cells and retina-specific gene expression is provided by the analysis of orbit formation in newborn pups. Orbits are the cavities in the skull that harbor the eyes. Figs. 2G and I show the eyeballs in the orbits of wild type mice. Figs. 2H and J show the empty orbits in *Rx*-deficient embryos. These findings demonstrate that the formation of orbits does not depend on the morphogenesis of the eyeball or on retina or lens-specific gene expression.

Taken together these results demonstrate that the development of auxiliary eye structures such as the conjunctiva, eyelid, eyelid muscle and lacrimal gland is a process that is initiated, and to a large degree executed, without the involvement of retinal cells.

Lens formation in the absence of retina

Our experiments have shown that in the absence of the retina the lateral head surface ectoderm develops into the ectoderm of the conjunctiva, lacrimal gland and eyelid, but not into lens ectoderm. This allows us to address what changes are necessary to divert the lateral head surface ectoderm into the lens. We have found previously that elimination of β-catenin expression in the head surface ectoderm resulted in the formation of ectopic lentoid bodies in the periocular ectoderm (Smith et al., 2005). In this study we investigated whether the loss of β-catenin expression in the lateral head surface ectoderm can lead to lens formation in *Rx*−/− embryos. For this purpose, we crossed *Pax6-LE-cre* mice with the fioxed allele of β-catenin and crossed these mice into the *Rx*−/− background. We argued that if the elimination of β-catenin in the head surface ectoderm is the sole requirement for lens formation, we would observe lens formation in *Rx*−/− embryos, even in the absence of retinal morphogenesis and retina-specific gene expression. However, if retina-specific gene expression or retinal morphogenesis is required for lens formation in addition to the elimination of β-catenin expression in the head surface ectoderm, we would not observe lens formation. We found that the genetic ablation of β-catenin in the head surface ectoderm of *Rx*−/− embryos leads to the activation of the lens-specific gene network and formation of a lens placode and lentoid bodies (Fig. 3). Specifically, we observed upregulation of *Pax6,* a

gene that has a key role in lens formation (Aota et al., 2003; Ashery-Padan et al., 2000; Chow et al., 1999; Fujiwara et al., 1994; Glaser et al., 1994; Hanson et al., 1994; Hill et al., 1991; Jordan et al., 1992; Kamachi et al., 2001; Ton et al., 1991). While *Pax6* is expressed at low levels in the head surface ectoderm of *Rx*−/− embryos as a part of a genetic program to form auxiliary eye structures (Fig. 3B), the elimination of β-catenin expression leads to an upregulation of its expression typical of lens formation (Figs. 3C, J). *Foxe3*, a gene that is typically expressed in early stages of lens induction (Fig. 3D) and is required for a proper lens development (Blixt et al., 2000; Brownell et al., 2000; Kenyon et al., 1999; Medina-Martinez et al., 2005; Medina-Martinez and Jamrich, 2007; Semina et al., 2001; Shi et al., 2006), is activated in the *Rx*-deficient embryos upon the elimination of β-catenin expression (Fig. 3F). This gene is not expressed in *Rx*−/− embryos (Fig. 3E), as it is not required for the formation of auxiliary eye tissues. Significantly, α-crystallin, a gene that is typically expressed in the differentiating lens fiber cells (Fig. 3G) (Andley, 2007; Cvekl and Duncan, 2007; Piatigorsky et al., 1994; Piatigorsky and Wistow, 1991; Wistow and Piatigorsky, 1987), is also activated upon elimination of β-catenin expression (Figs. 3I, K). α-crystallin is not expressed in *Rx*-deficient embryos, as its expression is specific for lens formation (Fig. 3H). All of these experiments show that elimination of β-catenin expression is sufficient to activate the lens-specific gene network in the head surface ectoderm arguing that a modification of β-catenin expression in the head surface ectoderm is a key process in lens formation. At the morphological level, we see formation of a lens placode that initiates invagination in the middle of *Pax6* expressing area (Fig. 3J). Lens-like structures form in these embryos despite the absence of retinal morphogenesis. This suggests that one of the central roles of retinal cells in lens induction is the elimination of β-catenin expression in overlying surface ectoderm.

Discussion

For the proper functioning of the eye, the development of the retina and the lens needs to be correlated with the formation of the conjunctiva, lacrimal glands, eyelids, and other auxiliary eye structures. How these processes are coordinated is a key question in the developmental biology of the eye. In this paper we report that auxiliary eye structures form even in the absence of retinal morphogenesis and retina-specific gene expression. These results, in combination with our previous finding that the lens does not form in embryos that do not display retina-specific gene expression and retinal morphogenesis (Brownell et al., 2000; Mathers et al., 1997; Swindell et al., 2006; Zhang et al., 2000), point to the existence of two separate developmental processes involved in the formation of the eye and its associated structures. The process that leads to the development of retinal structures and lens formation begins with the specification of the retinal progenitor cells in the anterior neuroectoderm. If functional retinal cells are not generated, as is the case in the *Rx*−/− embryos, the morphogenesis of the optic cup does not take place and the lens does not form. The exact role of the optic vesicle/cup in lens formation has been under intense discussion for many decades. Several investigators proposed that retinal cells are involved in lens induction (Faber et al., 2002; Furuta and Hogan, 1998; Kamachi et al., 1998; Mangold, 1931; Spemann, 1901; Wawersik et al., 1999). Others have argued that the optic vesicle/cup does not have a role in the induction of the lens, but rather it protects the presumptive lens cells from contact with cranial neural crest cells that have lens-repressing ability (Bailey et al., 2006; Sullivan et al., 2004; von Woellwarth, 1961). However, the formation of lenses in the medaka mutant *eyeless*, in which the morphogenesis of the optic cup does not take place because of a mutation in *Rx3* gene (Loosli et al., 2001; Rembold et al., 2006; Winkler et al., 2000), argues against the latter possibility. In this mutant, retina-specific gene expression takes place on the side of the brain, suggesting that it is the expression of retinal-specific genes that is essential for lens induction, rather than the morphogenesis of the optic vesicle/cup. Furthermore, in this paper we show that elimination of β-catenin expression in the head

surface ectoderm in *Rx*-deficient embryos leads to a formation of lens-like structures in the absence of the morphogenesis of the optic vesicle/cup. These two experiments indicate that the presence of the optic vesicle/cup is not an absolute requirement for lens formation. However, in both cases the lenses are smaller than in the wild type embryos. This suggests that the optic cup/vesicle plays a role in the determination of the size of the lens. In our view, during normal development presumptive retinal cells induce lens formation in head surface ectoderm. The developing optic vesicle/cup shields the developing lens placode from signals that promote formation of other tissues. In the absence of formation of presumptive retinal cells and retina-specific gene expression, the lens does not form. In the absence of retinal morphogenesis, but in the presence of presumptive retinal cells and retina-specific gene expression, the lens forms, but it is smaller than in the presence of retinal morphogenesis.

The development of auxiliary eye structures appears to be largely independent of the formation of the retina and the lens. Lateral head surface ectoderm is specified even in the absence of the retina or lens-specific gene expression, to initiate the formation of auxiliary eye structures such as eyelids, conjunctiva, eyelid muscles and lacrimal glands. This is not to say that the anterior lateral neuroectoderm does not have any effect on the patterning of the surface ectoderm. The lack of separation of the eye field into two domains concomitant with the fusion of the eye-associated structures in cyclopic embryos clearly suggests that correct patterning of the anterior neural plate plays a critical role in craniofacial patterning. However, while a linkage between the patterning of the anterior neuroectoderm and patterning of the surface ectoderm clearly exists, the formation of functional retinal cells is not an essential component in the craniofacial patterning of the eye region. The default development of the lateral head surface ectoderm into auxiliary eye structures is modified in the presence of retinal cells, as the central region of this ectoderm is induced to form a lens. Upon invagination/delamination, the lens modifies the presumptive ocular surface ectoderm further to form a cornea. The lens has been shown to be a key factor in the induction/differentiation of the cornea (Beebe and Coats, 2000; Lopashov and Stroeva, 1961), however the presence of the cornea in patients with primary aphakia is yet to be explained (Valleix et al., 2006). That auxiliary eye structures can form without eyeball formation was suggested previously by observations of extreme cases of BMP7-deficient mice, which do not have a morphologically visible optic cup, but develop auxiliary eye structures (Dimanlig et al., 2001; Dudley et al., 1995; Makarenkova et al., 2000; Wawersik et al., 1999). In this paper we show that retina-specific gene expression is not essential for the formation of auxiliary eye structures.

Our studies show that the initial changes in gene expression in the lateral head surface ectoderm visualized by the expression of the *Pax6-lacZ* reporter are related to the formation of eye associated structures. It is important to point out that lens forms in a part of this presumptive auxiliary ectoderm only upon interaction with the retina. Therefore, when analyzing gene expression in the head surface ectoderm, care should be taken not to attribute all changes in gene expression in the lateral head surface ectoderm to lens formation. Our data suggests that the presumptive ocular surface ectoderm can be changed into presumptive lens ectoderm by downregulation of β-catenin expression. It is not entirely clear how β-catenin mediates lens formation, as β-catenin is a bifunctional molecule. It can function as a signal transduction molecule that has important roles in development and tumorigenesis (Nusse, 1992; Wodarz and Nusse, 1998). β-catenin can also function in cell adhesion (Perez-Moreno et al., 2003) and affect morphogenesis. We have shown previously that the elimination of β-catenin expression has little effect on early lens development, but it does affect lens morphogenesis (Smith et al., 2005). In addition, elimination of β-catenin expression leads to the formation of ectopic lentoid bodies in the periocular ectoderm. One possibility is that β-catenin is involved in the default program of the presumptive ocular

surface ectoderm to form conjunctival ectoderm. The elimination of β-catenin expression is then necessary to convert presumptive conjunctival ectoderm into the presumptive lens ectoderm. β-catenin might not function through a canonical Wnt pathway, as we were not able to detect the Wnt pathway reporter TOPGAL activity in the lens placode (Smith et al., 2005), albeit it is possible that TOPGAL does not accurately report Wnt responsiveness. Alternatively, β-catenin might function through its role in cell adhesion. Disruption of β-catenin-mediated adhesion in the presumptive conjunctival ectoderm might sufficiently disrupt the default program of this ectoderm to divert the cells into its closest alternative cell fate, the lens. While in our experiments the change of cell fate was achieved by a targeted elimination of β-catenin expression in the head surface ectoderm, during normal development the elimination of β-catenin expression might be induced by signaling and/or contact with the evaginating retinal neuroectoderm.

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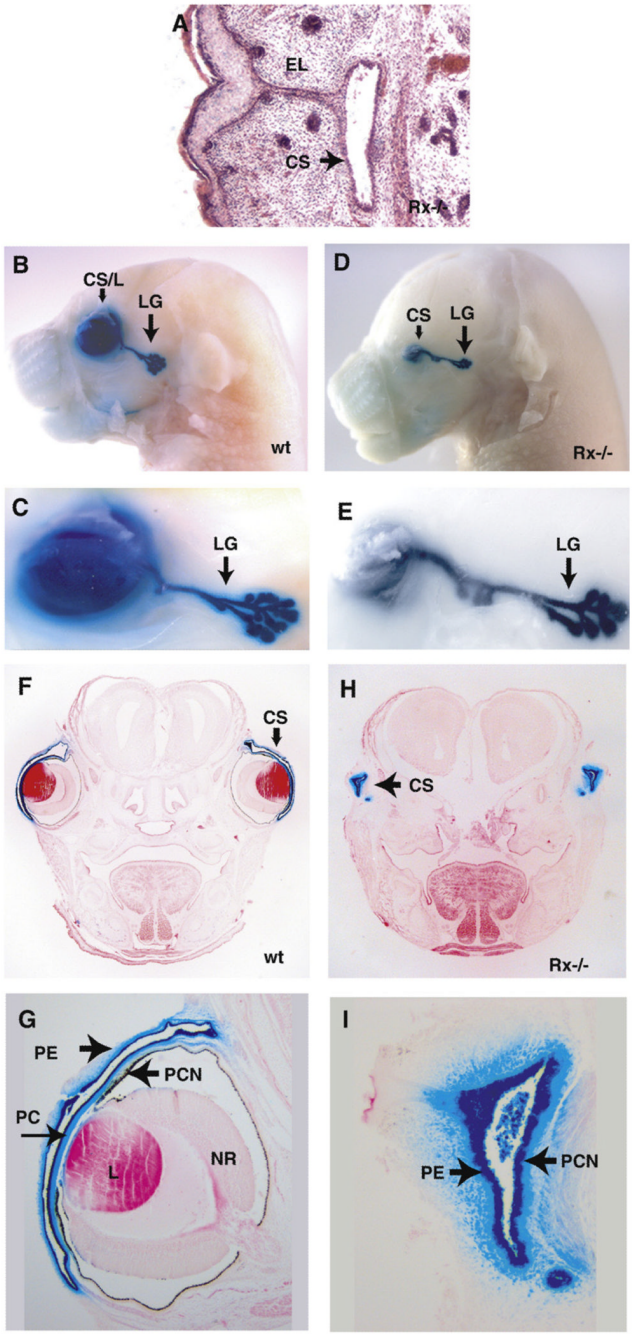

Fig. 1.

Formation of the lacrimal gland and conjunctival sack in wild type and *Rx*−/− embryos. (A) Section through the presumptive eye region of a newborn *Rx*−/− pup visualizing the conjunctival sack and the eyelids. (B) *lacZ* staining of the head of an E16.5 wild type embryo. (C) Detail of the *lacZ* staining from Fig. 2B visualizing the lacrimal gland. (D) *lacZ* staining of the head of an E16.5 *Rx*−/− embryo. (E) A detail of *lacZ* staining from Fig. 2D visualizing the lacrimal gland. (F) Cross section through the *lacZ* stained head of an E16.5 wild type embryo. (G) Detail of the *lacZ* staining from Fig. 2G, visualizing the double −layered conjunctival sack. (H) Cross section through the *lacZ* stained head of an E16.5 *Rx* −/− embryo visualizing the double-layered conjunctival sack. (I) Detail of the *lacZ* staining

from Fig. 2H visualizing the double-layered conjunctival sack. CS — conjunctival sack, EL — eyelid, L — lens, LG — lacrimal gland, NR — neuroretina, PC — presumptive cornea, PCN — presumptive conjunctiva, and PE — presumptive eyelid.

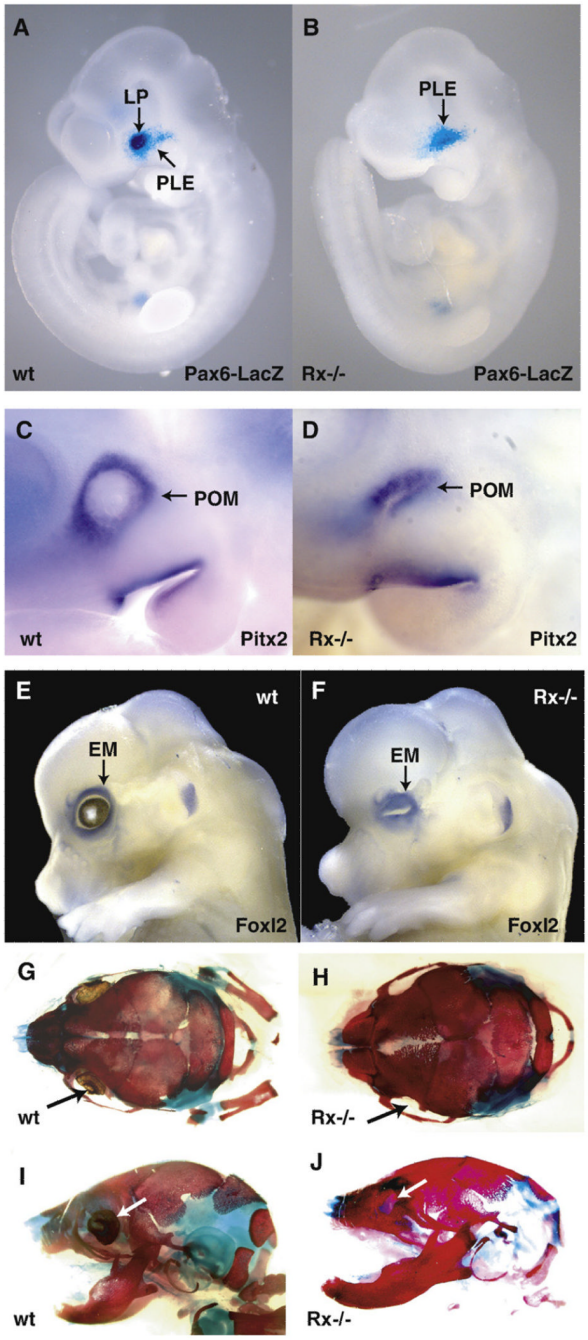

Fig. 2.

Formation of auxiliary eye structures in the *Rx*−/− and wild type mice. (A) *lacZ* staining of a E10 wild type embryo demonstrating the presence of the lens placode and the presumptive lens ectoderm. (B) *lacZ* staining of a E10 *Rx*−/− embryo demonstrating the presence of the presumptive lens ectoderm. (C) Whole mount in situ hybridization of the *Pitx2* probe to an E10 wild type embryo, demonstrating the presence of the periocular mesenchyme. (D) Whole mount in situ hybridization of *Pitx2* probe to an E10 *Rx*−/− embryo, demonstrating the presence of the periocular mesenchyme. (E) Whole mount in situ hybridization of the *Foxl2* probe to an E12.5 wild type embryo, demonstrating the presence of the presumptive eyelid muscles. (F) Whole mount in situ hybridization of the *Foxl2* probe to an E12.5 *Rx*−/−

embryo, demonstrating the presence of the presumptive eyelid muscles. (G) Dorsal view of a skull from a newborn wild type pup. Arrow points to the eye. (H) Dorsal view of a skull from a newborn *Rx*−/− pup. Arrow points to the empty eye cavity. (I) Lateral view of a skull from a newborn wild type pup. Arrow points to the eye. (J) Lateral view of a skull from a newborn Rx−/− pup. Arrow points to the empty orbit. EM — presumptive eyelid muscles, LP — lens placode, PLE — presumptive lens ectoderm, and POM — periocular mesenchyme.

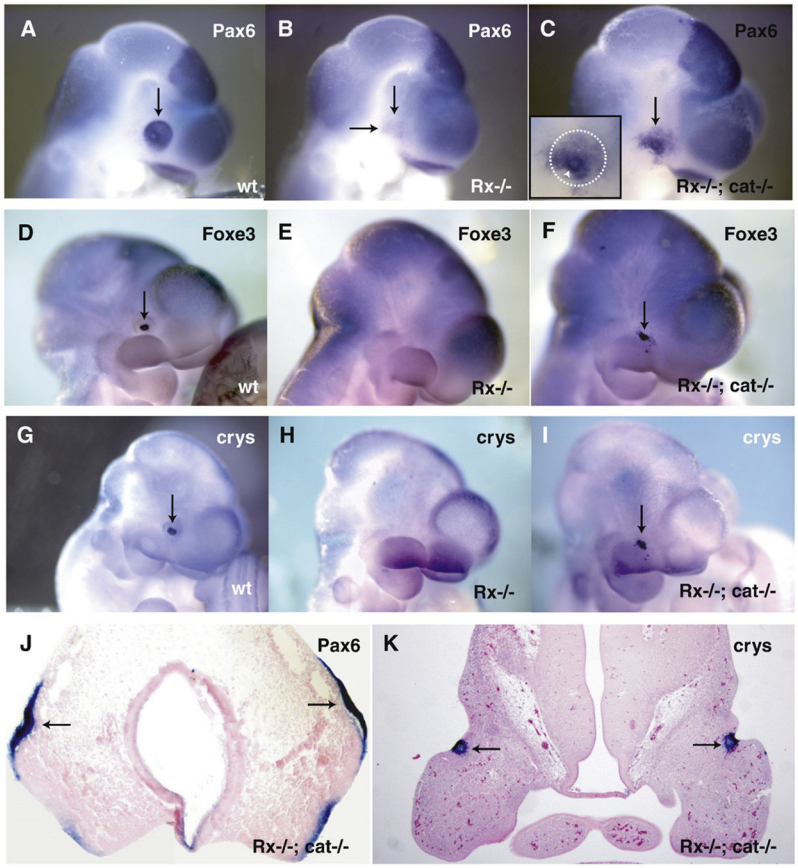

Fig. 3.

Lens induction in *Rx*−/− mice by a loss of β-catenin expression. (A–C) In situ hybridization of a *Pax6* probe to E10 mouse embryos. (A) Whole mount in situ hybridization of *Pax6* to a wild type embryo. Arrow points to *Pax6* expression in the optic cup and the lens. (B) Whole mount in situ hybridization to an *Rx*−/− embryo showing weak expression of *Pax6* in the presumptive conjunctival ectoderm (arrows). (C) Whole mount in situ hybridization of *Pax6* to an *Rx*−/−; β-*cat*−/− embryo. Inset in the C visualizes *Pax6* expression in the presumptive auxiliary ectoderm (dashed circle) and in the developing lentoid body (white arrowhead). (D–F) Whole mount in situ hybridization with a *Foxe3* probe to E10 embryos. (D) Whole mount in situ hybridization demonstrating *Foxe3* expression in a wild type embryo (arrow). (E) Whole mount in situ hybridization of *Foxe3* to an *Rx*−/− embryo demonstrating the absence of *Foxe3* expression. (F) Whole mount in situ hybridization of *Foxe3* to an *Rx*−/−; β-cat−/− embryo showing activation of *Foxe3* expression (arrow). (G–I) In situ hybridization of an α-crystallin probe to E10 mouse embryos. (G) Whole mount in situ hybridization demonstrating expression of α-crystallin in a wild type embryo (arrow). (H) Whole mount in situ hybridization of *Foxe3* to an *Rx*−/− embryo demonstrating the absence of α-crystallin expression. (I) Whole mount in situ hybridization of a−crystallin to an *Rx*-/−; β-cat−/−embryo showing activation of α-crystallin expression (arrow). (J) Section through an E10 embryo hybridized with *Pax6* demonstrating *Pax6* expression, thickening and invagination of the lens placodes in an *Rx*−/−; β-cat−/− embryo (arrows). (K) Section through an E11.5 embryo hybridized with α-crystallin demonstrating α-crystallin expression and formation of lentoid bodies in an *Rx*−/−; β-cat−/− embryo (arrows).