Int. J. Dev. Biol. 48: 761-770 (2004)
doi: 10.1387/ijdb.041878tb

Regulation of vertebrate eye development by *Rx* genes

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ABSTRACT The paired-like homeobox-containing gene *Rx* has a critical role in the eye development of several vertebrate species including *Xenopus*, mouse, chicken, medaka, zebrafish and human. *Rx* is initially expressed in the anterior neural region of developing embryos, and later in the retina and ventral hypothalamus. Abnormal regulation or function of *Rx* results in severe abnormalities of eye formation. Overexpression of *Rx* in *Xenopus* and zebrafish embryos leads to overproliferation of retinal cells. A targeted elimination of *Rx* in mice results in a lack of eye formation. Mutations in *Rx* genes are the cause of the mouse mutation *eyeless (ey1),* the medaka temperature sensitive mutation *eyeless (el)* and the zebrafish mutation *chokh.* In humans, mutations in *Rx* lead to anophthalmia. All of these studies indicate that *Rx* genes are key factors in vertebrate eye formation. Because these results cannot be easily reconciled with the most popular dogmas of the field, we offer our interpretation of eye development and evolution.

KEY WORDS: *retina, Rx, Rax, eye development*

Initial stages of vertebrate eye development

The formation of the vertebrate eye is an integral part of head formation and it requires the specification and regionalization of the anterior neural plate, evagination of the optic vesicles and finally, the cellular differentiation of the lens and retina. Vertebrate head formation and the commitment of cells towards ocular fates has been intensely studied for many decades, and several important interactions have been identified (Fig. 1). Early experiments in *Triturus* by Hilde Mangold and Hans Spemann (Spemann and Mangold, 1924) demonstrated that the dorsal blastopore lip, when transplanted to the ventral side of the amphibian embryo, can induce the formation of a secondary embryo. Because of the ability of the dorsal blastopore lip to organize surrounding tissue in this process, this region was named, by Spemann, the organizer. Later experiments by Spemann and Otto Mangold (Spemann, 1931; Mangold, 1933) demonstrated that the organizer can be divided into a head inducer and a trunk inducer. This division was made based on the observation that the anterior part of the organizer has the ability to induce secondary head structures, while the posterior part can induce trunk structures.

One of the major processes triggered by Spemann's organizer is neural induction that leads to the formation of the neural plate. A group of cells in the anterior end of the neural plate is then specified to form the retina. Neural induction is initiated during gastrulation and experimental evidence suggests that the eye field is specified to some degree by the midgastrula stage of development (Lupo *et al.,* 2002). The molecular mechanism leading to formation of the cephalic region is not yet fully understood, but several genes have been isolated that can, like the head organizer, induce formation of secondary head structures when injected into *Xenopus* embryos. Secreted proteins like *Cerberus* and *Dickkopf-1* (Bouwmeester *et al.,* 1996; Glinka *et al.,* 1998) are able to induce head formation when ectopically expressed on the ventral side of *Xenopus* embryos. *Chordin* and *noggin* can induce neural tissue by physically binding BMP4, a TGFβ-like molecule that needs to be repressed in order to convert uncommitted ectoderm into neuroectoderm (Sasai *et al.,* 1994; Zimmerman *et al.,* 1996). Complex interactions of *Cerberus, Dickkopf-1, chordin* and *noggin* with the components of the wnt, nodal, FGF and IGF signaling pathways lead to proper regionalization of the anterior neural plate (Houart *et al.,* 2002; Lagutin *et al.,* 2003; Lupo *et al.,* 2002; Pera *et al.,* 2001; Piccolo *et al.,* 1999).

The specification of the anterior neural plate is characterized at the molecular level by activation of several homeobox-contain-

Abbreviations used in this paper: BMP, bone morphogenetic protein; el, eyeless gene; ey1, eyeless 1 gene; RxMO, Rx morpholino; RX/RAX, human Rx gene; Rx morpholino.

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0214-6282/2004/$25.00

Printed in Spain
www.ijdb.ehu.es

ing genes including *Otx2, Pax6, Six3* and *Six6*. In *Xenopus,* these genes are activated almost simultaneously in a partially overlapping domain in the anterior neural plate (Mathers *et al.,* 1995) and some are involved in the patterning of the forebrain and eye development. One of the earliest genes expressed in the anterior neural region is the homeobox gene *Otx2*. *Otx2* is required for the formation of the anterior neural region, as mice lacking *Otx2* function form neither forebrain nor midbrain (Acampora *et al.,* 1995). The role of *Otx2* in the specification of retinal progenitors is not known, but *Otx2* is likely to play a permissive rather than an instructive role, as its activity is suppressed in the center of the presumptive eye field, possibly by the *Rx* protein. However, *Otx2* expression remains in the periphery of this field. This differential inactivation of *Otx2* is of functional significance, as the center of the eye field develops into the neuroretina, while the periphery of the eye field develops into the retinal pigment epithelium (Martinez-Morales *et al.,* 2003). In *Xenopus* there seems to be an important regulatory interaction between *Otx2* and the T-box containing gene ET (Li *et al.,* 1997; Zuber *et al.,* 2003), but a corresponding T-box gene with a similar function or expression has not yet been identified in higher vertebrates.

Pax6 is another homeodomain-containing transcription factor expressed in the anterior neural plate that plays a crucial role in vertebrate eye formation. Mutations in *Pax6* result in eye malformations known as *Aniridia,* Peter's anomaly, and cataracts in humans (Glaser *et al.,* 1992; Hanson *et al.,* 1994; Jordan *et al.,* 1992; Ton *et al.,* 1991) and *Small eye* syndrome in mice and rats (Fujiwara *et al.,* 1994; Hill *et al.,* 1991). The *Drosophila* homologue of *Pax6*, *eyeless,* is essential for *Drosophila* eye formation (Quiring *et al.,* 1994). *Six3* is also expressed in the anterior neural plate (Oliver *et al.,* 1995) and has a critical role in the formation of the forebrain as mutations in the human *Six3* cause holoprosencephaly (Pasquier *et al.,* 2000; Wallis *et al.,* 1999). Furthermore, mouse embryos lacking *Six3* function lack most of the head structures anterior to the midbrain (Lagutin *et al.,* 2003). The specific role of *Six3* in eye development is not yet known. *Six6 (Optx2)* is expressed in the early precursors of the eye (Jean *et al.,* 1999; Toy and Sundin, 1999) and its overexpression in *Xenopus* embryos results in the overproliferation of the retinal cells (Zuber *et al.,* 1999). The targeted elimination of this gene in mice confirmed that this gene has a role in the proliferation of retinal progenitor cells (Li *et al.,* 2002).

Recently a small family of paired-like homeobox genes that is critical for eye formation, the *Rx/Rax* (for Retinal homeobox) family, has been identified (Casarosa *et al.,* 1997; Eggert *et al.,* 1998; Furukawa *et al.,* 1997b; Loosli *et al.,* 2001; Mathers *et al.,* 1997; Ohuchi *et al.,* 1999).

Expression pattern of *Xenopus Rx* genes

We initially isolated the *Rx* genes from *Xenopus* ectoderm treated with 10mM NH_4Cl. When ectoderm from *Xenopus* blastulae (animal caps) is cultured in buffered saline, it forms epidermis. However, when the animal caps are treated with NH_4Cl, they begin to express genes specific for the anterior head region (Jamrich and Sato, 1989; Mathers *et al.,* 1995; Picard, 1975; Sive *et al.,* 1989). It is not fully understood how NH_4Cl is able to mimic neural induction, but *Xenopus* ectoderm cells undergo alkalinization in response to neural induction (Sater *et al.,* 1994). It appears that NH_4Cl can cause alkalinization of the ectoderm that leads to the formation of neural tissue (Uzman *et al.,* 1998). We made a cDNA library from NH_4Cl induced ectoderm and screened this library for homeobox-containing genes using degenerate primers and the polymerase chain reaction. We isolated several genes whose expression we analyzed by whole-mount *in situ* hybridization. One of the most interesting genes isolated by this approach was the novel, paired-like homeobox gene *Rx* (for Retinal homeobox) (Mathers *et al.,* 1997). *Xrx* was also independently isolated by Casarosa *et al.,* (1997) from a stage 24/25 *Xenopus* cDNA library by screening with the murine *Orthopedia* probe.

Xrx is first activated during gastrulation and its transcripts can be detected by *in situ* hybridization in late gastrula/early neurula embryos, demarcating a uniform field of cells in the anterior neural plate (Fig. 2A). *Xrx* expression is sharply delineated anteriorly from the cells of the cement gland anlage, which in *Xenopus* is the most anterior dorsal structure. The posterior border of *Xrx* expression is in the proximity of the forebrain midbrain boundary. Therefore, it appears that the *Xrx* early expression domain is primarily localized to the putative forebrain. This uniform domain becomes divided into two eye fields under the influence of the prechordal mesoderm. While today there is a general acceptance that the two eye fields of vertebrates are generated from a single eye anlage, the generation of two eye fields from one was controversial for many decades. For example, Spemann (Spemann, 1938) favored the hypothesis that the two eyes are generated from two independent eye fields, while his major opponent at that time, Adelmann (1929), pioneered the notion that the two eyes are generated from a single eye anlage. It is now known that during neurulation this field is divided along the midline of the embryo into two independent domains, which eventually give rise to the eyes of the embryo by the downregulation of eye-specific markers at the midline (Eggert *et al.,* 1998; Ekker *et al.,* 1995; Li *et al.,* 1997; Macdonald *et al.,* 1995). The signals that promote the division of the eye field emanate from the prechordal mesoderm that is located under the anterior neuroectoderm. In frogs, this midline signaling seems to suppress the retinal fate (Li *et al.,* 1997). In zebrafish the mechanism of eye field separation appears to be different in that the neural cells initially located posterior to the eye field migrate anteriorly and divide the eye field into two optic primordia (Fraser, 1999; Varga *et al.,* 1999). Mutations in genes that are involved in this midline signaling such as *ndr2* or *sonic hedgehog* lead to the lack of separation of the two domains and to the formation of cyclopic embryos (Chiang *et al.,* 1996; Hatta *et al.,* 1991).

During neurulation, the retina remains the primary site of *Rx* expression, but the pineal gland (epiphysis), and the ventral hypothalamus also express this gene (Fig. 2 B,C). Sections of neurula stage embryos show that initially the entire retinal neuroepithelium expresses *Xrx* to the same degree, but by the time the optic cup is formed, the *Xrx* RNA expression domain is restricted to the cells of the retinal ciliary margin (Mathers *et al.,* 1997). This is a very important finding as it had been shown that the retinal ciliary margin contains the multipotent retinal stem cells that continually generate the entire repertoire of retinal cell types throughout *Xenopus* life (Holt *et al.,* 1988; Stiemke and Hollyfield, 1995; Wetts and Fraser, 1988; Wetts *et al.,* 1989). Later in development, *Xrx1* is reactivated in the photoreceptor cells (Perron *et al.,* 1998).

Conservation of structure and expression of *Rx* genes

The structure of *Rx* genes is very conserved and since their discovery, they have been described in several vertebrate and invertebrate species including chicken, *Xenopus*, mouse, medaka, *Drosophila*, zebrafish, and human (Casarosa *et al.,* 1997; Eggert *et al.,* 1998; Furukawa *et al.,* 1997b; Loosli *et al.,* 2001; Mathers *et al.,* 1997; Ohuchi *et al.,* 1999). The number of *Rx* genes varies among different species, and generally ranges from one to three. The homeodomains of *Rx* proteins are extremely well conserved. They are for example identical between *Xenopus*, *Drosophila* and two of the three zebrafish proteins.

The expression pattern of *Rx* genes in different species is similar, but not identical. Like in *Xenopus*, the murine *Rx* (*Mrx*) is first activated in the anterior neural plate of E7.5 embryos. At E10.5 expression of *Mrx* is confined to the developing retina and ventral forebrain. There is a uniform expression in the entire neuroretina of E15.5 embryos. At later stages there is a progressive reduction of *Mrx* expression in the retina, which initiates in the ganglion cells and proceeds in concordance with the loss of proliferative activity in the retinal cell layers. By P6.5 *Mrx* transcripts are present only in the photoreceptor and inner nuclear layer (Mathers *et al.,* 1997).

While the single mouse *Rx* gene and the two *Xenopus Rx* genes have a very similar expression pattern, in the retina and ventral hypothalamus, the three zebrafish *Rx* genes display slightly different expression patterns. Initially, all three zebrafish *Rx* genes are activated in a similar area in the anterior neural plate, but later in development the *Zrx1* and *Zrx2* remain active exclusively in the retina, and the *Zrx3* continues to be expressed in the ventral hypothalamus (Chuang *et al.,* 1999; Chuang and Raymond, 2001; Mathers *et al.,* 1997). Interestingly, during the cellular differentiation of the retina, *Zrx1* and *Zrx2* are expressed in the adult cone cells, but not in the rod cells (Chuang *et al.,* 1999). *Zrx3* is expressed in the inner nuclear layer of the adult retina.

In medaka, *Rx3* is first expressed at late gastrulation and by the early neurula stages this gene is strongly expressed in a single field of the developing forebrain. By late neurula stages there is strong retinal expression in addition to the forebrain, but this retinal expression is progressively lost as the embryo proceeds through somitogenesis, leaving intense expression only in the ventral diencephalon. Adult fish show *Rx3* expression in the inner nuclear layer of the retina as well as the hypothalamus (Deschet *et al.,* 1999). Medaka *Rx2* expression begins several hours later than *Rx3* in the developing optic vesicle and then is maintained in the neuroretina, but not in the hypothalamus (Loosli *et al.,* 1998).

Two *Rx* genes were found in chicken, *cRax* and *cRaxL*. *cRax* is detectable in the ectoderm anterior to Hensen's node at stage 4. During neurulation, *cRax* is expressed similarly to mice in the anterior neural folds in the prospective retina, and in the ventral forebrain (Ohuchi *et al.,* 1999). *cRaxL* is expressed in the anterior neural ectoderm somewhat later than *cRax.* During the cellular differentiation of the retina, it is expressed in the initial stages of photoreceptor differentiation. *cRax* is not expressed in photoreceptor cells (Chen and Cepko, 2002). A review of *Rx* expression patterns in different species reveals that the most conserved aspect of vertebrate *Rx* expression is its early transcription in the anterior neural plate, followed by the expression in the eyes and ventral forebrain. This pattern of expression is conserved in the

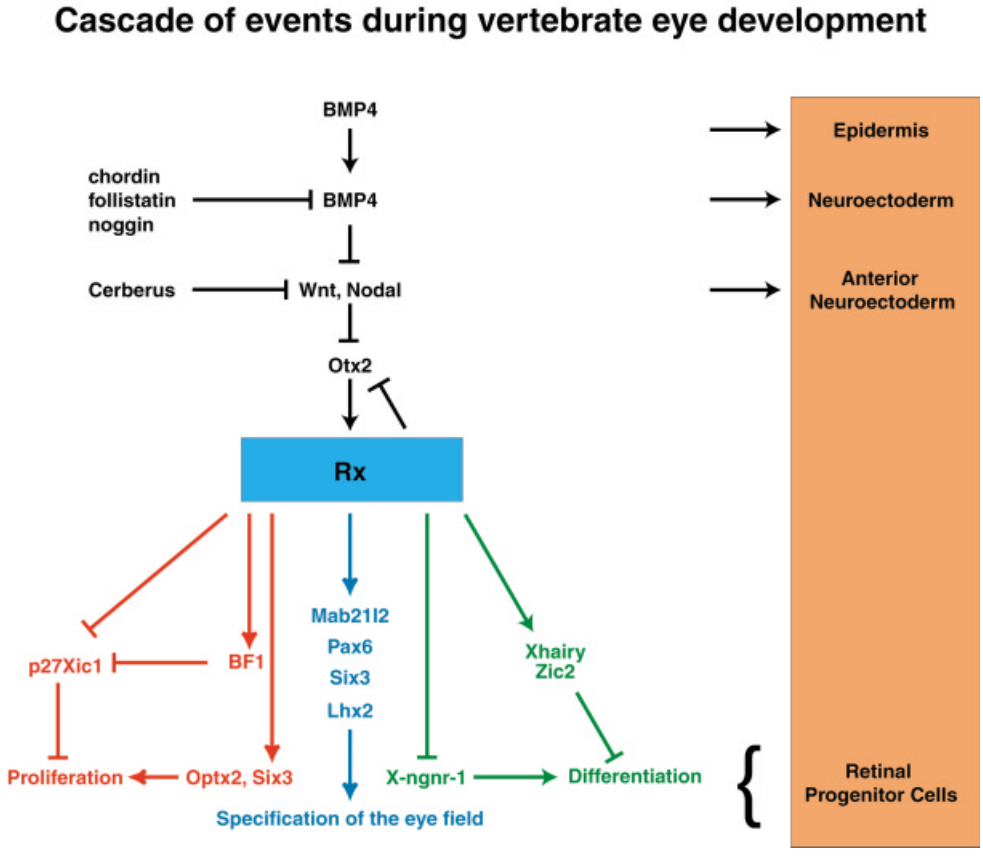

Fig. 1. A schematic diagram of the regulatory interactions taking place during the specification of the retinal field. *This simplified view shows that in the presence of BMP4 expression, the uncommitted ectoderm will form epidermis. As BMP4 is antagonized by chordin, follistatin or noggin, neural tissue will form. Additional inhibition of Wnt and/or nodal pathway is necessary to form anterior neuroectoderm. Anterior neuroectoderm expresses* Otx2 *that in turn, activates transcription of* Rx. Rx *performs several functions that are required for the formation of retinal progenitor cells.* Rx *promotes proliferation and inhibits differentiation of* Rx *expressing cells. At the same time, it increases transcription of several eye-specific genes like* Pax6, Six3 *and* Lhx2. *It also downregulates the transcription of* Otx2 *in the cells of the presumptive neuroretina. Since many of these regulatory interactions were not yet investigated in detail, it is important to emphasize that arrows between genes do not always imply direct regulatory interactions.*

two *Xenopus Rx* genes, in medaka *Rx3* and in the mouse *Rx* (Casarosa *et al.,* 1997; Loosli *et al.,* 2001; Mathers *et al.,* 1997). In zebrafish a duplication of the ancestral *Rx* gene allowed a progressive specialization of expression of the two *Rx* genes so that at later developmental stages one of them became preferentially expressed in the eyes (*Rx2*) and the other in the ventral hypothalamus (*Rx3*). The same separation of function occurs in the adult retina, with *Mrx* in both the photoreceptor layer and inner nuclear layer, while the zebrafish *Rx3* expression is confined to the inner nuclear layer and *Rx1* and *Rx2* are both expressed in the photoreceptor cells. An additional more recent duplication of the *Rx2* gene led to a presence of three *Rx* genes in zebrafish, of which two, *Rx1* and *Rx2*, remain active during retinal development, while the third *Rx* gene, *Rx3*, remains active only in the ventral hypothalamus. A similar situation is present in the medaka (Loosli *et al.,* 2001). The significance of this divergent evolution of *Rx* genes is not understood, but one could speculate that the differential expression of *Rx* genes in the retina and hypothalamus allows an independent regulation of proliferation in the retina and ventral hypothalamus. This in turn would allow modulation of eye development without affecting the development of the hypothalamus and vice versa.

The divergent expression pattern of *Rx* genes observed in zebrafish and medaka offers a unique opportunity to analyze the evolution of regulatory regions in this small gene family. It will allow identification of sequences that are responsible for the maintenance of *Rx* expression in the retina, and those responsible for the maintenance of *Rx* expression in the ventral hypothalamus. Identification of distinct elements that direct expression into the retina and into the ventral hypothalamus would have the advantage of being able to specifically modify gene expression only in the eyes or only in the hypothalamus. Comparison of regulatory elements from mouse, frog, medaka and zebrafish will reveal the molecular mechanism responsible for conserved and diverged aspects of *Rx* expression during evolution.

In contrast to the vertebrates, the *Drosophila Rx (drx)* is not expressed in the eye disk, but it is expressed in the part of the brain called the ellipsoid body and in the clypeolabrum (Eggert *et al.,* 1998). In planarians, the *Rx* gene was isolated in *G. tigrina,* but the *Gtrx* does not show any expression in the planarian eye cells (Salo *et al.,* 2002). In hemichordate, *Saccoglossus kowalevskii*, *Rx* is expressed in the anterior neuroectoderm. Since this acorn worm does not have eyes, *Rx* expression in these species cannot be associated with eyes either (Lowe *et al.,* 2003).

Functional studies with a mutated *Rx* gene

A. Targeted elimination of Rx *in mice*

The murine *Rx* (*Mrx*) was independently isolated by Mathers *et al.,* (1997) and by Furukawa *et al.,* (1997a) who gave this gene the name *rax*. Transcription of the *Mrx* begins around E7.5 in the anterior neural plate. At E10.5 expression of *Mrx* is confined to the developing retina and ventral forebrain. We have examined the effects of elimination of *Rx* function on the morphology of mouse embryos by targeted elimination of the *Mrx* gene (Mathers *et al.,* 1997). We have found that *Mrx-/-* embryos have no visible eye structures, while mice heterozygous for the *Rx* mutation are apparently normal (Fig. 2D). The abnormal phenotype of these embryos is apparent as early as E8.5 by the failure to form the optic sulci that give rise to the optic cups. This suggests that *Rx* is essential for initiation of eye development. At the morphological level, the primary problem is in the ventral forebrain. The ventral neuroectoderm is much thinner in mutants than in normal siblings, while the dorsal and lateral forebrain structures appear to be normal (Zhang *et al.,* 2000). We studied the expression of eye specific genes in *Mrx-/-* mutants. The primary focus of our investigation was the expression of *Otx2, Six3, Pax6* and *Foxe3*. In wild type embryos these genes are expressed in early stages of eye formation. *Otx2, Six3,* and *Pax6* are initially active in the anterior neural plate; and later in development their expression is prominent in the retinal progenitor cells (Blitz and Cho, 1995; Oliver *et al.,* 1995; Pannese *et al.,* 1995; Simeone *et al.,* 1993; Walther and Gruss, 1991). All three of the genes are also expressed in other areas of the embryo. *Foxe3* is initially expressed in the lens placode and later remains active in the cells of the anterior lens epithelium (Blixt *et al.,* 2000; Brownell *et al.,* 2000). When we analyzed expression of *Pax6, Otx2,* and *Six3* in *Rx-/-* embryos, we found that the initial activation of these genes in the anterior neural plate is not *Rx* dependent, but the specific upregulation of these genes in the retinal progenitor cells is *Rx* dependent. There are at least two possible interpretations of these findings. First, it is possible that the retinal progenitor cells do not form in these embryos and therefore there is no gene expression characteristic of the developing retina. Second, it is possible that some retinal cells are specified, but they fail to proliferate and because of their small number they are difficult to detect by *in situ* hybridization. Of special significance is the lack of *Pax6* expression in the retinal progenitor cells as it suggests that *Rx* is genetically upstream of *Pax6*. In contrast, there were no significant changes in *Rx* expression in the *Pax6*-/- background, demonstrating that both *Rx* expression and the initiation of eye development in mice is *Pax6* independent (Zhang *et al.,* 2000). Expression of *Foxe3,* an early marker of lens development, was not detected in the *Mrx-/-* mutant providing genetic evidence that the formation of the lens in mice depends on formation of retinal progenitor cells. In addition, we have investigated the expression of several other markers of eye development in this mutant, and we found no expression of these genes in the lateral part of the brain where the retina would evaginate. Our interpretation of this finding is that the retinal cells are not specified. An alternative explanation is that the retinal cells are specified, but they do not proliferate and because of their small number they are difficult to detect by *in situ* hybridization. We consider this possibility unlikely, as we can typically detect single cells by *in situ* hybridization.

The *Mrx* null embryos are unique in their failure to form an optic vesicle since even *Small eye* mutant embryos show optic vesicle formation, though it is abnormal morphologically (Grindley *et al.,* 1995; Hogan *et al.,* 1986). These observations demonstrate that *Mrx* function is essential for eye formation from its initial stages and that this gene has a unique role in eye development.

B. Analysis of eye development in embryos with mutated Rx *gene*

During last few years, several eye mutations have been identified that are due to the incorrect structure or regulation of the *Rx* gene. The *eyeless* mutation in mouse (*ey1*) displays severe eye and hypothalamic abnormalities. These abnormalities are due to a mutation in the *Mrx* gene that affects a conserved AUG codon

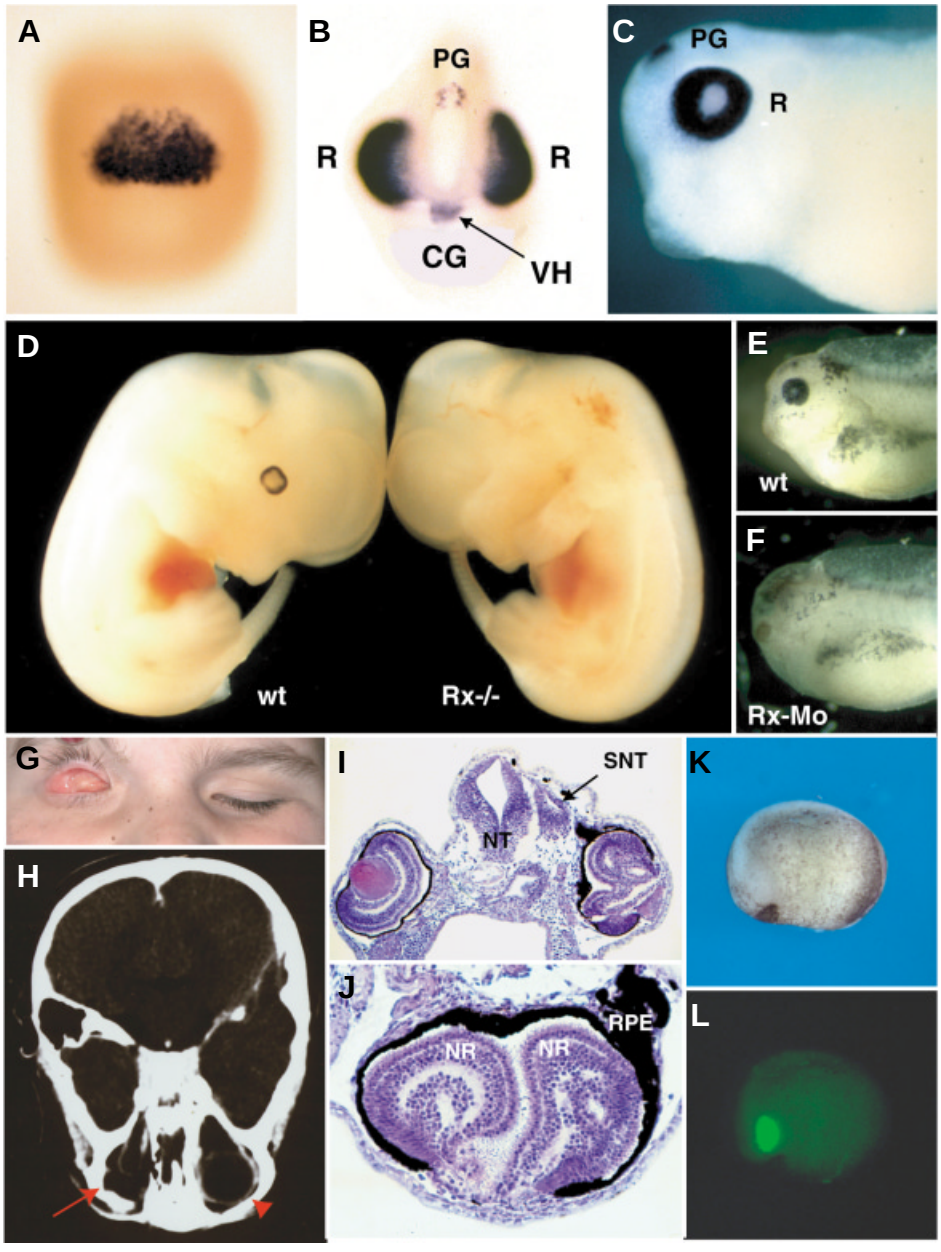

Fig. 2. *Rx* expression, phenotypes and regulation. (A-C) *Expression pattern of Xrx1 in Xenopus embryos.* **(A)** *Anterior view of an early neurula stage embryo showing expression of* Xrx1 *in a single field.* **(B)** *Anterior view of a tadpole showing* Xrx1 *expression in the pineal gland (PG), ventral hypothalamus (VH), and two developing retinas (R). The cement gland (CG) does not express* Xrx1. **(C)** *A lateral view of a tadpole showing* Xrx1 *expression in the retina (R) and pineal gland (PG). Notice the lack of expression in the lens.* **(D)** *Effects of elimination of* Rx *function in mouse embryos. Comparison of the* Rx*-/- mouse embryo (right) with* Rx*+/- sibling demonstrates that* Rx *function is required for eye formation. (E,F) Elimination of* Xrx1 *function by* Rx *specific morpholino* (RxMO) *in* Xenopus *embryos results in the absence of eye formation.* **(E)** *Uninjected side of embryo showing normal eye development, while the eye is completely missing on the opposite,* RxMO *injected side* **(F).** *(G,H) Mutations in the human* RX (RAX) *gene cause anophthalmia.* **(G)** *Absence of ocular tissue in a patient with a mutation in* RX *gene.* **(H)** *CT scan of the same patient showing anophthalmic orbit (red arrow) and other orbit (red arrowhead). (I,J) Overexpression of* Xrx1 *RNA in* Xenopus *embryos results in overproliferation of the cells of the retina and anterior neural tube.* **(I)** *A cross section through a* Xenopus *embryo injected on the right side with* Xrx1 *RNA showing a duplication of the anterior neural tube and overproliferation of retinal cells.* **(J)** *Both the retinal pigment epithelium (RPE) and the neuroretina (NR) show overproliferation in this eye from an embryo injected with* Xrx1 *RNA. As a result, the RPE and the neuroretina show additional folding of the cell layers. (K,L). Regulatory elements of the* Xrx1 *direct GFP expression into the developing retina of* Xenopus *embryos.* **(K)** *Lateral view of a* Xenopus *embryo under transmitted light.* **(L)** *The same embryo viewed under fluorescence optics shows GFP expression in the developing retina. Images (A-C) and (I,J) are modified from Mathers* et al., *(1997), and images (K,L) are from Zhang* et al., *(2003). (D-H) are our unpublished data. CG, cement gland; NR, neuroretina; NT, neural tube; PG, pineal gland; R, retina; RPE, retinal pigment epithelium; SNT, secondary neural tube; VH, ventral hypothalamus.*

that is used as an alternative translation initiation site. As a consequence of this mutation, the level of Rx protein is reduced (Tucker *et al.,* 2001).

In contrast, the temperature sensitive *eyeless* mutation in medaka is caused by an intronic insertion in the *Rx3* gene. This insertion leads to a transcriptional repression of the locus (Loosli *et al.,* 2001) that in turn leads to the lack of eye formation.

The zebrafish mutation *chokh* is caused by a mutation in the homeobox region of the zebrafish *Rx3* gene. This mutation introduces a stop codon into the homeodomain, severely truncating the Rx protein (Loosli *et al.,* 2003). Consequently, the mutant fish do not develop eyes.

In *Xenopus*, the function of *Rx* was eliminated by using dominant negative *Rx* constructs and by *Rx*-specific morpholinos. Injection of a dominant negative construct *Xrx1-EnR* or a morpholino directed against *Xrx1* into *Xenopus* embryos leads to a reduction or a loss of eyes and anterior head consistent with the phenotype observed in *Rx-/-* mice (Fig. 2 E,F - our unpublished observation; also see Andreazzoli *et al.,* 1999; Andreazzoli *et al.,* 2003).

In chicken, a putative dominant-negative allele of *RaxL* was introduced into the early chick eye using a retroviral vector. This construct caused a significant reduction in expression of early markers of photoreceptor cells. The reduction in numbers of photoreceptor cells was probably due to decreased survival of

developing photoreceptor cells, as there was increased apoptosis among cells of the retina expressing this construct (Chen and Cepko, 2002). In contrast, expression of the dominant-negative *RaxL* by Sakagami *et al.,* (Sakagami *et al.,* 2003) led to the conclusion that *RaxL* is involved in the regulation of ganglion cells. The reasons for these contradictory findings are not entirely clear.

In humans, *RX* has a critical role in eye formation as well. Mutations in human *RX* cause anophthalmia and sclerocornea (Fig. 2 G,H; Voronina *et al.,* 2004). In this report, a patient was identified with two different mutated alleles of *RX,* a truncation allele that prevents formation of the DNA-binding helix of the homeodomain and a missense mutation, within this helix, that reduces the ability of RX protein to bind to its DNA target.

C. Overexpression of Rx

The effects of overexpression of *Rx* genes were primarily examined by injection of *Xrx1* synthetic RNA into *Xenopus* embryos. Overexpression of *Xrx1* in the dorsal animal blastomeres of *Xenopus* embryos results in overproliferation of the neuroretina and retinal pigment epithelium (Fig. 2J). In some embryos ectopic retinal tissue was observed and the anterior neural tube was duplicated (Fig. 2J; Andreazzoli *et al.,* 1999; Mathers *et al.,* 1997). Similar results were obtained in zebrafish by Chuang and Raymond (Chuang and Raymond, 2001).

Regulation of *Rx*

At the molecular level, expression of *Rx* can be activated in *Xenopus* embryos by *chordin, noggin, Hedgehog*, and *wnt* pathways (Andreazzoli *et al.,* 2003; Rasmussen *et al.,* 2001; Zuber *et al.,* 1999), Neurogenin and retinoic acid represses *Rx* transcription (Andreazzoli *et al.,* 2003). *Rx* expression in the anterior neural plate is surrounded by expression of *X-ngnr-1* and *p27Xic1*. *X-ngnr-1* promotes neuronal differentiation and *p27Xic1* is a cell cycle inhibitor. It appears that *Xrx1* inhibits expression of *X-ngnr-1* and *p27Xic1*. In addition, *Rx* activates transcription of *XBF-1*. *XBF-1*, like *X-ngnr-1*, inhibits *p27Xic1* expression and therefore facilitates cell proliferation (Hardcastle *et al.,* 2000). As a result of all these interaction, the *Rx* expressing cells proliferate, but they do not differentiate. Another target of *Rx* is *Otx2*, a homeobox-containing gene that is essential for the specification of the anterior neural plate, but needs to be suppressed in the retinal territory. *Rx* protein seems to mediate this repression of *Otx2* transcription (Andreazzoli *et al.,* 1999).

The regulatory elements of the *Xenopus tropicalis* and *laevis Rx* gene are located in the 5' upstream region of the gene (Hirsch *et al.,* 2002; Zhang *et al.,* 2003). These sequences are able to direct gene expression into the developing eyes and ventral hypothalamus. Because of this, they are uniquely suited to manipulate gene expression in the developing eye and the ventral hypothalamus (Fig. 2 K,L). We used these sequences to investigate the role of FGF signaling mediated by the FGFR-4 in the specification of retinal cell types (Zhang *et al.,* 2003).

Implications for eye development and evolution

The exact function of *Rx* during eye formation is not yet fully understood, but there is an increasing body of evidence that *Rx* is primarily involved in the proliferation of cells in the retina and ventral hypothalamus. *Xenopus* embryos that overexpress *Xrx1* gene product show increased number of cells in the retina (Andreazzoli *et al.,* 1999; Mathers *et al.,* 1997). This increased number of retinal cells could be explained by the increased proliferation of cells or by increased recruitment of cells into retinal fate. However, Casarosa *et al.,* (2003) demonstrated that overexpression of *Xrx1* in *Xenopus* embryos lengthen the expression of markers of cycling cells such as cyclin D1, suggesting that proliferation plays an important role in this process. This is further supported by the observation of Andreazzoli *et al.,* (2003) that in *Xenopus, Rx* controls cell proliferation by inhibition of *X-ngnr-1,* a factor that promotes neuronal differentiation, and *p27Xic1*, a cell cycle inhibitor. The absence of eyes in *Rx-/-* mutants is compatible with the function of *Rx* in the determination and/or proliferation of retinal progenitor cells (Mathers *et al.,* 1997), but Zhang *et al.,* (2000) showed that the unusually thin ventral neuroectoderm that is present in *Rx-/-* mice is able to induce formation of the Rathke's pouch. This suggests that the ventral neuroectoderm in *Rx-/-* mice is at least partially specified and functional. However, there is no evidence in this experimental model that the retina is specified, as later markers of retinal development are not expressed and lens induction is not taking place in these animals (Brownell *et al.,* 2000; Zhang *et al.,* 2000). This would suggest that *Rx* is involved in the specification of retinal progenitor cells.

In contrast, in the medaka mutation *eyeless,* there is evidence that some retinal specification is taking place in the absence of *Rx3* protein (Loosli *et al.,* 2001; Winkler *et al.,* 2000). While the optic cups do not evaginate in this mutant, some gene expression characteristic of later stages of retinal development is observed. Lens induction also takes place. A similar situation was found in the zebrafish mutation *chokh* (Loosli *et al.,* 2003). Based on these observations it was suggested that the function of *Rx3* is in the evagination of the optic vesicle.

The discrepancy for the different phenotype in mouse and medaka and zebrafish is not fully understood, but it is important to point out that medaka and zebrafish have three *Rx* genes, while mouse has only one. Furthermore, only the mouse mutant is a true null mutant, complicating the comparison between these species further.

In summary, there is increasing evidence, mainly from *Xenopus* studies, that *Rx* acts as a cell type specific proliferation factor that is involved in the proliferation of cells from which the retina and the ventral hypothalamus are derived. Evidence from medaka and zebrafish suggests that *Rx* might be involved in the morphogenesis of the optic vesicle. Finally, observations from *Rx-/-* mice suggest that in addition to cell proliferation, *Rx* might have a role in the specification of the retinal progenitors. This is further supported by the finding that embryonic stem cells can be specified to form retinal cells by ectopic expression of *Rx* (Tabata *et al.,* 2004).

The requirement for *Rx* function is not universal in all species. While the development of the vertebrate eye is dependent on *Rx* function, the development of eyes in lower animals, including the insect, is not. The differential dependence of vertebrate and insect eye formation on *Rx* could have several reasons; one of them being that the vertebrate and insect eyes have a different evolutionary origin. Many different kinds of eyes are present in the animal kingdom and several theories have been proposed to explain this variety. Some believe that eyes appeared independently many times during evolution (Salvini-Plawen and Mayr, 1977), while others suggest that all eyes evolved from a common prototype

(Gehring and Ikeo, 1999). The currently favored hypothesis that all eyes developed from a common prototype is certainly appealing, but it is somewhat counterintuitive. The basic problem is created by the fact that the camera eye of vertebrates and the compound eye of insects not only look very different, but they are also generated through two entirely different developmental processes. The vertebrate retina is derived from the neuroectoderm, while the *Drosophila* eye is derived from the surface ectoderm (Wolff and Ready, 1991).

What supports the hypothesis that all eyes developed from a common prototype?

The initial support for this hypothesis came from the observation that orthologues of many genes involved in vertebrate eye development are involved in *Drosophila* eye development. The realization that flies lacking the function of *eyeless,* or mice lacking the function of the murine *eyeless* homologue *Pax6* do not develop visible eyes (Hill *et al.,* 1991; Quiring *et al.,* 1994), strengthens this argument. Finally, the finding that *eyeless* and *Pax6* can induce ectopic eye formation in *Drosophila* (Halder *et al.,* 1995), led to the suggestions that *Pax6* is a master control gene of eye development (Gehring, 1996) and that all eyes evolved from a common prototype (Gehring and Ikeo, 1999). Further support for these two proposals came from the observation that the overexpression of *Pax6* in *Xenopus* embryos can lead to the formation of ectopic eyes (Chow *et al.,* 1999). However, while the experiments leading to these proposals are convincing, there might be alternative explanations for these observations.

While in insects, *eyeless* truly might be the master gene of eye development, the role of its vertebrate homologue, *Pax6,* is less clear. First of all, the function of *Pax6* is not as essential for vertebrate eye development as originally believed. While it is true that embryos lacking *Pax6* function do not develop eyes, the eyes begin to form in *Pax6-/-* embryos, but they arrest at the optic vesicle stage (Grindley *et al.,* 1995; Zhang *et al.,* 2000). Therefore initiation of vertebrate eye development does not require *Pax6* function, arguing against *Pax6* being a master gene of vertebrate eye development.

Second, while it is true that the overexpression of *Pax6* can lead to the formation of ectopic eyes in *Xenopus*, this experiment does not necessarily show that *Pax6* is the gene initiating eye formation during normal *Xenopus* development. The interpretation of this experiment is complicated by the fact that genes involved in the regulation of eye development are involved in a complicated regulatory framework in which a feedback mechanism is frequently used (Chen *et al.,* 1997). For this reason, a dramatic overexpression of a certain gene product might activate upstream genes through a feedback loop that during normal development does not play a significant role. Indeed, it was observed that during overexpression of *Pax6, Rx* is activated ectopically (Chow *et al.,* 1999). This is somewhat troubling in the light of the fact that genetic evidence in mice shows that during normal development activation of *Rx* is not *Pax6* dependent (Zhang *et al.,* 2000). Therefore, it is hard to exclude in this experiment that the formation of ectopic eyes is due to an artificial activation of *Rx* or some other gene that is normally upstream of *Pax6.* It is therefore entirely possible that in some cases overexpression experiments show what can be done by a dramatic overproduction of a regulatory molecule, rather than demonstrating what is taking place during normal embryonic development.

Finally, while it is true that the overexpression of the vertebrate *Pax6,* like the overexpression of *eyeless,* leads to ectopic eye formation in *Drosophila*, this experiment only shows that the basic function of *Pax6* as a transcriptional activator/repressor is conserved to the degree that it can activate the insect eye-forming network. This is not surprising given the extremely high sequence conservation seen in the *Pax6* gene family.

Nevertheless, it is clear that many factors that are involved in the invertebrate eye formation have homologues in vertebrates and are expressed during vertebrate eye formation. Does this mean that all eyes developed from a common prototype? While many components of eye development are conserved in very distant species, recently several examples have been brought to light demonstrating that certain components that were believed to be critical for the eye-forming cascade are missing in a certain type of eyes. For example, the planarian eyes require neither *Pax6* activity nor *Six3* (Pineda *et al.,* 2002; Pineda and Salo, 2002). While *dachshund* is critical for invertebrate eye formation (Mardon *et al.,* 1994), it does not seem to have an important role in vertebrate eye formation (Davis *et al.,* 2001). And last, but not least, vertebrate eye formation is *Rx* dependent while invertebrate eye development is *Rx* independent (Davis *et al.,* 2003). We believe that the differential dependence of eye formation on *Rx* in insects and vertebrates reflects different evolutionarily origin of these two types of eyes.

We believe that the precursors of the vertebrate eye emerged in a region of the embryo where *Rx* was essential for the specification, survival or proliferation of cells. When considering this concept, it is important to realize that *Rx* is not only expressed in retinal progenitor cells. Rather, it is expressed in a field of cells from which the retinal progenitor cells, but also the cells of the ventral hypothalamus, will form. Therefore the expression of *Rx,* or for that matter *Pax6,* in the anterior neural plate is not sufficient for the formation of retinal progenitor cells. Indeed, there is no gene that is specifically expressed only in the presumptive retinal progenitor cells. For that reason, we do not believe that *Rx,* or any other gene can be called the “master gene” of eye development. In addition, the concept of a “master gene’ of eye development is not supported by the finding that essentially all the genes involved in eye formation are dispensable in one or the another species. Therefore, we consider it far more likely that specific regulatory interactions between several genes are necessary to assure that the retinal cells are formed.

In the ancestral eye, which eventually gave rise to the distinctive eye of *Drosophila*, *Pax6* might have played a critical role, but there is increasing evidence that the precursor of vertebrate eyes developed by the formation of a new regulatory network in *Rx* dependent cells.

Naturally, it is expected that the two regulatory networks involved in the formation of these two different types of eyes will show some similarities, since they were generated from a similar repertoires of active genes in the similar area of the embryo. However, the presence of superficially related regulatory networks cannot be easily used as a proof of a common evolutionary origin, as regulatory circuits are frequently reused during development and differentiation (Pichaud *et al.,* 2001). Some interactions among these genes result in eye formations, others do not. It is possible, indeed likely, that more than one combination of these

transcription factors can lead to eye formation. As a consequence of a different use of these factors, the development and the appearance of eyes vary drastically between species that developed independently. We believe that the different regulatory networks that are present in *Drosophila* and vertebrate eye formation reflect this fact. It is possible that the initial successful interactions that led to the insect eye formation were triggered by *Pax6*, while *Rx* triggered the successful interactions that led to the vertebrate eye formation.

One piece of evidence which directs us towards this alternative explanation of *Pax6*- and *Rx*- dependent eye evolution is the presence of two different types of photoreceptor cells which are present in many bilaterian groups (Arendt and Wittbrodt, 2001). It has been suggested that the rhabdomeric photoreceptors might be the manifestation of *Pax6* dependent eye evolution, while the ciliary photoreceptors are the result of *Rx* dependent eye evolution (Arendt, 2003). The hypothesis that *Rx* has an important function in the photoreceptor formation cannot be excluded. While the knockout of *Rx* in mouse shows only the dependence of formation of retinal progenitor cells on this gene, expression of *Rx* was observed in photoreceptor cells of several species (Perron *et al.,* 1998; Chuang *et al.,* 1999; Deschet *et al.,* 1999; Zhang *et al.,* 2003). Furthermore, it was shown that *Rx* binds to the photoreceptor conserved element-1 (PCE-1/Ret 1) in the photoreceptor cell-specific arrestin and IRBP promoter (Kimura *et al.,* 2000). Finally, the bovine gene related to *Rx*, *QRX*, was recently described as being capable of modulating photoreceptor gene expression (Wang *et al.,* 2004). Findings that show sequence changes in the human QRX gene of individuals with retinal degeneration (Wang *et al.,* 2004) indicate that *Rx* genes might be also involved in later steps of vertebrate eye formation.

Acknowledgements

We would like to thank Dr. Eric Swindell for a critical reading of the manuscript. This research was sponsored by NIH/NEI grant EY12505 to M.J. and by NIH/NEI training grant T32 EY07102 to T.J.B.

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