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# Medaka *eyeless* is the key factor linking retinal determination and eye growth

**Felix Loosli[1], Sylke Winkler[1], Carola Burgtorf[1], Elisa Wurmbach[2,*], Wilhelm Ansorge[2], Thorsten Henrich[1,‡], Clemens Grabher[1], Detlev Arendt[1], Matthias Carl[1], Annette Krone[1], Erika Grzebisz[1] and Joachim Wittbrodt[1,§]**

[1]European Molecular Biology Laboratory, Developmental Biology Programme, Meyerhofstr. 1, 69117 Heidelberg, Germany
[2]European Molecular Biology Laboratory, Biochemical Instrumentation Programme, Meyerhofstr. 1, 69117 Heidelberg, Germany
*Present address: Mount Sinai School of Medicine, New York, NY 10029, USA
‡Present address: Kondoh differentiation signalling project, Kyoto, Japan
§Author for correspondence (e-mail: Jochen.Wittbrodt@EMBL-Heidelberg.de)

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## SUMMARY

**The complete absence of eyes in the medaka fish mutation *eyeless* is the result of defective optic vesicle evagination. We show that the *eyeless* mutation is caused by an intronic insertion in the *Rx3* homeobox gene resulting in a transcriptional repression of the locus that is rescued by injection of plasmid DNA containing the wild-type locus. Functional analysis reveals that *Six3*- and *Pax6*- dependent retina determination does not require *Rx3*. However, gain- and loss-of-function phenotypes show that *Rx3* is indispensable to initiate optic vesicle evagination and to control vesicle proliferation, by that regulating organ size. Thus, *Rx3* acts at a key position coupling the determination with subsequent morphogenesis and differentiation of the developing eye.**

Key words: Retina determination, Morphogenesis, Proliferation, Size control, Zebrafish

## INTRODUCTION

The vertebrate eyes form from a single eye field, an anterior ectodermal territory containing a retina anlage and an abutting lens competence field. Under the influence of midline signals, this field splits into two bilateral regions each containing a neuroectodermal retina anlage and an ectodermal lens anlage. During neurulation, the retina anlage evaginates laterally from the forebrain, giving rise to the optic vesicles. These in turn invaginate distally to form the optic cups with neural retina (NR) and retinal pigmented epithelium (RPE) (Grainger, 1996; Jean et al., 1998). Several transcription factors that are required for optic vesicle development have been identified so far (Chow et al., 1999; Jean et al., 1998; Loosli et al., 1999; Porter et al., 1997). However, it is yet unknown in what manner these factors interact to initiate and control optic vesicle formation. What is the molecular link between the determination of the retina anlage and the formation of an optic vesicle?

The transcription factor *Pax6* is an essential regulator for eye development conserved in evolution (Quiring et al., 1994; Walther and Gruss, 1991). *Pax6* is expressed in the anterior neuroectoderm before optic vesicle formation in fish (Krauss et al., 1991; Loosli et al., 1998), frog (Hirsch and Harris, 1997), and chick (Li et al., 1994). *Pax6*$^{-/-}$ *Small eye* mutant mice develop small optic vesicles, which degenerate during subsequent development (Hill et al., 1991; Hogan et al., 1986). Overexpression of *Pax6* in *Drosophila* and *Xenopus* can lead to ectopic eye formation (Chow et al., 1999; Halder et al., 1995).

*Six3* plays a role in specifying early retinal identity (Bernier et al., 2000; Loosli et al., 1999). This evolutionarily conserved transcription factor contains a homedomain and a Six domain (Cheyette et al., 1994; Seimiya and Gehring, 2000; Toy et al., 1998). It is specifically expressed in the anterior neuroectoderm including the eye field and specific parts of the abutting surface ectoderm in all vertebrates examined (Bovolenta et al., 1998; Granadino et al., 1999; Loosli et al., 1998; Oliver et al., 1995; Seo et al., 1998; Zhou et al., 2000). Overexpression of *Six3* leads to expanded and ectopic retinal primordia in medaka (Loosli et al., 1999) and *Xenopus* (Bernier et al., 2000), and to an enlarged forebrain in zebrafish (Kobayashi et al., 1998). In a feedback loop, *Six3* thereby induces its own expression, and that of *Pax6* (Loosli et al., 1999). As *Pax6* also induces *Six3* (Chow et al., 1999), both factors activate each other (Loosli et al., 1999).

Vertebrate members of the Rx gene family are expressed in the early eye field, and, with the exception of medaka *Rx2*, which is exclusively expressed in the developing retina, also in medial regions of the prosencephalon (Casarosa et al., 1997; Chuang et al., 1999; Deschet et al., 1999; Furukawa et al., 1997; Loosli et al., 1999; Mathers et al., 1997; Ohuchi et al., 1999). Targeted inactivation of Rx in mouse leads to optic vesicle and forebrain deletions, indicating a function of Rx in the development of these structures (Mathers et al., 1997). Similar results have been reported for *Xenopus* embryos injected with a X*Rx1* dominant repressor construct (Andreazzoli et al., 1999). Conversely ectopic expression of *XRx1* in *Xenopus* embryos triggers overproliferation of retinal

tissue (Andreazzoli et al., 1999; Mathers et al., 1997, Zhang et al., 2000). Thus, the specific involvement of Rx genes in eye development with regard to the determination of the retina anlage and subsequent specification and morphogenesis of the retinal progenitor cells remains to be defined.

In the temperature-sensitive, early larval lethal medaka mutation *eyeless* (*el*) optic vesicles do not evaginate at the restrictive temperature (Winkler et al., 2000). This defect in morphogenesis leads to a complete lack of eyes. Temperature shift experiments indicate that gene activity is required before optic vesicle evagination.

We show that the gene affected by the *eyeless* mutation is *Rx3*, a member of the Rx gene family. We combine mutant analysis with gain-of-function approaches to place *Rx3* function into the genetic network of early eye development. *Rx3* is required to initiate and maintain optic vesicle morphogenesis and proliferation, but is dispensable for the formation of the retina anlage. These results provide a link between retinal determination, and the subsequent control optic vesicle morphogenesis and differentiation.

## MATERIAL AND METHODS

### Fish strains

The *el* mutation was crossed into and kept in two different inbred genetic backgrounds (*Cab* and *Kaga*) and kept as closed stocks at EMBL (Winkler et al., 2000). Embryos were staged according to Iwamatsu (Iwamatsu, 1994). Sex averaged linkage analysis showed a close linkage between *el* and the pigmentation locus *b* (1.3 cM).

### Cleaved amplified polymorphic sequences (CAPS) analysis

DNA of homozygous *el* hatchlings (three to five individuals pooled) or individual wild-type fish derived from brother-sister crosses of *el*/*Kaga* carriers was isolated (QIAamp kit, Qiagen). A 333 bp fragment was amplified using primers specific for the third exon of *Rx3* (up, ACACTCCCTCATCTTTGCTCTCCTTC; low, TGTTCCT-TGGCCTTCATCCTCA). PCR conditions were 35 cycles of 94°C for 30 seconds, 61°C for 30seconds, 72°C for 1 minute. PCR products were *Hae*III digested and separated on 3% agarose gels, showing cleavage of a *Cab*-specific 190 bp fragment into 100 bp and 90 bp fragments in *Kaga*.

### Isolation of *Rx3* full-length cDNA

A neurula stage λZAP cDNA library was screened with a 440 bp *Rx3* PCR fragment (Deschet et al., 1999). Positive clones were plaque purified, converted to pBluescript and sequenced (EMBL Accession Number, AJ298300)

### RT-PCR analysis of mutant and wild-type embryos

Total RNA was isolated from two-somite stage homozygous mutant embryos and wild-type siblings (Sambrook et al., 1989) and reverse transcribed using an oligo-dT and a *Rx3*-specific primer (ATGTTCCATTCTGGGCGTCTCAG). A *Rx3*-specific primer pair (up, TTAGACAAATGTGGCTCCTGGGATCAGCTTCA; low, TGT-TCCTTGGCCTTCATCCTCA) was used as follows: 35 cycles of 94°C for 45 seconds, 60°C for 1 minute, 72°C for 2 minutes. PCR products were analysed by gel electrophoresis.

### Isolation of a partial *Tbx2* and a *Tbx3* cDNA

A 440 bp medaka *Tbx2* fragment and a 600bp fragment of *Tbx3* were RT-PCR amplified from stage 18 RNA using degenerate primers (up, GAGGTIGAG GAYGAYCCIAARGT; low, CCIRTGTCYCTRAAI-CCYTTIGCRAA). PCR conditions were 5 cycles of 94°C for 1 minute, 53°C for 2 minutes, 72°C of 4 minutes, followed by 30 cycles with annealing at 58°C. PCR products were cloned (TopoTA, Invitrogen) and sequenced (EMBL Accession Numbers: *OlTbx2*, AJ298301; *OlTbx3*, AJ298302).

### Southern and northern blot analysis

Genomic DNA of adult homozygous mutant *el*, heterozygous *el* carrier and wild-type fish was prepared. The DNA was digested over night, separated on a 0.7% agarose gel and transferred to Hybond$N^+$ (Amersham) by capillary transfer. Filters were hybridised with $^{32}$P-labelled probes under high stringency conditions (Sambrook et al., 1989). Total RNA was isolated from wild-type embryos. Poly$(A)^+$ RNA was enriched (dynabeads, Dynal). 10 μg of $A^+$ RNA were separated on a formaldehyde agarose gel, blotted and hybridised (Sambrook et al., 1989).

### Genomic library construction and cloning of the mutant *el* locus

A lambda FixII (Stratagene) genomic library was generated from genomic DNA of homozygous *el*-mutant fish. A phage covering the 3′ part of the mutation was isolated using the *Rx3* cDNA as a probe under high stringency conditions. The remaining part of the mutant locus was cloned as PCR fragments, and the 5′ breakpoint of the insertion was isolated by splinkerette (Devon et al., 1995).

### Rescue plasmids

Bac and cosmid clones containing the *Rx3* locus were isolated from arrayed libraries (library 756, 73 and 74, RZPD Berlin) probed with *Rx3* cDNA. BAC and cosmid DNA was prepared following standard protocols. For injection the DNA was further purified on a Caesium chloride gradient (Sambrook et al., 1989). For the *Rx3* rescue plasmid, an 11 kb *Sna*BI fragment of the *Rx3* cosmid was cloned into pBluescript SK+ (Stratagene). The remaining cosmid was re-ligated resulting in the Δ*Rx3* plasmid. A frameshift was introduced into the second helix of the homeodomain by *Bsp*EI digestion of the *Rx3* rescue plasmid and re-ligation after filling the ends with Klenow polymerase (*Rx3*ΔHB plasmid).

### DNA injections

Plasmid DNA was injected into the blastomere of one-cell stage embryos from *el* carrier crosses. Injected embryos were kept under restrictive conditions. The following DNA concentrations were used for injection experiments: *Rx3* BAC, 50 ng/μl; control BACs, 50 ng/μl; *Rx3* cosmid, 40 ng/μl; *Rx3* plasmid, 5 and 20 ng/μl; Δ*Rx3* plasmid, 45 ng/μl; *Rx3*ΔHB plasmid, 50 ng/μl and pBluescript, 50 ng/μl.

### RNA injections

A 1.2 kb *Bam*HI-*Bgl*II fragment of the medaka *Rx3* cDNA cloned into pCS2+ was verified by sequencing and TNT transcription/translation (Promega). Linearized pCS*mouseSix3*, pCS*medakaSix3* (Loosli et al., 1999) and pCS*medakaRx3* plasmid DNA were in vitro transcribed and the purified RNA injected into one blastomere at the two-cell stage (Loosli et al., 1999). RNA concentrations in the injection solution were 200 ng/μl pCS*mouseSix3*, 200 ng/μl pCS*medakaSix3*, when injected into embryos of *el* carrier crosses; 100 ng/μl, when injected into wild-type embryos; and 50 ng/μl pCS*medakaRx3*. For controls, the respective concentration of *hGFP* RNA was injected. Injected embryos from *el* carrier crosses were kept at the restrictive temperature (18°C).

### In situ hybridisation and vibratome sectioning

Whole-mount in situ hybridisation using antisense riboprobes and vibratome sections of embryos were performed as described (Loosli et al., 1998).

## RESULTS

### *Rx3* expression is affected in *el*-mutant embryos

The *el* mutation, initially isolated in a strain from the Southern population of medaka, was crossed into and kept in the '*Cab*' inbred strain (Winkler et al., 2000). Crossing of *el* carrier fish into the *Kaga* strain revealed a close linkage of *el* and the pigmentation locus *b*. The *Kaga* strain is homozygous for the *B* allele that results in darkly pigmented melanophores. The *Cab* background carries the recessive *b* allele that leads to unpigmented melanophores. Analysis of the offspring of *el*/*Kaga* intercrosses resulted in 32 recombinant *el* embryos (*el*/*el*,*b*/*B*) in 2422 meiosis. This corresponds to a genetic distance of 1.3 cM and thus places *el* on linkage group 12 (LG 12; Naruse et al., 2000) in close proximity to the *b* locus.

*el* is a temperature-sensitive mutation. Optic vesicles of homozygous mutant embryos do not evaginate at the restrictive temperature (18°C) and subsequent differentiation is perturbed. At higher temperatures (28°C) in 52% of the *el*-mutant embryos optic vesicles of variable size form (Winkler et al., 2000). Temperature shift experiments showed that *el* activity is required at late gastrula/early neurula stages before optic vesicle evagination. We therefore examined genes that are expressed in the retina anlage before optic vesicle evagination. The expression of the retina determination genes *Six3* and *Pax6* is not affected in the retinal primordia of *el*-mutant embryos (Winkler et al., 2000). These genes have been mapped to different linkage groups (i.e. *Pax6*, LG 3; *Six3*, LG 19 (Naruse et al., 2000)), excluding *Pax6* and *Six3*, as candidates for *el*.

In the anterior neuroectoderm, the homeobox gene *Rx3* is expressed from late gastrula stages onwards (Deschet et al., 1999). The initially narrow, single expression domain widens and is subsequently split into two domains lateral to the forming axis (Fig. 1A). During neurulation and early somitogenesis stages, *Rx3* is expressed in the anterior ventral prosencephalon, the optic vesicles and the optic stalk, which connects these tissues (Fig. 1C). Expression in the retina and optic stalk is downregulated at later somitogenesis stages, such that *Rx3* expression finally is restricted to the hypothalamus. In the differentiating retina, *Rx3* is expressed in the inner nuclear layer (INL). In situ hybridisation showed that in *el*-mutant embryos, *Rx3* expression in all its wild-type expression domains is completely absent at all stages at the restrictive temperature (Fig. 1A-D). This indicates that either *Rx3* or an upstream regulator is affected by the *el* mutation.

### CAPS analysis of *el*

To examine whether *Rx3* itself or a regulator of *Rx3* expression is affected in the *el* mutation we examined whether *Rx3* maps to linkage group 12 and whether the mutation co-segregates with an *Rx3* specific polymorphism. We took advantage of the highly polymorphic genetic backgrounds of the inbred *Cab* and the *Kaga* strains. A PCR amplified 333 bp genomic *Rx3* DNA fragment contains a polymorphic *Hae*III restriction site (Fig. 2A). This cleaved amplified polymorphic sequence (CAPS) was used to determine the chromosomal location of the *Rx3* gene and to examine a potential linkage between *Rx3* and the *el* mutation.

*Cab*/*Kaga* back-cross analysis locates the *Rx3* gene to linkage group 12 in close proximity to the *b*-locus (Fig. 2D). *el* carrier fish of the *Cab* strain, in which the mutation has originally been identified, were crossed to *Kaga* fish and the wild-type, and mutant progeny of the resulting *el*/*Kaga* carrier intercrosses were examined for co-segregation by CAPS analysis. The *Cab*-specific *Hae*III polymorphism always co-segregated with the *el* phenotype in all 532 meiosis analysed (Fig. 2). Thus, the genetic distance of the *Rx3* gene and the *el* mutation is less than 0.19 cM, strongly suggesting that the mutation affects the *Rx3* gene proper.

### Genomic organisation of the wild type and mutant *Rx3* locus

To further analyse the nature of the *el* mutation we cloned a full-length *Rx3* cDNA. Genomic Southern blots probed with a 600 bp 5′ fragment of the *Rx3* cDNA suggested an insertion in the *Rx3* locus of *el*-mutant fish (Fig. 2B). *Rx3* containing BAC and Cosmid clones were isolated from respective wild-type libraries. The genomic organisation of the *Rx3* locus was examined by sequence analysis and Southern blot

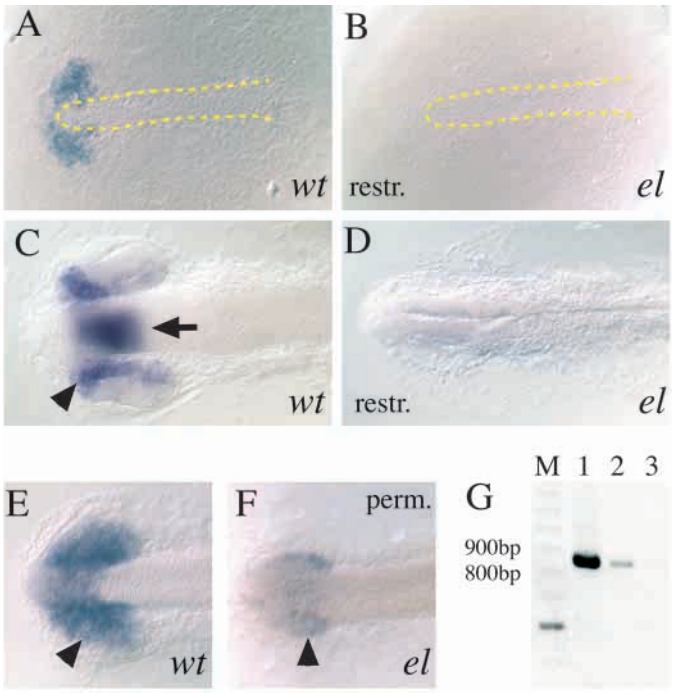



**Fig. 1.** *Rx3* expression in wild-type and *el*-mutant embryos. Dorsal views of whole-mount in situ hybridisation of late gastrula (stage 16; A,B) and four-somite stage (stage 20; C,D) wild type (A,C) and *el*-mutant embryos kept at restrictive temperature (B,D). Anterior is towards the left. Neural axis is indicated by a broken yellow line (A,B). (A) *Rx3* expression in anterior neural plate. (C) *Rx3* expression in hypothalamus (arrow) and optic cup (arrowhead). (B,D). Note complete loss of *Rx3* expression in mutant embryos kept under restrictive conditions. (F) Under permissive conditions, *Rx3* is weakly expressed in forebrain of *el*-mutant embryos at the two-somite stage (stage 19). Note reduced size of evaginating optic vesicles (arrowhead) at site of reduced *Rx3* expression compared with wild-type embryo of same stage (arrowhead in E). (G) Primers spanning entire open reading frame detect the wild-type transcript by RT-PCR in wild-type (lane 1) and *el*-mutant embryos (lane 2) under permissive conditions, while no transcript is detected in mutants under restrictive conditions (lane 3). M, 100 bp ladder. Abbreviations: perm., permissive temperature; restr., restrictive temperature.

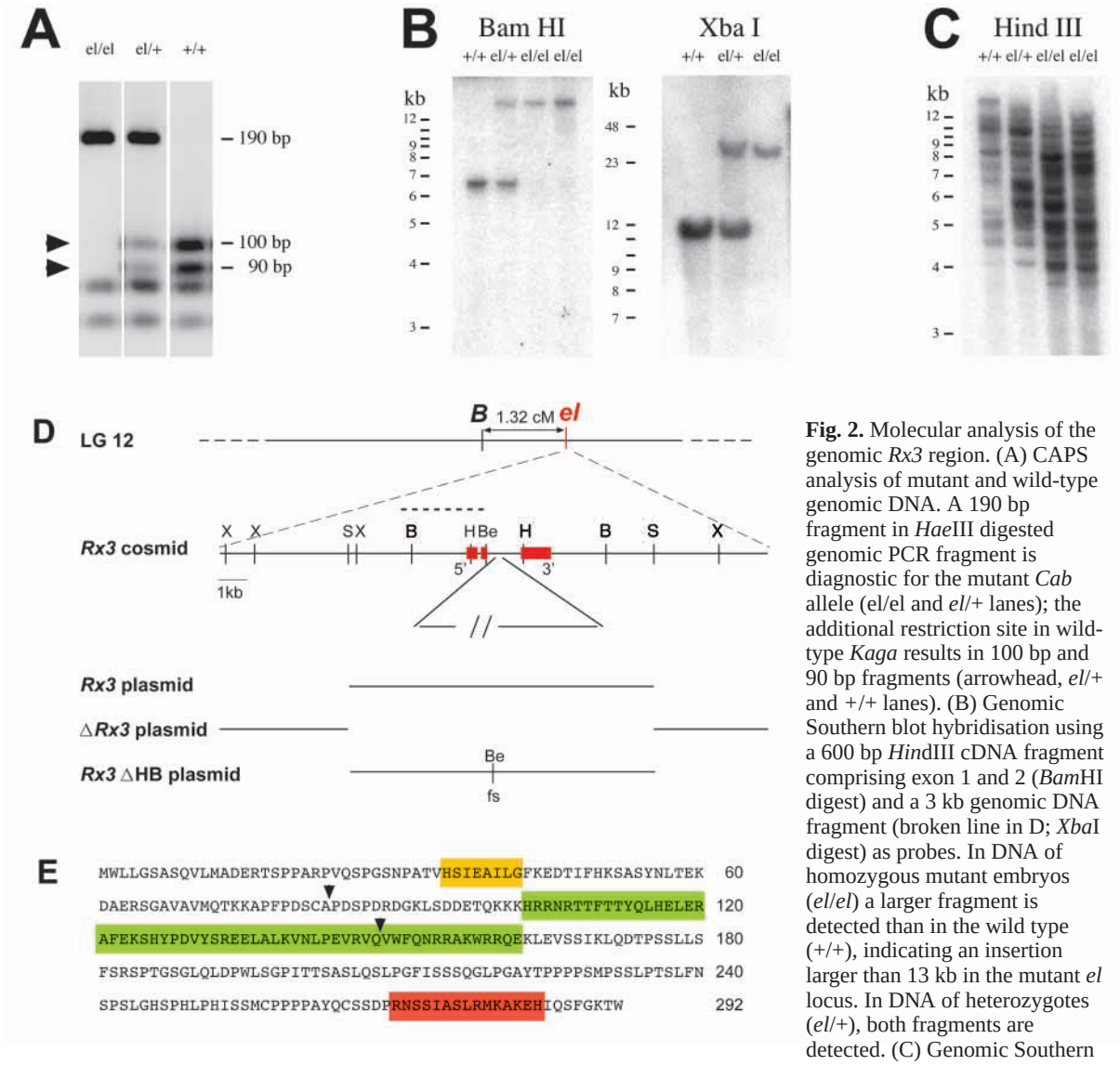



**Fig. 2.** Molecular analysis of the genomic *Rx3* region. (A) CAPS analysis of mutant and wild-type genomic DNA. A 190 bp fragment in *Hae*III digested genomic PCR fragment is diagnostic for the mutant *Cab* allele (el/el and *el*/+ lanes); the additional restriction site in wild-type *Kaga* results in 100 bp and 90 bp fragments (arrowhead, *el*/+ and +/+ lanes). (B) Genomic Southern blot hybridisation using a 600 bp *Hin*dIII cDNA fragment comprising exon 1 and 2 (*Bam*HI digest) and a 3 kb genomic DNA fragment (broken line in D; *Xba*I digest) as probes. In DNA of homozygous mutant embryos (*el*/*el*) a larger fragment is detected than in the wild type (+/+), indicating an insertion larger than 13 kb in the mutant *el* locus. In DNA of heterozygotes (*el*/+), both fragments are detected. (C) Genomic Southern blot hybridisation with a 4 kb insertion fragment reveals multiple copies in the genome, indicative of middle repetitive DNA. (D) Map of *Rx3* region indicating relative distance of *B* and *el* on linkage group 12 (LG 12). Red boxes represent three exons on *Rx3* cosmid; position of the insertion in intron 2 is shown. Regions comprised in different rescue plasmids and position of the frameshift (fs) are indicated. (E) Predicted amino acid sequence of the *Rx3* protein. Splice sites are indicated (arrowheads). Conserved octapeptide (yellow), homeodomain (green) and C-terminal region (red) are highlighted. Abbreviations: B, *Bam*HI; Be, *Bsp*EI; S, *Sna*BI; X, *Xba*I.

hybridisation. The *Rx3* open reading frame of 876 bp is split into three exons and spans a genomic region of about 2.9 kb (Fig. 2D). The predicted length of the RX3 protein is 292 amino acids. Sequence comparison with other homeobox genes of the Rx subclass shows that the medaka RX3 protein shares highest homology with zebrafish RX3. The RX3-specific octapeptide and the homeodomain are 100% identical and the conserved region in the C-terminal region (OAR; Furukawa et al., 1997) differ in only one amino acid between medaka and zebrafish RX3. However, the medaka paralogue RX2 differs by two amino acids in the octapeptide, one in the homeodomain and one in the OAR, compared with medaka RX3. In contrast to zebrafish, where three Rx genes have been cloned, we detected two paralogues in medaka.

The homeodomain is encoded by the 3′ part of exon 2 and the 5′ part of exon 3 (Fig. 2D,E). The mutant locus harbours an insertion in the intron intervening exon 2 and 3. The inserted DNA is middle repetitive as indicated by Southern blot hybridisation (Fig. 2C). The insertion of at least 13 kb has no significant homology to any known sequence. Apart from the insertion in intron 2, no further changes were detectable in the mutant *Rx3* locus. The open reading frame is unaffected and thus the locus has the potential to express a wild-type RX3 protein.

### Wild-type *Rx3* rescues optic vesicle evagination in the mutant embryo

The tight linkage of *el* and the pigmentation locus *b* was used to genetically mark the *el* mutation. The homozygous *el*-mutant offspring of an *el*/+,b/B carrier cross lacks the darkly

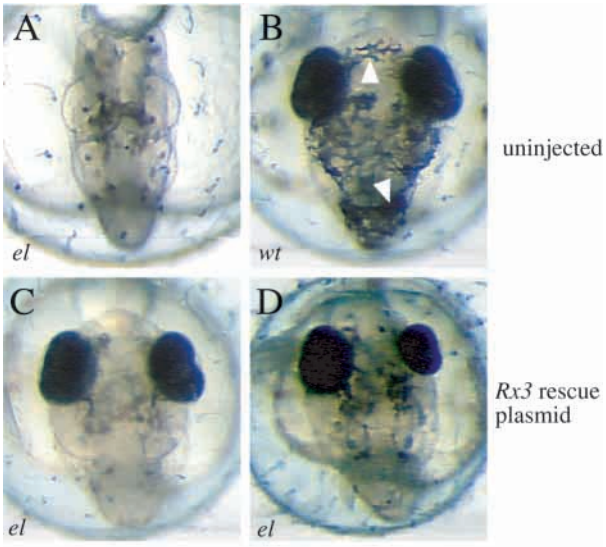
pigmented melanophores with the exception of less than 1.3% recombinants (Fig. 3A,B). The pigmentation becomes first visible at early somitogenesis stages. At the restrictive temperature, all mutant embryos lack eyes and do not form retinal pigmented epithelium (RPE), thus the mutant phenotype is fully penetrant and the expressivity not variable. Control injections into fertilised eggs affect neither penetrance nor expressivity (Fig. 3E). Mutant embryos injected with either the *Rx3* BAC or the *Rx3* cosmid form eyes of wild-type morphology in 25% and 39% of the cases, respectively (Fig. 3E).

The *Rx3* plasmid contains the *Rx3* locus (Fig. 2D) and rescues the *el* phenotype in 37% of the cases, while the remaining 8.2kb of the *Rx3* cosmid (Δ*Rx3* cosmid) have no rescuing activity (Fig. 3E). Northern blot analysis using the *Rx3* plasmid as a probe detects a single transcript of 1.8kb that corresponds to the *Rx3* mRNA (not shown) indicating that only the *Rx3* gene is expressed in the relevant time window from the *Rx3* plasmid. 50% of the rescued homozygous mutant embryos (n=8) identified by the lack of darkly pigmented melanophores show wild type morphology and behaviour (Fig. 3). This rescue is coupled with restored *Rx3* expression in mutant embryos (Fig. 4A,B). The finding that the rescued *Rx3* expression is confined specifically to the wild type expression domains indicates that all regulatory elements required for wild type expression are contained within the *Rx3* plasmid. To examine whether the rescue activity depends on a functional *Rx3* protein we introduced a frameshift in exon 2 of the *Rx3* plasmid. This results in a deletion of helices 2 and 3 of the homeodomain and the entire C-terminal portion (*Rx3*ΔHB plasmid, Fig. 2D). The frameshift completely abolishes the rescue activity (Fig. 3E) without affecting its expression (Fig. 4C,D) demonstrating that the rescue depends on the expression of a functional RX3 protein. On the other hand, expression from the rescue construct does not require *Rx3* activity (compare Fig. 4C with 4D), indicating that in contrast to *Pax6* and *Six3* functional RX3 protein is not required for its own expression.

## Expression of *Tbx* and *Vsx* genes in the developing retina require *Rx3*

The T-box-containing transcription factors *Tbx*2 and *Tbx*3 are expressed in the developing optic vesicles, ventral forebrain and the otic vesicles starting at early somitogenesis stages in wild-type medaka (Fig. 4H,K). In *el*-mutant embryos, however, cells of the retinal primordia do not express *Tbx*2 and *Tbx*3, whereas the other expression domains are not affected (Fig. 4I,L). Rescued presumptive retinae of mutant embryos express both *Tbx2* and *Tbx3* (Fig. 4G,J). This indicates that retinal *Tbx*2 and *Tbx*3 expression depend on *Rx3* activity and that the *Rx3* rescue plasmid can provide also this aspect of *Rx3* function. The paired like homeobox genes *Vsx1* and *Vsx2* are expressed in the differentiating retina (Winkler et al., 2000). In the retinae of rescued mutant embryos, expression of *Vsx1* and *Vsx2* that is lost in *el*-mutant embryos, is specifically restored (Fig. 4M-P).

Thus, the molecular and morphological analysis of rescued embryos shows that the *Rx3* plasmid fully rescues evagination of the optic vesicle and subsequent differentiation of the retina, substantiating that the *el*-mutant phenotype is caused by the intronic insertion in the *Rx3* gene.

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| DNA injected | N | *el* (*b/b*) embryos | eye formation | % |
|---|---|---|---|---|
| pBS | 116 | 38 | 0 | 0 |
| *Rx3* Bac | 186 | 43 | 11 | 25 |
| control Bac (*spt*) | 147 | 41 | 1 | 2 |
| *Rx3* cosmid | 175 | 36 | 14 | 39 |
| *Rx3* plasmid | 181 | 43 | 16 | 37 |
| Δ*Rx3* cosmid | 130 | 30 | 0 | 0 |
| *Rx3* ΔHB plasmid | 163 | 38 | 0 | 0 |

**Fig. 3.** Phenotypic rescue of *el*-mutant embryos. Uninjected (A,B) and injected (C,D) wild-type (B) and mutant embryos (A,C,D) raised for 8 days at restrictive temperature. Dorsal views, anterior is towards the top. Note dark pigmented melanophores in wild-type embryo (arrowhead in B), lacking in mutants (A,C,D). (A) No eyes form in mutants. (C,D) Rescued eye formation in mutant embryos injected with *Rx3* plasmid. (C) Note complete rescue of both eyes. (E) Table displaying results of different DNA and control injections. N, number of injected embryos; *el* (b/b) homozygous mutant embryos as judged by absence of dark melanophores. No rescue is observed in control injected embryos.

## Temperature sensitivity of *el* correlates with variable *Rx3* expression

At the restrictive temperature (18°C), *Rx3* is not expressed in *el*-mutant embryos (Fig. 1B,D). At the permissive temperature (28°C), however, we found weak *Rx3* expression at late neurula to early somitogenesis stages in 56% of the mutant embryos (Fig. 1F). Under these conditions, the mutant phenotype is variable, such that 52% of *el*-mutant embryos raised at the permissive temperature form small eyes. Thus, *Rx3* expression in *el*-mutant embryos kept at the permissive temperature closely correlates with the variable phenotype. Furthermore, RT-PCR analysis shows that at the permissive temperature a correctly spliced transcript is present in *el*-mutant embryos (Fig. 1G). Thus, the mutation affects the transcription of the *el* locus in a temperature-sensitive way, such that the *el* mutation is amorphic at the restrictive temperature and hypomorphic at the permissive temperature.

Taken together, three lines of evidence indicate that the locus

affected in the *el* mutation is the *Rx3* gene: (1) genetic mapping and the detection of an intronic insertion in the *Rx3* gene correlate; (2) the expressivity of the phenotype correlates with *Rx3* expression levels; and 3) the wild-type *Rx3* gene has rescuing activity.

### The regulatory interaction of *Six3* and *Pax6* is independent of *Rx3* activity

We had shown that overexpression of medaka or mouse *Six3* leads to retinal hyperplasia and the formation of ectopic retinal primordia in the mid- and hindbrain (Loosli et al., 1999). Ectopic retina formation in response to *Six3* or *Pax6* overexpression in *Xenopus* and medaka fish is preceded by a cross-regulatory interaction of these genes (Bernier et al., 2000; Chow et al., 1999; Loosli et al., 1999). It has been suggested that this interaction is a prerequisite for retinal determination. We examined whether *Rx3* functions in this regulatory interaction of *Six3* and *Pax6*.

To address this, we first investigated whether ectopic *Six3* expression in *el*-mutant embryos can relieve the transcriptional repression of the mutant locus at the restrictive temperature. At the two-somite stage, no *Rx3* expression was detected in the *el*-mutant embryos injected with *Six3* RNA (*n*=17, Fig. 5A,B). On the other hand, in all injected wild type siblings (n=67) *Rx3* expression was detected (not shown). Therefore, *Six3* overexpression does not relieve the transcriptional repression of the mutant *Rx3* locus under restrictive conditions. This allows examining the function of *Six3* in the absence of *Rx3*. The finding that optic vesicle evagination is not rescued in the *Six3* injected mutant embryos suggests a role of *Six3* upstream to or in parallel of *Rx3* (Fig. 5A,B).

Using the *el* mutation, we analysed whether *Rx3* activity is required for the crossregulatory interaction of *Six3* and *Pax6*. Ectopic medaka *Six3* expression in embryos that lack *Rx3* results in an enlarged *Pax6* expression domain (*n*=21/53, 39%) and in ectopic *Pax6* expression in the presumptive midbrain (*n*=9/53, 17%) at early somitogenesis stages (Fig. 5C,D). Similarly, in injected wild-type siblings used as internal control, expanded as well as ectopic *Pax6* expression was observed. Thus, ectopic *Pax6* expression in response to *Six3* overexpression does not require *Rx3* activity.

Mouse *Six3* overexpression results in the ectopic activation of the endogenous *Six3* gene, indicating that *Six3* functions in a regulatory feedback loop (Bernier et al., 2000; Loosli et al., 1999). To address whether this regulatory feedback loop of *Six3* will also function in the absence of *Rx3*, we injected mouse *Six3* mRNA at the restrictive temperature. We observed ectopic endogenous *Six3* expression at the early somitogenesis stages in 22% of injected *el*-mutant embryos (*n*=6/27, Fig. 5E,F). Consistent with expanded *Pax6* expression, endogenous *Six3* expression was also expanded in 40% (*n*=11) of the injected mutant embryos (not shown).

Thus, the crossregulatory interactions of *Six3* and *Pax6*, as well as the regulatory feedback loop of *Six3* are independent of *Rx3*. The finding that *Rx3* is not required for these regulatory

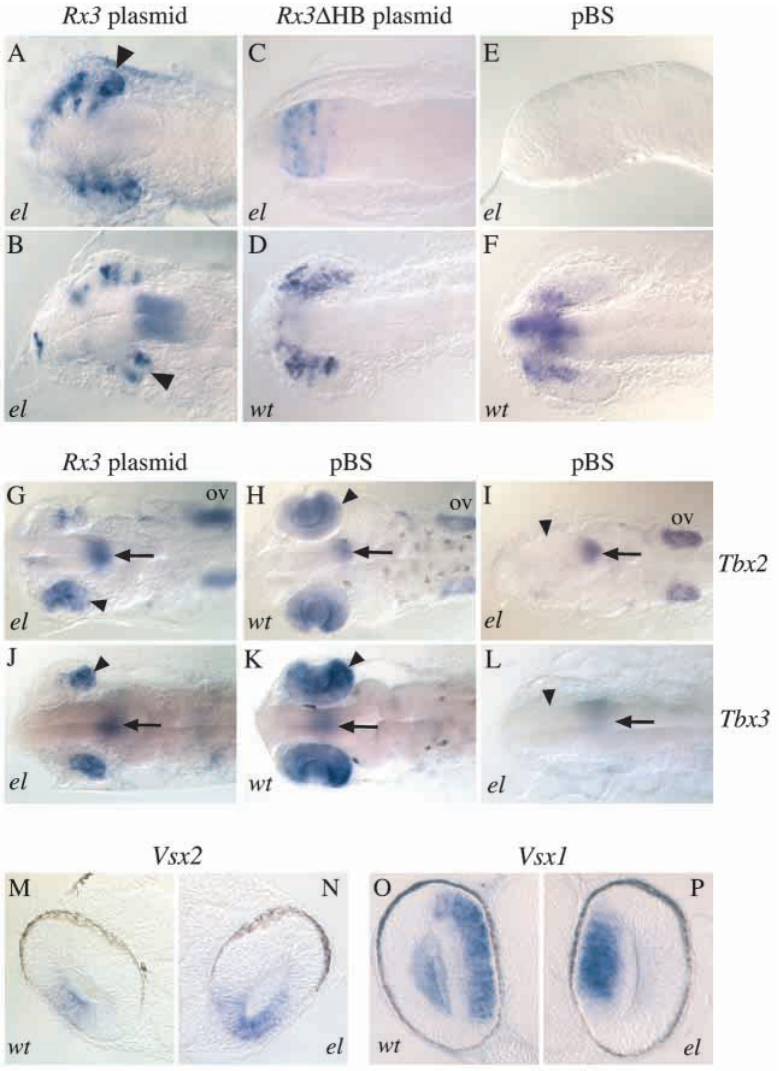


**Fig. 4.** Restored gene expression in rescued mutant embryos. (A-F) *Rx3* expression at the four-somite stage in mutant (A-C,E) and wild-type (D,F) embryos, dorsal views (A-D,F), injected with *Rx3* plasmid (A,B), *Rx3*ΔHB plasmid (C,D) and controls (E,F); lateral view (E). Anterior is towards the left. (A,B) Optic vesicle evagination correlates with level of restored *Rx3* expression (compare arrowheads in A,B). (C) Restored expression of nonfunctional *Rx3* protein does not rescue. (A-D) Note specific *Rx3* expression in mutant (A-C) and wild-type (D) embryos. Compare the homogenous wild-type *Rx3* expression in control injected (F) and clonal expression in *Rx3* plasmid injected mutant (A-C) and wild type (D) embryos. (E) Control injected mutant embryo lacks *Rx3* expression. (G-L) *Rx3* plasmid (G,J) and control injected (H,I,K,L) 12-somite mutant (G,I,J,L) and wild-type (H,K) embryos. *Tbx2* and *Tbx3* expression in the retina is rescued in *Rx3* plasmid-injected embryos (compare arrowheads in G-I for *Tbx2*, and J-L for *Tbx3*). Note that expression of *Tbx2* and *Tbx3* in the hypothalamus (arrows in G-I and J-L, respectively) and *Tbx2* expression in the otic vesicle (compare G-I) is not affected by the *el* mutation. (M-P) Transversal section of 22-somite (M,N) and 35-somite (O,P) uninjected wild-type (M,O) and *Rx3* plasmid-injected mutant embryos (N,P). Dorsal is towards the top. (M,N) *Vsx2* expression in wild-type (M) and rescued mutant embryo (N) in the ventral retina. (O,P) *Vsx1* expression in inner nuclear layer of the wild type retina (O) is rescued in injected mutant embryo (P). Abbreviation: ov, otic vesicle.

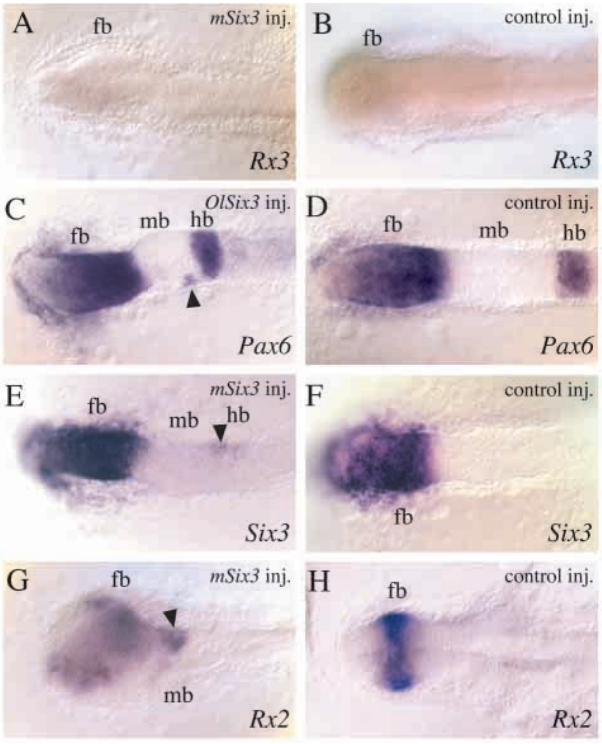


**Fig. 5.** *Rx3* is not required for retina determination. Dorsal views of two-somite stage (A-F) and six-somite stage *el*-mutant embryos (G,H). Anterior is towards the left. (A,E,G) Mouse *Six3* RNA injected, (C) medaka *Six3* RNA injected, (B,D,F,H) control injected *el*-mutant embryos raised at restrictive temperature. (A,B) *Six3* overexpression does not result in *Rx3* expression in *el*-mutant embryos. (B,C) Ectopic *Pax6* expression (arrowhead) in the midbrain in response to *Six3* overexpression. (E) *Six3* overexpression results in ectopic *Six3* expression in the presumptive midbrain (arrowhead) (G) Overexpression of *Six3* results in expanded and ectopic *Rx2* expression (arrowhead) in forebrain and presumptive midbrain, respectively. Abbreviations: fb, forebrain; hb, hindbrain; mb, midbrain.

interactions preceding the formation of ectopic retinal primordia indicates that *Rx3* is not essential for retina determination.

### *Six3* induces ectopic retinal primordia in an *Rx3* mutant background

To investigate whether *Rx3* is required for *Six3* mediated ectopic retina formation, we further analysed the effect of *Six3* overexpression in the *el*-mutant background. As a specific retina marker we used *Rx2* that in wild type embryos is exclusively expressed in presumptive neural retina.

In *el*-mutant embryos at early somitogenesis stages *Rx2* is expressed indicating that retinal determination is not affected by loss of *Rx3* activity (Fig. 5H). Furthermore, in the *Six3* injected mutant embryos we found strongly enlarged *Rx*2 expression domains ($n$=12/43, 28%) at early somitogenesis stages (Fig. 5G). Importantly, in injected mutant embryos ($n$=4/45, 9%) ectopic *Rx*2 expression was detectable in the presumptive midbrain, as in the wild type siblings ($n$=10/104, 10%; Fig. 5G arrowhead). This shows that also in the absence of *Rx3* activity, *Six3* can mediate respecification of presumptive midbrain to a retinal fate. This is in good agreement with a role of *Six3* in the determination of retinal primordia independent of *Rx3*, which acts subsequently in optic vesicle morphogenesis and proliferation.

### *Rx3* acts downstream of *Six3 and* controls proliferation in the optic vesicle

At late gastrula stages (stage 16), *Rx3* is expressed in a narrow domain across the anterior neuroectoderm. Using the forming axis as a morphological landmark, we found that *Rx3* expression overlaps with both the *Six3* and *Pax6* expression domains at this stage (Fig. 6A-C). The crossregulatory interactions of *Six3* and *Pax6* in early retina determination are not affected by the absence of *Rx3* activity in *el*-mutant embryos at the restrictive temperature, suggesting that *Six3* and *Pax6* expression do not depend on *Rx3*. To address whether *Rx3* acts downstream or in parallel to *Six3*, we examined the effects of *Six3* overexpression on *Rx3* expression and vice versa.

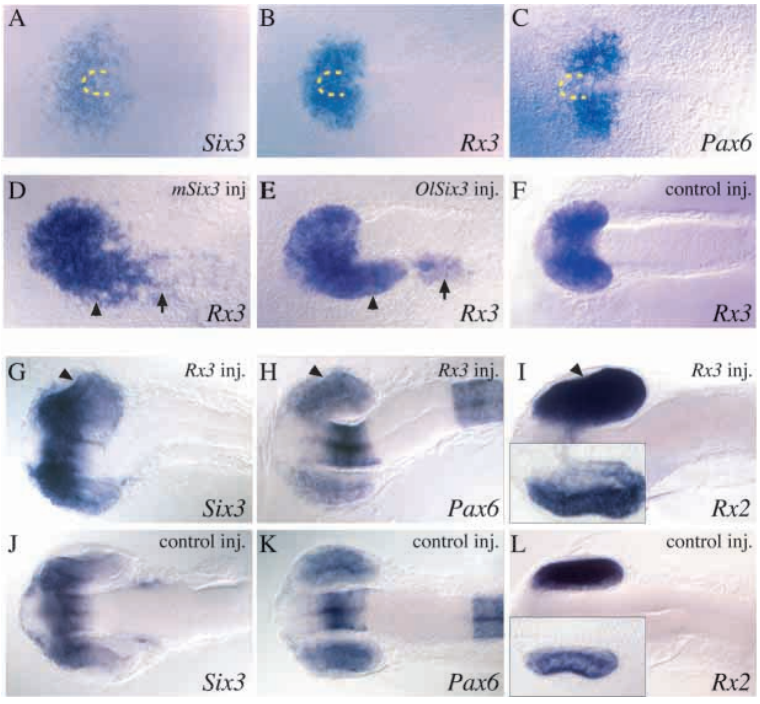


**Fig. 6.** Regulatory interactions of *Six3* and *Rx3*. Dorsal views of late gastrula (stage 16, A-D), two-somite stage (stage 19, E and F) and six-somite stage embryos (stage 21, G-L). Anterior is towards the left. (A-C) The expression of *Six3* (A), *Rx3* (B) and *Pax6* (C) partially overlap in presumptive forebrain. Anterior end of neural axis indicated by a broken line. (D,E) Overexpression of *Six3* results in expanded *Rx3* expression (arrowhead) and ectopic expression in the presumptive midbrain (arrow) at early neurula stage (D) and two-somite stage (E). (E) Note expanded expression in enlarged optic vesicle (arrow). (F) Control injected embryo showing wild-type expression. (G-I) *Rx3*-injected embryos with enlarged optic vesicles (arrowhead) show expanded *Six3* (G), *Pax6* (H) and *Rx2* (I) expression. (J-L) Control injected embryos at same stage. Note that anterior and posterior boundaries of respective expression domains are not shifted in response to *Rx3* overexpression (compare G with J,H with K and I with L). Insets in I and L show horizontal sections at comparable level. Note the increased cell number in the *Rx3*-injected eye (I).

Ectopic expression of mouse *Six3* results in an expansion of the *Rx3* expression domain (*n*=19/29, 65%) and in ectopic *Rx3* expression (*n*=8/29, 27%) already at late gastrula/early neurula stages (Fig. 6D). At late neurula/early somitogenesis stages an expansion of the *Rx3* expression domain (*n*=33/49, 67%) and ectopic *Rx3* expression in the presumptive midbrain of medaka *Six3* injected embryos (*n*=8/49, 16%) is observed (Fig. 6E). This strongly suggests that *Six3* acts as an upstream regulator of *Rx3* expression at these stages.

To test whether, in reverse, *Rx3* can activate *Six3* we overexpressed *Rx3*. Ectopic *Rx3* expression does not affect *Six3* expression at the time point when they are first co-expressed in wild-type embryos (late gastrula/early neurula stage) and at midneurula stages (not shown). Neither size nor morphology of the forebrain or optic vesicles is altered by *Rx3* overexpression at these stages (not shown). After optic vesicle evagination, however, the optic vesicles of injected embryos are often enlarged as visualised by *Six3* (*n*=13/36, 36%) and *Pax6* (*n*=15/41, 37%) expression (Fig. 6G-I), without affecting their expression domains in the forebrain. The finding that *Rx3* overexpression at somitogenesis specifically results in enlarged optic vesicles containing significantly more cells was confirmed by the expanded expression domain of *Rx*2 (*n*=34/101, 34%; Fig. 6I) that in wild-type embryos is expressed exclusively in the presumptive neural retina (Fig. 6L).

Thus, *Six3* overexpression results in ectopic *Rx3* expression. *Rx3* overexpression, however, does not alter *Six3* expression at stages before optic vesicle evagination, consistent with a role of *Rx3* downstream of *Six3*. The enlargement of optic vesicles caused by higher numbers of retinal cell in response to *Rx3* overexpression indicates a role of this gene in the control of proliferation in the optic vesicle.

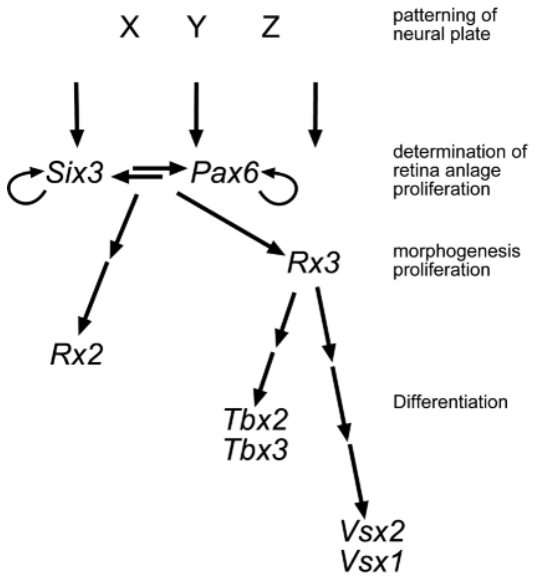


**Fig. 7.** Genetic hierarchy underlying early retina development. X, Y and Z represent factors that pattern the anterior neuroectoderm, leading to the overlapping expression of *Six3* and *Pax6* in the retina anlage, which results in the determination of retinal fate. The crossregulatory interaction and the feedback loops of *Six3* and *Pax6* (arrows) result in the maintenance of retinal fate. *Rx3* expression is regulated by these genes, but may also receive input of upstream factors X, Y and Z. *Rx3* activity is required for morphogenesis (optic vesicle evagination) and organ size regulation (proliferation in optic vesicle). The retina specific expression of *Rx2* does not depend on *Rx3* activity. Genes involved in subsequent differentiation steps require preceding *Rx3* activity (*Tbx2/3, Vsx1/2*).

## DISCUSSION

In this study, we show that the *eyeless* mutant phenotype in medaka fish is caused by an intronic insertion in the homeobox gene *Rx3*, resulting in a transcriptional repression of the locus. We show that determination of the retina anlage occurs normally in the absence of *Rx3* function, whereas subsequent morphogenesis and differentiation of the retinal primordia do not occur. Our studies indicate a role of *Rx3* downstream of the retina determination gene *Six3* in morphogenesis and growth control of the optic vesicle. Thus, *Rx3* plays a pivotal role in the manifestation of a fate that has been laid down in the organ anlage by upstream players.

Furthermore, we show that the regulatory interactions of *Six3* and *Pax6* that precede the formation of ectopic retinal primordia do not depend on *Rx3* activity. Our results provide novel insights into the regulatory interactions of key players involved in retina determination and subsequent steps of organ formation.

### Temperature-sensitive repression of the mutant *el* locus

The detailed phenotypic analysis of genetically marked *el*-mutant embryos shows that the penetrance is complete and the expressivity not variable at the restrictive temperature. At the permissive temperature, however, the expressivity is variable, while the penetrance is not affected. This temperature-sensitive expressivity of the mutant phenotype is tightly correlated with the expression levels of *Rx3* in the presumptive retina (Fig. 1). Furthermore, in our rescue experiments the degree of rescue correlates well with restored expression in the evaginating optic vesicles of *el*-mutant embryos (Fig. 4A,B). Thus, in both situations, optic vesicle evagination and *Rx3* expression correlate. In line with this, at the permissive temperature a correctly spliced transcript is detectable by RT-PCR. This strongly suggests that under permissive conditions a functional protein is made, which accounts for the formation of small eyes in the mutant embryos.

The nature of the transcriptional repression remains unclear. Database searches did not reveal any significant homology of the insertion to known sequences. It is conceivable that the insertion influences intronic enhancer elements near the insertion site, thereby resulting in a repression of the locus. However, preliminary data do not suggest the presence of regulatory elements in the second intron (not shown). This does not rule out the possibility that the insertion represses the *Rx3* locus by affecting its general accessibility for the transcriptional machinery. Temperature-sensitive splicing appears unlikely, as we cannot detect 5′ parts of the *Rx3* transcript using RT-PCR or whole-mount in situ analysis at the restrictive temperature. Further analysis of the regulatory elements of the locus will address the nature of the repression and its temperature sensitivity.

The *Rx3* plasmid rescues all aspects of the mutant

phenotype, namely optic vesicle evagination and retinal differentiation in embryos raised under restrictive conditions (Figs 3 and 4). The regulatory elements required for correct expression are contained in 11 kb of genomic DNA present in the *Rx3* plasmid as evidenced by specifically restored *Rx3* expression in the wild-type expression domains. A modified *Rx3* plasmid encoding a non-functional protein also results in specific *Rx3* expression in mutant and wild-type embryos. Therefore, a functional *Rx3* protein is not required for wild-type *Rx3* expression, in contrast to *Pax6* and *Six3*, which act in regulatory feedback loops (Chow et al., 1999; Loosli et al., 1999).

### *Rx3* functions downstream of the determination of the retina anlage

Our study shows that the *el* mutation genetically separates the determination of the vertebrate retina anlage from its subsequent morphogenesis, organ size control and neuronal differentiation.

*Rx3* is not involved in the determination of the retina anlage, as the expression of the retina determination genes *Six3* and *Pax6* are not affected by either gain or loss of *Rx3* activity. Furthermore, *Rx2* expression indicative of retinal fate is still present in *el*-mutant embryos. However, also in absence of *Rx3* activity, overexpression of *Six3* results in the formation of ectopic retinal primordia.

The targeted inactivation of a mouse *Rx* homologue results in anophthalmic embryos (Mathers et al., 1997). Similar to the situation in the medaka *eyeless* mutant, at the neural plate stage, early expression of *Six3*, *Pax6* and *Otx2* is not affected in *Rx* mutant mice (Zhang et al., 2000). This indicates that in $Rx^{-/-}$ mouse embryos, analogous to medaka, patterning of the anterior neural plate and thus the determination of the retina anlage is not affected.

However, at later stages (E9.0-10.5), upregulation of retina specific expression of *Six3* and *Pax6* is not detected in $Rx^{-/-}$ embryos, indicating that retinal progenitor cells are not present (Zhang et al., 2000). At the corresponding stage, the expression of the respective homologues is still detectable in the medaka mutant embryo. The difference is most probably due to a split of function of the medaka *Rx3* and *Rx2* genes, respectively (see below).

### *Rx2* and *Rx3* may have non-overlapping functions

The spatial and temporal expression patterns of the two medaka paralogues *Rx2* and *Rx3* differ significantly. Medaka *Rx3* is expressed already at late gastrula stages in the anterior neuroectoderm, and its expression domain comprises the ventral forebrain as well as the optic vesicle. *Rx2*, on the other hand, is first expressed several hours later at the late neurula stage in the evaginated optic vesicle, and is thus exclusively expressed in retinal progenitor cells. Thus, *Rx2* and *Rx3* in medaka are not co-expressed except for a short period in the retinal progenitor cells of the evaginated optic vesicle, suggesting mainly non-overlapping functions of these genes, while *Rx3* function is required in the evaginating optic vesicle *Rx2* is expressed independently and may play a role in later aspects of retinogenesis.

In zebrafish, three Rx homologues have been isolated. *Rx1* and *Rx2*, which are most similar to medaka *Rx2*, share the early expression domain with zebrafish *Rx3*, but show a slightly later onset of expression (Chuang et al., 1999). In the differentiating retina at late organogenesis stages, medaka and zebrafish *Rx3* are expressed in the inner nuclear layer overlapping with *Six3* expression. Expression of medaka *Rx2* is confined to the outer nuclear layer (photoreceptor layer) and the ciliary margin as is *Rx1* and *Rx2* in zebrafish (not shown).

### Genetic hierarchy underlying early retina development

Based on our study, we propose the following model for early vertebrate retina development (Fig. 7). Patterning of the anterior neural plate culminates in defined expression patterns of *Six3* and *Pax6*. This anterior neural plate patterning relies on the repression of *wnt* and BMP signalling (Niehrs, 1999), and requires the activity of the Otx transcription factors (Simeone, 1998). In the region where *Six3* and *Pax6* expression overlap, retinal fate is specified. An *Rx3*-independent regulatory feedback loop of these genes then ensures the maintenance of the retinal fate.

*Six3* overexpression in *el*-mutant embryos results in dramatically enlarged retinal primordia (Fig. 5G). This expansion does not occur on the expense of forebrain tissue, suggesting that *Six3* also affects cell proliferation independently of *Rx3* and thereby regulates the size of the retina anlage. Consistent with the suggested role of *Six3* in cell proliferation, the closely related *Xenopus Optx2* gene controls the size of the optic vesicles by regulating proliferation (Zhou et al., 2000; Zuber et al., 1999).

Under the influence of midline signalling, the retinal anlage is split into two retinal primordia (Varga et al., 1999). Mutations in *Six3* cause holoprosencephaly in humans, indicating a requirement for *Six3* in this process (Wallis and Muenke, 1999). The two retinal primordia then become localised to the lateral wall of the prosencephalon during neurulation.

Subsequent evagination of the primordia results in the formation of the optic vesicles. For this process, *Rx3* function is essential. Functional studies consistently argue for a regulatory role of vertebrate Rx genes in proliferation of retinal progenitor cells in the optic vesicle (Andreazzoli et al., 1999; Mathers and Jamrich, 2000), thus regulating its growth. In the absence of *Rx3* function, there is no sign of morphogenesis and the specified retinal precursors do not proliferate and eventually die. *Rx3* acts downstream of *Six3* and *Pax6* that determine the retina anlage. However, it is possible that *Rx3* initially also receives input from neural plate patterning genes.

Subsequent development divides the optic vesicle into specific regions that then give rise to neural retina (NR), retinal pigmented epithelium (RPE) and optic stalk. Several genes that are expressed during these later steps of retinal development require *Rx3* function directly or indirectly. Interestingly, the expression of *Tbx2* and *Tbx*3 is specifically affected in the retinal primordium, but not in the hypothalamus, where they are also co-expressed. This indicates a differential regulation of *Tbx2* and *Tbx3* in these tissues.

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