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# The conditional medaka mutation *eyeless* uncouples patterning and morphogenesis of the eye

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## SUMMARY

**In early vertebrate eye development, the retinal anlage is specified in the anterior neuroectoderm. During neurulation, the optic vesicles evaginate from the lateral wall of the prosencephalon. Here we describe the temperature-sensitive mutation *eyeless* in the Japanese medakafish. Marker gene analysis indicates that, whereas, specification of two retinal primordia and proximodistal patterning takes place in the mutant embryo, optic vesicle evagination does not occur and subsequent differentiation of the retinal primordia is not observed. The mutation *eyeless* thus uncouples patterning and morphogenesis at early steps of retinal development. Temperature-shift experiments indicate a requirement for *eyeless* activity prior to optic vesicle evagination. Cell transplantation shows that *eyeless* acts cell autonomously.**

Key words: Vertebrate, Eye, Medaka, *eyeless*, Optic vesicle, Patterning

## INTRODUCTION

Vertebrate eye formation starts during gastrulation with the determination of an eye field in the anterior ectoderm. Subsequently well-coordinated inductive events lead to the formation of a single retinal primordium in the anterior neuroectoderm and the presumptive lens placodes in the abutting head ectoderm (for review see Grainger, 1996; Jean et al., 1998). At late gastrula/early neurula stages, this single retinal field is split into two bilateral symmetrical retinal primordia by cell movements under the influence of signals emanating from the ventral midline (Gritsman et al., 1999; Li et al., 1997; Varga et al., 1999). Morphogenesis during neurula stages results in the evagination of the optic vesicles from the lateral wall of the forebrain, a process that requires changes in cell proliferation and programmed cell death (Schmitt and Dowling, 1994). Concomitantly, lens specification occurs when the optic vesicle contacts the overlying ectoderm of the lens placode (reviewed in Grainger, 1996). The optic vesicles invaginate under the influence of the developing lens to form the optic cups, in which differentiation of the neuroretina and the pigmented retinal epithelium (PRE) starts. The optic cup remains attached to the ventral diencephalon by the optic stalk, which eventually serves as a leading track for the projection of retinal axons to target regions in the midbrain.

Insect eye development has been analysed by a combination of genetic and molecular techniques. Surprisingly, key players, acting high up in the genetic pathway controlling *Drosophila* eye development, were found to be evolutionary conserved (Halder et al., 1995; Hammond et al., 1998; Oliver et al., 1995; Quiring et al., 1994; Xu et al., 1997) and to exert a comparable function during vertebrate eye development (Hill et al., 1991; Loosli et al., 1999; Oliver et al., 1996; Ton et al., 1991). In homozygous *Small eye* (*Sey*) mouse and rat, mutations in the transcription factor *Pax6* lead to the complete absence of the eyes, including lens, and the nose (Hill et al., 1991; Hogan et al., 1986; Matsuo et al., 1993). In these embryos, the optic vesicles initially evaginate, but degenerate during subsequent stages of development (Hogan et al., 1986). Recently, it was shown that overexpression of *Pax6* mRNA in *Xenopus* embryos can lead to the formation of an ectopic eye (Chow et al., 1999). *Six3*, a homeobox gene, is expressed early in the anterior neuroectoderm and subsequently in the optic vesicles and prosencephalon (Bovolenta et al., 1998; Kobayashi et al., 1998; Loosli et al., 1998; Oliver et al., 1995; Seo et al., 1998). Overexpression of *Six3* mRNA in medaka initiates the formation of ectopic retina tissue in the midbrain and anterior hindbrain involving a regulatory interaction of *Six3* and *Pax6* (Loosli et al., 1999). This indicates a role for *Six3* and *Pax6* in early steps of vertebrate retina formation.

Targeted inactivation of the transcription factor *Rx* in mouse results in the absence of eye structures and in severe defects of the anterior forebrain, demonstrating the necessity of this gene for eye development (Mathers et al., 1997). Mice lacking *sonic hedgehog* gene function exhibit cyclopia phenotypes, demonstrating an involvement of sonic hedgehog in the early proximodistal patterning of the retinal primordia (Chiang et al., 1996), as also suggested by overexpression experiments in fish embryos (Ekker et al., 1995; Macdonald et al., 1995).

Mutations affecting eye development have been isolated in

mutagenesis screens in zebrafish (Heisenberg et al., 1996; Malicki et al., 1996; Schier et al., 1996). Early steps of eye development are affected in the zebrafish mutation *masterblind* (*mbl*), which results in forebrain patterning defects and reduced or missing eyes (Heisenberg et al., 1996; Masai et al., 1997).

Here we present the spontaneous, recessive medaka mutation *eyeless* (*el*). *el* mutant (*el*$^{-}$) embryos do not develop morphologically visible eye structures and die at early larval stages. *el* is a temperature-sensitive mutation and its activity is required between the late gastrula and the early neurula stage prior to the evagination of the optic vesicle. Our analysis of the *el* mutation reveals that patterning of the eye primordia and optic vesicle morphogenesis are uncoupled by the *el* mutation.

## MATERIALS AND METHODS

### Fish stocks

The medaka mutant *eyeless* was originally identified by Hideo Tomita. The *el* mutation was crossed into and kept in two different genetic backgrounds of wild-type medaka (*O. latipes*), which are kept in closed stocks at the EMBL, as described (Köster et al., 1997). Embryos were staged according to Iwamatsu (1994). To identify *el* carrier fish, pairwise matings were set up and the embryos were kept at 28°C or 18°C, respectively, and screened for the *el* phenotype.

### Isolation of partial *Emx1*, *Vax1*, *Vsx1* and *Vsx2* cDNAs

A 550 bp medaka *Emx1* fragment was amplified by RT-PCR on stage19-21 total RNA, using degenerate PCR primers specific for *Emx1* (up, 5′ACIATCGAGWSIYTIGTIGGIAARGA; low, 5′CYGTTYTGRAACCAAACYTTIACYTG).

A 520 bp *Vsx2* fragment was amplified by RT-PCR stage 19-21 total RNA, using degenerate PCR primers specific for *Vsx2* (up, 5′GGITTYGGIATHCARGARATHYTIGG; low, 5′CCAYTTRGCTCKICKRTTYTGRAACC).

PCR conditions were 5 cycles at 94°C/1 minute, 48°C/2 minutes and 72°C/4 minutes, followed by 35 cycles with annealing at 53°C.

A 560 bp *Vax1* fragment was amplified by RT-PCR on stage 19-21 total RNA, using degenerate PCR primers specific for *Vax1* (up, 5′AGRATCCTIGTSAGRGAYGCIAARGG; low, 5′TIGTYCTICTGTAIGGCTCRAAIGC). PCR conditions were 5 cycles at 94°C/1 minute, 51°C/2 minutes and 72°C/4 minutes, followed by 35 cycles with annealing at 56°C.

A 300 bp *Vsx1* fragment was amplified by RT-PCR from stage 36 total RNA using degenerate PCR primers specific for *Vsx1* (up, 5′GARGARYTIGARAARGCYTTYMAYGA; low, 5′YTTYTTRTGCATICCSARSARCCA). PCR conditions were 5 cycles at 94°C/1 minute, 52°C/2 minutes and 72°C/4 minutes, followed by 35 cycles with annealing at 57°C.

A 1.3 kb *Vsx1* fragment was then isolated by PCR from a somitogenesis stage cDNA library (stage 23) using a Vsx1-specific primer (5′GCAAAGTGGCGAAAGCGTGAG) and a T7 primer (GTGAATTGTAATACGACTCACTATAG). Cycling conditions were 35 cycles at 94°C/1 minute, 62°C/1 minute and 72°C/4 minutes.

Resulting PCR products were cloned into the TopoTA vector (Invitrogen) and sequenced.

### Isolation of *Rx2* and *Vsx2* full-length cDNAs

A 560 bp *Rx2* fragment (Loosli et al., 1999) and a 520 bp *Vsx2* fragment were used to screen a 23-stage λZAP cDNA library at high stringency. Positive clones were plaque purified, converted to Bluescript SK plasmids and sequenced.

EMBL database accession numbers: *Vax1*, AJ250401; *Emx1*, AJ250402; *Vsx1*, AJ250403; *Vsx2*, AJ250404; *Rx2*, AJ250405.

### Whole-mount in situ hybridization and vibratome sectioning

Whole-mount in situ hybridization was performed as described (Loosli et al., 1998). The entire cloned cDNAs of α-*Crystallin*, *Emx1*, *Fgf8*, *Pax6*, *Rx2*, *Six3*, *Vax1*, *Vsx1* and *Vsx2* were transcribed for RNA riboprobes. In double-labeling experiments, the fast red detection (red) was performed first, followed by the NBT/BCIP (blue) staining. Vibratome sections of whole-mount stained embryos were done as described (Loosli et al., 1998).

### Staining and analysis of nuclei

Embryos were fixed in 4% paraformaldehyde in PBS, 0.5% Tween-20 and stored in 100% methanol at –20°C. Embryos were rehydrated and incubated in 2 mg/ml DAPI/PBS, washed (PBS) and mounted in 80% glycerol, 20% PBS/N-propyl gallate. 6 μm longitudinal sections of the whole embryo were scanned using confocal microscopy and nuclei in the optic stalk region of comparable longitudinal planes were counted. Two different wild-type embryos were analyzed in four focal planes each (average number of cells: 52 in the optic stalk region; 161 in both optic vesicles). Three different *el*$^{-}$ embryos were analyzed in 6 comparable focal planes each at the presumptive optic stalk region (average = 73 cells).

### Detection of apoptotic cells

Embryos fixed in 4% PF1 and stored in 100% methanol were rehydrated stepwise in PBST/methanol. Apoptotic cells were visualized using the ‘in situ cell death detection kit’ (Roche Molecular Biochemicals) in combination with the chromogens NBT/BCIP according to the manufacturers protocol.

### Transplantation

Cells transplantation was done as described (Ho and Kane, 1990; Hong et al., 1998) with the following modifications. Embryos were dechorionated enzymatically using proteinase K (10 mg/ml, 40 minutes at room temperature) followed by incubation in medaka hatching enzyme as described (Wakamatsu et al., 1993). Wild-type donor cells were taken at the blastula stage from a transgenic fish line stably expressing GFP under the control of the ubiquitous cytoskeletal actin promoter (T. H. and J. W., unpublished results). *el*$^{-}$ donor cells were derived from crosses of *el* carriers stably expressing GFP. Alternatively cells were labeled by injection of Rhodamine dextran (Sigma, 5% in 1× Yamamoto) into one blastomere at the 1-cell stage. At late blastula stage, 30-50 labeled cells from the animal pole region of donor embryos were transplanted into the same position of host embryos using a dissecting microscope equipped with UV illumination (Leica). Transplanted embryos were raised at the restrictive temperature of 18°C and analyzed from late neurula stage (stage 18) onwards.

## RESULTS

### Optic vesicles do not form in the medaka mutant *eyeless* (*el*)

The medaka mutant *eyeless* (*el*) was identified as a spontaneous, recessive mutation that affects eye development. The expressivity of the *el* phenotype is temperature sensitive. When raised at the permissive temperature of 28°C, homozygous *el*$^{-}$ embryos show a complete penetrance but variable expressivity of the phenotype at somitogenesis stages. The variability ranges from the complete absence of optic cups (Fig. 1, 48% of homozygous *el*$^{-}$ embryos) to the formation of one laterally located optic cup (data not shown) or of optic cups smaller than in the wild type (Fig. 2B, 52% of the homozygous *el*$^{-}$ embryos with one or two small optic cups). When raised

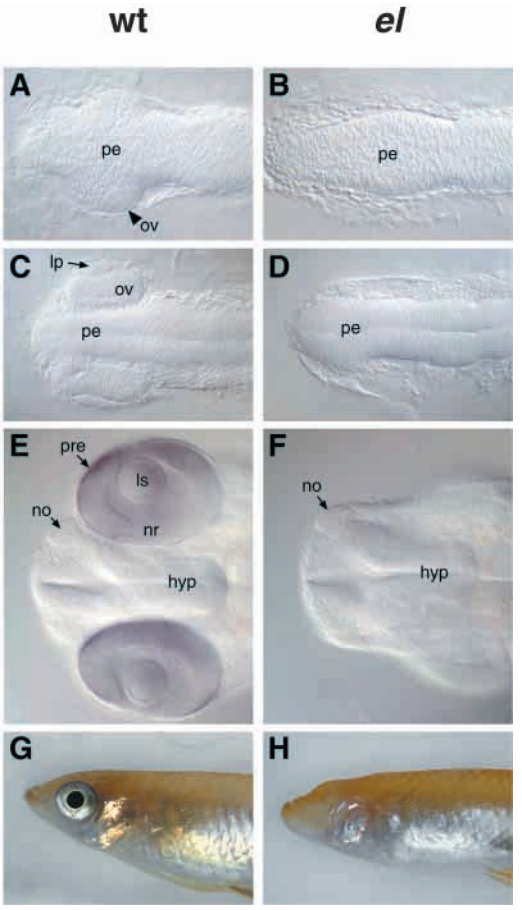


**Fig. 1.** Comparison of head development in wild-type and *el*⁻ embryos. (A,C,E) Wild type; (B,D,F) *el*⁻ embryos; (G) adult wild type and (H) adult *el*⁻ fish, anterior is to the left. (A,B) At mid neurula stage (stage 17) evagination of optic vesicles starts in wild type, but not in *el*⁻ embryos. (C,D) At 2-somite stage (stage 19), optic vesicles are fully evaginated in wild-type embryos and lens placodes start to thicken, *el*⁻ embryos show no sign of outbudding optic vesicles. At organogenesis stages, pigmentation of the PRE starts in wild-type embryos (E); no signs of pigmentation are found in *el*⁻ embryos (F). The nose is not affected in *el*⁻ embryos. (G,H) Adult wild-type and *el*⁻ animals, note complete absence of eye structures in mutant fish. hyp, hypothalamus; lp, lens placode; ls, lens; no, nose; nr, neuroretina; ov, optic vesicle; pe, prosencephalon; pre, pigmented retinal epithelium.

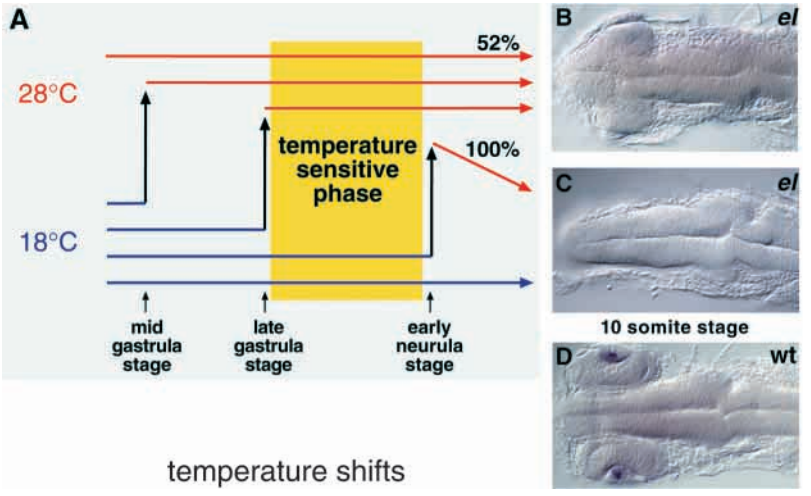


**Fig. 2.** *el* is a cold-sensitive mutation. (A) At the indicated stages, embryos were shifted from the restrictive to the permissive temperature (18°C to 28°C). The temperature-sensitive phase is indicated by yellow box. (B) *el*⁻ embryos kept at 28°C during the temperature-sensitive phase develop optic cups, albeit smaller than those of wild-type embryo at the 10-somite stage (D; stage 22); lens fibers express α-*crystallin* at this stage. (C) Embryos kept at restrictive temperature up to early neurula stages (stage 17) show full expressivity of the phenotype. No optic cups form in *el*⁻ embryos raised at the restrictive temperature (18°C) during temperature-sensitive phase.

at the restrictive temperature of 18°C, no morphologically visible optic cups form in homozygous *el*⁻ embryos. The results below refer to embryos raised under restrictive conditions.

The *el* phenotype becomes morphologically apparent at mid to late neurula stage (stage 17/18, Iwamatsu, 1994), when optic vesicle evagination from the lateral wall of the prosencephalon starts in wild-type embryos (Fig. 1A). This evagination is not observed in *el*⁻ embryos (Fig. 1B,D). In a

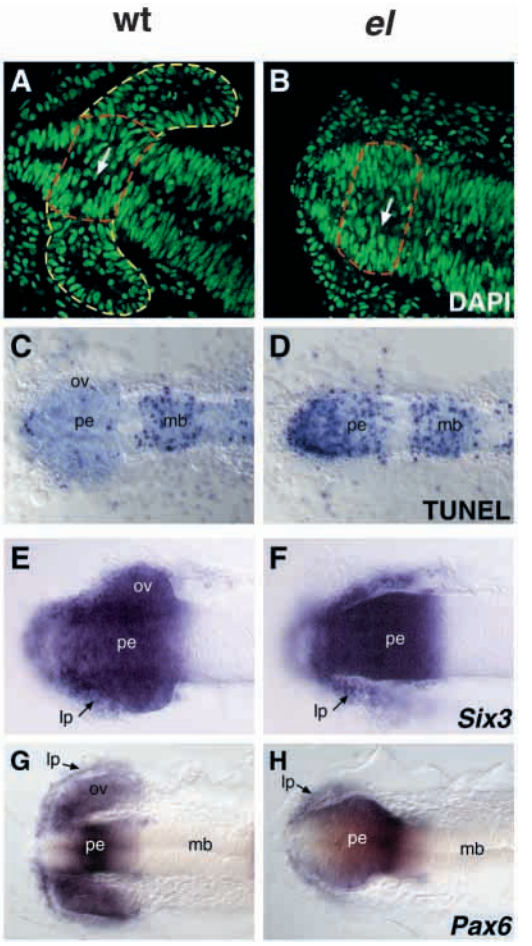


**Fig. 3.** Cell proliferation, apoptosis and marker gene expression at early stages of optic vesicle development. Dorsal views of neurula stage (stage 18, A-F) and early somitogenesis stage (stage 21, G,H) embryos. (A,B) Confocal image of a single focal plane of DAPI-stained nuclei at late neurula stages. Dashed red line marks presumptive optic stalk region in wild-type and corresponding region in *el*⁻ embryos; dashed yellow line outlines optic vesicles in wild-type embryos. Cell numbers are markedly lower in the wild-type optic stalk area (arrow). (C,D) TUNEL staining shows increased number of apoptotic cells in prosencephalon of *el*⁻ embryos. Midbrain shows comparable number of apoptotic cells in wild type and *el*⁻ and serves as internal control. Whole-mount in situ analysis of (E,F) *Six3* and (G,H) *Pax6* expression. Both are expressed in lens placodes of (E,G) wild-type and (F,H) *el*⁻ embryos. lp, lens placode; ov, optic vesicle; pe, prosencephalon; mb, midbrain.

few cases, minute lateral protrusions of the forebrain form transiently between late neurula and early somitogenesis stages (Figs 1B, 3H). The formation of other sensory organs, such as nose or ears, is not affected by the *el* mutation (Fig. 1F; data not shown). Homozygous *el* fish die at day 1 to 5 after hatching. Escapers (1.5%) can be raised to adulthood; males are sterile, but females are fully fertile and mate successfully. Adult *el* fish show no morphologically visible eye structures (Fig. 1H).

### *el* activity is required prior to optic vesicle evagination

The offspring of *el* carrier crosses were shifted at different stages of development from the restrictive temperature (18°C) to the permissive temperature (28°C), or vice versa. Embryos were analysed at later stages (10-somite stage, stage 22/23) when, in wild-type embryos, optic cups have formed (Fig. 2D). A temperature shift from 18°C to 28°C prior to the late gastrula stage does not affect the variable expressivity of *el* phenotype (Fig. 2B). However, when shifted at the early neurula stage (stage 17) or later, the variable expressivity is lost and all *el*$^{-}$ embryos exhibit a complete lack of morphologically visible optic cups (Fig. 2C). Conversely, a temperature shift from 28°C to 18°C prior to the late gastrula stage (stage 16) leads to a complete lack of optic cups in *el*$^{-}$ embryos, while a similar shift at the early neurula stage (stage 17) or later does not affect the variable expressivity of the *el* phenotype (data not shown). Thus, the phase between the late gastrula (stage 16) and the early neurula (stage 17) stage is the time window during which *el* activity is required.

### *el*$^{-}$ embryos have an increased number of prosencephalic cells

In *el*$^{-}$ embryos, optic vesicles do not evaginate from the prosencephalon at the neurula stage. To analyze whether this is due to the lack of tissue to be evaginated, we performed DAPI staining and used confocal microscopy to determine the cell distribution in this region of the brain. Optic vesicle evagination in wild-type embryos results in reduced cell numbers at the site of evagination (Fig. 3A, arrow), while *el*$^{-}$ embryos show a uniform distribution of cells along the lateral wall of the forebrain (Fig. 3A,B). Cell numbers in the total eye region of the prosencephalon at stage 18 of *el*$^{-}$ embryos were 70% less than the wild-type cell number (dashed yellow line; Fig. 3A,B). In contrast, counting cells in the lateral forebrain walls only (Fig. 3A,B, area surrounded by red dashed line), showed that *el*$^{-}$ embryos have 40% more cells than wild type, in good agreement with the more uniform distribution of cells in the lateral wall of the prosencephalon in the mutant (see above). This indicates that, in the mutant, cells instead of evaginating remain in the lateral wall of the prosencephalon.

To investigate whether the lower total cell number in the forebrain of *el*$^{-}$ embryos is caused by apoptosis, we used TUNEL labeling. The offspring of *el* carrier crosses were analyzed at different stages of development. At early stages (late gastrula to early neurula) no obvious differences in the number of apoptotic cells were detected in wild-type and *el*$^{-}$ embryos (data not shown). At stage 18 (mid neurula stage), however, 9 hours (18°C) after *el* activity is required, enhanced apoptosis is detected in the anterior prosencephalon of *el*$^{-}$ embryos during a short time period (2 hours, 18˚C; Fig. 3C,D). A second apoptotic wave is observed in *el*$^{-}$ embryos at the 12-somite stage (see below). Thus, the increase in cell numbers within the lateral walls of the prosencephalon of *el*$^{-}$ embryos due to the lack of evagination is in part counteracted by ectopic apoptosis affecting this region.

### Retinal tissue forms in the ventral diencephalon

The transcription factors *Pax6* and *Six3* are key players for early eye and forebrain development and are expressed in the presumptive eye and forebrain region during late gastrula stages (Chow et al., 1999; Hill et al., 1991; Hogan et al., 1986; Kobayashi et al., 1998; Loosli et al., 1999; Oliver et al., 1995). To examine if the expression of *Pax6* and *Six3* is affected in *el*$^{-}$ embryos, we performed whole-mount in situ hybridization. The expression patterns of *Pax6* and *Six3* were indistinguishable among sister siblings of an *el* carrier cross at late gastrula to early neurula stages (data not shown). Also, at late neurula stages, the expression patterns of *Pax6* and *Six3* in the forebrain are indistinguishable between wild-type and *el*$^{-}$ siblings (data not shown). Strikingly, the expression of *Pax6* and *Six3* in the presumptive lens placode of *el*$^{-}$ embryos is unaffected (Fig. 3E-H).

Altered expression of *Pax6*, however, was observed at the 12-somite stage. The dorsal diencephalic expression domain, which resembles an inverted V in wild type, is compressed in *el*$^{-}$ embryos (Fig. 4A,B; arrow). Ectopic *Pax6* expression is detected in the ventral diencephalon at the site of the optic stalk in wild-type embryos (arrowheads in Fig. 4A,B). Since this may reflect the presence of non-evaginated retinal precursor cells in the lateral wall of the diencephalon, we analyzed the expression of *Rx2*, a gene that is exclusively expressed in the retina of wild-type embryos from early somitogenesis (stage 19) onwards (Loosli et al., 1999). In *el*$^{-}$ embryos, *Rx2*-positive tissue is detected in two lateral domains located in the ventral forebrain (Fig. 4D) at the 6-somite stage. In some cases, the *Rx2*-positive domains exhibit a concave shape resembling an invaginating retina (arrowhead in Fig. 4F). This indicates the formation of retinal precursor cells in *el*$^{-}$ embryos, which are located in two ventral domains contiguous with the lateral wall of the diencephalon (Fig. 4E,F), at the site where the wild-type optic stalk connects.

Thus, except for the lack of evagination, morphogenetic movements of the developing diencephalon are normal. In mutant embryos, retina precursor cells initially born dorsally move with the rotating diencephalon to a ventral position during subsequent development and eventually end up at the attachment site of the optic stalk in the diencephalon. It is noteworthy that the split of the initial retinal field into two primordia is not affected by the *el* mutation, as two distinct domains of *Rx2*-positive retinal tissue form in the ventral diencephalon.

### Proximodistal patterning of retinal primordia in *el*$^{-}$ embryos

To investigate whether early patterning that generates proximal and distal identities in the optic vesicle occurs in homozygous *el*$^{-}$ embryos, we checked for the presence of optic stalk tissue.

In wild-type medaka embryos, the transcription factor *Vax1* is strongly expressed in the optic stalk and proximal retina and at lower levels in the ventral diencephalon and the olfactory

placodes at the 12-somite stage (Fig. 5A), similar to *Vax1* expression in mouse and *Xenopus* (Hallonet et al., 1998). In *el*$^-$ embryos, high levels of *Vax1* expression are found at a position where, in the wild-type embryos, the optic stalk is located (stage 23; Fig. 5B). The region expressing *Vax1* at lower levels is expanded anteriorly (Fig. 5A,B). *Vax1* expression in the olfactory placode is not affected in *el*$^-$ embryos (data not shown).

We also examined the expression of *Fgf8*, which is expressed in the optic stalk at somitogenesis stages (stage 27, Figs 5C, 7B) (Carl and Wittbrodt, 1999; Reifers et al., 1998). In *el*$^-$ embryos, *Fgf8* transcripts are detected in a structure reminiscent of the optic stalk at stage 27. As in the wild-type eye, where the *Fgf8*-expressing optic stalk connects to the retina (Figs 5C,7C), the *Fgf8* expression domain in *el*$^-$ embryos is located adjacent to the *Rx2*-positive retinal tissue in the wall of the ventral diencephalon (Figs 5D,7E). Thus, in *el*$^-$ embryos, the optic primordia are patterned and receive proximal and distal identities, which are maintained even though morphogenesis of the optic vesicles does not occur.

### Lens specification is not affected by the *el* mutation

Our data show that, in *el*$^-$ embryos, eye structures derived from the neuroectoderm, namely retina and optic stalk, form as parts of the lateral wall of the diencephalon. In amphibian embryos, lens formation in the head ectoderm is induced by the anterior neuroectoderm (Saha et al., 1989). To test whether the anterior neuroectoderm and the remnants of retinal tissue in *el*$^-$ embryos have the potential to induce lens formation and differentiation, we examined lens formation by morphological criteria, the expression of *Pax6* and *Six3* in the lens placode, and *α-crystallin* expression as a molecular marker for lens fiber differentiation (Loosli et al., 1998). At the 16-somite stage, lenses are detected in 80% of the *el*$^-$ embryos (Fig. 5G,H). The size of the mutant lenses is variable and smaller than in wild type (Fig. 5G,H). Remarkably these lenses, which express *Pax6* at this stage (data not shown), do not express *α-crystallin*, although, in wild-type embryos, *α-crystallin* expression is detected by the 10-somite stage (Fig. 2B). In *el*$^-$ embryos, *α-crystallin* expression is detected only in a few cases and only from the 18-somite stage onwards (Fig. 5F). In summary, lens induction as shown by wild-type *Pax6* and *Six3* expression (Fig. 3F,H) occurs, but, lens differentiation is delayed and, in most cases, incomplete, indicating that for proper differentiation the presence of an optic cup is required.

### Retinal tissue fails to differentiate in *el*$^-$ embryos

In the developing wild-type retina, *Rx2* is initially expressed in proliferating cells of the entire optic vesicle and is subsequently restricted to the neuroretina as opposed to pigmented retina epithelium (PRE) (Loosli et al., 1999). To investigate whether, in *el*$^-$ embryos, the non-evaginated *Rx2*-positive retinal tissue has the potential to differentiate further, we examined later stages of eye development for the presence of PRE. In wild-type embryos, PRE is first detected at the 30-somite stage (stage 28). In *el*$^-$ embryos, however, PRE is never detected at any stage of development (Figs 1F,H, 5F).

To investigate whether the *Rx2*-positive retinal tissue differentiates further into neuroretina cells, we performed whole-mount in situ hybridization with genes specific for the differentiating retina. In fish, two different homologues of murine *Chx10*, *Vsx1* and *Vsx2* (*Alx*), have been identified (Barabino et al., 1997; Levine et al., 1994, 1997; Passini et al., 1998). Similar to the situation in other fish species, medaka *Vsx2* is expressed prior to the onset of retinal cell differentiation in the temporal part of the ventral retina, starting at the 12-somite stage (stage 23) (Fig. 5G). As differentiation proceeds, *Vsx2* expression is confined to the temporally located marginal zone of the neuroretina. In *el*$^-$ embryos, *Vsx2* is not detectable in the *Rx2*-positive area in the ventral diencephalon (Fig. 5H). Medaka *Vsx1* is a marker for differentiated neurons of the retina. In the wild-type embryo, *Vsx1* is expressed in postmitotic cells in the inner nuclear layer of the neuroretina, where it is confined to the bipolar cells (Fig. 5I; Belecky-Adams et al., 1997). In *el*$^-$ embryos, *Vsx1* is not expressed in the domain defined by *Rx2* expression in the lateral wall of the ventral diencephalon (Fig. 5J). Taken together, even though retinal precursor tissue forms in *el*$^-$ embryos, this tissue remains undifferentiated and forms neither PRE nor neuroretina.

### Effects on forebrain morphogenesis

As indicated by the distorted *Pax6* expression domain in the dorsal diencephalon, it is likely that the retinal tissue in the ventral forebrain of *el*$^-$ embryos affects the morphogenetic movements of the diencephalon. To examine further the forebrain of *el*$^-$ embryos, we analyzed the expression of *Emx1* and *Emx2* as markers for specific regions of the developing forebrain (Boncinelli et al., 1993; Morita et al., 1995; Pannese et al., 1998; Loosli et al., 1999). The pattern of *Emx2* expression in the telencephalon and the ventral forebrain is unaffected in *el*$^-$ embryos (data not shown). *Emx1* is expressed dorsally in the telencephalon of wild-type and *el*$^-$ embryos. This indicates that the *el* mutation does not affect the formation of the telencephalon (Fig. 6A,B). However, the shape and location of the *Emx1* domain are altered in *el*$^-$ embryos. While the wild-type domain bends ventrally at the anterior tip of the brain during somitogenesis stages (stage 22), this occurs only partially in *el*$^-$ embryos (Fig. 6A,B), indicating a retained movement of the telencephalon. In contrast, more caudal structures such as the hypothalamus are not affected as demonstrated by the unaltered morphology and expression of *Emx2* (compare Fig. 6A and B; data not shown).

Analysis of apoptosis by TUNEL labeling revealed an increased number of apoptotic cells in the ventral forebrain and the lateral head ectoderm of *el*$^-$ embryos at the 12-somite stage (Fig. 6C,D, stage 23). Thus, in *el*$^-$ embryos, a second wave of ectopic apoptosis occurs in tissues that at later stages are affected in differentiation namely the retina and the lens.

### *el* functions cell autonomously in retinal precursor cells

To investigate whether *el* activity is required for the evagination of the optic vesicles in a cell-autonomous manner, we performed cell transplantation experiments. About 30-50 cells were transplanted at the late blastula stage from wild-type donors to isochronic offspring of an *el* carrier cross. To allow unambiguous identification of transplanted cells, a transgenic line stably expressing GFP in all cells was used as wild-type donor (T. H. and J. W., unpublished results). After transplantation, host embryos were raised at the restrictive

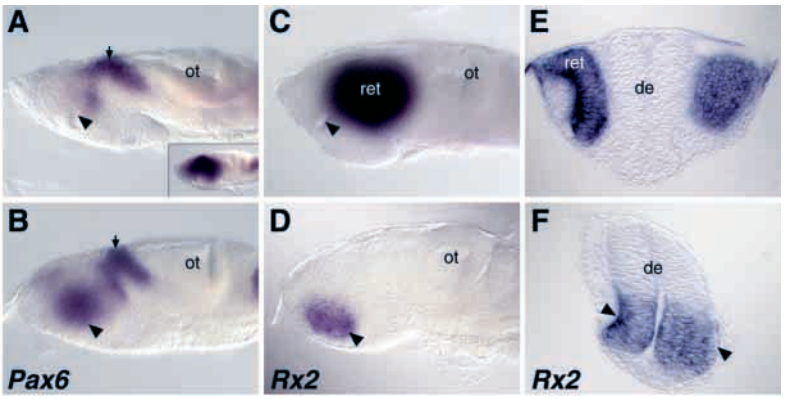


**Fig. 4.** *Pax6* and *Rx2* expression in the ventral forebrain of *el*⁻ embryos reveal formation of retinal primordia. In situ analysis of (A,B) *Pax6* and (C-F) *Rx2* expression in (A,C,E) wild-type and (B,D,F) *el* mutant embryos at 12-somite stages (stage 23). (A) Lateral view of wild-type embryo with eyes removed, showing *Pax6* expression. Inset: wild-type embryo prior to dissection of eyes. (B) In *el*⁻ embryos, ectopic *Pax6* expression in ventral diencephalon (arrowhead) at the location of optic stalk in wild-type embryos (compare arrowhead in A). The angle of the V-shaped *Pax6* domain in the dorsal diencephalon is narrower in *el*⁻ embryos (arrows in A, B). (C,D) Lateral view of *Rx2* expression in wild-type and *el*⁻ embryos. Ectopic *Rx2* expression domain (arrowhead) located in the ventral diencephalon of *el*⁻ embryos, comparable to that of ectopic *Pax6* (arrowheads in B,D, embryo in D is slightly tilted relative to B). (E,F) Transversal sections of eye region show *Rx2*-positive retinal tissue in two ventral domains (arrowheads), contiguous with lateral wall of diencephalon. de, diencephalon; ot, optic tectum; ret, retina.

temperature (18°C). The embryos were analyzed for 6 days from late neurula stage onwards.

Wild-type cells transplanted into *el*⁻ embryos are able to rescue the *el* phenotype and lead to the evagination of an optic vesicle and eventually to the formation of an eye (Fig. 6E,F). In all cases where optic vesicle formation was observed (*n*=3), evagination was unilateral and the tip of the outbudding optic vesicle exclusively comprised labeled wild-type cells, whereas the proximal part of the evaginating vesicle contained unlabeled mutant cells (2-somite stage, Fig. 6E). The contralateral side, however, showed complete failure of evagination. While in control transplantations, donor and host cells intermingle (Fig. 6H), wild-type cells transplanted into *el*⁻ embryos do not mix with mutant cells. The transplanted wild-type cells appear to initiate lateral outbudding of mutant cells. (Fig. 6E). However, at later stages, *el*⁻ cells do not contribute to the forming optic cup but are confined to more proximal regions (Fig. 6F).

To analyze the developmental potential of *el*⁻ cells in a wild-type background, 30-50 *el*⁻ cells stably expressing GFP were transplanted into wild-type embryos at the late blastula stage. Donor and corresponding host embryos were raised together (18˚C) and analyzed for 6 days from the late neurula stage onwards. In all cases (*n*=8) where transplanted *el*⁻ cells were found in the head region of wild-type hosts, they were excluded from the optic vesicles, but located in the forebrain and midbrain as well as the lens. Thus, the neighboring wild-type cells cannot rescue the mutant cells. Also, at later stages, no *el*⁻ cells were found in the optic cups (Fig. 6G). In summary, the cell transplantations show that *el* functions in a cell-autonomous manner.

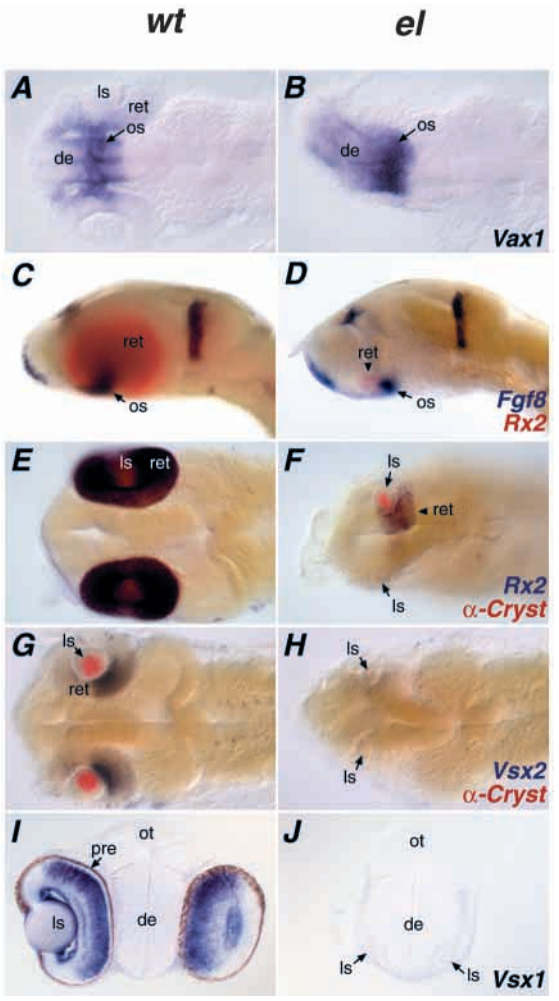


**Fig. 5.** Proximal optic vesicle structures and lenses form in *el*⁻ embryos. (A,B,E-H) Ventral views, (C,D) lateral views and (I,J) transversal sections of somitogenesis-stage (A,C,E,G,I) wild-type and (B,D,F,H,J) *el*⁻ embryos. (A,B) *Vax1* expression in optic stalk region and ventral diencephalon of wild-type and *el*⁻ embryos is expanded anteriorly in *el*⁻ embryos at 12-somite stages (stage 23, ventral view). Note anterior expansion of diencephalic *Vax1* expression in *el*⁻ embryos. (C,D) Optic stalk tissue expressing *Fgf8* (blue) located adjacent to *Rx2*-positive retina tissue (red) in ventral diencephalon of *el*⁻ and wild-type embryos at 30-somite stages. Other *Fgf8* expression domains are also not affected in *el*⁻ embryos. (E,F) Lenses in (E) wild-type and (F) *el*⁻ embryos located adjacent to *Rx2*-positive retinal tissue (blue). Not all lenses express α-*crystallin* (red) indicating retarded differentiation (see also H). (G,H) *Vsx2* (blue) expression is not detected in *el*⁻ embryos at the 16-somite stage. (I,J) *Vsx1* is not expressed in *el*⁻ embryos at the 35-somite stage (stage 30). de, diencephalon; ls, lens; os, optic stalk; ot, optic tectum; pre, pigmented retinal epithelium; ret, retina.

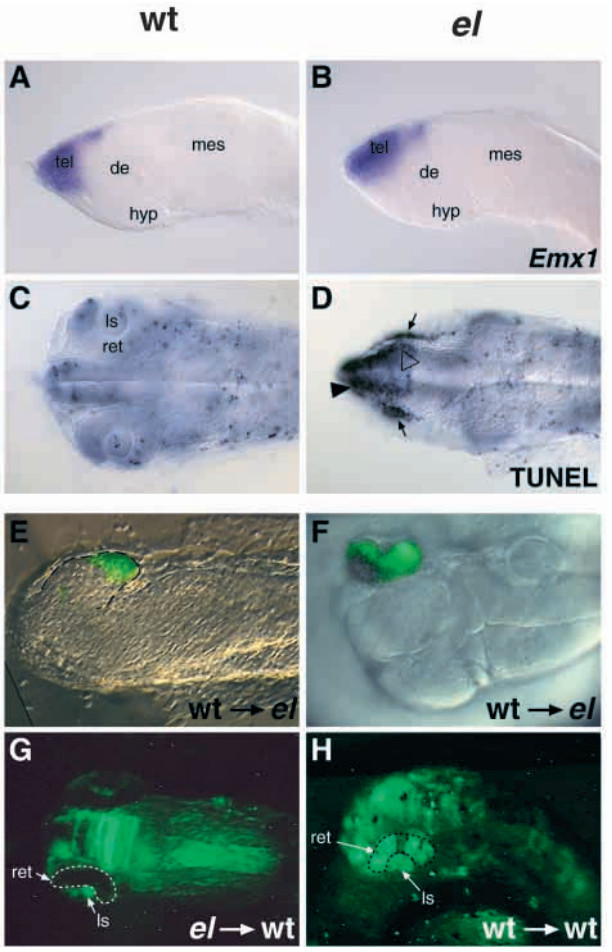


**Fig. 6.** Forebrain development at somitogenesis stages and chimera analysis. (A,B) Lateral view of telencephalic *Emx1* expression in wild-type and *el*⁻ embryos at 9-somite stages (stage 22). (A) In wild-type embryos, *Emx1* expression extends more ventrally. (C,D) Apoptotic cells visualized by TUNEL labeling in wild-type and *el*⁻ embryos at 12-somite stages (stage 23, ventral view). Note ectopic apoptosis in lens placode (arrow), ventral forebrain (arrowhead) and optic stalk region (open arrowhead) of *el*⁻ embryos. (E,F) Transplantation of wild-type cells (green) into *el*⁻ background. (E) Wild-type cells at the tip of optic vesicle (dashed line) at 2-somite stages. (F) Optic cup in same embryo at the 22-somite stage contains exclusively GFP-positive wild-type cells. (G) *el*⁻ cells (green) in wild-type background do not contribute to the optic cups (dashed white line) at 9-somite stage (stage 22). (H) Donor cells (green) contribute to all tissue in control transplanted embryos at 9-somite stage. de, diencephalon; hyp, hypothalamus; ls, lens; mes, mesencephalon; ret, retina; tel, telencephalon.

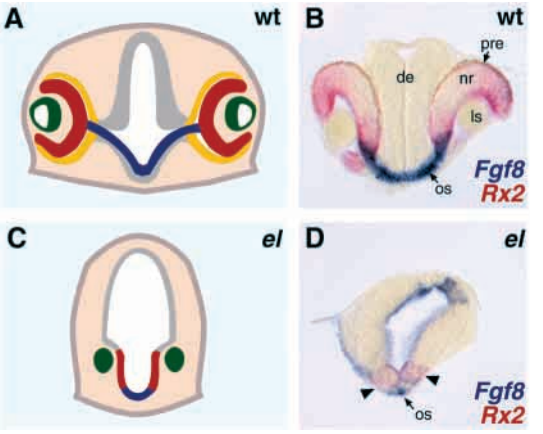


**Fig. 7.** Impaired morphogenesis results in a patterned retinal primordium in the ventral diencephalon of *el*⁻ embryos. (A,C) Schematic comparison of proximal (blue) and distal eye structures at organogenesis stages in wild-type and *el*⁻ embryos; from proximal to distal: blue, optic stalk; yellow, pigmented retina epithelium; red, neuroretina; green, lens. In *el*⁻ embryos, evagination of optic vesicle and subsequent differentiation of pigmented retina epithelium and neuroretina is impaired. (B) Transversal sections of eye region of wild-type embryos showing *Fgf8* (blue) and *Rx2* (red) expression in optic stalk and neuroretina at 30-somite stages (stage 28). (D) In *el*⁻ embryos *Fgf8* (blue) expression adjacent to *Rx2* (red, arrowheads) reveals formation of proximodistally patterned retinal structures in the diencephalic wall. de, diencephalon; ls, lens; nr neuroretina; os, optic stalk; pre, pigmented retinal epithelium.

## DISCUSSION

In this study, we show that optic vesicle morphogenesis is defective in the temperature-sensitive medaka *eyeless* mutant. Retinal tissue forms in the forebrain but does not evaginate (Fig. 7C,D), leading to increased cell numbers in the prosencephalon. The extra forebrain tissue affects later steps of brain morphogenesis such that telencephalic structures are retained in a more dorsal location. Proximal and distal identities as characteristic for wild-type optic vesicles are established in the absence of optic vesicle evagination (Fig. 7). Thus, early patterning occurs in the absence of optic vesicle formation, indicating that eye patterning and early eye morphogenesis are uncoupled by the *el* mutation.

The early expression of both *Pax6* and *Six3* during mid and late gastrula stages are unaltered. Thus, the determination of a single retina anlage and the early steps of lens induction are unaffected in *el*⁻ embryos. Temperature-shift experiments indicate a sensitive phase during the late gastrula and early neurula stage. Thus *el* activity is required prior to optic vesicle evagination, which in wild-type embryos starts 9 hours later (18°C).

Transplantation of wild-type cells into an *el* mutant background and the reciprocal experiment indicate a cell-autonomous requirement for *el* activity. Wild-type cells evaginate to form optic vesicles in *el* mutant embryos. Evagination on the contralateral side, when devoid of wild-type cells, is not observed. Mutant cells are found in the proximal region of optic vesicles rescued by transplanted wild-type cells but are not present in the resulting optic cups. This is consistent with a cell-autonomous nature of the *el* mutation where mutant cells are passively moved by the neighboring evaginating wild-type cells during initial stages of evagination. The reciprocal experiment shows that *el* mutant cells do not contribute to the optic cup confirming the cell-autonomous nature of the *el* mutation. Thus, neighboring wild-type cells cannot rescue the phenotype of *el* mutant cells.

Our data show that retinal primordia in *el*⁻ embryos do not form at an ectopic position, but rather fail to evaginate during development. These retinal primordia, as visualized by *Rx2* expression, are ventrolaterally located, adjacent to the position

where in the wild-type embryo the optic stalk emerges from the ventral diencephalon (Fig. 7). Furthermore, optic-stalk-like tissue as defined by the expression of *Fgf8* and *Vax1* is directly abutting the *Rx2* expressing tissue. Thus, in the absence of optic vesicle morphogenesis, retinal precursors with proximal and distal identities form within the lateral wall of the diencephalon. The presence of two retinal primordia and adjacent optic stalk like tissue consistently indicate that the split of the single eye anlage and subsequent patterning are unaffected. However, differentiation of the retinal primordia into pigmented retinal epithelium and neuroretina is lost.

At a stage when, in wild-type embryos, optic vesicles have already evaginated, a short phase of apoptosis is detectable in the prosencephalon of *el*$^{-}$ embryos in the region where the optic vesicles fail to evaginate. Since apoptosis in this region in *el*$^{-}$ embryos occurs later than optic vesicle evagination in wild-type embryos, ectopic cell death is the consequence rather than the cause for the lack of optic vesicle morphogenesis.

In *el*$^{-}$ embryos, a second apoptotic wave occurs at the stage when, in wild-type embryos, differentiation of the neuroretina is initiated. It results in ectopic cell death in the lens placode, the anterior part of the diencephalon and in the *Rx2*-positive retina precursor tissue of the mutant embryos; in wild-type embryos, no increase in the apoptotic index is detectable in these regions. This second wave of apoptosis suggests that the retinal precursor cells are either unable to respond to differentiation signals at this stage or do not receive the differentiation signals. This may in part be due to the lack of inductive signals between the developing lens and retina causing the observed apoptosis in both tissues.

In *el*$^{-}$ embryos, the lenses that form are reduced in size and often do not differentiate properly as indicated by a lack of α-*crystrallin* expression. The expression of *Pax6* and *Six3*, key players in the early steps of lens formation (Loosli et al., 1999; Oliver et al., 1996; Quinn et al., 1996), is unaltered in *el*$^{-}$ embryos indicating that lens determination occurs properly.

### *el* in comparison to other mutations affecting early eye development

Mutations affecting early steps of eye development have been described in other vertebrate species. All of these mutants exhibit a phenotype that differs significantly from that of the *el* mutation in medaka.

The zebrafish mutation masterblind (*mbl*) affects eye development to a variable degree. Severely affected embryos lack eyes and show an expansion of the dorsal diencephalon at the expense of the telencephalon (Heisenberg et al., 1996; Masai et al., 1997). Head sensory placode development is also affected in *mbl* mutant embryos, reflecting the proposed role of *mbl* in early forebrain patterning prior to the induction of the respective placodal structures. On the contrary, in *el*$^{-}$ embryos, formation of the telencephalon and sensory placodes is normal and early forebrain patterning, as revealed by the expression of *Pax6*, *Six3*, *Emx1* and *Emx2*, respectively, is not affected.

Absence of eye structures, mispatterning and, most severely, loss of entire forebrain was observed upon targeted inactivation of the mouse gene *Rx* (Mathers et al., 1997). Based on this phenotype, it has been proposed that *Rx* is required for eye formation from its initial stages (Mathers et al., 1997). The *el* mutation on the contrary affects optic vesicle evagination only. Marker gene analysis does not indicate a loss of forebrain structures in *el* mutant embryos and the initial steps of eye development are unaffected.

Mutations of the murine and rat *Pax6* gene, *Small eye* (*Sey*), lead to the absence of eyes and nose as well to severe mispatterning of the forebrain. Initially, however, optic vesicles do evaginate, but eventually degenerate later during development (Hill et al., 1991; Hogan et al., 1986; Matsuo et al., 1993). In *el*$^{-}$ embryos, in contrast, optic vesicle evagination does not occur, *Pax6* is expressed normally in the nasal and lens placode, and nose and lenses eventually form. Our data show that the *el* mutation interferes with optic vesicle morphogenesis rather than the determination of the retinal, lens and nose primordia. The gross morphology of the forebrain is only marginally affected in *el*$^{-}$ embryos, while *Pax6* mutant mice exhibit severe mispatterning of the forebrain.

In summary, analysis of the *eyeless* mutation showed that initial steps of retina formation and patterning as well as optic vesicle morphogenesis are two genetically separable processes. *el* function is required for proper evagination of the optic vesicle and for differentiation of retinal cells in the optic cup. Future experiments, using the temperature sensitivity of the medaka *el* mutation, will address the link between patterning and morphogenesis.

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