

Loss of eyes in zebrafish caused by mutation of *chokh/rx3*

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The vertebrate eye forms by specification of the retina anlage and subsequent morphogenesis of the optic vesicles, from which the neural retina differentiates. *chokh* (*chk*) mutant zebrafish lack eyes from the earliest stages in development. Marker gene analysis indicates that retinal fate is specified normally, but optic vesicle evagination and neuronal differentiation are blocked. We show that the *chk* gene encodes the homeodomain-containing transcription factor, Rx3. Loss of Rx3 function in another teleost, medaka, has also been shown to result in an eyeless phenotype. The medaka *rx3* locus can fully rescue the zebrafish mutant phenotype. We provide evidence that the regulation of *rx3* is evolutionarily conserved, whereas the downstream cascade contains significant differences in gene regulation. Thus, these mutations in orthologous genes allow us to study the evolution of vertebrate eye development at the molecular level.

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INTRODUCTION

The retina and lens of the vertebrate eye originate from an anterior territory, which comprises the neuroectodermal retina anlage and the abutting ectodermal lens-competence regions. During neurulation, the cells of the retina anlage converge at the midline in the prosencephalon, from where they then evaginate laterally to form the optic vesicles. These, in turn, induce lens formation in the adjacent competence regions.

Several genes required for the establishment of the retina anlage and its subsequent transition to optic vesicles have been identified. The homeodomain-containing transcription factors Six3 and Pax6 are essential for the determination of retinal fate in

the neuroectoderm (Carl *et al.*, 2002; Gehring, 2002; Loosli *et al.*, 1999) and also function in lens formation (Ashery-Padan & Gruss, 2001). The retinal homeobox transcription factor Rx has a crucial function in the morphogenesis of the optic primordia to form the optic vesicles (Loosli *et al.*, 2001; Mathers *et al.*, 1997). Both Six3 and Rx have been shown to control proliferation in the optic primordium (Andreazzoli *et al.*, 1999; Carl *et al.*, 2002; Loosli *et al.*, 2001; Mathers *et al.*, 1997). For proper morphology of the optic vesicle, the homeobox factor Vsx2/Chx10 is required (Barabino *et al.*, 1997).

Forward genetic screens in fish have been used to isolate an increasing number of mutations that cause defects in the developing eye (Malicki *et al.*, 1996; Loosli *et al.*, 2000; for a review, see Easter & Malicki, 2002). In zebrafish, two mutants have been described that affect early Wnt (Wingless/int-related)-signalling-dependent anterior–posterior patterning of the brain and, as a secondary consequence, result in eyeless or headless embryos (Kim *et al.*, 2000; Heisenberg *et al.*, 2001). These mutations are not specific to eye formation and also lead to severe patterning defects in other regions of the brain. So far, in the large-scale mutagenesis screens of zebrafish, surprisingly few mutations were identified that specifically affect the earliest stage of eye development.

Here, we describe the identification and analysis of a novel zebrafish mutant that lacks eyes from the earliest stages. The corresponding gene, named *chokh* (*chk*) after the Bangla word for ‘eye’, encodes Rx3. In view of the existing *rx* mutations in other vertebrate species, the isolation of a zebrafish *rx3* mutation closes an important gap in our understanding of conserved regulatory networks that underlie optic vesicle morphogenesis and differentiation. This now provides the opportunity to study the evolution of vertebrate eye development by cross-species comparison at the molecular level.

RESULTS

Zebrafish *chokh* mutants lack eyes

Mutants that are homozygous for the ethylnitrosourea-induced allele *s399* of the *chk* locus lack eyes at all stages of development (Fig. 1). This phenotype is similar to medaka *eyeless* (*el*) mutants (Winkler *et al.*, 2000). Both retinal pigmented epithelium and

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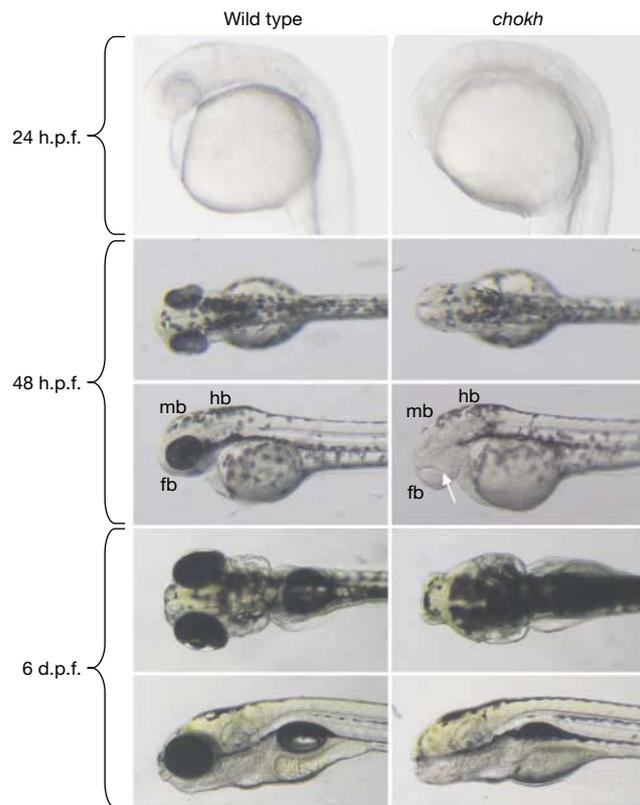


Fig. 1 | Morphological analysis of the *chokh* phenotype. Wild-type and mutant embryos are shown at 24 h post-fertilization (h.p.f.), 48 h.p.f. and 6 days post-fertilization (d.p.f.). Eyes are absent at all stages of development in *chokh* (*chk*) mutants. The overall morphology of the head and trunk is normal in mutants. At 48 h.p.f., a small lens is visible in *chk* mutants (arrow). fb, forebrain; hb, hindbrain; mb, midbrain.

neuroretina are missing. A lens forms, but it is markedly reduced in size. The overall morphology of the head and trunk is not affected by the mutation. In addition, all the main subdivisions of the brain (that is, the forebrain, midbrain and hindbrain), as well as the inner ear, seem normal. The *chk* mutation is recessive and fully penetrant. Mutants do not show visual responses, but respond to touch and vibration; they hatch and show apparently normal swimming, but die at ~3–4 weeks.

The *chokh* mutation disrupts *rx3*

The *chk* mutation was mapped close to the polymorphic CA-repeat marker *z10432* on chromosome 21 using 41 mutant embryos from a TL/WIK hybrid cross (see Methods). The zebrafish *rx3* gene had previously been mapped to the same position using the T51 radiation-hybrid panel (Geisler et al., 1999). The similar phenotypes of *chk* and medaka *el* strongly suggested that *rx3* might be a candidate gene. We therefore sequenced the coding region of *rx3* in our mutant and discovered a nonsense mutation in the homeodomain of the gene (Fig. 2A). This mutation is expected to convert the highly conserved tyrosine codon at position 479 to a stop codon (Fig. 2B). Therefore, two-thirds of the homeodomain (including the third helix, which is indispensable

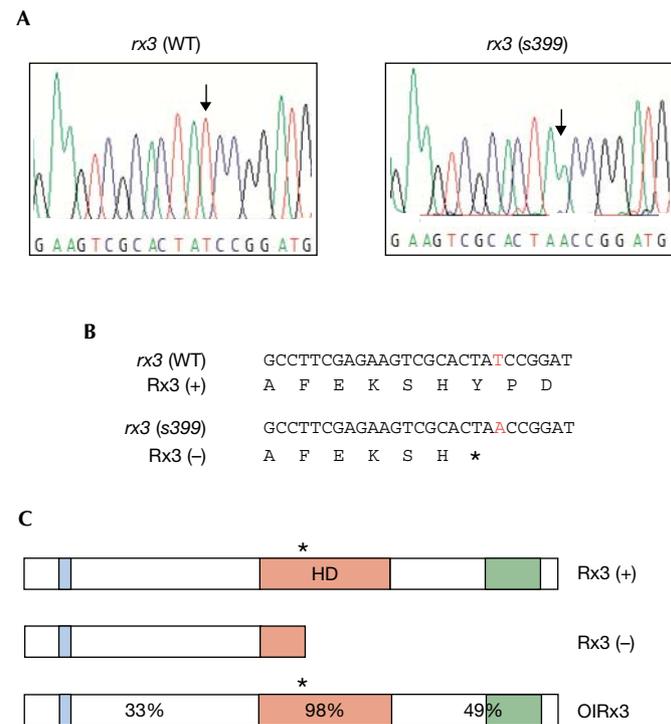


Fig. 2 | The *chokh* gene encodes Rx3. (A) Comparison of the sequencing trace data from wild-type and *chk* mutant *rx3* complementary DNA. Sequencing reveals a T to A point mutation (arrows). (B) The predicted translation of the wild-type and mutant *rx3* open reading frames. The mutation results in a premature stop codon (indicated by an asterisk) in the homeobox (red). (C) Comparison of zebrafish (Rx3) and medaka (OIRx3) proteins. The octapeptide (blue), homeodomain (HD; red) and the otp–aristaless–rx (OAR) domain (green) are indicated. Sequence identity at the amino-acid level is indicated for the amino- and carboxy-terminal regions and the homeodomain. The position of the nonsense mutation in the homeobox of *chk* and the frameshift mutation of the control plasmid (pOIRx3-fs) are indicated (asterisks). The mutation is predicted to cause a truncation of the protein.

for sequence-specific DNA binding) and the entire carboxy-terminal portion are deleted, resulting in a null allele (Fig. 2C).

Expression of *pax6* and *six3* are unaffected by loss of *rx3*

The homeobox transcription factors Six3 and Pax6 are key regulators of vertebrate retina specification and have been shown to function in a cross-regulatory interaction (Ashery-Padan & Gruss, 2001; Carl et al., 2002). Similar to other vertebrate species, in the zebrafish embryo Pax6 and Six3 are expressed in the eye field starting at late gastrula stages (Kobayashi et al., 1998; Püschel et al., 1992; Seo et al., 1998). We therefore used the expression of *six3a* (*six3.1*) and *pax6a* (*pax6.1*) as markers to examine the effects of loss of *rx3* function on early retina development in *chk* mutant embryos.

At 10.5 h post-fertilization (h.p.f.), just before optic vesicle evagination, *six3a* expression in the anterior neural plate comprises the retina anlage. Whole-mount *in situ* hybridization showed that *six3a* expression is unaltered in *chk* mutant embryos (data not

shown). At 11.5 h.p.f., the optic vesicles start to evaginate in wild-type embryos. This morphogenetic process does not occur in *chk* mutant embryos. At this stage, *pax6a* is expressed in the prosencephalon, which is adjacent to the head ectoderm, and more posteriorly in the hindbrain and spinal cord. In *chk* mutant embryos, *pax6a* is expressed normally in all of these domains (data

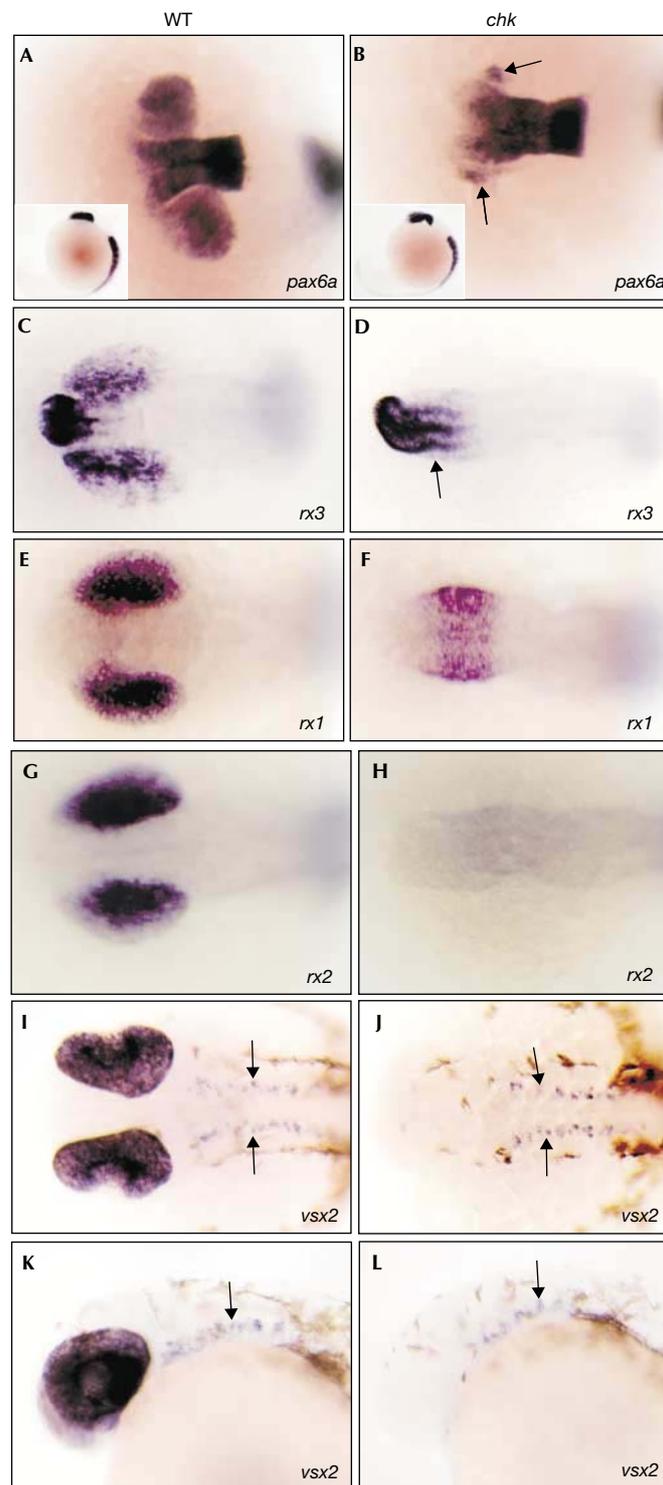
not shown). Hence, the normal early expression of *six3a* and *pax6a* indicate that forebrain patterning, and particularly retinal specification, occur normally in the absence of *rx3* function.

At 19 h.p.f., *pax6a* expression is prominent in the diencephalon, the evaginated optic vesicles and the abutting lens placodes (Fig. 3A). In *chk* mutant embryos, the diencephalic expression domain extends further anteriorly, which suggests that retinal progenitor cells remain in the forebrain instead of evaginating. The expression in the lens placodes is unaltered in mutant embryos, which indicates that *rx3* is not required for lens induction (Fig. 3B). Consistent with this, small lenses are visible at 48 h.p.f. (see also Fig. 1). More posterior *pax6a* expression in the hindbrain and spinal cord is unaffected at this stage, too, which is a further indication that central nervous system (CNS) regionalization is not affected by loss of *rx3*.

Rx3 is required for normal expression of Rx1 and Rx2

The enlarged *pax6a* expression domain in the forebrain of mutant embryos suggested that retinal progenitor cells, once specified, remain in the forebrain. To test this further, we used other marker genes that are expressed in retinal progenitor cells. *rx3* is initially expressed in the anteriormost neural plate, in a region which gives rise to the ventral forebrain and the optic primordia (Chuang et al., 1999). At 14 h.p.f., its expression is detectable in the presumptive anterior hypothalamus and the proximal optic vesicles (Fig. 3C). In *chk* mutant embryos, the *rx3* expression domain in the ventral forebrain extends posteriorly into the region from which the optic vesicles should evaginate, consistent with the abnormal location of retinal progenitor cells (Fig. 3D). Furthermore, the finding that *rx3* is expressed normally both in the presumptive hypothalamus and in retinal progenitor cells shows that *rx3* does not function in an autoregulatory feedback loop, similar to the situation in medaka (Loosli et al., 2001).

We used the retina-specific expression of the homeobox genes *rx1* and *rx2* to examine retina development in *chk* mutants in more detail. From 14 h.p.f. onwards, *rx1* and *rx2* are specifically expressed in the retinal progenitor cells of the optic vesicle (Fig. 3E,G). At that stage, *rx1* is expressed, although at reduced levels, in the ventral forebrain of mutant embryos at the position



◀ **Fig. 3** | Whole-mount *in situ* hybridization analysis of marker genes in wild-type embryos and *chokh* mutants. (A–J) Dorsal views. Lateral views are shown in (K) and (L), and in the insets in (A) and (B). Anterior is to the left in all panels. (A,B) Wild-type (WT) (A) and mutant (B) *pax6a* expression in the forebrain and optic vesicles at 19 h.p.f. Note the anteriorly extended *pax6a* expression in the mutant. Arrows indicate *pax6a* expression in the lens placodes. *pax6a* expression along the anterior–posterior axis is identical in wild-type and mutant embryos (insets in (A) and (B), respectively). (C,D) *rx3* expression in the ventral forebrain and optic vesicles of wild-type (C) and mutant (D) embryos at 14 h.p.f. Note the ectopic *rx3* expression in the forebrain of mutants (D) (arrow). (E,F) *rx1* is expressed in the optic vesicles of wild-type embryos (E) and in the forebrain of mutant embryos (F) at 14 h.p.f. Expression in the forebrain of the mutant (F) coincides with the position of the evaginated optic vesicles in wild-type embryos (E). (G,H) *rx2* is expressed in wild-type optic vesicles (G) but not in mutants (H) at 14 h.p.f. (I–L) *vsx2* expression in the optic cup and ventral midbrain and hindbrain (arrows) of wild-type (I,K) and mutant (J,L) embryos at 26 h.p.f. Midbrain and hindbrain expression is unaffected by the mutation.

from which the optic vesicles normally evaginate, whereas at 26 h.p.f. *rx1* expression is absent (Fig. 3F; and data not shown). *rx2* expression, however, is completely abolished at all stages in mutant embryos (Fig. 3H). Thus, the retinal progenitor-specific expression of *rx1* in the forebrain of *chk* mutant embryos provides further independent evidence for abnormally located retinal progenitor cells due to failure of optic vesicle evagination.

Retinal progenitors do not differentiate in *chk* mutants

We examined whether retinal progenitor cells in the ventral forebrain of *chk* mutant embryos undergo differentiation. At 26 h.p.f., the homeobox gene *vsx2* is expressed in mitotically active retinal progenitor cells before terminal differentiation. In the midbrain and hindbrain, *vsx2* is expressed in a ventrally located, paired row of cells (Barabino et al., 1997). Retinal expression of *vsx2* is not detectable at 26 h.p.f. in *chk* mutant embryos, whereas the expression in the double row of the midbrain and hindbrain is not affected (Fig. 3J,L). Thus, retinal progenitor cells do not reach this early stage of development in mutant embryos.

The basic helix–loop–helix gene *ath5* (*lak*, *atoh7*) is the earliest known marker for differentiating retinal ganglion cells and is essential for their development during the first differentiation wave in the retina (Kay et al., 2001; Masai et al., 2000). In wild-type embryos at 36 h.p.f., *ath5* is expressed in the proximal retina in presumptive ganglion cells (Fig. 4D). In mutant embryos, *ath5* expression is not detectable, indicating that the first step of retinal differentiation is already disrupted in *chk* mutant embryos (Fig. 4E).

The *chk* mutant is rescued by medaka *rx3*

We analysed whether injection at the one-cell stage of a plasmid containing medaka *rx3* and its regulatory regions (Loosli et al., 2001) is able to rescue the *chk* phenotype. As a control, we injected a frameshift derivative of the plasmid (Loosli et al., 2001). Because a conserved *BspEI* restriction site was used to introduce the frameshift mutation, the same portion of the medaka Rx3 protein was deleted in the control plasmid and by the *chk* mutation (Fig. 2C). Wild-type embryos injected with the control plasmid were analysed for medaka *rx3* (*Olr3*) expression at 12 h.p.f. by whole-mount *in situ* hybridization. For both plasmids, specific and strong *Olr3* expression was detectable in the ventral forebrain and evaginating optic vesicles at this stage (Fig. 4A–C). Thus, both plasmids contain the essential regulatory elements for stage- and region-specific *rx3* expression in zebrafish embryos.

In the rescue experiments, we genotyped the injected embryos by PCR, followed by digestion with *BspEI*, which cuts the wild-type DNA, but not the *chk* mutant DNA. In a representative experiment, on injecting the pOIRx3 plasmid, we obtained 15 eyeless (non-rescued) mutants among 68 injected embryos at 48 h.p.f. We genotyped the 53 embryos that had eyes and found that 7 of them had homozygous mutant genotypes. This corresponds to a rescue rate of 32% (7 out of 22 injected mutants), which is similar to the result obtained with the same plasmid in medaka *el* mutants (37%; Loosli et al., 2001). Injection of the control plasmid, pOIRx3-fs (which contains a frameshift mutation in *rx3*), did not rescue any of the mutants. For this plasmid,

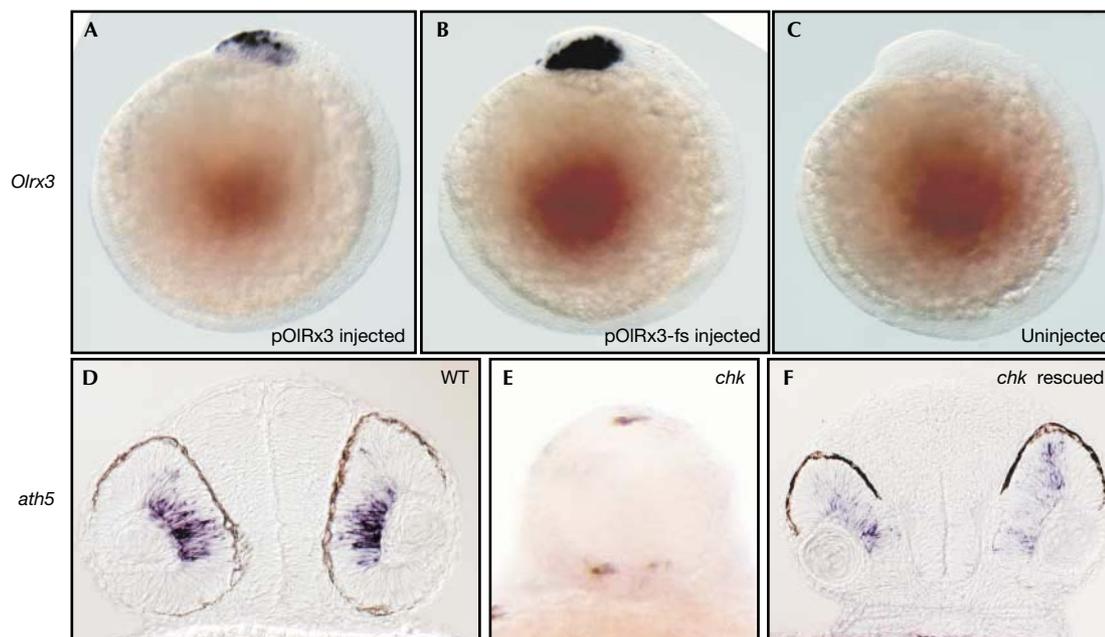


Fig. 4 | Rescue of *chk* mutants by the medaka *rx3* locus. (A–C) Lateral views, with the anterior to the left. (D) and (F) show transverse sections at the level of the eye. (E) is a frontal view. (A,B) Medaka *rx3* (*Olr3*) expression at 12 h post-fertilization in wild-type (WT) embryos injected with the rescue plasmid (pOIRx3; A) and control plasmid (pOIRx3-fs; B). Note the specific expression in the ventral forebrain and optic vesicles of injected embryos. (C) The same *Olr3* probe does not cross-hybridize with uninjected control embryos. (D–F) *ath5* is expressed in ganglion cells of wild-type retinæ (D) at 36 h.p.f., but not in the mutant (E) (whole-mount, to show the absence of staining). The dark spots in (E) are due to pigmentation. Injection of pOIRx3 restores morphology and wild-type *ath5* expression (F, compare with D).

all 34 genotyped embryos (with eyes) were either heterozygous or were homozygous wild-type.

Interestingly, most rescued mutants (six out of seven in the experiment described above) seemed to be morphologically normal. The size and morphology of their eyes were indistinguishable from wild-type embryos. This result was reproducible and was obtained by double-blind scoring of rescued, control-injected and uninjected embryos.

We analysed *ath5* expression in *chk* mutant embryos that were injected with the pOIRx3 plasmid and found that *ath5* expression is restored in the optic cups of rescued embryos at 36 h.p.f. (Fig. 4F). Thus, molecular analysis confirms the result of our morphological inspections, showing that morphogenesis and differentiation of retinal progenitor cells in *chk* mutants is fully restored by the medaka *rx3* locus.

DISCUSSION

We have identified a novel point mutation in zebrafish that results specifically in a lack of eyes. In summary, map position, sequence analysis and rescue by complementation show that the *chk* gene encodes the zebrafish orthologue of the homeodomain transcription factor Rx3. The nonsense mutation that we identified is predicted to lead to a complete loss of function, as it deletes most of the DNA-binding homeodomain and the entire C-terminal portion. We showed that retinal progenitor cells fail to evaginate as optic vesicles, and that subsequent differentiation of the optic primordia is blocked. Other regions of the brain are not affected. Thus, in contrast to other zebrafish genes that affect anterior brain development, *chk/rx3* is specific for the developing optic primordia.

Our analysis of marker gene expression shows that *rx3* requirement is limited to the portion of the neural plate that will give rise to the optic primordia. Retinal specification and the formation of the lens placode are normal, consistent with neural-plate-specific expression of *rx3*.

Recently, a loss-of-function mutation of *rx3* has been described in the teleost medaka that also results in the complete absence of retinae (Loosli et al., 2001). In cross-species rescue experiments, we show that injection of the medaka *rx3* locus results in stage- and region-specific expression of this gene in the zebrafish embryo that resembles the endogenous pattern. This indicates that, in addition to the open reading frame of *rx3*, the respective regulatory elements are also highly conserved. This is further supported by the high frequency of complete rescues that were obtained. Therefore, the regulatory region of *rx3* can be expected to share conserved motifs, an issue that will be addressed in the future.

In zebrafish, all three *rx* paralogues are expressed in the evaginating optic vesicles (Chuang et al., 1999), whereas in medaka only *rx3* is expressed during optic vesicle evagination. Medaka *rx1* and *rx2* are first expressed in the fully evaginated optic vesicle just before the transition to optic cups (Loosli et al., 2001; F.L. and J.W., unpublished data). Thus, there is considerably more temporal overlap of *rx* gene expression during the critical period in zebrafish than in medaka. However, the phenotypic consequences of loss of *rx3* are equally dramatic in both species. This suggests that the weak and transient *rx1* expression in *chk* mutants is not sufficient for optic vesicle evagination because either the expression level is too low or, alternatively,

rx1 and *rx3* have non-redundant functions. Future experiments will address this issue.

The closely related genes *rx1* and *rx2* are specifically expressed in retinal progenitor cells of both species. Interestingly, *rx1* and *rx2* are differentially affected by the respective *rx3* mutations. Whereas in the medaka *el* mutation both *rx1* and *rx2* are still expressed, albeit at reduced levels (Loosli et al., 2001; F.L. and J.W., unpublished data), only *rx1* is initially expressed in the zebrafish *chk* mutant, whereas *rx2* expression is completely abolished. Hence, only in zebrafish are *rx1* and *rx2* completely dependent on *rx3* function. This finding shows a different transcriptional control of orthologues in these two vertebrates, which predicts an interesting divergence of the respective regulatory elements.

In contrast to *rx1* and *rx2*, retinal *vsx2* is lost in both *el* and *chk* mutants, suggesting that residual *rx1* and *rx2* expression cannot rescue *vsx2* expression in the medaka embryo. This indicates that, probably indirectly, *vsx2* depends mainly on *rx3* function.

Although they are highly conserved in both structure and expression pattern, several genes of the downstream cascade are differentially affected by the medaka and zebrafish *rx3* mutations. The availability of *rx3* mutations in both species offers a unique paradigm to study the divergence of gene function by changes in gene regulation. The ongoing whole-genome sequencing projects of medaka and zebrafish will provide the tools that are needed to compare the regulatory elements of these genes and address the evolution of regulatory interactions at the molecular level.

METHODS

Fish stocks and mapping. The *chk*^{s399} mutation was originally discovered in D. Stainier's laboratory in an unrelated screen for ethylnitrosourea-induced mutations that disrupt organ development (H.A. Field, E.O., H.V. and D. Stainier, unpublished data) and is now maintained in a TL background. The mutant name *chokh* (*chk*) was submitted to the Zebrafish Information Network database (<http://www.zfin.org>). For mapping, an s399 TL carrier was crossed to a WIK wild-type fish, and heterozygous F₁ progeny were identified in the next generation. Bulk-segregant analysis was performed on 41 homozygous mutants and 48 siblings from one cross to determine the linkage group, using a panel of 192 simple-sequence repeat markers (CA repeats), which were chosen for their high rate of polymorphisms between TL and WIK strains (K.F.-B. and H.B., unpublished data). Single-embryo analysis was used to confirm bulk linkage.

Cloning of zebrafish *rx3*. Total RNA was isolated from 2–3-day wild-type and *chk* mutant embryos using Trizol (Gibco BRL). The BD Biosciences SMART RACE complementary DNA amplification kit was used to generate 5' cDNA. The coding region of *rx3* was amplified by nested PCR (outer primers: NUP primer and 5'-AGTGGCCAGCTGCATTGT-3'; 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1.5 min; inner primers: 5'-CTTTTCTGC CGGGACAGTCT-3' and 5'-AAAACGGATCCAGACCACTG-3', 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min). The PCR product was gel extracted and sequenced using internal primers of the nested PCR reaction.

DNA injections and genotyping of rescued embryos. One-cell embryos from an incross of two heterozygous *chk* carriers were injected with 200 ng μl⁻¹ of a plasmid that contains medaka *rx3* and regulatory regions (pOIRx3) or a control plasmid that

contains a frameshift mutation in *rx3* (pOIRx3-fs). pOIRx3 carries the entire *rx3* locus. This plasmid fully rescues the medaka *el* mutant, whereas the pOIRx3-fs plasmid has no rescue activity (Loosli et al., 2001). Embryos were scored for the presence or absence of eyes at 48 h.p.f., and DNA was isolated from individual embryos. A 426-bp fragment of *rx3* was amplified by PCR with the following primers: 5'-GGATGATGAAAACCCGAAGA-3' and 5'-TGGGCTGAATAACAAACG-3' (35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min). PCR products were digested with *BspE1* overnight and run on a 2% agarose gel.

In situ hybridizations and vibratome sections. Whole-mount *in situ* hybridizations using digoxigenin-labelled antisense RNA probes and vibratome sections were carried out as described in Loosli et al. (2001).

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