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ORIGINAL ARTICLE

Lens gene expression analysis reveals downregulation of the anti-apoptotic chaperone αA-crystallin during cavefish eye degeneration

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Abstract We have conducted a survey of the expression patterns of five genes encoding three different classes of major lens proteins during eye degeneration in the blind cavefish *Astyanax mexicanus*. This species consists of two forms, an eyed surface-dwelling form (surface fish) and a blind cave-dwelling (cavefish) form. Cavefish form an optic primordium with a lens vesicle and optic cup. In contrast to surface fish, however, the cavefish lens does not differentiate fiber cells and undergoes massive apoptosis. The genes encoding the lens intrinsic membrane proteins MIP and MP19 and the divergent βB1- and γM2-crystallins are expressed during cavefish lens development, although their levels are reduced because of a smaller lens, and the spatial distribution of their transcripts is modified because of the lack of differentiated fiber cells. In contrast, the *αA-crystallin* gene, which encodes a heat shock protein-related chaperone with antiapoptotic activity, is substantially downregulated in the developing cavefish lens. The results suggest that suppression of αA-crystallin antiapoptotic activity may be involved in cavefish eye degeneration.

Keywords Cavefish · Lens apoptosis · MIP · MP19 · βB1-Crystallin · γM2-Crystallin · αA-Crystallin

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Introduction

Cave-dwelling animals, including flatworms, arthropods, amphibians, and teleosts, have lost or reduced their visual system as a consequence of life in the absence of light. Many different theories have been proposed to explain the convergent loss of eyes in cave animals (Barr 1968). Among these theories, neutral mutation (the accumulation of hypomorphic mutations in the absence of selection), natural selection, and genetic drift seem to be the most promising explanations for eye regression (Culver 1982; Wilkens 1988; Jeffery 2005). A recent QTL analysis of morphological traits supports natural selection as the mechanism for eye loss in cavefish (Protas et al. 2007). If selection is indeed operating to prevent eye formation, what are the benefits of losing eyes? We study the teleost *Asytanax mexicanus* as a model system to understand the genetic and developmental mechanisms of eye degeneration and the evolutionary mechanisms responsible for their adaptation to cave life.

A. mexicanus consists of two conspecific forms: an eyed surface-dwelling (surface fish) and a blind cave-dwelling (cavefish) form (Wilkens 1988; Jeffery 2001; Yamamoto 2004). Cavefish embryos develop an eye primordium consisting of an optic cup and lens vesicle. However, the cavefish lens does not differentiate fiber cells; instead, it undergoes massive apoptosis and eventually disappears in adults (Jeffery and Martasian 1998; Soares et al. 2004). Normal lens fiber differentiation involves an abbreviated apoptosis-like pathway in which nuclei and other organelles are removed from the cell (Wride 1996; Dalm 1999). In the cavefish lens, apoptotic nuclei are retained in fiber cell progenitors that are arrested before terminal differentiation. Other optic tissues are also affected in cavefish: photoreceptor cells degenerate, the retina becomes disorganized

and ceases growth, and the cornea, iris, and ciliary body are not induced (Langecker et al. 1993; Yamamoto and Jeffery 2000; Alunni et al. 2007; Strickler et al. 2007). The microophthalmic cavefish eye eventually sinks into the orbit and is overgrown by connective tissue and epidermis. Transplantation of a surface fish lens vesicle into the cavefish optic cup during embryogenesis can rescue eye development (Yamamoto and Jeffery 2000), suggesting that the regulation of lens apoptosis is a key factor in eye degeneration.

Changes in gene expression in the lens and surrounding tissues may play a role in cavefish eye degeneration. Expression of *hedgehog* (hh) genes and their downstream targets is expanded along the anterior embryonic midline in cavefish embryos resulting in a decrease in the *pax6*-expressing eye domain (Yamamoto et al. 2004; Menuet et al. 2007). Hyperactivity of the Hh signaling pathway appears to be a cause of eye degeneration because lens apoptosis can be specifically induced by *sonic hedgehog* overexpression in surface fish (Yamamoto et al. 2004). Furthermore, *hsp90α*, which encodes a heat shock protein chaperone with a role in lens apoptosis, is activated specifically in the cavefish lens (Hooven et al. 2004). The importance of Hsp90α in lens apoptosis has been established by pharmacological inhibition, which suppresses cell death and improves lens development in cavefish (Hooven et al. 2004). In addition, a reduction in *αA-crystallin* expression has been reported in the developing cavefish lens (Behrens et al. 1998), but the extent of downregulation was not quantified, and because other lens genes were not studied, it remained possible that the decrease is a general casualty of lens degeneration.

Many regulatory genes encoding transcription factors and cell signaling components are known to function in the complex pathway leading to vertebrate lens differentiation (Lang 2004). Most of the transcription factors involved in lens development (e.g., Pax6, Prox1, Sox, and Maf family members) are also important in determining other parts of the vertebrate embryo. Therefore, gross changes in their function are unlikely to affect cavefish lens differentiation. Accordingly, it has been shown that Pax6 and Prox1 are expressed normally in the developing cavefish lens (Behrens et al. 1997; Jeffery et al. 2000; Strickler et al. 2001). The genetic pathways underlying eye degeneration may be difficult to identify by a candidate regulatory gene approach. As an alternative strategy, we have examined expression of downstream genes involved in lens fiber cell differentiation. It is possible that genes at the termini of developmental pathways, which are expressed exclusively or primarily in the lens, are more susceptible to evolutionary changes and may be better candidates to reveal the dysfunctional genetic pathways involved in eye degeneration.

In this paper, we compare three different classes of genes that are expressed primarily or exclusively in the lens. We demonstrate that the *Major Intrinsic Protein* (*MIP*) and *MP19* genes, which encode lens water-transporting channels (Gorin et al. 1984; Kumar et al. 1993; Shiels and Bassnett 1996), and the *β1-* and *γM2-crystallin* genes, which encode lens structural proteins (Graw 1997), are expressed during surface and cavefish lens development. In contrast, the *αA-crystallin* gene, which encodes an hsp-related chaperone with potent antiapoptotic activity (Mao et al. 2004; Morozov and Warousek, 2005; Liu et al. 2007), is substantially downregulated in the cavefish lens. The results suggest that αA-crystallin suppression may regulate lens apoptosis and eye degeneration in the blind cavefish *Astyanax*.

Materials and methods

Biological materials

Astyanax surface fish were collected at Balmorhea State Park, TX. The Pachón cavefish used in this investigation were collected from Cueva de El Pachón, Tamaulipas, Mexico. Fish were raised on a 14-h light and 10-h dark photoperiod at 25°C and allowed to spawn naturally (Jeffery et al. 2000). Embryos were raised at 25°C.

Gene cloning

Ribonucleic acid (RNA) was isolated from surface fish at 36–72 h postfertilization (hpf) using the RNeasy Maxi Kit (Qiagen, Valencia, CA) or the Oligotex Direct messenger RNA (mRNA) Midi/Maxi Kit (Qiagen). RNA was made into complementary deoxyribonucleic acid (cDNA) using the First Strand cDNA Synthesis Kit for reverse transcriptase polymerase chain reaction (RT-PCR; AMV; Roche Applied Science, Indianapolis, IN). RT-PCR using degenerate primers was performed using the PCR Master Kit (Roche Applied Science) under the following cycling conditions: one cycle for 2 min at 94°C, five cycles for 1 min at 94°C, 2 min at 35°C and 3 min at 72°C, five cycles for 1 min at 94°C, 2 min at 45°C and 3 min at 72°C, 20 cycles for 1 min at 94°C, 2 min at 55°C and 3 min at 72°C, and one cycle for 10 min at 72°C.

The degenerate primers used to amplify MIP were designed from *Xenopus*, rat, mouse, and bovine MIP. The degenerate MP19 primers were designed from zebrafish, rat, mouse, and human MP19. The β-crystallin degenerate primers were designed from zebrafish (βB1), chicken (βB1), *Bos taurus* (βB1), mouse (βB2), rat (βB1), and human (βB) crystallins. The γ-crystallin degenerate primers were designed from carp (M2 and M3), *Xenopus*, rat (γA), and mouse (γF) crystallin sequences deposited in GenBank. *MIP* DNA was amplified using MIP1 (5′-CCAGTRTAATA

CAWCCCAAAGAGGTG-3′) and MIP2 (5′-AACCCWGC RGTCACTTTTGCCTTCC-3′) primers. *MP19* was amplified using LIM1 (5′-CCCGTGGAGATACTGCACGCC-3′) and LIM2 (5′-CTGCGGGCTGTCCATGCATCC-3′) primers. *βB-crystallin* DNA was amplified with β1 (5′ GAGTACCC RCGHTGGGAYACSTGG-3′) and β2 (5′-GTAGCCRCGC TASCCDGGYTACTG-3′) primers, and *γM-crystallin* DNA was amplified with γ1 (5′-TCATCTTCTACGAGGAYA AGG-3′) and γ2 (5′-TGATACGCCTCATGCTCATG-3′) primers. The primers used to amplify *αA-crystallin* were α1 (5′-ATGGATATTGCCATCCAGCACC-3′) and α2 (5′-CTA ACCGCCAAACCTACTGGTG-3′), which were selected by referring to the published *Astyanax αA-crystallin* sequence (Behrens et al. 1998; GenBank accession number Y11301).

The PCR products were gel extracted and ligated into either the pPCR-Script AMP SK (+) vector (Stratagene, La Jolla, CA) or the pSTBlue-1 vector (Novagen, Madison, WI). Ligated vectors were transformed, and putative positive colonies were grown in liquid culture. Vector DNA was extracted from these cultures using the QIAPrep Spin Miniprep Kit (Qiagen) and sequenced. The sequences were subjected to basic local alignment search tool and phylogenetic analysis (see below) to confirm that the expected DNA fragments had been cloned. The GenBank accession numbers for the cloned DNA sequences are as follows: *MIP*, AF264702; *MP19*, DQ492288; *βB-crystallin*, AF195949; and *γM-crystallin*, AF195948.

Phylogenetic analysis

Sequence alignment was performed using ClustalX (Thompson et al. 1997). Phylogenetic trees were constructed using the neighbor-joining method in the MEGA2 version 2.1 software (Kumar et al. 2001) with distances calculated using the *p* distance method and 1,000 bootstrap pseudoreplications.

In situ hybridization

Antisense and sense riboprobes were generated from cloned DNA sequences using the DIG RNA Labeling Kit (SP6/T7; Roche Applied Science). In situ hybridization was performed on samples fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) according to Yamamoto et al. (2004). Samples were viewed as whole mounts or subsequently embedded in Paraplast, sectioned at 8 μm, mounted on gelatin-subbed slides, and viewed by light microscopy.

Quantitative real-time RT-PCR

For quantitative real-time RT-PCR (qPCR), total RNA was extracted from cavefish and surface fish at 48 hpf using the Rneasy Mini kit (Qiagen) according to the manufacturer's protocol. Extracted RNA was quantified using a Ribogreen assay. We used 1 μg of RNA to create the cDNA using the stated protocol for Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo(dt) primer (5′-CGGAATTCTTTTTTTTTTTTTTTTTTV-3′, Sigma Genosys). Blank cDNA was created with total RNA as described but with no reverse transcriptase, to serve as a negative control to measure genomic contamination. mRNA levels were quantified using iQ SYBR Green SuperMix for the iCycler instrument (Bio-Rad, Hercules, CA) and analyzed according to the manufacturer's stated protocol for the iCycler iQ Real-Time PCR Detection System (Bio-Rad).

Primers were designed using Primer Express (v 2.0, Applied Biosystems) and either a known *A. mexicanus* sequence (see below) or the homologous region between zebrafish and *Tetraodon nigroviridis* cDNAs (for β-actin). The qPCR products were verified for the appropriate size by dissociation curve analysis and gel electrophoresis. Primers were 18–30 nucleotides in length with a melting temperature between 60 and 64°C. The forward and reverse primer sequences were as follows. The *αA-crystallin* sense primer was 5′-GGGACTCTATGCGCCCTTGT-3′, and the antisense primer was 5′-CACCGGTCACATCTGCAAGA-3′. The *MP19* sense primer was 5′-GTCACTGCTCGCGTGC TTCT-3′ and the antisense primer was 5′-GTGTTCCAGCT GCGAAGGTT-3′. The *β-actin* sense primer was 5′-CACA CMGTGCCCATCTAYGA-3′, and the antisense primer was 5′-CRGCYARATCCAGACGCAGRAT-3′. The *α-actin* sense primer was 5′-CACGGCATCATCACCAACTG-3′, and the antisense primer was 5′-CCACAGGGAGCTCGTT GTAGA-3′.

The qPCR output provided a Ct value for the threshold cycle. The threshold cycle is representative of fluorescence derived from the binding of SYBR green to the double-stranded PCR product. Data were transformed to a ΔCt value by subtracting the sample Ct value from the sample with the highest expression level to control for amplification efficiency. The ΔΔCt value was then calculated by normalizing gene expression to α- and β-actin using the geNorm software and methods (GeNorm v3.4, Vandesompele et al. 2002).

Statistical analysis of qPCR results was done using a one-way analysis of variance with cavefish and surface fish as the independent variables and relative mRNA levels as the dependent variable. Values reported are means + SE, and $p<0.05$ was required for significance.

Antibody staining

Samples were fixed overnight in 4% paraformaldehyde in PBS, embedded, and sectioned as described above. Anti-

body staining was carried out using γ-crystallin antibody (provided by Dr. Robert Grainger, University of Virginia). The sections were washed twice for 5 min in PBS, subjected to antigen denaturation for 15 min in 5 M urea, blocked in 5% nonfat dry milk for 30 min, rinsed twice in PBS for 10 min, incubated in primary antibody (diluted 1:100 in 5% nonfat dry milk in PBS) for 45 min, rinsed twice for 10 min in PBS, incubated in secondary antibody (biotin-conjugated goat anti-rabbit diluted 1:60 in 5% nonfat dry milk in PBS) for 60 min, rinsed twice in PBS for 10 min, incubated in Avidin-TRITC (diluted 1:50 in 5% non-fat dry milk in PBS) for 60 min, and rinsed twice for 10 min in PBS. All procedures were performed at room temperature. Finally, samples were mounted and viewed by fluorescence microscopy.

Results

MIP and *MP19* gene expression

Astyanax MIP and *MP19* DNA sequences were verified by phylogenetic analysis. *Astyanax MIP* was placed in a clade with zebrafish and killifish *MIP* DNA sequences supported by a bootstrap value of 100% (Fig. 1i). All other vertebrate *MIP* genes were positioned as an outgroup of the teleost *MIP* gene clade. *Astyanax MP19* was clustered in a clade with zebrafish and pufferfish *MP19* supported by a bootstrap value of 96% (Fig. 2i). Mammalian *MP19* sequences were placed in an outgroup of this clade. These results indicate that we have cloned parts of *Astyanax MIP* and *MP19* genes.

The expression patterns of the *MIP* and *MP19* genes were determined by in situ hybridization in 24- and 48-hpf surface fish and cavefish embryos (Figs. 1a–h, 2a–h). Lens fiber cell differentiation is initiated in surface fish at about 24 hpf, and lens apoptosis is first observed in cavefish at 36 hpf (Jeffery and Martasian 1998; Yamamoto and Jeffery 2000; Hooven et al. 2004). As described in other vertebrates (Chepelinsky et al. 1991; Church and Wang 1993), in situ hybridization with antisense RNA probes showed that the *Astyanax MIP* and *M19* genes are expressed in the lens (Figs. 1a–d, 2a–d). In these and other in situ hybridization studies described below, sense RNA probes showed no activity above background (data not shown). The patterns of *MIP* and *M19* expression determined by in situ hybridization were similar and are described together below.

At 24 hpf, both genes were expressed in the posterior sector of the surface fish and cavefish lens (Figs. 1e, f, 2e, f), which contains the precursors of primary lens fiber cells. The expression domains were smaller in the cavefish relative to the surface fish probably because of reduced

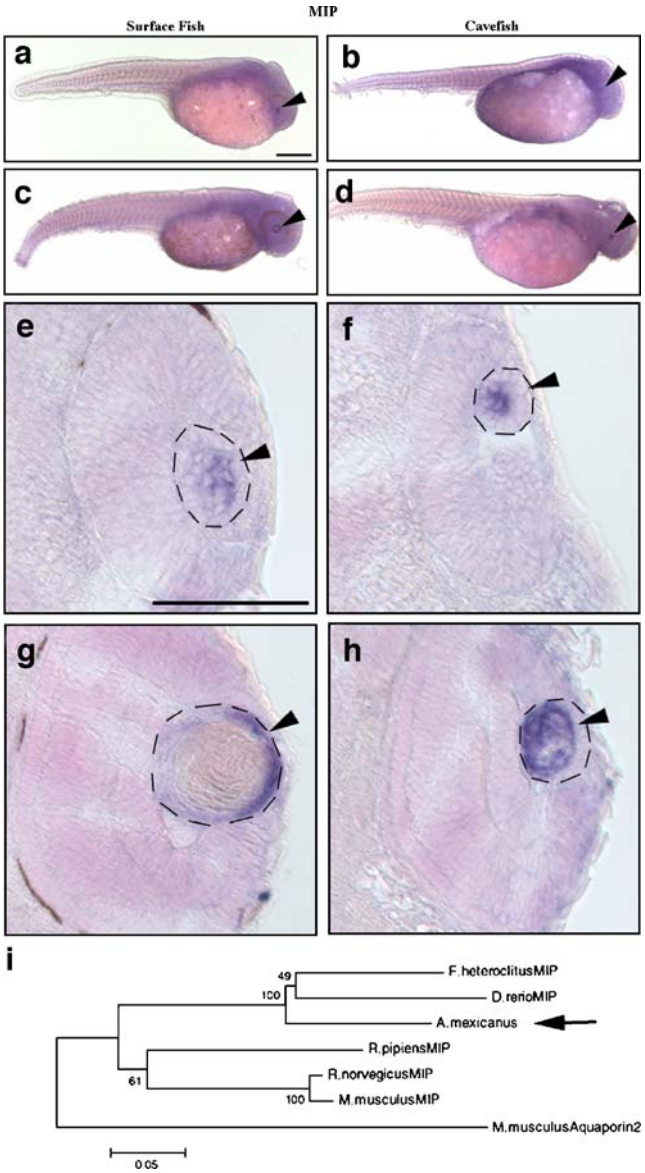


Fig. 1 *MIP* gene expression during surface fish and cavefish lens development. **a**–**h** *MIP* expression determined by in situ hybridization in 24- (**a**, **b**, **e**, **f**) and 48-hpf (**c**, **d**, **g**, **h**) surface fish (**a**, **c**, **e**, **g**) and cavefish (**b**, **d**, **f**, **h**) embryos. **a**–**d** Whole mounts. **e**–**h** Sections of optic areas. *Arrowheads*: lens. *Dashed lines* outline the lens in **e**–**h**. *Scale bar* in **a** is 200 μm; magnification is the same in **a**–**d**. *Scale bar* in **e** is100 μm; magnification is the same in **e**–**h**. **i** Phylogenetic tree of *MIP* DNA sequences constructed by the neighbor-joining (NJ) method. The branch lengths are proportional to phylogenetic distance. The *scale bar* represents an evolutionary distance of 0.05 nucleotide substitutions. Percentage values appearing at the nodes indicate bootstrap values for 1000 replicates. Arrow: *Astyanax MIP* sequence

overall size of the cavefish lens vesicle. At 48 hpf, differences in the pattern of gene expression were apparent between surface fish and cavefish. In surface fish, *MIP* and *M19* transcripts were restricted to epithelial cells surrounding the central sphere of differentiated fiber cells, which did not express either gene (Figs. 1g, 2g). The distribution of transcripts did not change between 24- and 48-hpf in

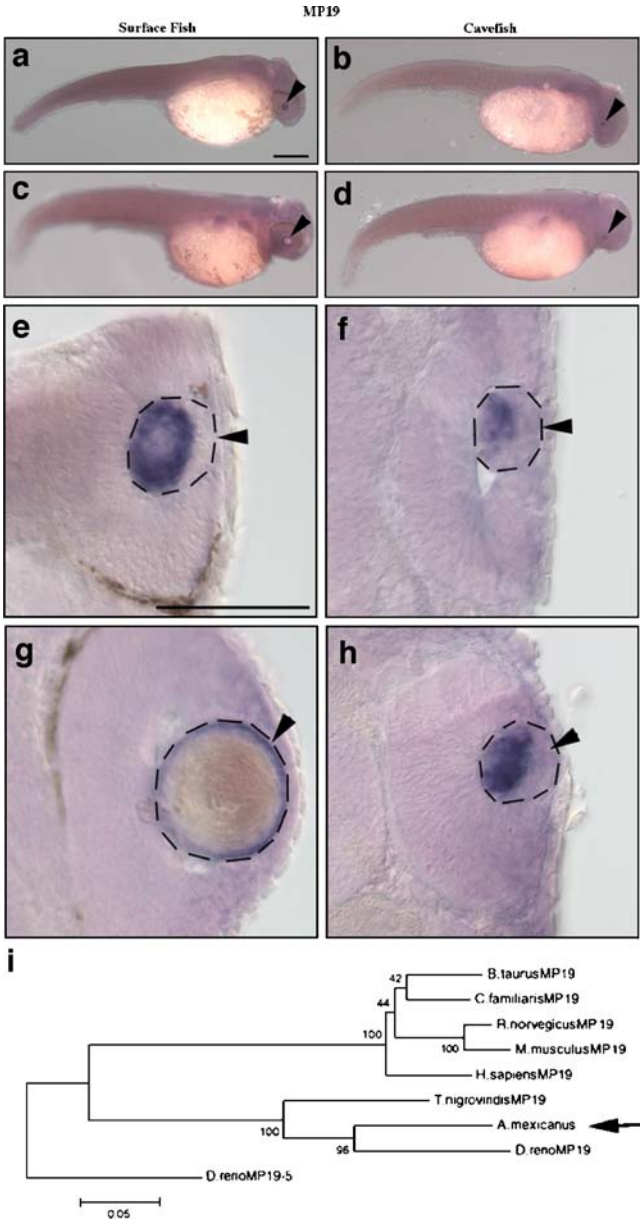


Fig. 2 *MP19* gene expression during surface fish and cavefish lens development. **a**–**h** *MP19* expression determined by in situ hybridization in 24- (**a**, **b**, **e**, **f**) and 48-hpf (**c**, **d**, **g**, **h**) surface fish (**a**, **c**, **e**, **g**) and cavefish (**b**, **d**, **f**, **h**) embryos. **a**–**d** Whole mounts. **e**–**h** Sections of optic areas. *Arrowheads*: lens. *Dashed lines* outline the lens in **e**–**h**. *Scale bar* in **a** is 200 μm; magnification is the same in **a**–**d**. *Scale bar* in **e** is 100 μm; magnification is the same in **e**–**h**. **i** Phylogenetic tree of *MP19* DNA sequences constructed by the NJ method. The branch lengths are proportional to phylogenetic distance. Details are the same as described for Fig. 1i. Arrow: *Astyanax MP19* sequence

cavefish (Figs. 1h, 2h): Both transcripts were restricted to lens fiber cell precursors in the posterior lens vesicle. The spatial differences in *MIP* and *M19* expression in 48-hpf surface fish and cavefish are likely to be a consequence of the failure of primary lens fiber cells to differentiate in the degenerating cavefish lens vesicle. The results show that the *MIP* and *M19* genes are expressed during cavefish lens development.

βB1- and *γM2-crystallin* gene expression

The *Astyanax β*- and *γ-crystallin* DNA sequences were verified by phylogenetic analysis (Fig. 3). *Astyanax γ-crystallin* DNA (*A. mexicanus* G; Fig. 3) grouped with teleost *γM2-crystallin* sequences to the exclusion of other vertebrate *γ-crystallin* genes, a clade of *βB-crystallin* genes and the *Astyanax αA-crystallin* (*A. mexicanus*A; Fig. 3) gene. We also obtained a second *Astyanax γ-crystallin* DNA (not used in these studies), which was placed with *γS-crystallins* of other fish species. The *Astyanax β-crystallin* DNA (*A. mexicanus* B; Fig. 3) grouped with vertebrate *βB1-crystallin* sequences in a clade distinct from those containing the *γM*- and *αA-crystallin* DNA sequences. The clade containing the teleost *γ*- and *β-crystallin* genes was supported by bootstrap values of 92 and 88%, respectively. The results indicate that we have cloned partial *Astyanax βB1- and γM2-crystallin* genes.

The expression of *βB1*- and *γM2-crystallin* genes was examined in sections of surface fish and cavefish optic primordia between 24 and 72 hpf (Figs. 4 and 5). The patterns of *βB1*- and *γM2-crystallin* expression were similar and resembled those of *MIP* and *M19*. At 24 hpf, *βB1*- and *γM2-crystallin* were expressed in the posterior regions of surface fish and cavefish lens vesicles (Figs. 4a, b, 5a, b). The expression domains were smaller in cavefish, consistent with the smaller size of the lens. Between 36 and 72 hpf, *βB1*- and *γM2-crystallin* expression was restricted to epithelial cells surrounding differentiated lens fiber cells in surface fish (Figs. 4c, e, g, i, 5c, e, g, i). Expression was stronger in the anterior portion of the lens, which consists of undifferentiated lens epithelial cells. Despite apoptosis, expression of the *βB1*- and *γM2-crystallin* genes persisted from 36 to 72 hpf in the small cavefish lens (Figs. 4d, f, h, j, 5d, f, h, j). The area of *βB1*- and *γM2-crystallin* expression increased from 24 to 48 hpf but then decreased from 36 to 72 hpf, presumably as a consequence of lens apoptosis. The results show that the *βB1*- and *γM2-crystallin* genes are expressed during cavefish lens development.

To determine whether γ-crystallin protein is produced in the cavefish lens, sections of 48- and 72-hpf surface fish and cavefish optic areas were stained with a γ-crystallin antibody (Fig. 6). At both stages, the surface fish lens was positive for γ-crystallin protein expression (Fig. 6a, b, e, f). The cavefish lens also showed γ-crystallin staining at both stages, although the stained area is smaller than the surface fish lens and decreased from 24 to 72 hpf (Fig. 6c, d, g, h), probably because of protein degradation during apoptosis. Cross-reacting antibodies were not available to assess MIP, MP19, and other crystallin proteins.

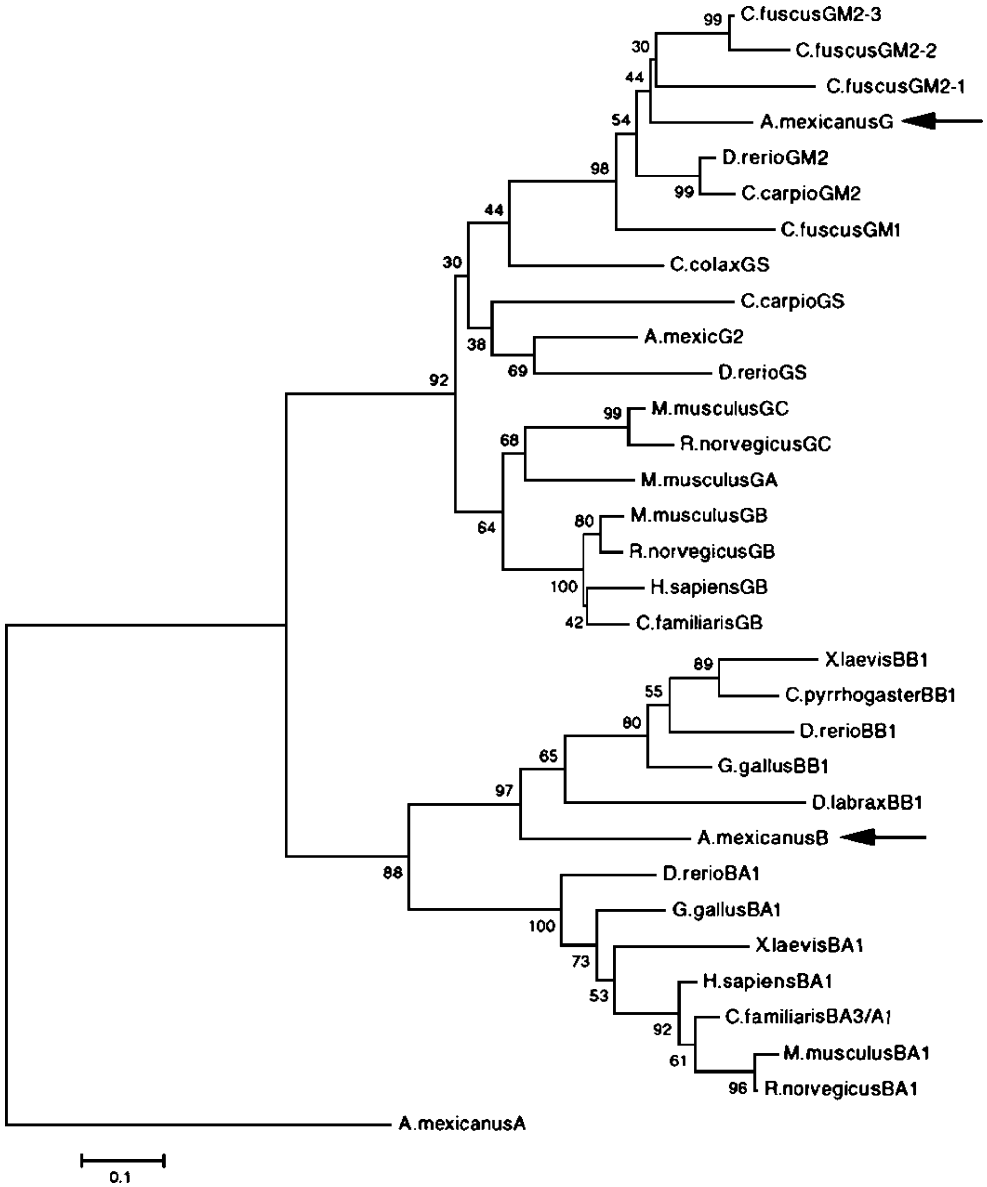
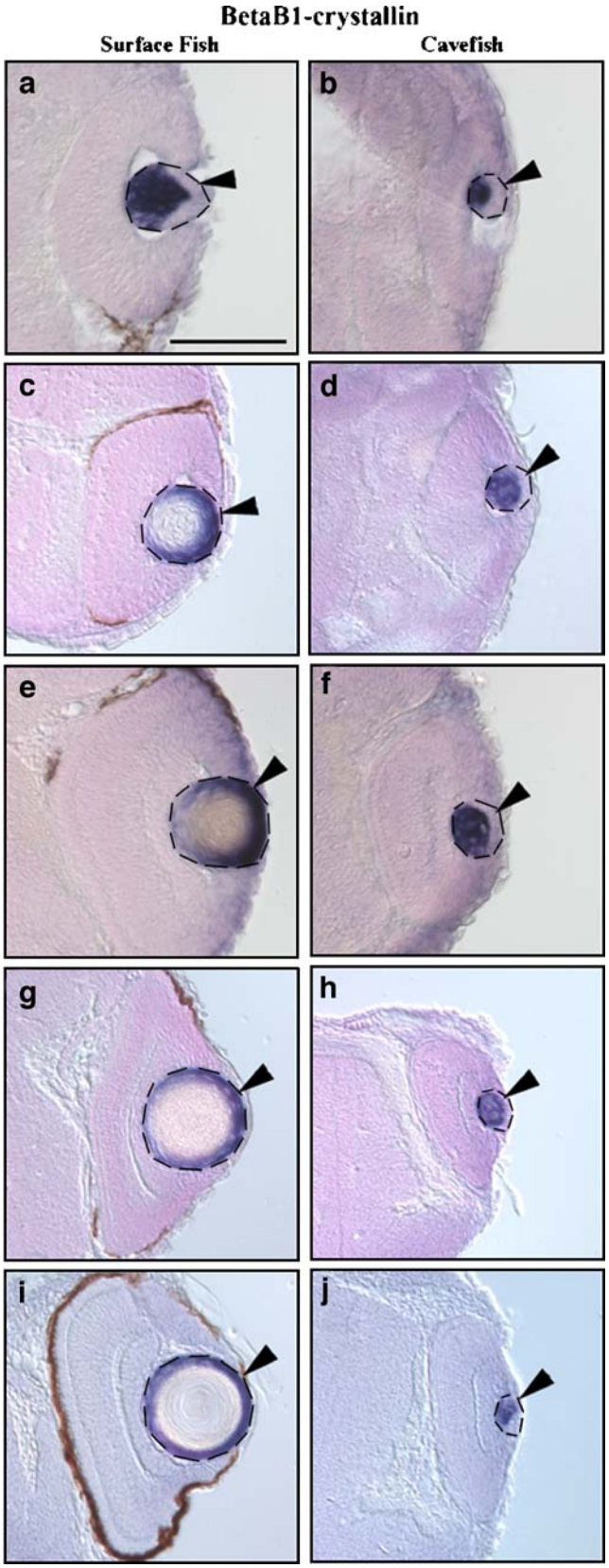


Fig. 3 Phylogenetic trees of *β*- and *γ-crystallin* DNA sequences constructed by the NJ method. *Astyanax γM2-crystallin* groups with other *γM2-crystallin* sequences, whereas *Astyanax βB1-crystallin* groups with other *β1B-crystallin* sequences. Details are the same as described for Fig. 1i

αA-crystallin gene expression

The *Astyanax αA-crystallin* DNA we isolated was identical to part of the coding region of the same gene in the Genbank database (Behrens et al. 1998). The expression pattern of *αA-crystallin* was compared in sections of surface fish and cavefish optic primordia at the same developmental stages as the other crystallin genes (Fig. 7). In zebrafish (Posner et al. 1999; Runkle et al. 2002), *αA-crystallin* is expressed primarily although not exclusively in the developing lens. At 24 hpf, there was no accumulation of *αA-crystallin* transcripts in the surface fish or cavefish lens (Figs. 7a, b). In surface fish, *αA-crystallin* mRNA was observed from 36 to 72 hpf in epithelial cells surrounding the differentiated lens fibers (Fig. 7c–i). The delayed onset of *αA-crystallin* expression relative to other crystallin genes has also been reported in the amphibian lens (Mikhailov et al. 1997). In contrast to surface fish, *αA-crystallin* mRNA was not detected in the lens of most cavefish embryos during the same developmental period (Fig. 7f, h, j). At 36 hpf, about 25% of the cavefish embryos analyzed here showed very weak *αA-crystallin* expression in the posterior lens (Fig. 7d), but this low level of expression was not seen in most 36-hpf embryos (data not shown) or at later stages of development. The results suggest that the *αA-crystallin* gene is downregulated during cavefish eye degeneration.

To further investigate the possibility of *αA-crystallin* downregulation, we used qPCR to compare transcript levels in surface fish and cavefish. In these experiments, we compared *αA-crystallin* mRNA levels with those of M19



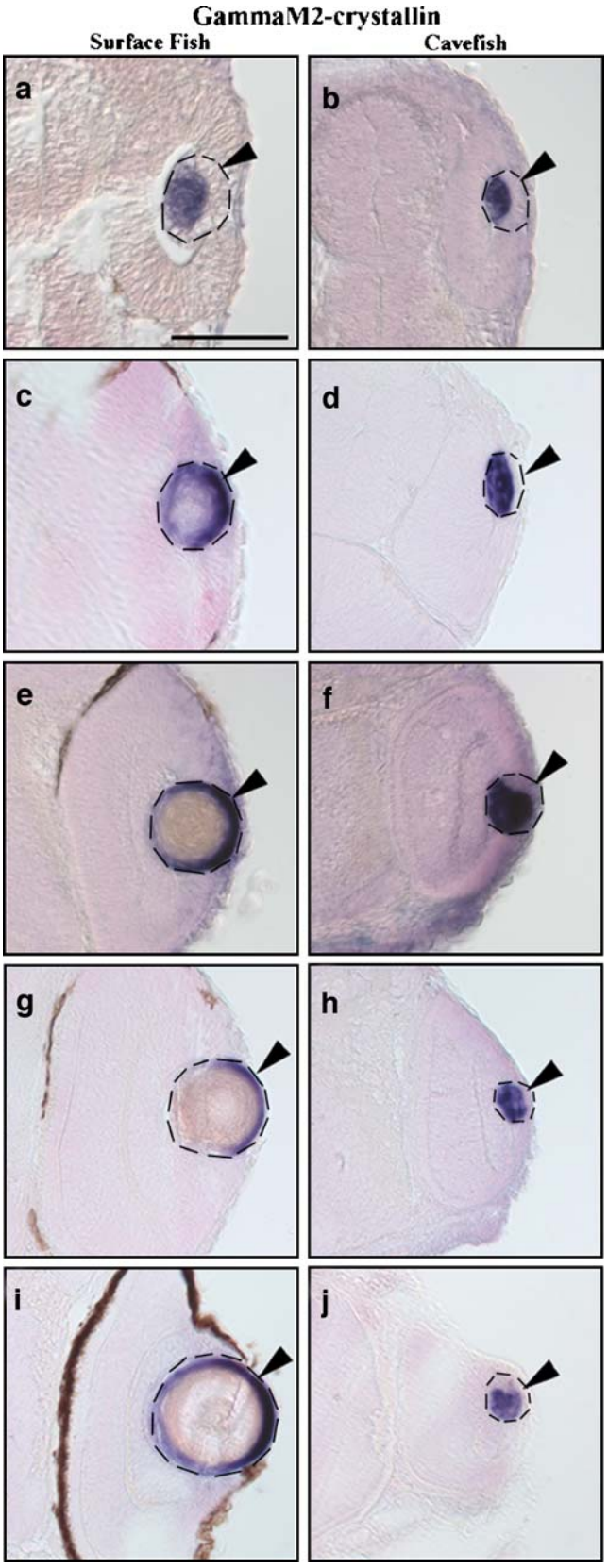
◀ **Fig. 4** *βB1-Crystallin* gene expression during surface fish and cavefish lens development. Sections through the optic areas of surface fish (**a**, **c**, **e**, **g**, **i**) and cavefish (**b**, **d**, **f**, **h**, **j**) embryos after in situ hybridization at 24 (**a**, **b**), 36 (**c**, **d**), 48 (**e**, **f**), 60 (**g**, **h**), and 72 hpf (**i**, **j**). *Arrowheads*: lens. *Dashed lines* outline the lens. *Scale bar* in **a** is 100 μm; magnification is the same in **a**–**j**

mRNA, which is expressed in the cavefish lens (Fig. 2), and β-actin mRNA, which is expected to show the same abundance in cavefish and surface fish (Fig. 8). The results showed that MP19 mRNA in cavefish was about half the level present in surface fish (Fig. 8b). In contrast, *αA-crystallin* was barely detectable in cavefish relative to surface fish (Fig. 8a). Finally, as expected, there was no significant difference in the level of β-actin transcripts in cavefish and surface fish (Fig. 8c). Based on these results, we conclude that the *αA-crystallin* gene is strongly downregulated during cavefish lens development.

Discussion

This study was designed to determine the identity of genes whose expression is substantially downregulated during abnormal development of the cavefish lens. We focused on known genes at the termini of genetic cascades leading to lens differentiation because it was reasoned that they are most likely to be modified under conditions of relaxed selection for vision in the dark cave environment. We have shown that the *MIP*, *MP19*, *γM2-crystallin*, and *βB1-crystallin* genes, which encode lens structural proteins, are expressed during surface fish and cavefish development, suggesting that they do not have a role in lens apoptosis and eye degeneration. In contrast, we have demonstrated that the *αA-crystallin* gene, a chaperone with antiapoptotic activity, is strongly downregulated in the cavefish lens, suggesting loss of function. The results suggest that suppression of the *α-crystallin* gene may induce and/or sustain lens apoptosis during eye degeneration in the blind cavefish *Astyanax*.

The *MIP*, *MP19*, and *crystallin* genes were selected for analysis based on the following reasoning. First, these genes are expressed either exclusively (*MIP* and *M19*) or primarily (*crystallins*) in the lens. Thus, changes in their expression in cavefish would not be predicted to interfere with the development of other tissues and organs. Second, these genes encode very abundant lens proteins: The crystallins alone are estimated to comprise about 90% of the total water-soluble lens proteins (Bloemendal et al. 2004). Because the synthesis of these proteins must require a significant allocation of metabolic resources, their loss of function in cavefish could be an adaptive strategy to conserve energy in the resource-poor cave environment



◀ **Fig. 5** *γM2-crystallin* gene expression during surface fish and cavefish lens development. Sections through the optic areas of surface fish (**a**, **c**, **e**, **g**, **i**) and cavefish (**b**, **d**, **f**, **h**, **j**) embryos after in situ hybridization at 24 (**a**, **b**), 36 (**c**, **d**), 48 (**e**, **f**), 60 (**g**, **h**), and 72 hpf (**i**, **j**). *Arrowheads*: lens. *Dashed lines* outline the lens. *Scale bar* in **a** is 100 μm; magnification is the same in **a**–**j**

(Protas et al. 2007). Third, these genes are expressed at the termini of gene networks leading to lens fiber cells, which fail to differentiate in cavefish embryos (Langecker et al. 1993). During relaxed selection on eyes, genes that function at the ends of developmental pathways are more likely to accumulate hypomorphic mutations than upstream regulatory genes, which generally function in development of multiple tissues and organs.

We have selected a group of genes for this analysis that are most likely to show changes in cavefish according to two of the current ideas for the evolution of eye degeneration: neutral mutation and positive selection for energy conservation (Wilkens 1988; Jeffery 2005; Protas et al. 2007). Contrary to the expectations of either theory, however, expression of *MIP*, *M19*, *βB1-crystallin*, and *γM2-crystallin* was found in the developing cavefish lens. Moreover, γ-crystallin protein was also present, indicating that functional *γ-crystallin* mRNA is transcribed, processed, and translated in the cavefish lens. The β- and γ-crystallins are necessary for lens transparency, which is neither established nor required in the blind cavefish. These results suggest that the function of these four genes has been conserved in cavefish, perhaps because of an unknown direct or indirect selective advantage or role in development of nonoptic tissues. Similar results have been reported in blind subterranean mammals. Even after several million years of evolution in darkness, *β-* and *γ-crystallins* are strongly expressed in the lens of the blind mole, *Talpa europea*, and the blind mole rat, *Spalax ehrenbergi* (Quax-Jeuken et al. 1984).

In striking contrast to the *M19*, *MIP*, *βB1-crystallin*, and *γM2-crystallin* genes described above, which show modestly reduced expression levels in cavefish, we have shown by in situ hybridization and qPCR that the *αA-crystallin* gene is strongly downregulated in the developing cavefish lens. The present results in Pachón cavefish support those of Behrens et al. (1998), who showed that *αA-crystallin* gene expression is decreased at two developmental stages in Piedras, another *Astyanax* cavefish population. Genetic studies indicate that multiple genes are responsible for cavefish eye degeneration (Wilkens 1988; Borowsky and Wilkens 2002; Protas et al. 2007). The downregulation of *αA-crystallin* in two different but phenotypically similar cavefish populations suggests that *αA-crystallin* or an upstream regulator is a candidate for one of these genes. Despite its downregulation, however, nucleotide differences resulting in loss of function have not been detected either in

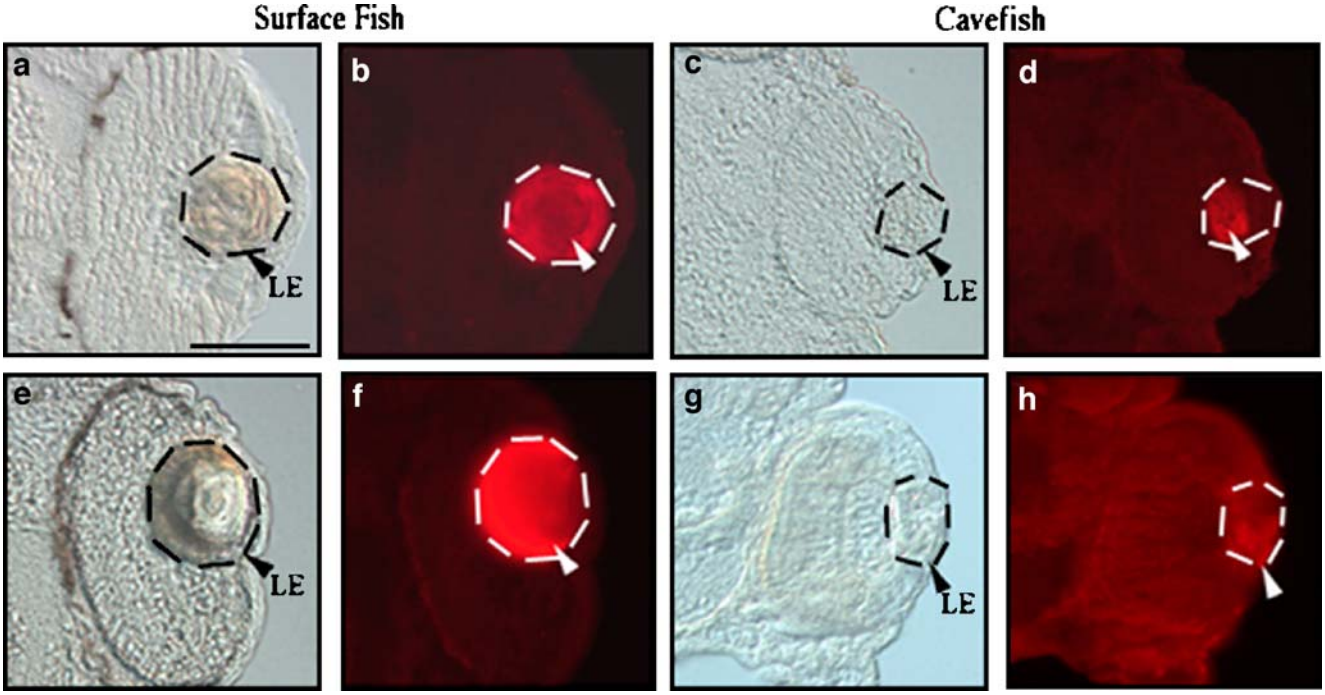


Fig. 6 γ-Crystallin expression during surface fish and cavefish lens development. Sections through the optic areas of surface fish (**a**, **b**, **e**, **f**) and cavefish (**c**, **d**, **g**, **h**) at 48 (**a**–**d**) and 72 hpf (**e**–**h**) stained with γ-crystallin antibody. Bright-field images (**a**, **c**, **e**, **g**). Fluorescence images (**b**, **d**, **f**, **h**). *LE* and *arrowheads*: lens. *Dashed lines* outline the lens. *Scale bar* in **a** is 100 μm; magnification is the same in **a**–**j**

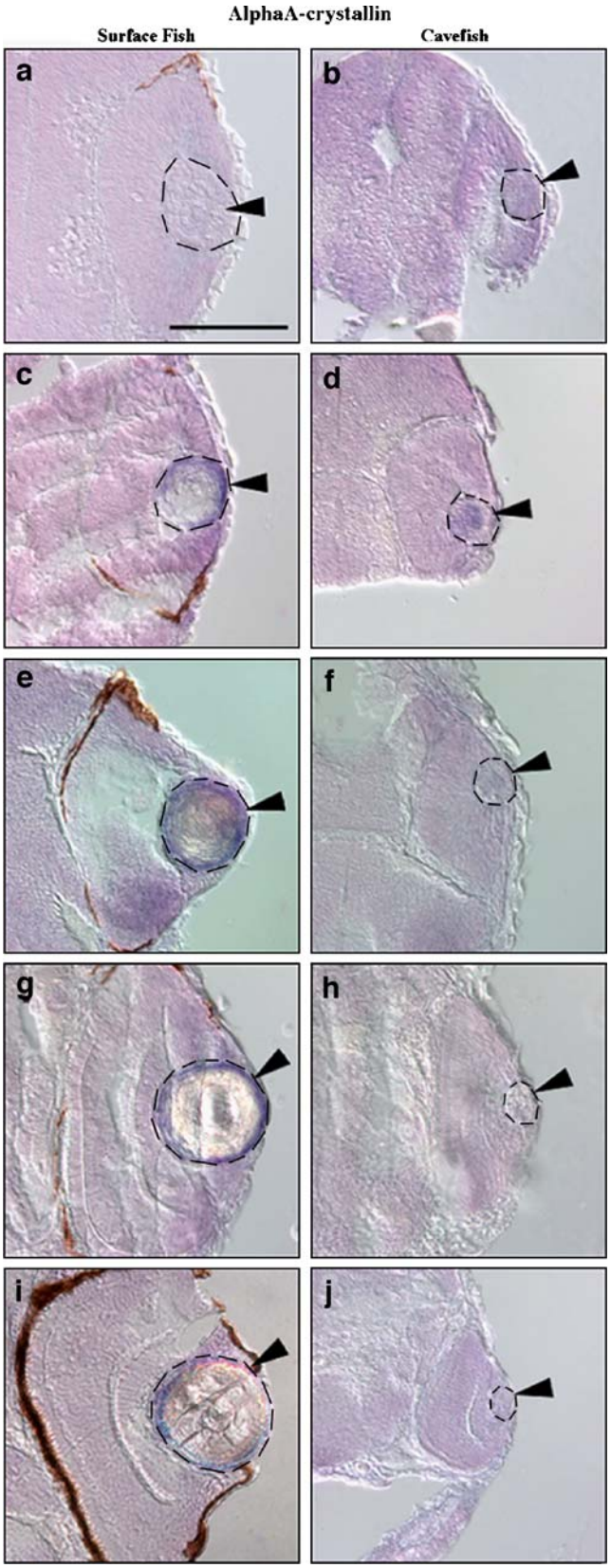
the coding region or the putative upstream regulatory region of cavefish *αA-crystallin* relative to its surface fish counterpart (Behrens et al. 1998).

Recent analysis of the zebrafish *cloche* mutant has shown that αA-crystallin is required for normal lens development (Goishi et al. 2006). In the absence of αA-crystallin, γ-crystallin is not solubilized, and lens fiber cells do not differentiate, which affects lens transparency and produces cataracts. The cavefish lens phenotype is similar in some ways to the lens phenotype of the zebrafish *cloche* mutant (Goishi et al. 2006): *αA-Crystallin* mRNA is downregulated, *γ-crystallin* mRNA is present, and lens fiber cells are not formed.

The vertebrate *αA-crystallin* genes are regulated by a complex array of transcription factors, including Pax6, retinoic acid receptors, members of the Sox, Maf, and CREB families, AP-1, and Prox1 (Cevkl et al. 1994; Kamachi et al. 1995; Yang et al. 2006). Some of these (e.g., Pax6, Sox, Maf, and Prox1) also regulate *MIP*, *βB-crystallin*, and *γM-crystallin* expression (Civil et al. 2002; Cui et al. 2004: Yoshida and Yasuda 2002; Reza et al. 2007) and are therefore not likely candidates for factors whose modification would specifically affect *αA-crystallin* expression. If *αA-crystallin* promoters are indeed unchanged in the two forms of *Astyanax* (Behrens et al., 1998), then one or more unidentified trans-acting regulatory factors may be responsible for *αA-crystallin* downregulation in cavefish. Recently, Shi et al. (2005) have shown that the transcription factor Pitx3 is necessary for lens development in zebrafish. Morpholino knockdown of *pitx3* in the latter study produced a similar phenotype to the one in cavefish lens. Therefore, the Pitx3 pathway may be a potential upstream regulator of *αA-crystallin* expression in cavefish.

The α-crystallin protein consists of αA-crystallin and αB-crystallin subunits, which interact in an approximate 3:1 ratio (Graw 1997). In addition to its role as a chaperone, α-crystallins can protect cells from stress-induced apoptosis. Although their antiapoptotic function is not completely understood, αA- and αB-crystallins are known to block initiation of the cell death pathway by sequestering the proapoptotic factors Bax, Bcl-X, p53 (Mao et al. et al. 2004; Liu et al. 2007), and caspase 6 (Morozov and Warousek 2005). In a key study, Morozov and Wawrousek (2005) showed that the knockdown of both α-crystallin subunits causes elevated caspase activity in mouse lens fiber cells. The lens phenotype of the double knockdown mice also resembles the cavefish lens phenotype: fiber cell precursors become apoptotic and do not lose their nuclei. An additional point is that *α-crystallin* expression is conserved in blind subterranean mammals (Hendriks et al. 1987), and their lenses are small but apparently not apoptotic (Sanyal et al. 1990). These results and observations strongly imply that α-crystallin functions to protect the developing lens from apoptosis.

The downregulation of αA-crystallin and its possible role in lens apoptosis in cavefish can be explained in two ways. First, the *αA-crystallin* gene could be suppressed



◀ **Fig. 7** *αA-crystallin* gene expression during surface fish and cavefish lens development. Sections through the optic areas of surface fish (**a**, **c**, **e**, **g**, **i**) and cavefish (**b**, **d**, **f**, **h**, **j**) embryos after in situ hybridization at 24 (**a**, **b**), 36 (**c**, **d**), 48 (**e**, **f**), 60 (**g**, **h**), and 72 hpf (**i**, **j**). *Arrowheads*: lens. *Dashed lines* outline the lens. *Scale bar* in **a** is100 μm; magnification is the same in **a**–**j**

transcriptionally at an early stage of lens development, thereby unleashing proapoptotic factors and inducing full-scale apoptosis as an alternative to lens fiber cell differentiation. Second, DNA and/or mRNA hydrolysis associated with apoptosis may secondarily cause *αA-crystallin* down-regulation. The second possibility is plausible because *αA-crystallin* mRNA is first detected late in surface fish lens development, relative to transcripts of the four other genes we have studied, which are not as greatly downregulated, and this is the same time at which apoptosis is observed in the cavefish lens. Continuous cell division acts to produce new lens cells in concert with apoptosis of older cells. As development proceeds, the newer cells also die until the cavefish lens almost completely disappears later in the adult (Strickler et al. 2002; Soares et al. 2004). Therefore, even if

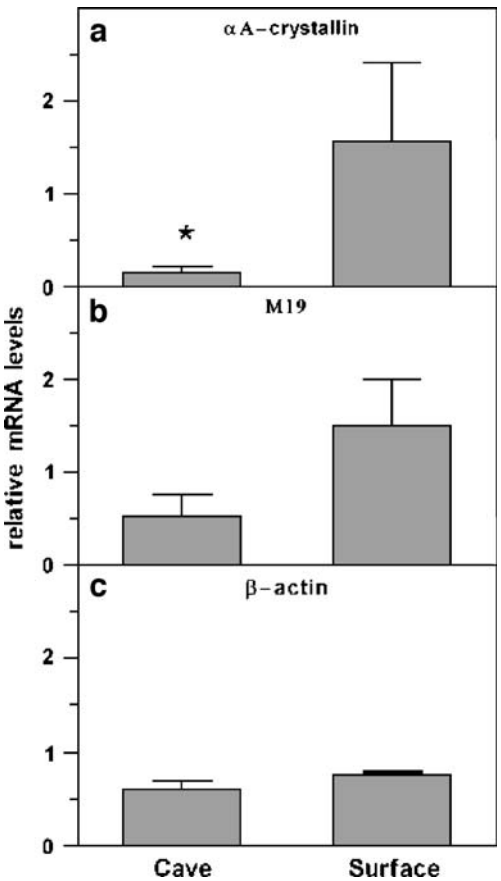


Fig. 8 Relative surface fish and cavefish mRNA levels at 48 hpf measured by quantitative real time RT-PCR. **a** *αA-crystallin* mRNA. **b** *M19* mRNA. **c** *β-actin* mRNA. *Error bars*: Mean+ SE. *Asterisk* in **a**: $p<0.5$ in a one-way ANOVA comparing cavefish ($n=3$) and surface fish ($n=4$) *αA-crystallin* mRNA levels

α-crystallin is not instrumental in initially triggering apoptosis, its downregulation could reinforce or sustain lens cell death during the gradual degeneration of the cavefish eye.

It has recently been shown that lens protects the retina from programmed cell death in *Astyanax* (Strickler et al. 2007). Thus, the early degeneration of the lens may have evolved to inhibit overall eye growth and development in cavefish. However, a retina phenotype has not been observed after downregulation of *αA-crystallin* in the zebrafish lens (Goishi et al. 2006). These results might suggest that *αA-crystallin* downregulation alone is not sufficient to produce retina degeneration.

Together with previous results showing activation of the proapoptotic factor *hsp90α* in the lens (Hooven et al. 2004), the present study showing downregulation of the antiapoptotic factor *αA-crystallin* supports an alternative hypothesis of the loss of eyes in blind cavefish. Eyes may be actively destroyed by selection for optic cell death as an adaptation to cave life. This idea can be considered as a variation of the energy conservation hypothesis: If it is costly to make eyes, cavefish may benefit by lacking them. Alternatively, instead of serving as a means to conserve energy, it may be adaptive to inhibit the growth of the eye primordium and bury the microophthalmic eye within the orbit simply because this could minimize any deleterious risks of maintaining an exposed nonfunctional eye on the surface of the animal.

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