

## ORIGINAL ARTICLE

## Differentially expressed genes identified by cross-species microarray in the blind cavefish *Astyanax*

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### Abstract

Changes in gene expression were examined by microarray analysis during development of the eyed surface dwelling (surface fish) and blind cave-dwelling (cavefish) forms of the teleost *Astyanax mexicanus* De Filippi, 1853. The cross-species microarray used surface and cavefish RNA hybridized to a DNA chip prepared from a closely related species, the zebrafish *Danio rerio* Hamilton, 1822. We identified a total of 67 differentially expressed probe sets at three days post-fertilization: six upregulated and 61 downregulated in cavefish relative to surface fish. Many of these genes function either in eye development and/or maintenance, or in programmed cell death. The upregulated probe set showing the highest mean fold change was similar to the human ubiquitin specific protease 53 gene. The downregulated probe sets showing some of the highest fold changes corresponded to genes with roles in eye development, including those encoding gamma crystallins, the guanine nucleotide binding proteins *Gnat1* and *Gant2*, a BarH-like homeodomain transcription factor, and rhodopsin. Downregulation of gamma-crystallin and rhodopsin was confirmed by *in situ* hybridization and immunostaining with specific antibodies. Additional downregulated genes encode molecules that inhibit or activate programmed cell death. The results suggest that cross-species microarray can be used for identifying differentially expressed genes in cavefish, that many of these genes might be involved in eye degeneration via apoptotic processes, and that more genes are downregulated than upregulated in cavefish, consistent with the predominance of morphological losses over gains during regressive evolution.

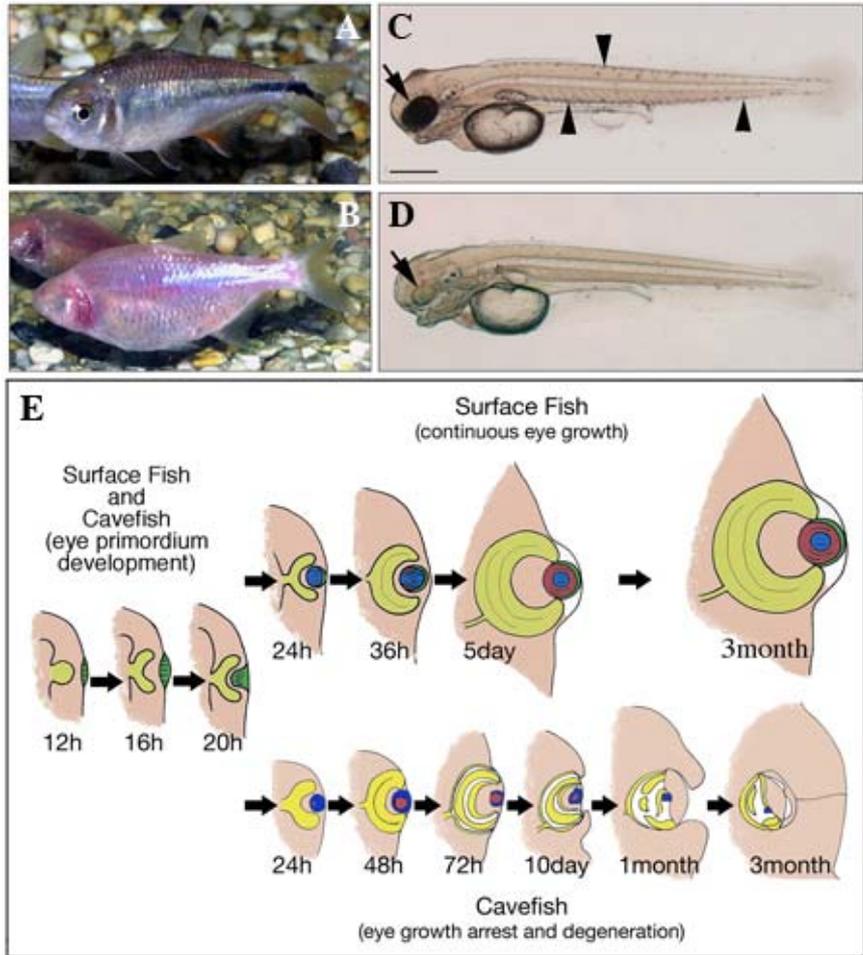
**Key words:** apoptosis, blind cavefish, cross-species microarray, differentially expressed genes, eye development.

### INTRODUCTION

The teleost *Astyanax mexicanus* De Filippi, 1853 is an emerging model system for studying the evolution of development (Jeffery 2008). This species consists of a

pigmented surface dwelling form with eyes (surface fish) and a blind albinitic cave dwelling (cavefish) form (Fig. 1A, B). Many traits have been gained and lost during the evolution of cavefish from its surface fish ancestor, but the most prominent regressive features are the absence of eyes and pigmentation. Recent studies have shown that melanin-containing pigment cells do not develop because of a mutation in the *oca2* gene (Protas *et al.* 2006). The *oca2* gene normally functions in supplying sufficient amounts of L-DOPA, a tyrosinase substrate and melanin

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**Figure 1** (A–D) *Astyanax* surface fish (A,C) and cavefish (B,D) adults (A,B) and 3-days post-fertilization larvae (C, D). Cavefish have no eyes and lack body melanin pigmentation. Cavefish larvae have small degenerating eye primordia lacking pigmentation. Arrows: eye primordia. Arrowheads: body pigment cells in surface fish. Scale bar in (C): 200  $\mu$ m; magnification is the same in (C) and (D). (E) Diagram showing eye development in surface fish and eye degeneration in cavefish. Left: similar eye primordia are formed in surface fish (above) and cavefish (below) embryos. Middle and right: eyes differentiate and grow continuously during surface fish development; whereas they initially increase in size, subsequently degenerate and eventually sink into the orbit in cavefish.

precursor. Loss of *oca2* function causes a block in melanin synthesis and failure of melanophores to differentiate. Eye degeneration is a more complex phenotype controlled by at least 12 different genes (Protas *et al.* 2007). None of these mutated eye genes have been identified. Therefore, development of effective means to identify the genes involved in eye degeneration is of major importance in understanding blind cavefish evolution and development.

Despite the absence of eyes in adults, embryonic cavefish develop eye primordia consisting of a lens and optic cup (Fig. 1E). Instead of differentiating, the cavefish lens undergoes massive apoptosis and eventually disappears (Jeffery & Martasian 1998; Soares *et al.* 2004). Normal lens fiber differentiation involves the removal of

nuclei and other organelles during an abbreviated apoptosis-like event (Wride 1996; Dalm 1999). In the cavefish lens, however, apoptosis runs to completion with degraded nuclei being retained in lens fiber cell progenitors arrested prior to terminal differentiation. The development of other optic tissues is also affected in cavefish: the retina becomes disorganized and ceases to grow, photoreceptor cells degenerate, and the cornea, iris and ciliary body are not induced (Langecker *et al.* 1993; Yamamoto & Jeffery 2000; Alunni *et al.* 2007; Strickler *et al.* 2007a). The microphthalmic cavefish eye eventually sinks into the orbit and is overgrown by connective tissue and epidermis. Eye formation can be rescued during embryogenesis by transplantation of a surface fish lens into the cavefish optic cup (Yamamoto & Jeffery 2000), sug-

gesting an important role for the lens in normal eye development.

Thus far, three different genes or gene systems have been implicated in controlling cavefish eye degeneration: two whose expression is upregulated and one whose expression is downregulated. Expression of *hedgehog* and downstream target genes is increased along the anterior midline in cavefish embryos, resulting in decreased activity of the negatively controlled *pax6* gene in eye primordia (Yamamoto *et al.* 2004). Hyperactivity of the Hedgehog signaling pathway appears to be a cause of eye degeneration because lens apoptosis can be specifically induced by *sonic hedgehog* overexpression in surface fish embryos (Yamamoto *et al.* 2004). In addition, *hsp90 $\alpha$* , which encodes a molecular chaperone, is activated in the cavefish lens (Hooven *et al.* 2004). Thus far, the only known downregulated gene during cavefish eye development encodes the anti-apoptotic factor  $\alpha$ A-crystallin (Behrens *et al.* 1998; Strickler *et al.* 2007b).

High throughput searches for genes showing a change in expression levels compared to surface fish is one way to identify genes underlying the loss of eyes and other trait changes in cavefish. DNA microarray technology is useful in identifying differentially expressed genes within a single species (Chauhan *et al.* 2002; Michaut *et al.* 2003). However, producing DNA chips for microarray analysis involves isolation of a large number of transcribed sequences, limiting its application to organisms for which many genes have already been identified: namely, human, yeast, mouse, *Drosophila* and *Danio rerio* Hamilton, 1822 (zebrafish), a teleost closely related to *Astyanax*. However, microarray analyses have also been successful when the research organism does not match the organism used to produce the microarray chip (Kayo *et al.* 2001). Here we report the results of a cross-species microarray approach that has successfully identified many differentially expressed genes in blind cavefish.

## MATERIALS AND METHODS

### Biological materials

*Astyanax* surface fish were originally collected at Balmorhea State Park, Texas, USA. Cavefish were collected from Cueva de El Pachón, Tamaulipas, Mexico. Fish were maintained in the laboratory on a 14:10 h L:D photoperiod at 25°C and spawned naturally. Embryos were collected and raised at 25°C.

### RNA isolation

Total RNA was extracted from 3-day post-fertilization (dpf) surface fish and cavefish larvae using the RiboPure RNA Isolation kit (Ambion, Austin, Texas, USA). RNA was converted into double stranded cDNA, as described previously (Strickler *et al.* 2007b). Biotin labeled antisense cRNA was produced from cDNA to use as a microarray probe.

### Microarray analysis

Surface fish and cavefish cRNA was hybridized to Affymetrix Zebrafish Genome Array chips with 16 oligonucleotide pairs representing approximately 14900 transcripts (Affymetrix, Santa Clara, California, USA). Five separate pair-wise hybridizations were performed. The pair-wise hybridizations used surface fish and cavefish cRNA extracted from three different clutches of 3-dpf larvae. The three pairs of cRNA probes were used individually for the first three hybridizations. In the last two hybridizations, cRNA probes prepared from equal amounts of each of the three-paired RNA samples were combined. The probes were hybridized to the arrays overnight at 45°C and subsequently washed, stained and scanned according to the Affymetrix Gene Chip Expression Analysis Technical Manual. Array chip scans were stored as digital cell intensity (CEL) files for analysis purposes.

Microarray chip analysis was performed using the DNA-Chip Analyzer (dChip; Li & Wong 2001a). Surface fish and cavefish CEL images from each of the five replications were loaded into dChip. CEL files were read and normalized using the invariant set normalization method (Li & Wong 2001a). Samples were then analyzed by applying the model-based expression method using the PM/MM difference model (Li & Wong 2001b). A comparison analysis was performed using the five cavefish chip arrays as the baseline and the five surface fish arrays as the experimental samples. To perform the analysis, group means of intensity with standard error for each probe set were determined for both baseline (surface fish) and experimental (cavefish) samples. Comparison criteria of at least a two fold change (FC) between the means using the lower 90% confidence bound of FC were used. During the analysis, a random permutation comparison was run 50 times to determine the false discovery rate of putative significant genes. This 50-permutation analysis, represented as a mean percentage value, was repeated 20 times, yielding a mean false discovery rate value of 13.4%.

The main analysis results were further interpreted by entering the probe set identification numbers into an expression batch query to determine known gene homologies (<http://www.affymetrix.com/index.affx>). This analysis provided gene ontology information, including descriptions of biological processes and molecular and cellular functions. Probe sets not identified by the Affymetrix expression batch query were subjected to further analysis. Similarities or possible homologies for these probe sets were determined by performing a BlastX analysis on genome and probe sequences from the Affymetrix website.

### Polymerase chain reaction amplification of gene sequences

RNA extraction, cDNA synthesis, and reverse transcription polymerase chain reaction (PCR) amplification techniques were performed according to Strickler *et al.* (2007b). Gamma-M crystallin DNA was amplified using the degenerate oligonucleotide primers  $\gamma$ C1 (5'-TCA TCTTCTACGAGGAYAAGG-3') and  $\gamma$ C2 (5'-TGAT ACGCCTCATGCTCATG-3') designed from carp (M2 and M3), *Xenopus*, rat ( $\gamma$ A), mouse ( $\gamma$ F) crystallin sequences deposited in GenBank. The *Astyanax* rhodopsin gene sequence (GenBank U12328), originally cloned by Yokoyama *et al.* (1995), was used to design the oligonucleotide primers Rho1 (5'-ATGAA CGGGACA-GAGGTCCATAC-3') and Rho2 (5'-TTATGCCGG GGACACGGAGGAGAC-3'). The PCR products were purified by agarose gel electrophoresis. Bands of the expected size were sequenced, subjected to Blast analysis to verify their identity, and inserted into the pPCR-Script AMP SK(+) vector (Stratagene, La Jolla, California, USA) to prepare RNA probes for *in situ* hybridization (Strickler *et al.* 2007b).

### *In situ* hybridization

*In situ* hybridization was performed on whole mounts of larvae fixed in 4% paraformaldehyde, as described by Strickler *et al.* (2007b). After hybridization, the stained larvae were postfixed overnight at 4°C in 4% paraformaldehyde, dehydrated through an ethanol series, embedded in Paraplast, and sectioned at 8  $\mu$ m. The sections were mounted on glass slides and viewed by light microscopy.

### Immunostaining

For immunostaining, larvae were fixed in 4% para-

formaldehyde overnight at 4°C, dehydrated through an ethanol series, embedded in Paraplast, and sectioned at 8  $\mu$ m. Sections were incubated with a polyclonal antibody to gamma crystallin (kindly provided by Dr Robert Grainger, University of Virginia) or rhodopsin (Leinco Technologies, St. Louis, Missouri, USA), and staining was visualized using DAB substrate, as described by Yamamoto and Jeffery (2000). The sections were counterstained with hematoxylin, mounted, and viewed by light microscopy.

## RESULTS AND DISCUSSION

### Microarray analysis

To identify differentially expressed genes, RNA from 3-dpf surface fish and cavefish larvae was hybridized to Affymetrix Zebrafish Genome Array chips. This stage of development was selected for analysis because it is the first time that widespread eye degeneration and growth arrest is visibly apparent in cavefish (Fig. 1C–E). Microarray analysis revealed 67 differentially expressed probe sets: 6 whose expression is increased and 61 whose expression is decreased. In Table 1, each probe set is listed according to its change in level of expression, with positive values indicating upregulation and negative values indicating downregulation. According to the Affymetrix sequence database, 31 probe sets have homologies or similarities to previously identified zebrafish genes. The 36 remaining probe sets that could not be identified using the Affymetrix database were further investigated by BLAST and web searching, which yielded information on the identity of 14 additional probe sets. Therefore, microarray analysis identified 50 known and 17 unknown genes that are potentially subject to differential regulation in cavefish embryos.

To be certain that genes identified by microarray analysis are actually differentially expressed, it is necessary to use additional means to quantify their mRNA levels. Thus far, two of these genes, *gamma crystallin* and *rhodopsin*, have been confirmed in this way (see below), suggesting that cross-species microarray analysis can predict differentially expressed genes in *Astyanax*. The genes identified by microarray analysis are not a random selection: a large number are known to be associated with two processes: eye development and programmed cell death. The identification of genes involved in these two processes account for some of the major differences between cavefish and surface fish. As might be expected in cases of regressive evolution, the microarray analysis detected mostly downregulated genes.

**Table 1** Differentially expressed genes in 3-days post-fertilization cavefish relative to surface fish determined by microarray analysis

| Gene homology  | Probe set          | GenBank Accession Number | Fold change |
|--|--------------------|--------------------------|-------------|
| <i>Upregulation</i>                                    |                    |                          |             |
| Ubiquitin specific protease 53                         | Dr.14618.1.A1_at   | BM185013                 | +1474.3     |
| Unknown  | Dr.20823.1.A1_at   | BI326634                 | +42.0       |
| Transmembrane protein 33                               | Dr.7488.1.S1_at    | BC044544.1               | +10.8       |
| Neurologin   | Dr.18973.1.A1_at   | BI709496                 | +8.5        |
| Unknown  | Dr.18477.1.A1_at   | BM154222                 | +4.0        |
| Unknown  | Dr.16871.3.A1_at   | AI544824                 | +2.8        |
| <i>Downregulation</i>                                  |                    |                          |             |
| Similar to gamma B crystallin                          | Dr.25729.1.S1_at   | BM573934                 | -61.2       |
| Gamma M4 crystallin                                    | Dr.19583.1.S1_at   | AL915133                 | -33.5       |
| Guanosine nucleotide binding protein 1                 | Dr.9899.1.S1_at    | NM_131868.1              | -29.5       |
| Similar to BarH  | Dr.16934.1.A1_at   | BI325578                 | -25.9       |
| Rhodopsin  | Dr.354.1.S1_at     | NM_131084.1              | -23.0       |
| Guanosine nucleotide binding protein 2                 | Dr.9881.1.S1_at    | NM_131869.1              | -17.2       |
| Unknown  | Dr.11355.1.A1_at   | BG308730                 | -15.5       |
| Werner helicase interacting protein 1                  | Dr.16690.1.A1_at   | BI980401                 | -13.5       |
| Neurofilament protein M                                | Dr.19653.1.S1_at   | AL919725                 | -12.3       |
| Unknown  | Dr.3789.1.A1_at    | BI890279                 | -9.8        |
| Similar to Six3/Six6/Optx2.                            | Dr.26486.1.S1_at   | BC053123.1               | -9.1        |
| Similar to F-actin capping protein                     | Dr.10440.1.A1_at   | BE605864                 | -8.0        |
| Heparin binding EGF-like growth factor protein alpha-1 | Dr.16053.1.S1_at   | BM531631                 | -7.6        |
| Topoisomerase I  | Dr.7467.1.S1_at    | BC044159.1               | -7.5        |
| c-Fos interacting transcription Factor 2               | Dr.2401.1.A1_at    | CD604147                 | -7.0        |
| Bobby sox homolog                                      | Dr.22157.1.A1_at   | AW019059                 | -7.0        |
| Cytokine inducible SH2-containing protein              | Dr.9911.1.A1_at    | AW826769                 | -6.7        |
| egl-9 homolog 3  | Dr.9457.1.A1_at    | BI671269                 | -6.2        |
| Unknown  | Dr.25949.1.A1_at   | CD605589                 | -6.3        |
| Gamma M2c crystallin                                   | Dr.20815.2.S1_at   | BQ783659                 | -6.2        |
| Protocadherin A  | Dr.21025.1.A1_at   | AF043903.1               | -6.1        |
| Unknown  | Dr.12482.1.S1_x_at | BM036408                 | -6.0        |
| Bromodomain-containing protein 8                       | Dr.11534.2.A1_at   | AL729194                 | -6.0        |
| APTase type 13A  | Dr.11388.1.S1_at   | BI889248                 | -5.9        |
| Unknown  | Dr.16478.1.A1_at   | BI705297                 | -5.9        |
| Similar to F-box leucine rich repeat protein 2         | Dr.7543.1.A1_at    | AW019494                 | -5.9        |
| Stomal cell derived factor receptor I isoform a/3      | Dr.10103.1.A1_at   | CB890995                 | -5.9        |
| Unknown  | Dr.11073.1.A1_at   | AI588427                 | -5.8        |
| Clathrin H chain                                       | Dr.26329.1.A1_at   | BG728815                 | -5.8        |
| Unknown  | Dr.16610.1.S1_at   | BI983598                 | -5.5        |
| ATPase V1-D  | Dr.14072.1.S1_at   | BC045370.1               | -5.4        |
| Unknown from retina cDNA                               | Dr.12806.1.A1_at   | BI879720                 | -5.3        |
| RAD54-like DNA repair protein                          | Dr.5408.1.S1_at    | BC046050.1               | -5.1        |
| Unknown  | Dr.2197.1.A1_at    | AI721729                 | -4.9        |
| Similar to RAD 21 homolog                              | Dr.15478.1.A1_at   | BM316135                 | -4.8        |
| Putative XXC 177 protein                               | Dr.19902.2.A1_x_at | AW116196                 | -4.8        |
| ATP binding cassette transporter G-2a                  | Dr.22153.1.A1_at   | AW019053                 | -4.7        |
| cGMP-gated cation channel rod photoreceptor            | Dr.4773.1.A1_at    | AL715316                 | -4.7        |

(continued next page)

|  |                    |             | (continued) |
|--|--------------------|-------------|-------------|
| Hepatic transcription factor 2                 | Dr.14662.1.S2_at   | NM_131880.1 | -4.6        |
| Polyhomeotic pH 1 homolog                      | Dr.19425.1.S1_at   | AB064939.1  | -4.5        |
| Signal recognition particle receptor B         | Dr.9914.1.S1_at    | BM181827    | -4.3        |
| Jun B proto-oncogene                           | Dr.10326.1.S1_at   | BC053234.1  | -4.3        |
| Unknown  | Dr.1002.1.S1_at    | BM858769    | -4.2        |
| Unknown  | Dr.14191.1.A1_at   | BQ109774    | -4.2        |
| Unknown  | Dr.22021.1.A1_at   | AI957759    | -4.1        |
| Geranyl diphosphatesynthetase 1                | Dr.25151.1.S1_at   | BC052115.1  | -4.0        |
| F-box leucine rich repeat protein              | Dr.16658.1.S1_at   | BF937803    | -4.0        |
| Unknown  | Dr.12300.1.A1_at   | BG728602    | -3.9        |
| Similar to Hsp90 $\alpha$                      | Dr.25536.1.A1_at   | BG306502    | -3.8        |
| MHC class II receptor activity                 | Dr.9662.1.S1_at    | BQ261450    | -3.8        |
| Unknown  | Dr.14840.1.A1_at   | BM071859    | -3.8        |
| Unknown  | Dr.18540.3.A1_at   | BI883285    | -3.8        |
| Hydroxymethylbilanesynthetase                  | Dr.3338.1.S1_at    | BC053268.1  | -3.6        |
| Unknown  | Dr.26420.1.A1_at   | CD606196    | -3.5        |
| Adducin 3                                      | Dr.10904.1.S1_a_at | BC050486.1  | -3.4        |
| Similar to SRR1-like protein                   | Dr.16362.1.A1_at   | BI430090    | -3.4        |
| Similar to acetyl-coenzyme A dehydrogenase VL  | Dr.3523.1.A1_at    | AI964241    | -3.3        |
| Similar to protein tyrosine phosphatase CRYP-2 | Dr.9411.1.A1_at    | AL909228    | -3.3        |
| Synaptophysin-like protein                     | Dr.25595.1.A1_at   | BM958030    | -3.2        |
| Similar to phosphodiesterase 4B                | Dr.16127.1.S1_at   | BI887495    | -3.2        |
| Zinc finger protein subfamily 1A5              | Dr.25707.1.A1_at   | CD605002    | -3.1        |

The upper list shows probe sets predicted to be upregulated in cavefish (positive values). The lower list shows probe sets predicted to be downregulated in cavefish (negative values). Entries are listed in descending order by absolute value fold change (FC) with the first entry representing the probe set with the highest positive FC and the last entry representing the probe set with the highest negative FC. EGF, epidermal growth factor.

Indeed, 61 out of 67 genes (91%) are downregulated in 3-dpf cavefish relative to surface fish embryos. Together, these results suggest that microarray analysis using a DNA chip from the closely related zebrafish is useful for discovering genes that are differentially expressed during cavefish evolution and development.

We next describe further studies on two of the genes predicted to be downregulated in cavefish that confirm the microarray analysis. Then, in the succeeding paragraphs, we discuss some of the most important genes identified in the microarray analysis and their potential roles in cavefish.

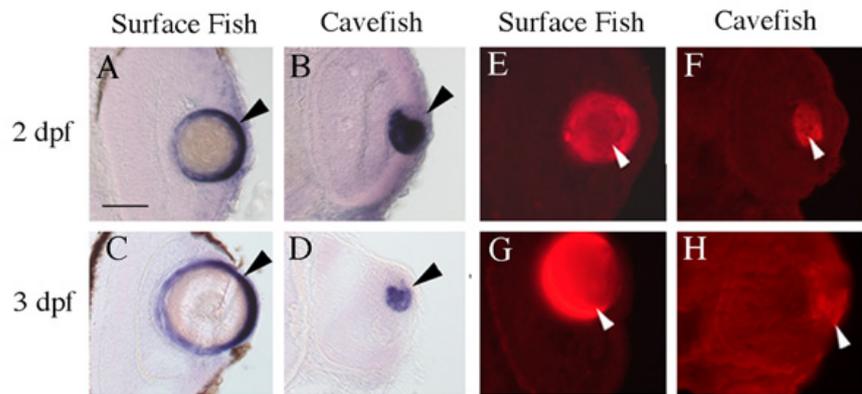
### **Gamma crystallin and hsp90 $\alpha$ downregulation in the cavefish lens**

According to microarray analysis, three different gamma crystallin genes may be downregulated in cavefish. Confirmation of this result was sought by investigating mRNA levels by *in situ* hybridization and protein

accumulation by immunostaining. As expected, the cloned *gamma-M crystallin* gene and gamma crystallin protein are expressed only in the *Astyanax* lens (Fig. 2). *Gamma crystallin* transcripts are prevalent in the epithelial layer of the 2-dpf and 3-dpf surface fish lens (Fig. 2A, C). Although transcripts are also detectable in cavefish at these developmental stages, they are confined to a small area within the lens core, which may contain undifferentiated lens fiber cells (Fig. 2B, D). By 3dpf, the lens core area in which *gamma crystallin-M* mRNA is expressed has become even smaller, this explaining its downregulation. Immunostaining with a gamma crystallin antibody also shows lower amounts of protein accumulation in the cavefish lens, particularly by 3 dpf (Fig. E–H). These results support the microarray analysis in showing that a *gamma-M crystallin* gene is differentially expressed in cavefish.

Our data, in conjunction with other studies (Behrens *et al.* 1998; Strickler *et al.* 2007b), indicates that at least three ( $\alpha$ -crystallin,  $\beta$ -crystallin, and *gamma-M* crys-

**Figure 2** Gamma crystallin mRNA and protein expression. Surface fish. (A, C, E, G) Cavefish (B, D, F, H). Sections showing *gamma-M* crystallin mRNA expression in the surface fish and cavefish lens determined by whole mount *in situ* hybridization at 2 (A, B) and 3-days post-fertilization (dpf) (C, D). (E–H) Sections showing gamma crystallin protein expression in the surface fish and cavefish lens (arrowheads) determined by antibody staining at 2 (E, F) and 3 dpf (G, H). Scale bar in A is 50  $\mu$ m; magnification is the same in each frame. A–D after Strickler *et al.* (2007b).



tallin) and possibly as many as six (including the three gamma crystallin genes predicted to be downregulated by microarray analysis) crystallin genes are downregulated during cavefish lens development. Therefore, there might be widespread downregulation of lens structural proteins during cavefish eye primordium development. This downregulation is interesting in light of the case of *hsp90 $\alpha$* , a gene previously shown to be upregulated in the cavefish lens (Hooven *et al.* 2004), and provides further support that the latter gene may be important in eye degeneration.

The *hsp90 $\alpha$*  gene was predicted by microarray to be downregulated rather than upregulated (Table 1), as would have been expected from previous results (Hooven *et al.* 2004). Perhaps the peak of *hsp90 $\alpha$*  expression in the lens, which occurs at approximately 1.5–2 dpf (Hooven *et al.* 2004), has subsided by 3 dpf. Alternatively, because this gene is expressed in many tissues under non-stress conditions (Sass *et al.* 1996), including most of the developing musculature, its overall large signal in microarray analysis might mask smaller changes in the lens.

### Rhodopsin downregulation

Microarray analysis predicted that the *rhodopsin* gene, which encodes a key visual pigment in the photoreceptor layer of the retina, is downregulated in cavefish. The microarray results were confirmed by *in situ* hybridization. At 3 dpf, an appreciable number of *rhodopsin*

mRNA expressing cells were observed in the surface fish photoreceptor layer (Fig. 3A). In contrast, only a few photoreceptor cells containing *rhodopsin* mRNA were detected in 3 dpf cavefish (Fig. 3B). At 10 dpf, an uninterrupted layer of *rhodopsin* mRNA-expressing cells was observed in the photoreceptor layer of the surface fish retina (Fig. 3C). Only a few cells containing *rhodopsin* mRNA could be found in the cavefish retina at this stage (Fig. 3D). Immunostaining studies provided similar results to those obtained for mRNA distribution: rhodopsin was present in many cells distributed throughout the photoreceptor layer in surface fish (Fig. 3E), whereas it was restricted to just a few widely spaced clusters of cells in the cavefish retina (Fig. 3F). The results show that rhodopsin downregulation can be explained by much fewer numbers of rod cells in the cavefish retina.

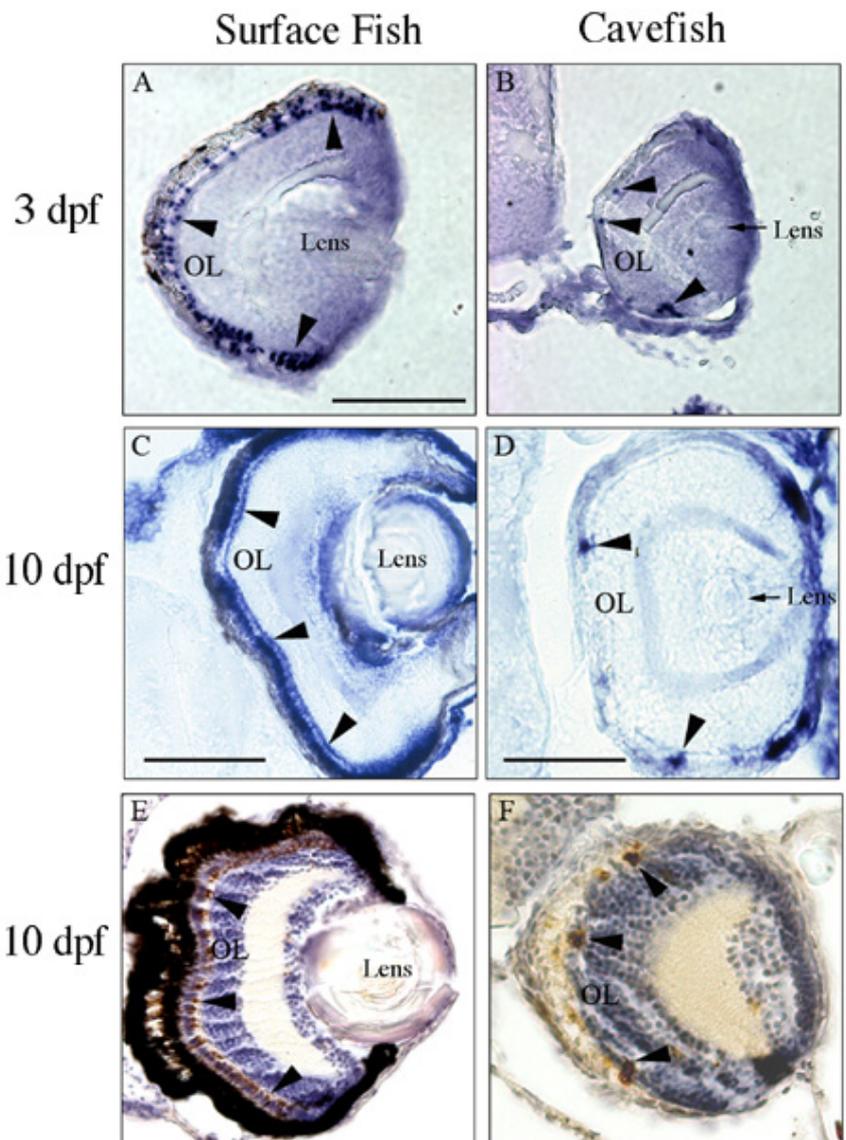
### Eye development and maintenance genes

Many additional genes predicted by microarray analysis to be downregulated in cavefish are known to function in eye development and/or maintenance. Several of the genes with high-fold downregulation in cavefish are involved in photoreceptor function (Table 1). The *gnat1* and *gnat2* genes that encode guanine nucleotide binding proteins comprise the transducing subunit of the rod and cone G-proteins, respectively (Ray *et al.* 1997; Shen & Raymond 2004) and a differentially expressed gene encodes a protein similar to the receptor cGMP-gated ca-

tion channel in rod cells. The gene encoding protocadherin was also identified as downregulated in cavefish. Protocadherin is necessary for the intercalation of photoreceptor cells with the retinal pigment epithelium (Seiler *et al.* 2005). The genes encoding BarH and Six3/Six6/Optx2 homeodomain transcription factors were recognized as downregulated in cavefish by microarray analysis. In the developing mammalian eye, *BarH* is expressed in a subset of retinal cells including photoreceptors (Saito *et al.* 1998). The *six3*, *six6* and *optx* genes, members of the Six3 gene family, have been im-

plicated in lens development, retina development, or both processes (Lopez-Rios *et al.* 1999). It is possible that one or more of these genes might be a cause or consequence of eye degeneration.

Two other downregulated genes are of interest with regard to eye development, protein tyrosine phosphatase (CRYP-2) and the middle molecular weight neurofilament gene (NF-M), which function in axon outgrowth and guidance (Stepanek *et al.* 2001). NF-M expression has been correlated with projection length and neural arborization (Zopf *et al.* 1990).



**Figure 3** Rhodopsin mRNA and protein expression. (A, C, E) Surface fish. (B,D,F) Cavefish. (A–D) Sections are shown of 3- (A, B) and 10- (C, D) days post-fertilization (dpf) larvae in which *rhodopsin* mRNA was determined by whole mount *in situ* hybridization. (E, F) Sections of 10-dpf larvae in which rhodopsin protein was determined by antibody staining. Arrowheads indicate cells stained for rhodopsin mRNA (A–D) or protein (E,F). OL, retinal outer nuclear (photoreceptor) layer. Scale bar in (A): 100µm; magnification is the same in (A) and (B). Scale bar in (C): 200µm; magnification is the same in (C) and (E). Scale bar in (D): 100µm; magnification is the same in (E) and (F).

There are significantly fewer axon bundles in the cavefish optic nerve (Soares *et al.* 2004). Therefore, these genes may be downregulated in cavefish as a cause of or a response to the existence of fewer retinal-tectal projections.

### Programmed cell death genes

Microarray analysis resulted in the identification of many differentially expressed genes involved in programmed cell death. Of the genes with highest FC, five are downregulated, and one (ubiquitin specific protease 53) is upregulated. Because of massive apoptosis in the lens (Jeffery and Martasian 1998), it seems that programmed cell death genes would be downregulated rather than upregulated in cavefish. However, genes have been discovered that promote as well as inhibit programmed cell death (St. Clair *et al.* 1997). Accordingly, the pro-apoptotic genes are expected to be upregulated, whereas anti-apoptotic genes may be downregulated in cavefish. One of the latter,  $\alpha A$ -crystallin, has already been identified by other means (Strickler *et al.* 2007b), although it was not highlighted in the microarray analysis.

The five genes related to apoptosis that microarray analysis predict to be downregulated in cavefish are: *RAD21*, *phosphodiesterase 4B*, *HB-EGF*, *Egl-9/SM-20* and *Rad54*. *RAD21* was the highest-fold downregulated of all genes linked with programmed cell death. The precise role of the *RAD21* gene in apoptosis is unclear, but it appears to be involved in DNA stabilization and DNA strand break repair, which are both required for normal cell maintenance as well as programmed cell death (Pati *et al.* 2002). The gene encoding phosphodiesterase 4B was also predicted to be downregulated in cavefish. Suppression of this gene has been linked to growth inhibition and apoptosis (Ogawa *et al.* 2002). Cells with more phosphodiesterase might be resistant to programmed cell death, and conversely a decrease in expression of this gene might lead to apoptosis in cavefish. A gene encoding heparin-binding epidermal growth factor (*HB-EGF*) also showed appreciable downregulation. This gene has been shown to prevent apoptosis (Zhang *et al.* 2004). *HB-EGF* can be activated by reactive oxygen species as a result of oxidative stress (Kim *et al.* 2005), an event leading to programmed cell death. Furthermore, *HB-EGF* is important in nervous system development, including cell migration, survival and differentiation (Xian & Zhou 1999). Presumptive neural cells of the cavefish retina die by programmed cell death

(Strickler *et al.* 2007a), and downregulation of *HB-EGF* could be important in this process. *Egl-9* is a homolog of the vertebrate *SM-20* gene, which has been implicated in caspase-dependent cell death (Straub *et al.* 2003). *Rad54* is involved in DNA stabilization (Wesoly *et al.* 2006). DNA damage and fragmentation can be a direct cause, as well as an effect, of apoptosis (Belyaev 2005; Janssens *et al.* 2005). It is possible that *RAD54* is instrumental in controlling DNA fragmentation during cavefish apoptosis. Perhaps cavefish have a natural propensity for undergoing programmed cell death as a result of stress encountered in the cave environment.

The gene showing the highest FC of any probe set in this study codes for ubiquitin specific protease 53 (Table 1). Ubiquitin tags proteins for degradation (Li *et al.* 2002), and protein degradation by the ubiquitin-mediated proteasome is the major form of eukaryotic proteolysis. One of the functions of the ubiquitin proteasome system is the breakdown of proteins that have already been cleaved by caspases during apoptosis (Melino 2005). Therefore, genes that function in this system could be upregulated during massive programmed cell death in the cavefish lens.

### CONCLUSIONS

Several conclusions can be drawn from the present study. First, cross-species microarray analysis using a zebrafish gene chip and *Astyanax* RNA can be used to discover differentially expressed genes during cavefish development. Second, based on microarray, *in situ* hybridization and immunological analysis, the expression of gamma-M crystallin and rhodopsin are strongly downregulated in the cavefish lens and photoreceptor layers of the retina, respectively. These results are consistent with apoptotic cell death of lens pre-fiber cells and the inhibition of photoreceptor cell differentiation in the cavefish retina. Third, the microarray results suggest that the vast majority of differentially expressed genes are downregulated in cavefish. This is in contrast to what has been described for the few differentially expressed genes identified by other means, such as *in situ* hybridization and quantitative PCR, which are upregulated in cavefish (Hooven *et al.* 2004; Yamamoto *et al.* 2004; Jeffery 2005). Fourth, a large proportion of the downregulated genes have roles in eye development and/or in programmed cell death. Finally, the present study has provided a large number of candidate genes that can be tested for possible roles in eye degeneration. Although many of these candidates may not be the mutated genes

themselves, they could be part of the downstream pathways affected by these genes and, therefore, provide insights into their identity.

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