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αA-crystallin expression prevents γ-crystallin insolubility and cataract formation in the zebrafish *cloche* mutant lens

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Cataracts, the loss of lens transparency, are the leading cause of human blindness. The zebrafish embryo, with its transparency and relatively large eyes, is an excellent model for studying ocular disease in vivo. We found that the zebrafish *cloche* mutant, both the *cloche*^{m39} and *cloche*^{s5} alleles, which have defects in hematopoiesis and blood vessel development, also have lens cataracts. Quantitative examination of the living zebrafish lens by confocal microscopy showed significant increases in lens reflectance. Histological analysis revealed retention of lens fiber cell nuclei owing to impeded terminal differentiation. Proteomics identified γ-crystallin as a protein that was substantially diminished in *cloche* mutants. Crystallins are the major structural proteins in mouse, human and zebrafish lens. Defects in crystallins have previously been shown in mice and humans to contribute to cataracts. The loss of γ-crystallin protein in *cloche* was not due to lowered mRNA levels but rather to γ-crystallin protein insolubility. αA-crystallin is a chaperone that protects proteins from misfolding and becoming insoluble. The *cloche* lens is deficient in both αA-crystallin mRNA and protein during development from 2-5 dpf. Overexpression of exogenous *αA-crystallin* rescued the *cloche* lens phenotype, including solubilization of γ-crystallin, increased lens transparency and induction of lens fiber cell differentiation. Taken together, these results indicate that *αA-crystallin* expression is required for normal lens development and demonstrate that cataract formation can be prevented in vivo. In addition, these results show that proteomics is a valuable tool for detecting protein alterations in zebrafish.

KEY WORDS: Cataract, Crystallin, Chaperone, *Cloche*, Eye, Lens, Zebrafish, *cryaa*

INTRODUCTION

Cataracts, the loss of eye lens transparency, are the leading cause of blindness in humans (Resnikoff et al., 2004), with about 17 million cases worldwide per year (Foster and Resnikoff, 2005). Understanding the molecular mechanisms responsible for cataract formation could be useful for designing treatments other than surgical intervention, which is presently the only available recourse. Genetic studies in human and rodents have implicated crystallin defects as major contributory causes of cataract formation (Graw, 2004). Crystallins are a large family of proteins that constitute 90% of the soluble protein in lens fiber cells (Bloemendal et al., 2004; Graw and Loster, 2003). There are three classes of these proteins, including α-, β- and γ-crystallins. Each crystallin is expressed in the lens with spatial and temporal expression patterns characteristic for each class. Mutations in human *γC-crystallin* (*CRYGC*) or *γD-crystallin* (*CRYGD*) are associated with protein misfolding and protein aggregation within the lens (Graw, 2004). Whereas β- and γ-crystallins are structural proteins, αA-crystallin is a member of the small heat shock protein family and is also known as HSPB4 (Franck et al., 2004). αA-crystallin acts as a molecular chaperone that protects proteins from misfolding (Horwitz, 2003). Defects in this gene are associated with autosomal dominant congenital cataracts (Litt et al., 1998).

Zebrafish embryos are excellent models for studying the genes that regulate embryonic development (Talbot and Hopkins, 2000; Thisse and Zon, 2002). Zebrafish embryos are small, can be maintained on a large scale and develop rapidly within days. Unlike mice, the embryos are extracorporeal and transparent, allowing ready visualization of organs during development. The transparency and relatively large eyes make zebrafish embryos a particularly excellent model for studying eye development and ocular diseases (Glass and Dahm, 2004; Malicki, 2000). Functional vision is developed by 3 days post-fertilization (dpf) (Easter and Nicola, 1996). Chemical mutagenesis screening has identified mutants with an opaque lens, e.g. *lop* (Vihtelic et al., 2005b). However, the cause of the opacity has not been established.

Forward genetics have been used to identify zebrafish genes involved in normal development and genetic disorders, and to elucidate their function. For example the *cloche* mutant lacks blood cells and blood vessels (Qian et al., 2005; Stainier et al., 1995; Sumanas et al., 2005; Weber et al., 2005). However, a limitation of a strictly genetic approach is the inability to study protein function. Proteomics is an alternative approach that identifies proteins, measures their abundance, detects post-translational modifications, and provides sequence data for cloning purposes. To date there have been very few attempts to analyze zebrafish protein profiles by proteomics. We chose to study protein defects in *cloche* as it is not clear what the genetic defect is. Surprisingly, we noted that *cloche* not only had defects in hematopoiesis and vasculogenesis, but also had lens opacity, a characteristic of cataracts.

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We report here that γ-crystallin protein is insoluble in *cloche* lens, resulting in lens opacity. Furthermore, the *cloche* lens is deficient in αA-crystallin, a chaperone protein that keeps proteins soluble. Overexpression of exogenous *αA-crystallin* (*cryaa* – Zebrafish Information Network) rescues to a great degree the *cloche* lens phenotype; for example, γ-crystallin protein becomes more soluble, the lens becomes more transparent in vivo and lens fiber cell differentiation is induced. It was concluded that αA-crystallin expression is required for normal lens development and that these results are significant as they show, possibly for the first time, that cataract formation can be prevented in vivo.

MATERIALS AND METHODS

Materials

Anti-newt γ-crystallin guinea pig antiserum and anti-mouse αA-crystallin rabbit antiserum were kindly obtained from Dr M. Okamoto (Nagoya, Japan) and from Dr J. J. Liang (Harvard Medical School, Boston, MA). Horseradish peroxidase (HRP)-conjugated anti-guinea pig antibody was purchased from Jackson ImmunoResearch Laboratories. HRP-conjugated anti-mouse antibody was purchased from Amersham Biosciences UK.

Zebrafish maintenance

All animal protocols were approved by the Children's Hospital Institutional Animal Care and Use Committee. Zebrafish were maintained as described (Westerfield, 2000), and staged as described (Kimmel et al., 1995). Two independent *cloche* alleles, *cloche*^{m39} and *cloche*^{s5}, were used in this study. Embryo age is defined as the number of days post-fertilization (dpf). Euthanasia of zebrafish was accomplished by treatment with tricaine (Sigma-Aldrich) before protein or RNA extraction. The homozygotes were separated based on visual criteria such as cardiac edema, enlarged hearts and lack of blood cell circulation.

Light and confocal microscopy

Living embryos in embryo media including tricaine were analyzed by stereomicroscopy (Olympus). For confocal microscopic analysis, embryos were embedded in 3% methyl cellulose in embryo media including tricaine. Stacks of confocal micrographs were recorded from anesthetized zebrafish lens using a Leica TCS SP2 (Leica Microsystems) confocal laser-scanning microscope fitted to a DM IRE 2 inverted microscope with a 488 nm argon ion laser. Serial sections were obtained at 1 μm intervals under the reflected light mode. Images recorded under transmitted light were used as a general locator for the quantitative analysis of selected reflected light images. Reflected light was analyzed by the digital image analysis program (Leica Confocal Software and ImageJ).

Protein extraction

Approximately 100 zebrafish embryos were transferred into 1.5 ml microcentrifuge tubes. Centrifugation at 15,000 ***g*** for 5 minutes resulted in two phases. The lower phase contained the yolk, whereas the upper phase contained zebrafish without yolk. The bottom of the tube was pierced with a 26 G needle to remove the bottom yolk layer. To obtain total embryo extracts containing soluble protein and cell membrane-associated proteins, embryos were washed with ice-cold PBS three times and lysed in 8 M urea, 4% CHAPS and 60 mM dithiothreitol by sonication for 20 seconds on ice, and the lysates were left on ice for an additional 30 minutes. To avoid protein degradation, proteinase inhibitor (Roche) and phosphatase inhibitor 1 and 2 (Sigma) were added to embryo lysates. Precipitates were removed by centrifugation at 15,000 ***g*** for 20 minutes at 4°C. To prepare soluble and insoluble protein, zebrafish embryos (2.5 dpf) were homogenized in detergent-free 20 mM Tris-HCl (pH 7.5). Soluble and insoluble fractions were separated by centrifugation.

Two dimensional gel electrophoresis

Total embryo extracts were analyzed by two-dimensional gel electrophoresis. A BioRad protein IEF cell was used for the first dimension. Embryo extracts (80 μg) were applied to immobilized pH gradient gel strips, 11 cm, linear (pH 3-10). IEF was carried out at 20°C for 2 hours at 250 V (linear ramp), and for 5.5 hours at 8000 V (linear ramp). The second dimension was a 10-20% gradient SDS-PAGE for 1.5 hours at 150 V. Proteins were visualized by SYPRO Ruby fluorescent staining (BioRad). The gels were photographed and the intensities of gel spots were analyzed by a digital image analysis program (ImageJ). The intensities of spots were normalized relative to wild-type samples being 100%. Gel spots were excised using a spot cutter (The Proteome Works, BioRad). Obtaining sufficient amounts of protein for sequencing (20 pmol) required excision from two or three spots. Samples were analyzed by electrospray tandem mass spectrometry. These spectra were correlated with known protein sequences using the Sequest algorithm and other programs developed in the Harvard Microchemistry Lab (Chittum et al., 1998; Eng et al., 1994).

Western blots

Total embryo extracts, or extracts fractionated into soluble and insoluble protein, were analyzed by western blot. Two μg of embryo extracts were analyzed by 12% SDS-PAGE. The membranes were incubated with anti-mouse αA-crystallin antisera (1/2000 dilution) or anti-newt γ-crystallin antisera (1:2000 dilution) followed by HRP-conjugated anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, 1:5000) or anti-rabbit IgG (Amersham Pharmacia Biotech; 1: 10,000), and visualization with enhanced chemiluminescence (Perkin Elmer Life Science). The specificity of each antibody was confirmed by western blotting of lysates prepared by transiently transfecting Chinese Hamster Ovarian cells (American Type Culture Collection) with expression constructs of zebrafish *αA-* (Runkle et al., 2002) (GenBank Accession Number, NM 152950), *αB1-* (Posner et al., 1999; Smith et al., 2006) (GenBank Accession Number, NM 131157), *βB1-* (Chen et al., 2001) (GenBank Accession Number, NM 173231) and *γ-crystallin* genes. Cell maintenance and gene transfection procedures were as described previously (Goishi et al., 2003). Protein bands were analyzed by the digital image analysis program (ImageJ). Results were normalized by setting the densitometer of the wild-type samples as 100%.

Cloning of crystallin (Cry) genes

Sixteen peptide sequences from tandem mass spectrometry were analyzed in a BLAST search. Twelve of the peptide sequences were completely identical to several EST clones. Oligonucleotide primers for PCR were generated based on matched EST clone sequences. Using the SMART-RACE cDNA Amplification Kit (BD bioscience), 5′ and 3′ zebrafish *γ-crystallin* cDNA ends were obtained by RT-PCR from 48 hpf embryo total RNA.

Sense primers for 3′RACE are follows: CryG-S1, 5′-GGCGACTGTG-GTGACTTCTCCTCCTACA-3′; CryG-S2, 5′-CATGAAGGTCACCTTC-TTTGAGGA-3′; CryG-S3, 5′-GCAAACCATGATGGGCAAAGGTC-3′; CryG-S4, 5′-AGTAACCATGATGGGCAAGGTCAT-3′; CryG-S5, 5′-CGCAACCATGATGGGCAAGGTCGTT-3′, CryG-S6, 5′-CGCAAACAT-GATGGGCAAGGTCGCC-3′; CryG-orf1-s, 5′-AACATGAAGGTCACC-TTCTTTGAGGACA-3′.

Antisense primers for 5′RACE are follows: CryG-As1, 5′-CCGAA-GTGCCACATCCTGCCTCTGTAGT-3′; CryG-As2, 5′-GTACCAAGA-GTCCATGATACGCCTCA-3′; CryG-orf1-AS, 5′-CTAGTACCAAGAGT-CCATGATACGCCTCA-3′.

PCR products for *γ-crystallin* genes were subcloned into the pCRII/TOPO vector (Invitrogen). *γ-crystallin* open reading frames were generated by RT-PCR. PCR products were subcloned into the pCRII/TOPO vector and the expression vector pcDNA3.1/V5-His/TOPO (Invitrogen). An EST database BLAST search with mammalian αB-crystallin protein sequences revealed a second zebrafish *αB-crystallin* gene that was cloned by PCR. The second *αB-crystallin* gene was amplified using the following primers: CRY-ABb-S, 5′-TTGCAGAAGAGGCCCAGACTCA-3′; CRY-ABb-As-stop, 5′-CACAAACATTTGGCCGTCAGTAGG-3′.

Whole-mount in situ hybridization and histology

Whole-mount in situ hybridization was performed as previously described (Bovenkamp et al., 2004; Lee et al., 2002). Briefly, crystallin cDNAs were used as a template to synthesize digoxigenin-labeled sense and antisense riboprobes. Embryos were fixed in 4% paraformaldehyde overnight and then permeabilized by proteinase K treatment prior to hybridization. After color was detected with alkaline phosphatase

substrates, the embryos were mounted in glycerol and photographed. Paraformaldehyde fixed, paraffin-embedded embryos were used for histology (Hematoxylin and Eosin).

RT-PCR

Two μg of total RNA from 2 dpf, 3 dpf and 4 dpf zebrafish were isolated and reverse transcribed by using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's suggestions. Zebrafish *elongation factor 1α* (*ef1a*; GenBank Accession Number, NM 131263) was used as a loading control.

Each sample was amplified with the following primers. *αA-crystallin*: CRYAA-S, 5′-TATGGATATTGCGATCCAACACCCTT-3′; CRYAA-AS, 5′-AGAGCCTGAGTTGCTCTTGTCCTCG-3′. *αB1-crystallin*: CRYAB-S, 5′-TGTCAGCTCCAAACTAAGGTACAATG-3′; CRYAB-AS, 5′-TGCAGCTCTGGACAGGTTCAT-3′. *βB1-crystallin*: CRY-BB1-S, 5′-CACCATGTCTCAGACCGCCAAA-3′; CRY-BB1-As, 5′-CTGTTTAGCGGCGGTCATGTTGA-3′. *αBb-crystallin* (GenBank Accession Number AY939876): CRY-ABb-S; CRY-ABb-As, 5′-CTTCTGAGGGCCAGCGACGGT-3′. *γ-crystallin*: Cry-G-S1 and Cry-G-As1. *flk1* (GenBank Accession Number, NM 131472): Flk1-1098S, 5′-TGCCATCGAACCAGAAAGACCAAGAG-3′; Flk1-1299As, 5′-TGCCCTTGTCCTCCATTGTCACATTA-3′. *ef1a*: Ef1-S, 5′-GCCCCTGCCAATGTA-3′; Ef1-As, 5′-GGGCTTGCCAGGGAC-3′.

Microinjection

Zebrafish *αA-* and *γ-crystallin* cDNA were subcloned into pXT7, which was obtained from Dr S. Sokol (Beth Israel, Boston). To generate capped mRNA, plasmids were linearized by *Sal*I and transcribed with T7 RNA polymerase using the mMESSAGE mMACHINE (Ambion) according to the manufacturer's instructions. For microinjection of RNA into zebrafish, 100 pg of synthesized mRNA were injected into the one- to four-cell stage embryos obtained from crosses between *cloche* heterozygotes. The *αA-crystallin* promoter-EGFP (Kurita et al., 2003) was injected (100 pg) into one- to four-cell stage embryos. The injected embryos were examined under a fluorescence microscope (Olympus).

Statistical analysis

The Mann-Whitney *U* test was used to test for differences between two groups. Accepted level of significance for all tests was $P<0.05$. Box-and-whisker plots were generated to show the levels of reflectance intensity. The line in the box corresponds to the median value. The lower and upper whiskers correspond to 10th and 90th percentiles, respectively.

Photoreceptor staining

Embryos were fixed in 4% paraformaldehyde/PBS (pH 7.4) for 2 hours at room temperature, incubated in 30% sucrose/PBS overnight, and embedded in Neg50 frozen section medium (Richard-Allen Scientific). Sections of 10 μm section were prepared, rehydrated and blocked in 0.1% Tween20, 10% goat serum and 0.5% TritonX-100 in PBS. Immunohistochemistry was carried out with an antibody zpr-1 that detects zebrafish photoreceptors, a gift from Dr J. Malicki (Massachusetts Eye and Ear Infirmary, Boston) (Pujic and Malicki, 2001). Analysis was carried out by confocal microscopy.

RESULTS

Two-dimensional gel electrophoresis reveals diminished crystallin levels in *cloche* embryos

cloche mutant and wild-type zebrafish embryos extracts were compared by proteomics to identify possible altered protein profiles that might reveal clues about the mechanisms contributing to the *cloche* phenotype. Two dimensional (2D) gel analysis of total extracts, as described in Material and methods, showed consistently that one spot (20 kDa, pI of 8.8) was strongly reduced by about 75% in *cloche* compared with wild-type embryos (Fig. 1A). Tandem mass

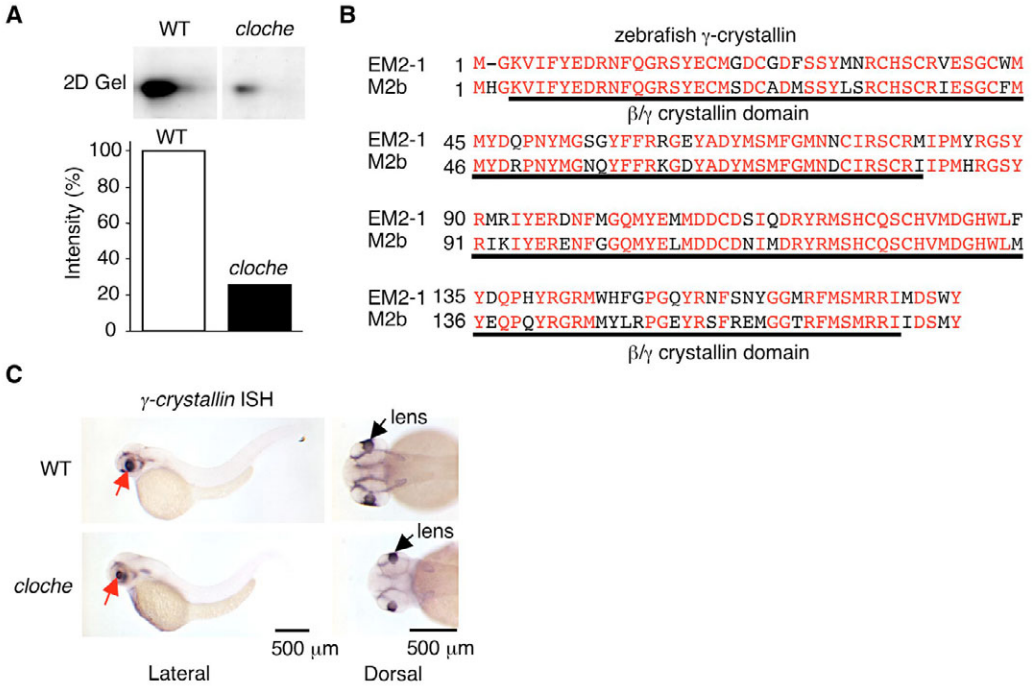

Fig. 1. Crystallin proteins levels are diminished in *cloche* embryos. (**A**) 2D-gel electrophoresis: Total 2.5 dpf extracts as described in the Materials and methods were analyzed. A spot (20 kDa, pI of 8.8) was identified that was strongly reduced in *cloche* compared with wild-type (WT) embryos. Spot intensities were analyzed by an image analysis program (ImageJ) and the values were normalized to wild type (100%). (**B**) The spot was cut out of the gel and identified by MS/MS spectrometry sequencing to be a member of the γ-crystallin family. This protein, designated as embryonic γ-crystallin M2 type1 (EM2-1) was 78% homologous to a recently described zebrafish γ-crystallin M2b (M2b) that had been cloned from adult lens tissue (Wistow et al., 2005). The two sequences were aligned. Identical amino acids are in red. The Reverse Position Specific BLAST program showed two β/γ-crystallin domains that are underlined in black. (**C**) *γ-crystallin* gene expression was analyzed by in situ hybridization at 2.5 dpf. Both lateral and dorsal views are shown. *γ-crystallin* is expressed solely in the lens.

spectrometry (MS/MS) and subsequent gene cloning identified this altered zebrafish embryonic protein to be γ-crystallin (Fig. 1B). Eight different γ-crystallin cDNAs were isolated from 2 dpf embryos and the encoded proteins were 95-100% identical at the amino acid level (GenBank Accession Numbers: DQ294939, DQ294940, DQ288267, DQ294941, DQ294942, DQ294943, DQ298448, DQ298449). One of these cDNAs was designated as embryonic zebrafish *γ-crystallin M2 type1* (*γEM2-1-crystallin*, GenBank Accession Number, DQ294939) (Fig. 1B, top, EM2-1). This zebrafish cDNA encodes a protein of 174 putative amino acids with two β/γ-crystallin domains, the same as in other species. It is 78% homologous in amino acid sequence to a very recently described zebrafish *γ-crystallin M2* gene (*crygm2b*; GenBank Accession Number, NM001020783) that had been cloned from adult lens tissue (Vihtelic et al., 2005a; Wistow et al., 2005) (Fig. 1B, bottom, M2b). However, our crystallin gene appears to be embryonic and was found only in embryonic cDNA databases. We also isolated a second *αB-crystallin* gene, which we called *αBb-crystallin* (*cryabb*; GenBank Accession Number, AY939876). This gene is identical to *αB2-cystallin* (GenBank Accession Number; DQ113417) (Smith et al., 2006) and similar to a previously reported *αB1-crystallin* (GenBank Accession Number, NM 131157) (Posner et al., 1999).

Crystallins are expressed mostly in lens and constitute 80-90% of lens protein (Bloemendal et al., 2004; Graw and Loster, 2003). The spatiotemporal expression pattern of *γ-crystallin* was analyzed by whole-mount in situ hybridization using our cloned zebrafish *γ-crystallin EM2-1* cDNA. At 2 dpf, *γ-crystallin* was expressed entirely in the lens (Fig. 1C). However, unlike the protein, there was no significant difference between *cloche* and wild-type embryo *γ-crystallin* gene expression.

αA-crystallin gene expression is down-regulated in *cloche*

There are several classes of crystallins, including *α*-, *β*- and *γ-crystallin*, and each class has several different genes (Chen et al., 2001; Posner et al., 1999; Runkle et al., 2002; Vihtelic et al., 2005a; Wistow et al., 2005; Smith et al., 2006). The expression levels of *αA*-, *αBb*-, *βB1*- and *γ-crystallin* were analyzed by semi-quantitative RT-PCR (Fig. 2A). This analysis showed that *αA-crystallin* gene expression was strongly downregulated in *cloche*, whereas gene expression of the other crystallins was not altered. The *cloche* mutant is defective in hematopoiesis and blood vessel development (Qian et al., 2005; Stainier et al., 1995; Sumanas et al., 2005; Weber et al., 2005). As a control to confirm the *cloche* phenotype, the RNA levels of *flk1*, a VEGF receptor expressed by endothelial cells, were diminished in *cloche*. As a loading control, the levels of *ef1a*, which are known to remain constant from 8 hours to 72 hours of zebrafish development (Qian et al., 2005; Xu et al., 2003), did not change.

To show that *αA-crystallin* mRNA levels decrease in vivo as well, an *αA-crystallin* promoter-EGFP construct was injected into *cloche* embryos (*n*=470; homozygous=120, wild-type siblings=350) (Fig. 2B). The specificity and reliability of this had been described previously (Kurita et al., 2003). At 2.5 dpf, the wild-type siblings showed strong and uniform fluorescent intensity in the lens and

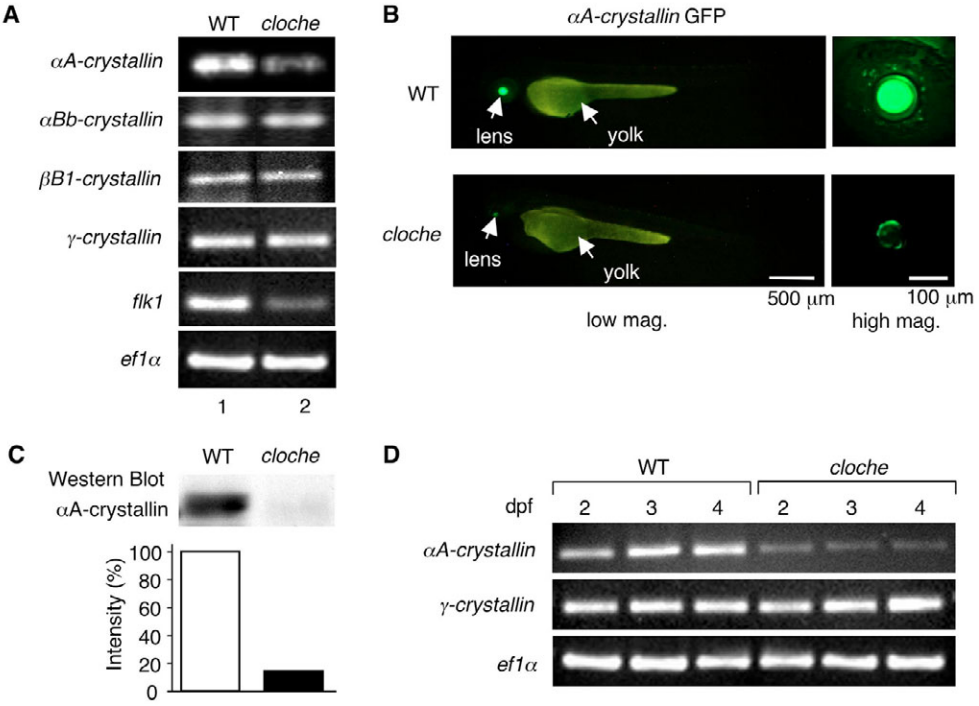

Fig. 2. *crystallin* gene expression. (**A**) Zebrafish embryo (2.5 dpf) mRNA expression levels were analyzed by semi-quantitative RT-PCR. Lane 1, wild type; lane 2, *cloche*. Wild-type versus *cloche* gene expression is shown for *αA-crystallin*, *αBb-crystallin*, *βB1-crystallin*, *γ-crystallin* and *flk1* (*vegfr2*). *ef1a* was used as a loading control. (**B**) *αA-crystallin* promoter-EGFP was injected into one- to four-cell stage embryos and fluorescence (green) was measured at 2.5 dpf. Lateral views are shown at low and high magnifications. Fluorescence was found only in the lens and was greatly diminished in the *cloche* lens. (**C**) Western blot. Total protein as described in Fig. 1A were extracted and lysates (2 μg) were separated by SDS-PAGE and analyzed by western blotting with anti-αA-crystallin. The bands were analyzed by an image analysis program (ImageJ) and the values were normalized to wild-type bands being 100%. (**D**) Crystallin expression as function of development. Zebrafish embryo (2-4 dpf) mRNA expression levels were analyzed by semi-quantitative RT-PCR. Wild-type versus *cloche* gene expression is shown for *αA-crystallin* and *γ-crystallin* expression. *ef1a* was used as a loading control.

nowhere else (Fig. 2B, top). However, in *cloche* there was only faint fluorescence intensity in the lens and no expression in the lens core at 2.5 dpf (Fig. 2B, bottom) and even at 5 dpf (not shown).

Western blotting of total extract with specific anti-mouse αA-crystallin antibody showed an 85% reduction in the levels of αA-crystallin protein (Fig. 2C) consistent with the RNA results. A time course revealed that the level of *αA-crystallin* mRNA in *cloche* were diminished and these low mRNA levels remained constant from 2-5 dpf (Fig. 2D). However, the levels of *γ-crystallin* were the same developmentally in both wild type and *cloche* (Fig. 2D).

The *cloche* embryo lens has cataracts

Crystallin defects are associated with cataracts, which are characterized by light scattering opacity of the lens (Bloemendal et al., 2004; Graw, 2004). Zebrafish embryos are transparent and thus the gross morphology of the eye and lens can be examined readily by bright-field microscopy of living zebrafish embryos. At 2.5 dpf, lenses of wild-type embryo siblings are completely transparent (0 opaque/300 embryos, 0% opacity) (Fig. 3A, top), whereas the *cloche* embryo lenses are cloudy with light scattering in 428 of 633 embryos (68% opacity) (Fig. 3A, bottom). By 3 dpf, the cloudy lens phenotype was more apparent, ranging from 84% (533/633) opacity in one analysis to 100% opacity (34/34) in another. In another approach, lenses were analyzed by stereomicroscopy with highly oblique (80° off-axis) illumination. There was no light scattering in wild-type lens, whereas strong reflectance was observed in the *cloche* lens (not shown).

During normal lens development, the nuclei of lens fiber cells that express crystallins are readily detected by histology at 2 dpf, diminished by 3 dpf and absent by 4 dpf, at which point the lens fiber cells had become terminally differentiated and the lens was transparent (Fig. 3B, top panels). By contrast, in the *cloche* embryo, lens fiber cell nuclei were still detectable at 4 dpf (Fig. 3B, bottom panels) and even at 5 dpf (not shown). The presence of nuclei in the *cloche* lens was confirmed by staining with Hoechst 33342 which visualizes nuclei (not shown). A quantitative analysis shows an 85-90% loss of wild-type nuclei by 3 dpf but persistent appearance of nuclei in *cloche* at 2-4 dpf (Fig. 3C, n=14). The persistence of nuclei in the *cloche* mutant suggests that this phenotype was not due to delayed development.

Lenses were also analyzed by confocal microscopy to measure lens transparency in a non-invasive and more quantitative manner. At 2-4 dpf, wild-type sibling lens were totally transparent (Fig. 3D, top panels), whereas the *cloche* lenses displayed intensive light scattering in the lens core (Fig. 3D, bottom panels). The intensity of reflectance in the live *cloche* embryo lens increased with time. The median value of intensity was 37-fold higher in *cloche* lens compared with the wild-type lens at 4 dpf (P=0.0209) (Fig. 3E).

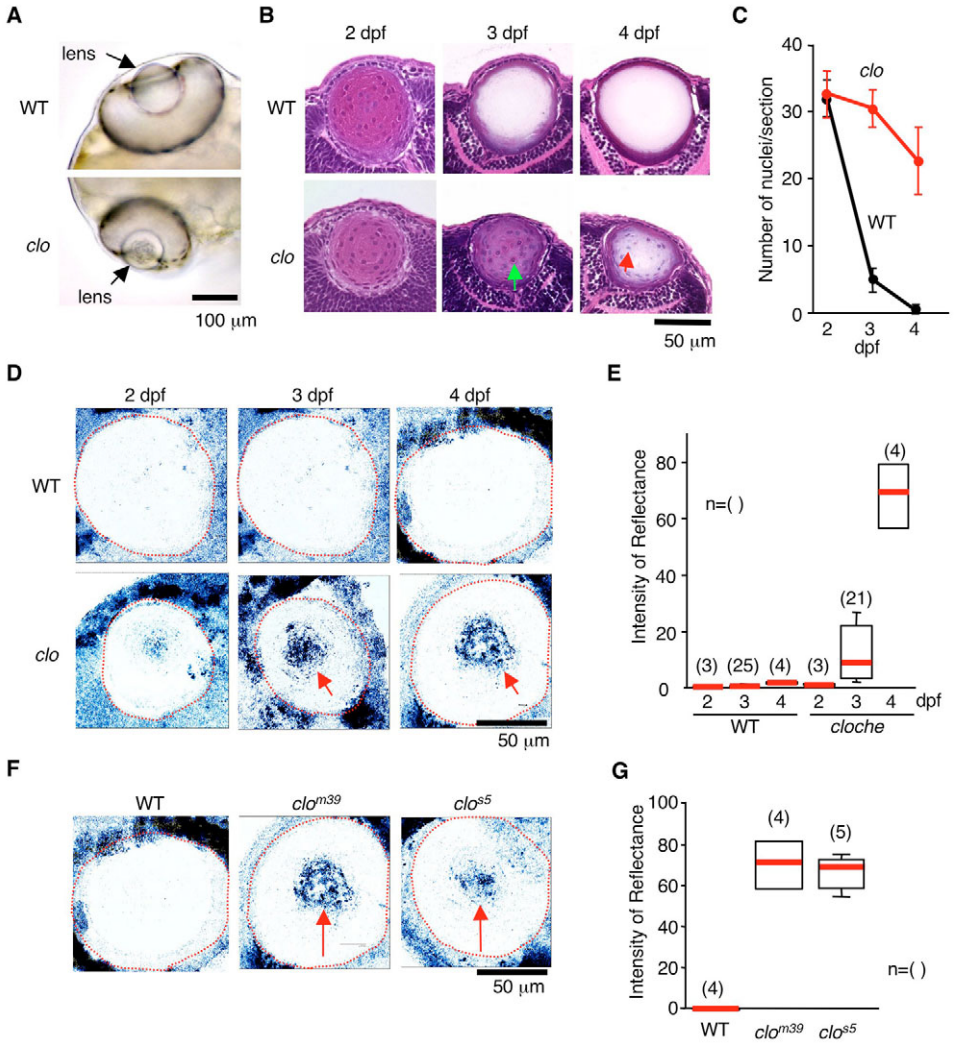

Fig. 3. The cloche lens has cataracts. (**A**) The morphology of the eye was analyzed by bright-field microscopy at 2.5 dpf. (**B**) Sections were prepared from 2-4 dpf embryos and analyzed by Hematoxylin and Eosin. The green arrow (3 dpf) and red arrow (4 dpf) point to nuclei. (**C**) The number of lens fiber cell nuclei per section was analyzed at 2-4 dpf. Three sections of each lens were analyzed by an image analysis program (ImageJ). Fourteen embryos for each time point were analyzed. The mean±s.d. is shown. (**D**) The reflected light (reflectance) of the lens was detected by confocal microscope at 2-4 dpf. The red arrows point to areas of reflected light. (**E**) Statistical analysis of reflectance intensity. The Mann-Whitney *U* test was used to test for differences between two groups. The red line in the box corresponds to the median value. The differences between wild-type and *cloche*^{m39} at 3 dpf (P=0.0339) and 4 dpf (P=0.0209) are significant. (**F**) The reflected light (reflectance) of the lens in *cloche*^{m39} (*clo*^{m39}) and *cloche*^{s5} (*clo*^{s5}) was compared by confocal microscopy at 4 dpf. Reflectance is comparable between the two. The red arrows indicate areas of reflected light. (**G**) Statistical analysis of reflectance intensity. The Mann-Whitney U test was used to test for differences between two groups. The red line in the box corresponds to the median value. The differences between wild type and *cloche*^{m39} (*clo*^{m39}) (P=0.0209) or *cloche*^{s5} (*clo*^{s5}) (P=0.0143) are significant.

Another allele of a zebrafish *cloche* mutant, *cloche*^{s5} (Brown et al., 2000; Qian et al., 2005; Weber et al., 2005), was also analyzed for the presence of cataracts. As in *cloche*^{m39}, the lens was opaque (not shown). However, confocal microscope analysis showed a quantitative high degree of reflectance in *cloche*^{s5} comparable with *cloche*^{m39}(Fig. 3F,G).

cloche is a mutant defective in blood vessel formation. However, lack of blood vessels does not appear to contribute to the cataract phenotype. When wild-type embryos were treated with VEGFR2 kinase inhibitor, blood vessels were not formed, but neither were the cataracts (not shown).

Besides the lenses other parts of the zebrafish eye were examined, in particular the retina, by Dr L. Smith (Children's Hospital, Boston, MA, USA) and Dr J. Malicki (Massachusetts Eye and Ear Infirmary, Boston, MA, USA) who specialize in retinal disease (Smith, 2003; Malicki, 2000). Immunohistochemistry with a monoclonal antibody zpr-1 that detects photoreceptors (Larison and Bremiller, 1990; Pujic and Malicki, 2001) demonstrated that the photoreceptors (green) develop normally in *cloche* even at 5 dpf. The thickness of the retinal layers at 3.5 dpf and 5 dpf were similar to wild-type retina (see Fig. S1 in the supplementary material).

Formation of *cloche* lens cataracts is prevented by overexpressing *αA-crystallin*

αA-crystallin is a small heat shock protein that acts as a chaperone, inhibits aggregation of several target proteins and maintains the solubility of crystallin proteins in vitro (Dahlman et al., 2005; Horwitz, 2003). Accordingly, zebrafish *αA-crystallin* mRNA was injected into zebrafish embryos at the one- to four-cell stage and the effects on opacity were analyzed by bright-field microscopy, confocal microscopy and histology (Fig. 4). Wild-type embryo lenses were clear (0% opaque, *n*=300) (Fig. 4A, lane 1). Uninjected *cloche* embryos had a cloudy and opaque lens (84% opaque, *n*=633), as measured by bright-field microscopy (Fig. 4A, panel 2). However, *cloche* embryos injected with *αA-crystallin* mRNA showed a much lower frequency of opacity (19.8% opaque, *n*=96) at 4 dpf (Fig. 4A, panel 3). Conversely, overexpression of *γ-crystallin,* which has no chaperone activity, in *cloche* embryos had no protective effect on lens opacity (100% opaque, *n*=35) (Fig. 4A, panel 4).

The efficacy of rescue was also measured by confocal microscopy (Fig. 4B). There was no reflectance in wild-type embryos (Fig. 4B, panel 1) and a great degree of reflectance in *cloche* lens (Fig. 4B, panel 2). However, overexpression of *αA-crystallin* resulted in a significant decrease in reflectance. The median lens reflectance (*n*=4) of *cloche* lens was 70 units but when *αA-crystallin* was overexpressed the median reflectance decreased to 41 units (*n*=4, *P*=0.0433, Fig. 4B, panel 3). However, as a control for possible non-specific effects resulting from mRNA injections, overexpression of *γ-crystallin* had no protective effect (Fig. 4B, panel 4). The median reflectance value was 85 units, more than *cloche* but not statistically significant (*n*=4). The differences between overexpression of *αA-crystallin* compared with overexpression of *γ-crystallin* were substantial and significant (*P*=0.0209).

Histology indicated that overexpression of *αA-crystallin* (4 dpf) also rescued the lens fiber nuclei phenotype (Fig. 4C). Unlike *cloche* at 4 dpf (Fig. 4C, panel 2) nuclei were not detected when *αA-*

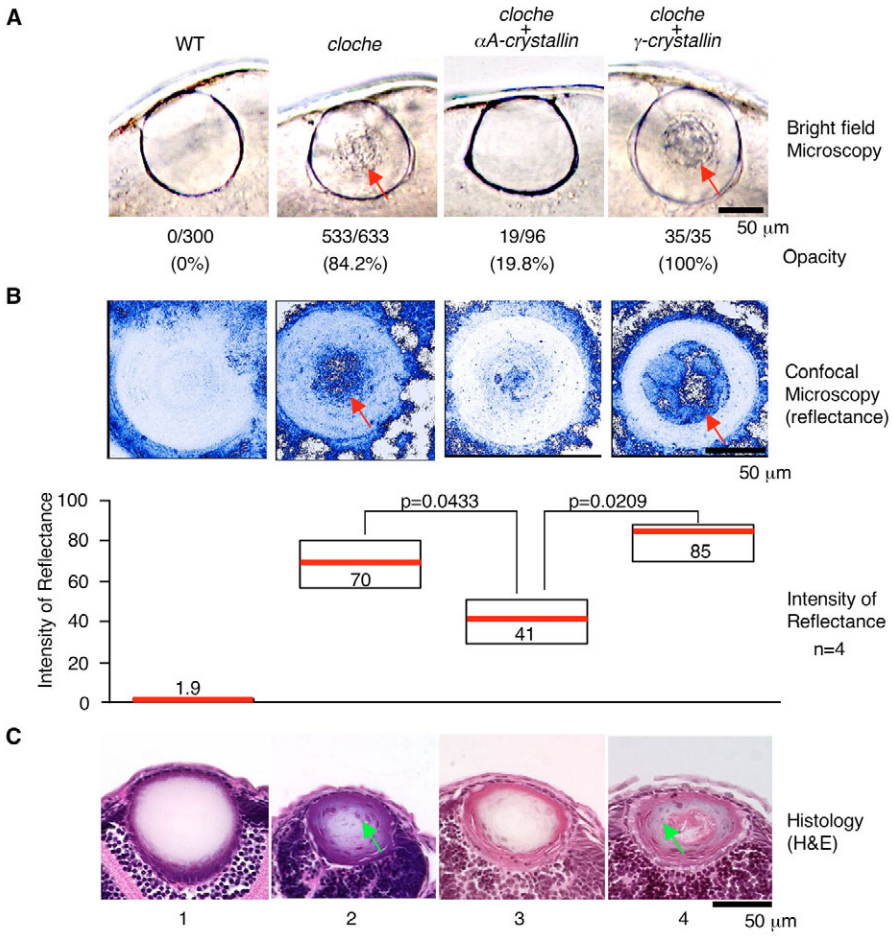

Fig. 4. αA-crystallin expression prevents cataract formation in cloche. *αA-* (panel 3) or *γ-crystallin* (panel 4) mRNA was injected into embryos at the one- to four-cell stage and phenotypes were compared with uninjected wild type (panel 1) and *cloche* lens (panel 2). (**A**) Living embryos were analyzed by stereomicroscopy and the number of opaque lenses was counted. Red arrows point to scattered light. The percent of lenses that demonstrate opacity is shown in parentheses. (**B**) Living embryos lenses were analyzed by confocal microscopy and the intensity of reflected light was measured. Red arrows indicate areas of reflected light. The median reflectance was measured as in Fig. 3D. The Mann-Whitney *U* test was used to test for differences between two groups. The differences in the lens between *cloche* embryos and *cloche* embryos overexpressing *αA-crystallin* (*P*=0.0433) and the differences between *cloche* overexpressing *αA-crystallin* versus *γ-crystallin* (*P*=0.0209) are significant. (**C**) Sections were prepared from 4 dpf embryos and analyzed by Hematoxylin and Eosin staining. The green arrows indicate nuclei. The number of lens fiber cell nuclei per section was analyzed at 4 dpf. Three sections of each lens were analyzed by an image analysis program (ImageJ). Fourteen embryos were analyzed. At 4 dpf, wild type and *cloche* had 0.0±0.0 and 22.6±5.0 nuclei, respectively. In the rescue experiment, *αA-crystallin* overexpression reduced the nuclei from 22.6±5.0 to 3.0±0.0 (mean±s.d.).

crystallin was overexpressed (Fig. 4C, panel 3) and the lens appeared more like the wild-type lens at 4 dpf. (Fig. 4C, panel 1). However, overexpression of *γ-crystallin* in *cloche* embryos had no protective effect on lens nuclei (Fig. 4C, panel 4).

αA-crystallin overexpression maintains *cloche* γ-crystallin solubility

The increased transparency as a result of overexpression of *αA-crystallin* could be due to its preventing γ-crystallin from becoming insoluble. Accordingly, the solubility of γ-crystallin protein was analyzed in *cloche* with and without *αA-crystallin* overexpression (Fig. 5). Protein was extracted from 2.5 dpf zebrafish embryos in detergent-free 20 mM Tris-HCl (pH 7.5), centrifuged to separate soluble and insoluble fractions, and analyzed by western blot. Of the total protein, 83% of wild-type embryo γ-crystallin was soluble and 17% was insoluble, whereas 20% of *cloche* γ-crystallin was soluble and 80% was insoluble (Fig. 5A). However, when *αA-crystallin* was overexpressed in *cloche*, there was a significant increase in soluble γ-crystallin levels, with over 60% of the protein being soluble (Fig. 5B). These results show that αA-crystallin rescues the insolubility phenotype and indicates that the lower levels of *cloche* γ-crystallin shown by 2D gel electrophoresis (Fig. 1A) were due to protein insolubility.

Similar results were found in the *cloche*^{s5} allele (see Fig. S2 in the supplementary material). αA-crystallin was not expressed at the protein and mRNA levels, γ-crystallin was insoluble, and the cataract phenotype was rescued by overexpression of *αA-crystallin* mRNA.

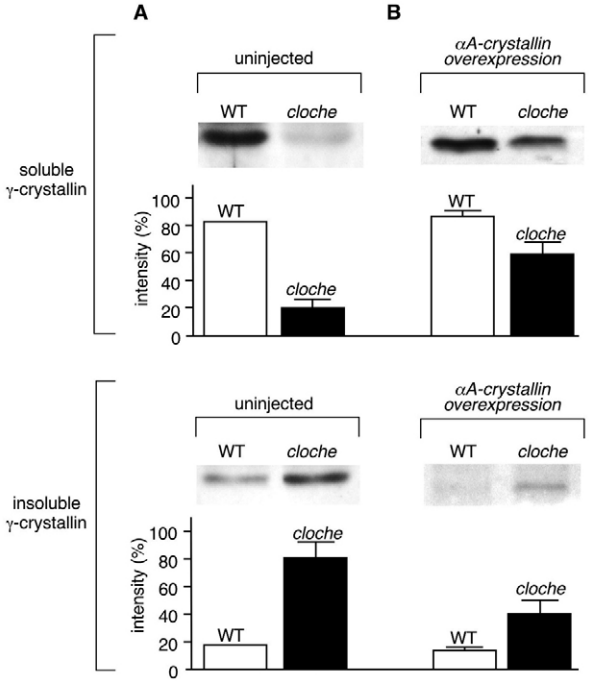

Fig. 5. *αA-crystallin* overexpression prevents γ-crystallin insolubility. (**A**) Uninjected embryos. (**B**) Embryos injected with *αA-crystallin* mRNA. Zebrafish embryos (2.5 dpf) were lysed detergent-free in 20 mM Tris-HCl (pH 7.5) and centrifuged. Soluble (top) and insoluble (bottom) fractions were separated, and were each analyzed by western blot with anti-γ-crystallin antibody. The intensity of the bands in the western blots was measured by an image analysis program. Three independent experiments using *cloche*^{m39} were performed. The bar graph represents the mean±s.d.

DISCUSSION

The zebrafish *cloche* mutant, noted for defects in hematopoiesis and vascular development, has surprisingly an additional phenotypic characteristic, cataract formation. The cataracts are characterized by insoluble, aggregated protein, a high degree of reflectance, and a lack of lens fiber cell differentiation. We propose an in vivo mechanism for cataract formation in which αA-crystallin, a chaperone for γ-crystallin, is not expressed, the result being insolubility of γ-crystallin, and lack of terminally differentiated mature lens fiber cells. The net results of these defects are lens opacity and loss of transparency.

The evidence for this mechanism combines protein and zebrafish analysis. *cloche γ-crystallin* is expressed at the same mRNA levels as wild-type *γ-crystallin* and is localized to the lens in a normal manner as shown by in situ hybridization. However, the *cloche* γ-crystallin protein is mostly insoluble as shown by 2D-gel electrophoresis and western blot. Thus, there appears to be a γ-crystallin protein defect in *cloche*. However, *αA-crystallin* mRNA expression, promoter reporter activity in the lens and protein levels are all greatly diminished. αA-crystallin is not only a structural lens protein but is also a functional chaperone that prevents protein denaturation in vitro (Dahlman et al., 2005; Horwitz, 2003). Evidence that αA-crystallin acts as a chaperone is that the overexpression of *αA-crystallin* in *cloche* mutants prevented cataract formation in 80.2% of the embryos as measured by bright-field microscopy. Quantitative analysis in the living embryo lens by confocal microscopy showed that reflectance was reduced by 41%. Biochemical analysis revealed that the insolubility of γ-crystallin was reduced by 60%. In addition, overexpression of *αA-crystallin* induced lens fiber cell denucleation as shown by histological staining, contributing to an increase in lens transparency. As a control for crystallin specificities, *γ-crystallin* overexpression had no such protective effects. These results in vivo are consistent with previous reports that zebrafish αA-crystallin functions as a chaperone for target substrates in vitro (Dahlman et al., 2005).

Our results are consistent with a number of reports in mammals that crystallin defects are associated with cataracts. For example, in mice, targeted deletion of *αA-crystallin* leads to cataracts (Brady et al., 1997). Missense mutations in *αA-crystallin* have been found in mice with cataracts (Chang et al., 1999; Graw et al., 2001). Mouse *αA/αB crystallin* double knockouts show that both α-crystallin genes are necessary for proper fiber cell formation (Boyle et al., 2003). At least 20 missense, nonsense, deletion and insertion mutations in mouse *γ-crystallin* have been identified (Graw, 2004). In humans, missense mutations in *αA-crystallin* have been found in human autosomal dominant congenital cataracts (Litt et al., 1998). A nonsense mutation in *αA-crystallin* is associated with autosomal recessive congenital cataracts (Pras et al., 2000). There are also human *γ-crystallin* defects associated with cataracts (Graw, 2004). Missense, nonsense and insertion mutations associated with cataracts have been found in *γC-crystallin* and *γD-crystallin* (Graw, 2004). These studies have provided good evidence linking crystallin defects to mammalian cataracts. However, a mechanism for cataract formation and prevention of cataracts in the mammalian lens in vivo has been lacking so far.

There is the possibility that the cataract phenotype is a non-specific consequence of developmental delay. However, this is unlikely for several reasons. One is that *αA-crystallin* expression is very diminished compared with wild type and these low mRNA and promoter levels remain constant from 2-5 dpf. The relatively high levels of *γ-crystallin* do not change in this time period. These results suggest that *γ-crystallin* will be irreversibly insoluble throughout

development. Furthermore, during normal lens development, the nuclei of lens fiber cells that express crystallins were readily detected by histology at 2 dpf, greatly diminished by 3 dpf and absent by 4 dpf, at which point the lens fiber cells had become terminally differentiated and the lens was transparent. However, in the *cloche* embryo, lens fiber cell nuclei were still detectable at 5 dpf. The persistent presence of nuclei and concomitant lack of transparency in the developing *cloche* mutant is inconsistent with delayed development. In addition, we find the same lens cataract phenotype and rescue by *αA-crystallin* mRNA in two different alleles of zebrafish *cloche*, *cloche*^{m39} and *cloche*^{s5} (see Fig. S2 in the supplementary material), that have different genetic alterations, making it unlikely that cataract formation in *cloche* is non-specific. A degree of specificity for the lens phenotype is indicated in that the photoreceptors in *cloche* retina appear structurally normal in development at 3.5-5 dpf (see Fig. S1 in the supplementary material). Finally, we examined a zebrafish mutant, *crash&burn*, that is developmentally delayed because of loss of *bmyb* function, resulting in decreased cyclin B1 expression and mitotic arrest (Shepard et al., 2005; Stern et al., 2005). In these mutants, there are no cataracts and the lenses are totally transparent (Fig. S3 in the supplementary material). Thus, developmental delay is insufficient to induce cataracts. Taken together, these results suggest that the *cloche* lens phenotype represents a specific progressive pathological disorder rather than a delay in physiological development.

Zebrafish *cloche* was originally described as a mutant that had defects in hematopoiesis and blood vessel development (Stainier et al., 1995). Whether there is a link between the cataract phenotype and this blood/blood vessel phenotype is not clear, the lens being avascular. VEGF is a major regulator of angiogenesis. VEGF acting through VEGF receptor tyrosine kinases is necessary for axial vessel and intersegmental vessel development (Goishi and Klagsbrun, 2004). These interactions can be disrupted by administration of VEGFR2 kinase inhibitors or morpholino antisense oligos (MO) to zebrafish embryos (Chan et al., 2002; Lee et al., 2002; Nasevicius et al., 2000). However, neither a VEGF receptor kinase inhibitor nor an anti-VEGF MO induced a cataract phenotype in wild-type zebrafish embryos, even at 5 dpf. Another plausible candidate could be FGF2, one of the first angiogenesis factors to be identified (Shing et al., 1984). Endothelial cells in culture respond to FGF2 by migrating and proliferating mainly via FGF receptor 1 (FGFR1). In addition to a role in angiogenesis, FGFR1 is expressed by hematopoietic/endothelial precursor cells (Magnusson et al., 2005) and FGF signaling regulates hematopoietic development (Faloon et al., 2000). FGF also initiates lens fiber cell differentiation in mice (Lovicu and McAvoy, 2005). Whether FGF defects are linked to both loss of blood/blood vessels and cataracts remains unclear. Our preliminary data showed that FGFR kinase inhibitor did not induce a cataract phenotype in wild-type zebrafish lens.

There are several *cloche* alleles, one being *cloche*^{m39}, which is a spontaneous mutant, and another being *cloche*^{s5}, which is chemically induced mutant (Brown et al., 2000; Qian et al., 2005; Weber et al., 2005). The original studies carried out here have used *cloche*^{m39} embryos. However, *cloche*^{s5} also shows diminished *αA-crystallin* levels, insoluble γ-crystallin protein, and evidence of cataracts. In both alleles, overexpression of *αA-crystallin* in *cloche* rescued the cataract phenotype.

Our results are significant as they may constitute the first demonstration of blocking cataract formation in vivo. There may be some practical consequences of using zebrafish as a cataract model. Zebrafish are amenable to large-scale, systematic screens to identify small molecules that can suppress disease phenotypes (Peterson and Fishman, 2004; Zon and Peterson, 2005). For example, the zebrafish mutation *gridlock* disrupts aortic blood flow in a region and manner akin to aortic coarctation in the human. A zebrafish small molecule screen has been used to discover a class of compounds that suppressed the coarctation phenotype (Peterson et al., 2004). The zebrafish lens is very visible, transparent and relatively large. It is possible to administer drugs to zebrafish in their swim medium (Peterson and Fishman, 2004; Zon and Peterson, 2005); thus, zebrafish *cloche* may be useful for screening anti-cataract drugs.

Finally, our results demonstrate the value of proteomics in detecting protein alterations in zebrafish mutants. Most studies in zebrafish are based on genetic analysis. However, gene expression and mRNA abundance do not necessarily correlate with protein abundance. Another major limitation of mRNA-based approaches is the inability to detect post-translational modifications that may determine protein function. In our study, *γ-crystallin* mRNA was normal but the protein was not soluble. We propose that zebrafish proteomics will provide a new dimension in zebrafish research.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/13/2585/DC1

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